Granulocyte-Colony Stimulating Factor Therapy to Induce Neovascularization in Ischemic Heart Disease

Rasmus Sejersten Ripa
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This dissertation is based on the following original publications. These publications are referred to by their roman numerals:


PREFACE

The thesis is based on research performed during my time as research fellow at the Department of Cardiology, Rigshospitalet, from 2003 to 2007. I am indebted to Jens Kastrup, MD, DMSc who introduced me to cardiac neovascularization therapy. His continuous interest in my work and never failing optimism despite negative results encouraged me to move on. It has always been a pleasure working with Jens. Erik Jørgensen, MD has provided brilliant ideas and constructive criticism in all phases of our trials. His clinical approach and broad view of things when I tended to be lost into the details has improved the work substantially.

The remaining members of the "Cardiac Stem Cell Laboratory", Wang Yongzhong, MD, PhD; Mandana Haack-Sørensen, MSc; Lene Bindslev, MSc, PhD; Tina Friis, MSc, PhD; Stig Lyngbæk, MD; Steen Mortensen, Tech; and Sandra Miran, RN have provided an innovative and enthusiastic atmosphere.

I was originally introduced to research during medical school by Marie Luise Bisgaard, MD. The years of basic laboratory work has been a steady foundation for my continued interest in research. Later, I became involved in clinical research during my stay as research fellow at Duke University Medical Center, North Carolina, USA. Galen S. Wagner, MD; Peter Clemmensen, MD, DMSc; and Peer Grande, MD, DMSc were devoted mentors (and pleasant travel companions) and have since been following my work with great interest and inspiring comments.

I wish to thank all my co-authors who contributed with their expertise to complete the studies. A special gratitude to Jens Christian Nilsson, MD, PhD and Lars Sandergaard, MD, DMSc who taught me cardiovascular MRI, and to Birger Hesse, MD, DMSc and Andreas Kjær, MD, DMSc who introduced me to nuclear medicine. Finally, I want to express my appreciation to all the research fellows and study nurses at the 14th floor. Space was limited, but we have had plenty of coffee, fruitful discussions and exiting trips to conferences around the world - it was never boring. A special thanks to Jens Jakob Thune, MD, PhD who besides conducting all the STEMMI echoes also was a daily source of inspiration, and to Matias Lindholm, MD, PhD who initiated the “kageklub” and explored Boston shopping with me.

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Rasmus Sejersten Ripa
February 2011

Til Maria,
Alvin og Josefine
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Granulocyte-Colony Stimulating Factor Therapy to Induce Neovascularization in Ischemic Heart Disease

Rasmus Sejersten Ripa

ABSTRACT

Cell based therapy for ischemic heart disease has the potential to reduce post infarct heart failure and chronic ischemia. Treatment with granulocyte-colony stimulating factor (G-CSF) mobilizes cells from the bone marrow to the peripheral blood. Some of these cells are putative stem or progenitor cells. G-CSF is injected subcutaneously. This therapy is intuitively attractive compared to other cell based techniques since repeated catheterizations and ex vivo cell purification and expansion are avoided. Previous preclinical and early clinical trials have indicated that treatment with G-CSF leads to improved myocardial perfusion and function in acute or chronic ischemic heart disease.

The hypothesis of this thesis is that patients with ischemic heart disease will benefit from G-CSF therapy. We examined this hypothesis in two clinical trials with G-CSF treatment to patients with either acute myocardial infarction or severe chronic ischemic heart disease. In addition, we assessed a number of factors that could potentially affect the effect of cell based therapy. Finally, we intended to develop a method for in vivo cell tracking in the heart.

Our research showed that subcutaneous G-CSF along with gene therapy do not improve myocardial function in patients with chronic ischemia despite a large increase in circulation bone marrow-derived cells. Also, neither angina pectoris nor exercise capacity was improved compared to placebo treatment. We could not identify differences in angiogenic factors or bone marrow-derived cells in the blood that could explain the neutral effect of G-CSF.

Next, we examined G-CSF as adjunctive therapy following ST segment elevation myocardial infarction. We did not find any effect of G-CSF neither on the primary endpoint - regional myocardial function - nor on left ventricular ejection fraction (secondary endpoint) compared to placebo treatment. In subsequent analyses, we found significant differences in the types of cells mobilized from the bone marrow by G-CSF. This could explain why intracoronary injections of unfractionated bone marrow-derived cells have more effect that mobilization with G-CSF.

A number of other factors could explain the neutral effect of G-CSF in our trial compared to previous studies. These factors include timing of the treatment, G-CSF dose, and study population. It is, however, remarkable that the changes in our G-CSF group are comparable to the results of previous non-blinded studies, whereas the major differences are in the control/placebo groups. We found that ejection fraction, wall motion, edema, perfusion, and infarct size all improve significantly in the first month following ST-segment myocardial infarction with standard guideline treatment (including acute mechanical revascularization), but without cell therapy. This is an important factor to take into account when assessing the results of non-controlled trials.

Finally, we found that ex vivo labeling of cells with indium-111 for in vivo cell tracking after intramyocardial injection is problematic. In our hand, a significant amount of indium-111 remained in the myocardium despite cell death. It is difficult to determine viability of the cells after injection in human trials, and it is thus complicated to determine if the activity in the myocardium tracks viable cells.

Cell based therapy is still in the explorative phase, but based on the intense research within this field it is our hope that the clinical relevance of the therapy can be determined in the foreseeable future. Ultimately, this will require large randomized, double-blind and placebo-controlled trials with “hard” clinical endpoints like mortality and morbidity.
BACKGROUND AND AIM
The concept of adult stem cells within the bone marrow was introduced in 1960 by identification of cells capable of reconstituting hematopoiesis in mice.\textsuperscript{1} Asahara et al\textsuperscript{2,3} extended this concept almost 40 years later by showing that bone marrow-derived circulating endothelial progenitor cells incorporated into sites of angiogenesis in animal models of ischemia. In 2001, Orlic et al\textsuperscript{4} published a ground-breaking but also very controversial\textsuperscript{5,6} trial challenging the paradigm of the heart as a post-mitotic organ thereby igniting the notion of cardiac regeneration. In the following decade an increasing number of animal and small clinical studies have indicated an effect of cell based therapies for ischemic heart disease.

Ischemic heart disease
Ischemic heart disease is caused by a pathological mismatch between the supply to and demand for oxygen in the left ventricle. The pathology is most commonly stenotic or obstructive atherosclerotic disease of the epicardial coronary artery. The normal coronary circulation supplies the heart with sufficient oxygen to prevent underperfusion. This is accomplished by the ability of the coronary vasculary bed to rapid adaptation of the coronary blood flow by varying its resistance.

An atherosclerotic stenosis increases epicardial resistance and thus limits appropriate increases in perfusion when the demand for oxygen is augmented (e.g. during exercise). In severe stenosis, small changes in luminal diameter (e.g. by spasm or thrombi) can produce significant hemodynamic effects and even reduce myocardial perfusion at rest. Myocardial ischemia can also occur with normal oxygen supply if myocardial demands are markedly increased by left ventricular hypertrophy or during exercise.

The symptoms of myocardial ischemia range from silent ischemia to stable angina pectoris to unstable angina pectoris to non-ST- and ST-segment elevation myocardial infarction (STEMI).

Patients with STEMI or patients with moderate to severe but stable angina pectoris (Canadian Cardiovascular Society (CCS) angina class II-IV) have been included into the majority of trials with gene- or cell-therapy. The pathology in these two populations has many similarities but also some important differences.

First, patients with STEMI (usually patients with previous myocardial infarction are excluded) have a single, or occasionally a few, severe ischemic events caused by one coronary occlusion whereas the patients with chronic ischemia suffer from intermittent myocardial ischemia (often through years) usually caused by stenotic lesions in several coronary arteries.

Second, patients with chronic ischemia typically have reversible ischemia not leading to myocardial necrosis, whereas patients with STEMI develop irreversible myocardial damage. We thus have difference in the therapeutic goals in the two patient populations. Patients with STEMI need new myocytes and vascular support for both new myocytes and for hibernating myocytes within the necrotic area, whereas patients with chronic ischemia primarily need improved perfusion of the reversible ischemic area. This also affects the endpoint assessment in the two populations. Patients with chronic ischemia will be expected to have no change or even a slow deterioration in heart function with their current anti-ischemic treatment whereas patients after STEMI are expected to have a recovery in function due to recovery of hibernating myocardium following balloon angioplasty and coronary stenting. A significant placebo effect can be expected in both populations underscoring the need for a proper control group.

Many early phase clinical trials of new therapies for patients with ischemic heart disease have safety as primary endpoint and efficacy as secondary exploratory endpoint. These early trials are often without control-groups or with non-blinded, non-placebo treated controls. This warrants for extreme caution in data interpretation since both a significant placebo effect as well as a significant change due to ‘the natural course’ must be accounted for.

Biological intervention in myocardial regenerative medicine
Based on our pathogenetic understanding, previous trials of biological intervention in myocardial regeneration can roughly be divided into three main groups, vascular growth factor proteins, genes encoding vascular growth factors, and stem/progenitor cell therapy. Only a few trials have combined these modalities.

Protein therapy
The list of known vascular growth factors with
angiogenic potential is long and includes vascular endothelial growth factor (VEGF) A,B,C,D,E; fibroblast growth factor (FGF) 1,2,4,5; angiopoietin 1,2; hepatocyte growth factor, monocyte chemotactic protein 1, platelet derived growth factor BB, e-nitric-oxide synthase, i-nitric-oxide synthase, and many more. So far, mainly VEGF-A and FGF have been used in human trials since these seem to be most important in adult vessel growth.

The trials hypothesized that increased supply of vascular growth factors increases neovascularization and thus improve symptoms. The primary goals of the trials were to develop an administration strategy that provided optimal local tissue concentration for an optimal period of time without high systemic concentrations.

The VIVA trial and the FIRST trial were the two largest randomized trials using VEGF-A and FGF, respectively. Despite encouraging earlier trials with fewer patients and often without controls both the VIVA trial and the FIRST trial were neutral without any improvement beyond placebo. The explanations for these disappointing results could be several, first VEGF-A and FGF might not have any significant clinical effect, second dose and route of administration may be insufficient in achieving optimal concentration of the growth factor within the heart. The second hypothesis is supported by the short half-life of the administered protein, but administration of a higher dose was not possible due to dose-limiting toxicities resulting from systemic exposure.

Trials with growth factor gene therapy were then initiated to enhance myocardial expression for a sustained period of time and to minimize systemic effects.

**Gene therapy**

Gene therapy is introduction of genetic material into an organism in order to obtain a therapeutic result by production of proteins. The advantages over protein therapy are primarily less systemic concentrations and prolonged period of expression. Some of the pitfalls are to achieve optimal tissue expression and to prevent expression in other tissues. The gene needs a transfection vector to get into the cells; this can be viruses, liposome particles or naked plasmids. Naked plasmid is the most simple to use, but also a method with low transfection rate.

Several minor safety and efficacy trials using both the VEGF-A and the FGF genes have been published. Naked plasmid, liposomes, and viruses have been used as transfection vector, and both intracoronary and intramyocardial (during thoracotomy or percutaneously) administration has been used.

The REVASC Trial randomized 67 patients with severe angina pectoris and coronary artery disease to intramyocardial AdVEGF-A gene transfer (N=32) or continued maximum medical therapy (N=35). The treatment was open-label, and the control group did not receive placebo treatment. The primary endpoint of change in time to ST-segment depression on exercise ECG after 12 weeks was not statistically significant compared to controls. Several secondary endpoints including exercise test at 26 weeks, and CCS angina class did reach a statistical significant difference.

Our group initiated a multicenter, randomized, double-blind, and placebo controlled trial of plasmid VEGF-A gene therapy in patients with stable severe angina pectoris (The Euroinject One Trial). Intramyocardial injections of the plasmids or placebo were given via the left ventricular cavity using a catheter-based guiding and injection system (the Noga-Myostar system). Eighty patients with severe stable ischemic heart disease and significant reversible perfusion defects assessed by single photon emission tomography (SPECT) were included. The prespecified primary end point was improvement in myocardial perfusion defects assessed by single photon emission tomography (SPECT) were included. The prespecified primary end point was improvement in myocardial perfusion defects at the 3-months follow-up SPECT and patients were followed with clinical examinations, SPECT, NOGA, exercise test, angiography and echocardiography. Disappointingly, the VEGF-A gene transfer did not significantly improve the stress-induced myocardial perfusion abnormalities compared with placebo. However, local wall motion disturbances (secondary endpoints) improved assessed both by NOGA (p = 0.04) and contrast ventriculography (p = 0.03). Finally, no gene-related adverse events were observed.

The next step from protein/gene therapy to cell therapy was promoted by these rather discouraging clinical results with protein/gene treatment, and very positive preclinical studies utilizing bone marrow-derived stem- or progenitor cells.

**Stem cell therapy**

The rigorous definition of a stem cell requires that it possesses self-renewal and unlimited potency. Potency (differentiation potential) is divided into totipotent...
(differentiate into embryonic and extraembryonic cell types), pluripotent (differentiate into cells derived from any of the three germ layers), multipotent (produce only cells of a closely related family of cells), and unipotent (can produce only one cell type); strictly only totipotent and pluripotent cells are stem cells whereas multipotent or unipotent cells with self-renewal capacity should be referred to as progenitor cells. It is a matter of ongoing and hectic debate whether committed hematopoietic progenitor cells can undergo transdifferentiation into cardiac myocytes or not.4-6,30 Human studies have indicated that mobilization of progenitor and stem cells is a natural response to myocardial injury31-33 correlating to endogenous concentration of granulocyte-colony stimulating factor (G-CSF).34 The degree of mobilization seems to predict the occurrence of cardiovascular events and death.35 Animal studies showed that bone marrow-derived endothelial precursor cells could induce new blood vessel formation (vasculogenesis) and proliferation from existing vessels (angiogenesis) after myocardial infarction.4,36 After a quick translation from bench to bedside, several small human safety trials have been conducted in patients with both chronic myocardial ischemia37-43 and acute myocardial infarction44-49. Five larger trials with intracoronary infusion of bone marrow-derived mononuclear cells after acute myocardial infarction were published with diverging results.50-54 The Norwegian ASTAMI trial (n=100)51, the Polish REGENT trial (n=200)53, and the Dutch HEBE trial (n=200)54 were randomized, but without placebo treatment in the control-arm, whereas the German REPAIR-AMI (n=204)50 and a Belgian trial (n=67)52 were both randomized, double-blind, and placebo-controlled. Only REPAIR-AMI showed a significant improvement in the primary endpoint ejection fraction in the active arm (48.3±9.2% to 53.8±10.2%) compared to the control arm (46.9±10.4% to 49.9±13.0%; p=0.02). The trial was not designed to detect differences in cardiac events, but the prespecified secondary combined endpoint of death, recurrence of myocardial infarction, or revascularization at one year follow-up was significantly reduced in the cell group compared with the placebo group (p=0.009).55 In addition, there was a trend towards improvement of individual clinical endpoint such as death, recurrence of myocardial infarction, and rehospitalization for heart failure.55 The 2-year follow-up of the REPAIR-AMI trial demonstrated a sustained reduction in major cardiovascular events. In a subgroup of 59 patients magnetic resonance imaging (MRI) showed a higher regional left ventricular contractility and a non-significant difference in ejection fraction.56 In comparison, 18 months follow-up data from the randomized BOOST trial indicate that a single dose of intracoronary bone marrow cells does not provide long term improvement in left ventricular function when compared to controls.57 The REPAIR-AMI Doppler Substudy (n=58) has provided insight into the mechanism of intracoronary cells infusions by measuring a substantial improvement in minimal vascular resistance during adenosine infusion 4 months after treatment indicating an improved microvascular circulation.58 The hitherto largest published trial of intramyocardial bone marrow-derived cell injection for chronic myocardial ischemia included 50 patients into a double-blind, placebo-controlled trial.59 The authors reported a significant improvement in stress score by SPECT 3 months after treatment (treatment effect of -2.44 points, p<0.001).

The designs of the trials have so far often been driven by pragmatic solutions, and while some questions have been answered many more have been raised. This has opened for a reverse translation from bedside to bench in order to clarify some of the unknown factors such as optimal cell type and number, optimal route of administration, optimal time of therapy, optimal patient selection, usefulness of repeated or combined treatments etc.60

The use of pharmacological mobilization of stem and progenitor cells from the bone marrow into the blood is an attractive alternative to intracoronary or intramyocardial injection because the treatment is noninvasive and does not require ex-vivo purification of the cells. G-CSF is an appealing candidate since it is well known from clinical hematology and thus has an established safety profile.61

**Granulocyte-Colony Stimulating Factor**

Endogenous G-CSF is a potent hematopoietic cytokine which is produced and released by monocytes, fibroblasts, and endothelial cells. G-CSF regulates the production of neutrophils within the bone marrow and stimulates neutrophil progenitor proliferation, maturation, and functional activation. G-CSF binds to the G-CSF cell surface receptor expressed on myeloid...
progenitor cells, myeloid leukemia cells, leukemic cell lines, mature neutrophils, platelets, monocytes, and some lymphoid cell lines. Ligand binding induces activation of a variety of intracellular signaling cascades ultimately affecting gene transcription, cell survival and differentiation.

G-CSF is involved in mobilization of granulocytes, stem, and progenitor cells from the bone marrow into the blood circulation. The process of mobilization has mainly been investigated for hematopoietic stem and progenitor cells and is not fully understood, but seems to be mediated through binding of G-CSF to the G-CSF receptor, leading to a subsequent digestion of adhesion molecules by enzyme release from myeloid cells, and through trophic chemokines. Stem cell derived factor-1 (SDF-1, also named CXCL12) and its receptor CXCR4 seem to play a central role in regulation of hematopoietic stem cell trafficking in the bone marrow and in mobilization by G-CSF. SDF-1 is a potent chemoattractant for hematopoietic stem cells produced in the bone marrow by stromal cells and its receptor CXCR4 is expressed on the surface of hematopoietic stem cells.

SDF-1 protein concentrations in the bone marrow decline sharply during G-CSF treatment. SDF-1 mRNA expression decreases during G-CSF mobilization, and the magnitude of the decline correlates well with the magnitude of mobilization. Studies of CXCR4 deficient mice have shown that this gene is necessary for sufficient retention of myeloid precursors in the bone marrow and neutralizing CXCR4 or SDF-1 antibodies significantly reduced stem cell mobilization. In addition, inhibition of SDF-1 binding to CXCR4 (by AMD3100) leads to rapid mobilization of hematopoietic cells (CD34+) from the bone marrow. The opposite effects of AMD-3100 and neutralizing CXCR4 antibodies are puzzling and could reflect differences in the binding properties of the two molecules.

Several other adhesion molecules are known to regulate hematopoietic stem cell trafficking, such as VCAM-1/β-1 integrin, hyaluronic acid/CD44, kit/kit ligand, and several selectins. G-CSF induces through an unknown mechanism, a proteolytic microenvironment in the bone marrow by release of a number of proteases including neutrophil elastase, cathepsin G, and matrix metalloproteinase 9. These proteolytic enzymes are capable of cleaving the key adhesion molecules within the bone marrow, SDF-1, VCAM-1, and kit ligand. However, neutrophil elastase, cathepsin G or matrix metalloproteinase 9 deficient mice have normal G-CSF induced mobilization, and thus the precise mechanism for G-CSF induced cell mobilization remains to be determined.

It has recently been shown that the G-CSF receptor is expressed in cardiomyocytes and that G-CSF activates signaling molecules in cardiomyocytes and hydrogen peroxide-induced apoptosis was significantly reduced by pre-treatment of cardiomyocytes with G-CSF. These results suggest that G-CSF has direct anti-apoptotic effect in cardiomyocytes besides mobilization, differentiation and proliferation of stem or progenitor cells. The proposed molecular mechanisms of these G-CSF induced cardioprotective effects in the subacute-chronic phase are through the Janus kinase 2 / Signal transducer and activator of transcription 3 (Jak2/STAT3) pathway activated by the G-CSF receptor. STAT3 is a transcriptional factor known to activate numerous growth factors and cytokines and has been shown to protect the heart during stress (e.g. in patients with myocardial infarction and during treatment with cytotoxics). The cardioprotective effects of G-CSF on post-myocardial infarction hearts were abolished in mice overexpressing dominant-negative mutant STAT3 protein in the cardiomyocytes.

Also recently, G-CSF has been proposed to have an acute “postconditioning-like” effect on the reperfusion injury. G-CSF administration started at onset of reperfusion in a Langendorff-perfused rat heart led to myocardial activation of the Akt/endothelial nitric oxide synthase pathway leading to increased nitric oxide production and ultimately to reduction in infarct size. Finally, G-CSF has been reported to be an anti-inflammatory immunomodulator by inhibition of main inflammatory mediators such as interleukin-1, tumor necrosis factor-alpha, and interferon gamma. Thus, G-CSF could attenuate left ventricular remodeling following acute myocardial infarction by a direct anti-inflammatory effect.

It remains to be determined whether the beneficial effect of G-CSF on cardiac function in animal studies is primarily caused via cell recruitment or via a more direct effect on the myocardium. Filgrastim is a recombinant methionyl human granulocyte colony-stimulating factor (r-metHuG-CSF) of 175 amino acids. Neupogen® is the Amgen Inc. trademark for filgrastim produced by Escherichia coli (E...
coli) bacteria. The protein has an amino acid sequence identical to the natural sequence, but the product is nonglycosylated because Neupogen® is produced in E coli, and thus differs from G-CSF isolated from human cells.

Filgrastim has been used to mobilize hematopoietic stem cells from the bone marrow to the peripheral circulation for the treatment of patients with hematologic diseases for several years, thus Filgrastim treatment has been proven safe and effective in both hematological patients and healthy donors.83,84 Mild side effects are very frequent (typically bone pain, myalgia, artralgia or headache) but they almost never leads to discontinuation of treatment. Rare side effects (0.01-0.1%) are interstitial pneumonitis, respiratory distress syndrome, thrombocytopenia and reversible elevations in uric acid. Very rare side effects (<0.01%) are spleen rupture and allergic reactions.

The current clinical indications of Filgrastim in Denmark are to (1) reduce the duration of neutropenia in patients with nonmyeloid malignancies undergoing myeloablative chemotherapy followed by marrow transplantation, (2) reduce time to neutrophil recovery following chemotherapy, (3) mobilize stem cells to the peripheral blood, (4) for chronic administration to reduce the incidence and duration of sequelae of severe neutropenia.

Pathogenesis of myocardial regeneration

This section gives a short review of the mechanisms and variables of importance for clinical biological intervention. It is focused on vascular regeneration and the impact of cellular components, growth factor and cytokines.

Embryonic development and subsequent postnatal adaptation of the vascular system to changes in functional needs occur by three different processes: (1) vasculogenesis, (2) angiogenesis, or (3) arteriogenesis (review in 85). This nomenclature is not always strictly followed, and some even uses the term ‘angiogenesis’ to summarize all types of vascular formation. All tree processes involves a cascade of different cell types, numerous soluble and cell-bound factors, transcription factors, and cell receptor expression in a complex coordinated interaction that is still not completely described. The below description is an overview of the processes and some of the most important steps involved. The mechanism of how bone marrow-derived cells influence neovascularization remains debated (page 15): Do the cells incorporate into the tissue (e.g. as endothelial or smooth muscle cells) or do they primarily act through paracrine signaling to support the vessel growth and/or maturation?

Vasculogenesis is the first process in embryonic vascular development and denotes an in situ differentiation of endothelial precursor cells (hemangioblasts86) into blood vessels. The mesoderm-derived angioblasts migrate into clusters (blood islands) and mature into endothelial cells that assemble into a primitive vascular network in both the yolk sac and the embryo (review in 87). The process is regulated by a cascade of growth and transcriptional factors, proteases and receptor expressions. The initiating signal for vasculogenesis in embryology is probably tissue ischemia due to rapid tissue growth. CXCR4 and SDF-1 are expressed during embryonic development88 and a role in angioblasts migration to ischemic areas could be assumed. FGF-2 and VEGF-A appear paramount in subsequent blood island formation, cell differentiation and vascular maturation.89-91

Tissue ischemia and exogenous granulocyte macrophage-colony stimulating factor (GM-CSF) or VEGF-A has been shown, in animal studies, to stimulate postnatal vasculogenesis by mobilization and differentiation of endothelial precursor cells.92,93 Like angiogenesis, the process of postnatal vasculogenesis within ischemic tissue is driven by hypoxia-induced production of cytokines and growth factors like VEGF-A94 and SDF-195. Postnatal vasculogenesis requires extravasation and migration of the progenitor cells as described on page 23.

Angiogenesis is the capillary growth (sprouting) from existing vessels. The term also involves division of existing vessels by transendothelial cell bridges or pillars of periendothelial cells. Angiogenesis is initiated by hypoxic stabilization of the transcription factor hypoxia-inducible factor (HIF)-1α.96 This leads to a local upregulation in expression of VEGF-A and a number of other angiogenic factors.97 The new sprouting vessel is initiated in one endothelial cell lining the native vessel (the ‘tip cell’). The endothelial cell exposed to the highest VEGF-A concentration is selected as the endothelial tip cell.98,99 Furthermore, this tip cell seems to gain competitive advantage over neighboring endothelial cells by VEGF-A induced upregulation of
'delta-like 4'. Delta-like 4 activates Notch receptors on the neighboring cells leading to a down-regulation of delta-like 4 expression in these cells. VEGF-A exerts its effect in angiogenesis primarily through binding to the VEGFR2. The tip cell becomes a polarized non- or low-proliferative cell with filopodia extending towards and 'sensing' the angiogenic stimuli and environment. The sprout elongates by migration of the tip cell and proliferation of endothelial 'stalk cell' trailing behind the tip cell. The stalk cells form junctions from the tip cell to the native vessel and form a lumen in the new sprout. The migration of the tip cell is an invasive process requiring proteolytic degradation of the extracellular matrix, especially the 'membrane type-1 matrix metalloproteinase' appears paramount for the invasion. Eventually the sprout connects with another sprout by tip cell fusion. The new tubular structure is stabilized into a mature vessel by tightening of cellular junctions, recruitment of pericytes and deposition of extracellular matrix. Normoxia of the tissue once the new vessel is perfused lowers the local VEGF-A concentration leading to quiescent of the endothelial cells (named 'phalanx cells') and vascular homeostasis.

Arteriogenesis denotes the formation of muscular arterioles from preexisting capillaries or small arterioles. Postnatal arteriogenesis is widely studied in collateral vessel circulation following arterial occlusion. The temporal sequence of arteriogenesis is divided into the initiation phase, the growth phase, and the maturation phase.

In contrast to angiogenesis, arteriogenesis seems initiated by physical forces experienced by the cell independent of ischemia. A pre-existing network of small caliber collateral anastomoses exists in humans. Arterial occlusion (e.g. by atherosclerotic plaque) result in a drop in pressure distal to the occlusion. This new pressure gradient across the occlusion drives the flow along the smaller pre-existing bridging arteries to circumvent the occlusion. Increased flow in the collateral arteries creates a shear stress and circumferential tension at the wall sensed by the smooth muscle cells and endothelial cells. The physical stimuli in the smooth muscle cells seem to increase expression of the proarteriogenic molecule, 'monocyte chemotactic protein-1' via the mechanosensitive transcription factor 'activator protein-1'. The mechanical stimuli of endothelial cells modulates endothelial gene expression and gene expression analysis following hindlimb ischemia in mice have identified differential expression of more than 700 genes. Very fast surface expression of adhesion molecules on the endothelial cells as well as expression of inflammatory cytokines leads to recruitment of bone marrow-derived cells and differentiation of collateral artery smooth muscle cells to a synthetic phenotype. The next 'growth' phase of arteriogenesis result in luminal expansion. This is accomplished by a degradation of the basal membrane and outward migration and proliferation of the vascular cells triggered by a number of signaling pathways involving both growth factors and paracrine signaling from recruited bone marrow-derived cells. As luminal diameter increases, shear stress decreases, and expression of inflammatory cytokines decreases. In this 'maturation' phase, collateral vessels can either mature and stabilize or undergo neointimal hyperplasia and regression. The fate of the vessel is probably determined by the hemodynamic forces, that is, the largest and most developed vessels will stabilize and the smaller and less developed vessels will regress.

A number of cell populations from the bone marrow play a role in arteriogenesis. These participate in a temporally coordinated process in the different phases of arteriogenesis. Neutrophil leukocytes are the first cells to infiltrate the vessel during the initial phase (within a few hours) through binding to the adhesion molecules expressed by the endothelial cells, but the neutrophils are only present in the first few days of the process. The neutrophils seem to recruit inflammatory monocytes to the growing vessel perhaps mediated by VEGF-A release. The monocyte has a paramount role in arteriogenesis and accumulates in the vessel shortly after the neutrophil recruitment that is, in the growth phase of arteriogenesis. Depletion of macrophages seems to eliminate flow-induced remodeling of the vessel in mice. The origin of the inflammatory cells involved in arteriogenesis remains controversial. An experiment in rats could indicate that inflammatory leukocytes and monocytes/macrophages at least in the first days of the process comes from proliferation of tissue resident cells rather than from the circulation.
Bone marrow-derived stem- and progenitor cells
This paragraph aims to give a brief overview of the bone marrow-derived cells potentially involved in cardiac cell-based therapy. Three cell populations from the bone marrow are typically described in cardiac cell-based therapies: the hematopoietic stem/progenitor cells, the endothelial progenitor cells (EPC), and the multipotent mesenchymal stromal cells (MSC). Irrespective of the cell type, several potential mechanisms of cell-based therapies can be hypothesized. These mechanisms can be both direct by incorporation and differentiation of the cells into cardiac or/and vascular cells, or indirect by secretion of paracrine factors, cytoprotection, or immunomodulatory effects (page 15). The main source of progenitor cells is thought to be the bone marrow, but cells from other tissues like fat most likely also contribute.116,117 A number of resident cardiac stem/progenitor cell has been identified and also appear involved in cardiac myogenesis (review in 118). These cells will not be described further in this overview.

Hematopoietic stem/progenitor cells is multipotent cells that can differentiate into all the blood cell types, both in the myeloid and the lymphoid cell lineage and has unlimited capacity of self-renewal. Numerous studies of bone marrow transplantation in hematological patients have documented the possibility of restoration of bone marrow and hematopoietic function 119; however the precise phenotype and characteristic of the hematopoietic stem cells remain debated.

Hematopoietic stem cells have been isolated from bone marrow and peripheral blood as cells expressing CD34 and/or CD133. The number of cells expressing CD34 predicts hematopoietic recovery after blood stem cell transplantation 120 and are thus used to assess the numbers of peripheral blood hematopoietic progenitor/stem cells in the clinic.

The interest in myocyte-differentiation potential of the hematopoietic stem cells was motivated by the still controversial publication in Nature by P. Anversas group.4 The authors found that transplantation of hematopoietic stem cells into infarcted mice hearts led to myocardial regeneration apparently through transdifferentiation of hematopoietic stem cells to functional myocytes. These results were later reproduced by the same group 26,121, whereas other groups could not.5,6,122 Endothelial progenitor cells are found in the bone marrow and in peripheral blood. There has been and is a continued debate over the phenotype and functional characteristics of EPC.

The term EPC has typically been cells in the blood or the bone marrow co-expressing a hematopoietic (CD34, CD133) and endothelial markers (e.g. VEGFR, CD31, Tie-2). However, this phenotype is not exclusive to EPC. Another approach to EPC isolation is to plate peripheral blood mononuclear cells to give rise to colonies. This result in two cell populations: the ‘early outgrowth EPC’ (also called proangiogenic haematopoietic cells) and the extremely rare ‘late outgrowth EPC’ (also called endothelial colony-forming cells).123 The late outgrowth cells have rapid proliferation and seem to include true stem/progenitor cells. They are reported to have a CD34+CD45− phenotype and express VEGFR2 but not CD133 or CD14.124 The majority of published studies of EPC have used early outgrowth EPC.

The number of circulating EPC following acute myocardial ischemia increases31,125 whereas patients with 3-vessel disease undergoing diagnostic cardiac catheterization have low numbers of circulating EPC.126 Several drugs used in patients with myocardial ischemia increases the concentration of EPC in the blood e.g. ACE-inhibitors and statins.127,128

Circulating putative EPC were first isolated by Asahara et al. 3 who cultured cells expressing CD34 or VEGFR2. The cells differentiated into an endothelial-like phenotype and incorporated into areas with vasculogenesis/angiogenesis where the cells appeared integrated into the capillary wall.3 Shi et al. found in a similar study that a subset of CD34+ cells could differentiate into endothelial cells in vitro in the presence of FGF, insulin-like growth factor 1, and VEGF-A.129

The mechanism of EPC contribution to adult angiogenesis and arteriogenesis is not clarified but the prevailing belief is a paracrine rather than a direct incorporation and differentiation of the cells (page 15). This is supported by their capability of releasing angiogenic growth factors including VEGF-A, SDF-1, and insulin-like growth factor 1.130 Transdifferentiation of EPC into cardiomyocytes has been reported by the group of S. Dimmeler,131,132 however, like in the case of hematopoietic stem cells these results have been difficult to reproduce by others.133
**Multipotent Mesenchymal Stromal Cells:** Nearly 40 years ago Friedenstein et al. described that fibroblast-like (stromal) cells from the bone marrow were capable of reconstituting the hematopoietic microenvironment at ectopic sites. Later, research identified the multipotent bone marrow stromal cells (MSC) that can differentiate into mesodermal cell lines. The group of Verfaillie has even described a *pluripotent* cell-type (termed multipotent adult progenitor cells (MAPC)) purified from the bone marrow. Noteworthy though, evidence for pluripotency of MAPC has been difficult to reproduce by others.

MSC is often isolated from the bone marrow, but has been identified in a number of tissues, including fetal and umbilical blood, lung, liver, kidney and adipose tissue. It has recently been shown that pericytes (cells surrounding epithelial cells in capillaries and microvessels) and cells residing in the tunica adventitia share antigenic markers and behave similarly to MSC in culture. It has thus been proposed that the natural MSC niche is perivascular both within bone marrow and other tissues.

Both the defining characteristics and the isolation procedure of MSC differ among investigators due to a lack of simple sensitive and specific markers. MSC is often isolated by plastic adherence and a fibroblastic appearance. Flow cytometry is an easy approach for cell phenotyping based on cell-surface antigens. Unfortunately, no sensitive and specific marker-set of MSC has been found – in contrary a huge list of markers expressed or not-expressed by MSC isolated by different groups from different tissues exist making comparisons of published results difficult. In addition, often MSC phenotypes are described after in vitro culture and little is known about the in vivo phenotype. To complex matters more, the nomenclature is ambiguous. Terms like colony forming units fibroblasts, mesenchymal stem cells, marrow stromal cells, mesenchymal progenitor cells, mesodermal progenitor cells, skeletal stem cells, multipotent mononuclear stem cell, non-hematopoietic stem cell, and multipotent adult progenitor cell probably name the same cell population (at least to some extent).

The International Society for Cellular Therapy recommended in 2005/2006 ‘multipotent mesenchymal stromal cell’ (MSC) as the designation for plastic-adherent cells isolated from bone marrow and other tissues. The following three minimal criteria for defining MSC were suggested: (1) plastic-adherent when maintained in standard culture conditions, (2) Specific surface phenotype (must express CD105, CD73, CD90 and must lack expression of CD45, CD34, CD14 or CD11b, CD79α or CD19, HLA-DR), and (3) In vitro differentiation into osteoblasts, adipocytes and chondroblasts.

MSC has been shown to differentiate into both endothelial cells, vascular smooth muscle cells and cardiomyocytes. However, another study indicate that MSC cannot acquire a mature cardiomyocyte phenotype. MSC has been shown to express anti-apoptotic, angiogenic and arteriogenic factors like interleukin 6, VEGF-A, leukemia inhibitory factor, and matrix metalloproteinase 2. Enzyme-linked immunosorbent assay of MSC medium contained secreted VEGF-A, insulin-like growth factor 1, hepatocyte growth factor, adrenomedullin, placental growth factor and interleukin 6. These characteristics of MSC could indicate both a potential direct (by cell engraftment and differentiation) and indirect (by paracrine) effect.

MSC are reported to express a number of functional chemokine receptors allowing for their migration in response to chemokine gradients in damaged tissue. However, some controversy exist e.g. over the expression of the CXCR4 receptor. Myocardial infarctions, bone fractures, and renal injury are examples where transplanted MSC has been shown to home to the damages area. Passage of the endothelial barrier is essential for tissue homing of circulating cells. MSCs has been shown in vitro to interact by P-selectin and VCAM-1/β1-integrin with endothelial cells under shear flow, thus allowing egress from the bloodstream. The SDF-1/CXCR4 signaling axis is a strong candidate for MSC migration although one recent study could not show an effect of CXCR4 inhibition on MSC migration to ischemic tissue and another study indicate that ‘monocyte chemotactic protein 3’ is an important MSC homing factor. Numerous studies have described a positive effect of MSC therapy on ischemic tissue (e.g. increased capillary density in infarcted area or reduce scar formation after myocardial infarction). The majority of engraftment studies show, that only a small fraction of intravenous MSC engraft, and of these, only a small fraction differentiates. A growing number of studies support the hypothesis that the benefit of MSC
transplantation comes from release of paracrine molecules. These effects could potentially be angiogenic, anti-apoptotic, anti-inflammatory or perhaps through a paracrine effect on resident cardiac stem cells.

Peripheral blood multipotent mesenchymal stromal cell (PBMSC): The existence of MSC in the peripheral blood under homeostatic conditions remains controversial. It is also unclear where PBMSC originates and where they go. As with bone marrow-derived MSC, terminology and isolation procedures differ among investigators (review in ), this may contribute to the mixed results regarding PBMSC. PBMSC are often isolated as adherent, clonogenic, and fibroblast-like and thus also termed colony-forming units-fibroblastic (CFU-F). CFU-F from peripheral blood (typically following G-CSF treatment) has been claimed identified by several groups. The frequency of CFU-F from peripheral blood varies widely among studies but is low (or even absent compared to the frequency in bone marrow-derived mononuclear cells. A trial by Kassis et al. comparing isolation of PBMSC by plastic adherence with fibrin microbeads-based isolation could indicate that a suboptimal isolation procedure enhances the low yield of PBMSC in many trials. The immunophenotype of CFU-F from peripheral blood share many similarities with bone marrow-derived MSC but also some differences. They lack CD34, CD45, and HLA-DR and express CD90 and CD106 as bone marrow-derived MSC do. In contrary to marrow-derived MSC, CD133 has been reported expressed, and CD105 are not always expressed. These differences open the question if PBMSC are bone marrow-derived MSC mobilized to the blood or a distinct cell population.

Potential mechanisms of cell-based therapy
Improved myocardial function after cell based therapies was initially ascribed vascular and/or myocardial regeneration by a direct action of transplanted cells through myogenesis and/or vasculogenesis. Different lines of stem- and progenitor cells were repeatedly demonstrated to differentiate into endothelial cells, vascular smooth muscle cells and myocytes. However, an increasing number of studies have shown a remarkable lack of sustained engraftment and differentiation of the transplanted cells. Another observation is the absent correlation between the number of transplanted cells and functional improvement. These observations have led to the hypothesis that the improved function after cell therapy may – at least in part – be caused by secretion of paracrine factors rather than differentiation. Potential paracrine effects could be neovascularization (potentially vasculogenesis, angiogenesis and arteriogenesis), improved remodeling and contractility as well as myocardial protection and/or cardiac regeneration by resident cells. The importance of neovascularization was confirmed by Yoon et al who demonstrated in a very elegant design that vascular differentiation (endothelial and smooth muscle lineage commitment) of bone marrow-derived mononuclear cells is critical in left ventricular recovery following acute myocardial infarction. Elimination of cardiac-committed cells in the same study did not affect ejection fraction.

A growing body of evidence for the paracrine hypothesis exist (review in ). Some of the most notably studies have shown that conditioned medium from stem/progenitor cells can reproduce the functional results observed after cell transplantation. Shabbir et al. found in an unusual setup, that injection of MSC into skeletal muscle improved cardiac function although the transplanted cell appeared to be trapped in the skeletal muscle. The authors found evidence that MSC-derived interleukin 6 activated skeletal muscle-cells Jak/STAT3 pathway. Skeletal muscle then increased expression of VEGF-A and hepatocyte growth factor that supposedly had a positive effect on heart failure. This study could indicate a very complex cascade from transplanted cell to target organ involving several cell-types and trophic factors.

The paracrine mechanism opens the opportunity for protein-based rather than cell-based therapy once the paracrine factors are identified. However, the temporal and spatial co-operation between several beneficial factors could be so complex that a cell-based strategy would still be most optimal.
Aim and hypothesis
With this background it has been the aim of this translational programme to establish and evaluate cell based therapies using G-CSF as a treatment modality for patients with ischemic heart disease. It has been our hypothesis that clinical effective cardiac regeneration requires cellular components and exogeneous/endogeneous modulating molecules in symphoni. Therefore, we
• Evaluated safety and effects of combined treatment with G-CSF and VEGF-A-gene therapy in patients with chronic ischemic heart disease. I
• Investigated if inherent differences in patients could serve as markers for selecting patients for gene- or cell-therapy. II, III
• Evaluated the clinical effect and safety of treatment with G-CSF following STEMI IV and reasons for failed effect of G-CSF. V
• Determined the recovery in left ventricular function and morphology after current guideline treatment of STEMI VI
• Evaluated a method for intramyocardial in vivo cell tracking. VII
This review will aim at presenting the implications and conclusions of our studies in relation to other investigations.

TRIAL DESIGN
Measures of efficacy
The optimal and conclusive efficacy endpoint in a cardiovascular trial is allcause mortality or perhaps morbidity. However, this would require a huge patient population which is neither ethically nor economically justifiable for neovascularization trials at present. One key issue in our trial designs has thus been to find the best surrogate endpoint available.

For patients with stable chronic ischemia, one approach is the patient’s subjective assessment of symptoms and wellbeing since we would expect only minor changes in the disease without new intervention. For some patients with chronic disease this endpoint may be more important than prolongation of life. 183 However, this evaluation of ‘quality of life’ will require a strict control for the substantial placebo-effect instituted by our invasive treatment and by the close follow-up of our tendering study nurses.

To diminish the significance of influence from the placebo effect, a number of more objective measures of cardiac function and perfusion can be considered.

Myocardial volumes and function
Myocardial function and left ventricular volumes are traditionally assessed using echocardiography, but also ventriculography, SPECT, positron emission tomography (PET), computed tomography (CT), and MRI can be used. 184 Most often, myocardial function is assessed at rest, but it can be visualized during pharmacological or even physiological stress. Change in left ventricular ejection fraction is often used as primary endpoint. This seems reasonable since ejection fraction has been shown to predict mortality. 185 However, ejection fraction at rest can be preserved despite large infarctions due to hypercontractility of non-infarcted myocardium. 186 Regional function may be more informative and the wall motion score index has been found to be superior to ejection fraction in predicting prognosis following myocardial infarction. 187

2D echocardiography is widely used in clinical practice and research because it is fast, easily accessible, and contains no radiation exposure but is also dependent on the operator and the acoustic window. In addition, quantification of left ventricular volumes rely on some geometric assumptions that are not always met especially in ischemic cardiomyopathy. 188 These limitations result in an only moderate accuracy (median limits of agreement from ±16 to ±19%) when compared to radionuclide or contrast ventriculography. 189

ECG gated SPECT allows assessment of left ventricular volumes 190 using an automated 3-D reconstruction of the ventricle and the method has a good reproducibility. 191 The primary drawbacks are the use of ionizing tracers, the long acquisition time and the low temporal resolution. In addition, low spatial resolution limits the assessment of regional wall motion. At present, most investigators consider MRI as the gold standard for assessing global and regional left ventricular function due to high accuracy and reproducibility combined with high spatial resolution.

The advantages of MRI for functional evaluation compared to other imaging techniques are its non-invasiveness, the use of non-ionizing radiation, independence of geometrical assumptions and acoustical windows, and no need of contrast media. The primary drawbacks are low (but improving) temporal resolution, and low accessibility. The examination of patients with
Tachycardia, especially irregular, (e.g. atrial fibrillation) or implanted ferromagnetic devices such as implantable cardioverter-defibrillator and pacemaker is problematic or impossible. Furthermore, MRI scanners may cause claustrophobia in many patients. The STEMMI trial included 78 patients and MRI was not feasible in 20 patients (25%) primarily due to claustrophobia. This is more than usually expected, but the patients were psychologically fragile due to the very recent STEMI. Another recent MRI trial early after acute myocardial infarction showed an even higher drop-out rate. The cinematographic MRI technique used for the measurements of cardiac volumes also poses problems. Image information for each frame in a given position is sampled over a number of consecutive heart cycles (15 in our trials) within a set time-window (50 ms in our trials); the process is termed segmented k-space sampling. This temporal resolution can 1) cause problems in defining the frame with endsystolic phase, 2) cause blurring of the endo- and epicardial borders since the myocardium is contracting in the 50 ms time window, and 3) the required breath-hold during the 15 heart cycles can be difficult for the patients. Furthermore, only one short axis slice could be obtained within a single breath-hold (in end-expiration) with our equipment. Thus, if the point of end-expiration varies from slice to slice, this affects the position of the diaphragm, resulting in non-consecutive slices. Partial volume effect can be a problem near the base and apex, since each slice has a thickness (8 mm in our trials). This may result in imprecise border definitions. Despite these problems several studies have reported high accuracy and reproducibility in determining left ventricular volumes and thus function. Still, echocardiography will fulfill the clinician’s needs in the vast majority of cases, whereas MRI is a sophisticated alternative primarily indicated for research purposes.

**Myocardial perfusion**

Regional myocardial perfusion is another important endpoint in trials of cardiac neovascularization since these therapies are hypothesized to induce capillaries and small arterioles not visible by coronary angiography. Gamma camera imaging and PET have been used for perfusion assessment for more than a decade and more recently CT, contrast echocardiography and MRI have advanced within this field. Perfusion can be visualized during both rest and stress (pharmacological or physical).

SPECT is probably the most available clinical method for perfusion assessment. The myocardial uptake of the radioactive tracers’ thallium 201 and technetium 99m labeled sestamibi/tetrofosmin is proportional to the
blood flow. The method is limited by high ionizing radiation, low spatial resolution (aprox. 10 mm with our equipment) and frequent image artifacts. In comparison PET has better spatial resolution (6-10 mm) but is still insufficient to detect minor subendocardial defects. With PET absolute perfusion can be quantified by dynamic imaging of radioactive isotopes as they pass through the cardiovascular system. PET is less prone to attenuation artifacts than SPECT since accurate attenuation correction can be done. However, PET is expensive and has low accessibility.

CT and contrast echocardiography is emerging as modalities for perfusion assessments. The great advantage is the high spatial resolution (<1 mm) but more validation and optimization of the methods remains.

MRI can quantify the myocardial perfusion by dynamic imaging of the first pass of a paramagnetic (non-ionizing) contrast agent through the heart (Figure 1). The modality has an acceptable spatial (2-3 mm) and temporal (0.5-1.0 s) resolution, but is not widely validated and it is cumbersome to assess the absolute perfusion using this method. The method is further limited by recent accumulating evidence that MRI contrast media containing gadolinium (especially gadodiamide) can cause irreversible nephrogenic systemic fibrosis in patients with renal insufficiency.

To date, nephrogenic systemic fibrosis has only been reported in patients with severe renal impairment.

Conclusion

There are several surrogate endpoints and methods with clinical relevance for neovascularization trials. PET offers accurate measure of perfusion and left ventricular volume during stress and rest with higher spatial resolution than SPECT. Echocardiography is very accessible and has excellent temporal resolution for volume assessment. The MRI technology offers a range of high-quality endpoints with very high spatial resolution within a single examination without a need for radiation. In the design of each trial it remains important to choose the primary endpoint with most clinical relevance.

Ethical considerations in trial design

Treatment with gene or cell therapy is a new area of research warranting for caution in study design. The primary concern is and must be the safety of the treatment and the secondary concern is the efficacy of the treatment. This is not different from traditional drug-trials, but this being a new treatment modality should probably demand for an even higher bar of safety than usually required. The real question is how to gain this knowledge or assumption of safety? Ultimately, we need large double-blinded and randomized patient groups followed for a long period of time. Obviously, this is not possible or even ethical with a new treatment modality where we need to base our initial safety assumption in theoretical knowledge of the treatment (what side effects do we expect knowing the potential effect of the treatment?), early animal trials, early phase clinical trials with few (perhaps healthy) individuals, and the gradual increase in patient number if the treatment still seems safe and effective. In the ethical consideration, it is also important to account for the morbidity of the patients before inclusion. Very ill patients with poor prognosis and without any treatment options will probably accept higher risk than patients with a more benign disease.

The trials included in this thesis have primarily focused on treatment with one pharmacon (G-CSF) in patients with either severe chronic myocardial ischemia or following acute myocardial infarction. G-CSF is a registered drug used for years in healthy donors and patients with hematological diseases. The drug is generally well tolerated with few and mild side effects. However, the drug was not formally tested in patients with ischemic heart disease. At the time of the trial design there was increasing evidence from animal and small clinical trials that autologous bone-marrow derived cells (mononuclear cells) led to improved myocardial function via neovascularization and perhaps myogenesis. It was believed that hematopoietic stem or progenitor cells were the ‘active substance’. Mobilization of cells from the bone marrow seemed like an attractive alternative to intracoronary or intramyocardial injection that would not require bone marrow aspiration or cardiac catheterization. G-CSF was known to mobilize hematopoietic cells from the bone marrow into the circulation. Animal studies had shown that circulating stem and progenitor cells are attracted to ischemic myocardium and incorporates into the formation of new blood vessels. On this background Orlic et al injected mice with recombinant rat stem cell factor and recombinant human G-CSF to mobilize...
stem cells for 5 days, then ligated the coronary artery, and continued the treatment with stem cell factor and G-CSF for 3 days. Afterwards, the ejection fraction progressively improved as a consequence of the formation of new myocytes with arterioles and capillaries.207

Our initial studies with G-CSF included patients with severe chronic ischemic heart disease208,1 since we found more evidence that bone marrow derived cells would promote neovascularization, than neogeneration of myocytes. We thus hypothesized that patients with severe chronic ischemia would potentially benefit more from the treatment compared to patients with acute myocardial infarction or heart failure. In addition, the clinical experience with G-CSF to patients with ischemic heart disease was at that time limited. Despite good long term safety results from hematology, we initially included patients only with severe morbidity without any options for further conventional treatment. All patients included went through a strict screening procedure including a renewed evaluation by independent cardiologists and thoracic surgeons to ensure that no conventional treatment was possible.

Later, accumulating evidence31,37,44,45,209,210 of both safety and efficacy of G-CSF lead us to initiate a trial with G-CSF to patients with STEMI.IV,211

G-CSF FOR CHRONIC ISCHEMIA

Hill et al212 and our group208 have treated patients with chronic myocardial ischemia due to stable severe occlusive coronary artery disease with G-CSF to induce myocardial vasculogenesis and angiogenesis. Both trials were small, non-randomized safety trials with few patients (n=16 and n=13). Three other trials have included patients with intractable angina to treatment with G-CSF and subsequent leukopheresis and intracoronary cell infusion.213-215

Hill et al212 showed no change in left ventricular ejection fraction, in resting or dobutamine stressed left ventricular wall motion or perfusion measured by MRI, nor in treadmill exercise test despite a huge increase in hematopoietic progenitor cells (CD34+ and CD34+/CD133+ cells).

We showed a similar increase in CD34+ cells in the blood following G-CSF treatment.208 The perfusion defects at rest and stress assessed with SPECT demonstrated unchanged number of segments from baseline to 2 months follow-up. This was confirmed with MRI where myocardial perfusion during pharmacological stress was unchanged in the ischemic myocardium from baseline to follow-up. Left ventricular ejection fraction decreased from baseline to follow-up measured with MRI (from 57±12 to 52±11, p=0.01), and the trend was the same with SPECT (from 48±10 to 44±12, p=0.09), whereas the ejection fraction was unchanged by echocardiographic evaluation. This finding could indicate an adverse effect of G-CSF on the myocardium, maybe by an inflammatory response in the microcirculation by the mobilized leucocytes and subsequent development of myocardial fibrosis.

The change in subjective clinical outcomes were more positive, CCS class improved from 2.7±0.6 to 1.7±0.6 (p=0.01), nitroglycerin consumption from 1.5±2.1 to 0.5±1.2 per day (p<0.05), and number of angina pectoris attacks per day from 1.7±1.7 to 1.0±1.6 (p<0.05).208 The interpretation of these subjective measures is not easy. On one hand this endpoint is most important for the patient (who does not care about improvement in SPECT); on the other hand this is a non-randomized study with only historical controls making placebo effect a potential confounder. However, the clinical improvement seems restricted to patients with a pronounced mobilization into the peripheral circulating of CD34+ stem suggesting a causal relationship.208 It can be speculated if the treatment with G-CSF led to deterioration of perfusion and thus infarction of previously ischemic myocardium, this might explain the deterioration in ejection fraction and the diminished symptoms of ischemia.

G-CSF and VEGF-A gene therapy

G-CSF therapy increased the vascular supply of bone marrow-derived cells to the myocardium but did not improve myocardial perfusion and function.208,212 We hypothesized that this could be caused by a lack of signals from the myocardium to engrgt the cells into the ischemic myocardium. VEGF-A165 has been demonstrated to be of importance for the differentiation of stem cells into endothelial cells participating in the vasculogenesis93 and is also important in the homing of cells to ischemic areas (page 23). Animal studies further suggest that a combination of treatment with VEGF-A gene transfer followed by G-CSF mobilization of stem cells might be superior to either of the therapies.216,217
On this background, we performed a clinical study to evaluate the safety and clinical effect of VEGF-A165 gene transfer followed by bone marrow stimulation with G-CSF in patients with severe occlusive coronary artery disease. Sixteen patients were treated with direct intramyocardial injections of the VEGF-A165 plasmid followed 1 week later by subcutaneous injection of G-CSF for 6 days. Two historic control groups from the Euroinject trial were included in the study: 16 patients treated with VEGF-A gene transfer alone and 16 patients treated with blinded placebo gene injections. The treatment was well tolerated and seemed safe with no serious adverse events during the combined VEGF-A165 gene and G-CSF treatment or in the follow-up period. Also we had no serious procedural events during intramyocardial injection of the VEGF-A165 gene in these 16 patients. However, it is known that NOGA mapping and injection is not a risk free procedure. Five patients (6%) in the Euroinject One Trial had serious procedure-related complications, two of these were at our institution. Similar events following the NOGA procedure has been reported by others. It is our impression that most of these events can be avoided with increased experience by the staff. Approximately 100 NOGA procedures have been performed at our institution without any serious events since the two described events (J. Kastrup, personal communication). We have also detected significant (but usually only minor) release of cardiac markers (CKMB and troponin T) following the NOGA procedure.

The treatments lead to a significant increase in circulating CD34+ cells as expected after the G-CSF treatment (Figure 2). The prespecified primary efficacy endpoint of change in perfusion defects at stress SPECT came out neutral after 3 months follow-up (Figure 3), this result was confirmed with MRI measurement of myocardial perfusion during adenosine stress (baseline 62%±32 to 74%±32 at follow-up, p=0.16). In addition, there was no significant difference in changes in CCS classification, angina pectoris attacks, nitroglycerin consumption, or exercise time between the three groups (Figure 4). In opposition to the trial with G-CSF as monotherapy, there was no deterioration in resting left ventricular ejection fraction after G-CSF treatment neither with MRI nor with SPECT. This trial has several limitations, and primarily it must be considered if this trial was underpowered to detect a difference especially since we included few patients with short follow-up period into an open-label design with only historical controls. Furthermore, we were unable to
analyze all patients for all endpoints due to technical difficulties and in some instances poor image quality. In favour of our results are the facts that multiple endpoints using different methods consistently have shown virtually identical results from baseline to follow-up. In conclusion, we found no indication of clinical effects or improved myocardial function following combined treatment with VEGF-A165 gene transfer and G-CSF.

Of interest, a trial (clinicaltrials.gov, NCT NCT00747708) is currently being conducted in patients with congestive heart failure secondary to ischemic heart disease. The investigators aim to include 165 patients into several treatment arms to investigate G-CSF alone or in combination with intracoronary/intramyocardial cell injections.

Safety of G-CSF to patients with chronic ischemia
Our group has treated a total of 29 patients with severe chronic myocardial ischemia with G-CSF without any serious vascular adverse events. Serious vascular adverse events have been reported in two patients (13%) by Hill et al212 and one patient (20%) by Boyle et al213. In a rigorous trial by Kovacic et al214 4 patients (20%) had episodes of cardiac ischemia with elevated troponin I and either ECG changes or elevated CKMB, in addition troponins were elevated in 17 other occasions but without ECG changes or elevated CKMB after G-CSF. The patients described the episodes as typical for their usual angina pattern. The authors speculate if these troponin elevations reflect the natural history of refractory angina, or the G-CSF treatment, since the trial was not placebo controlled.213

Patients with multi-vessel chronic ischemic heart disease are potentially susceptible to the G-CSF-induced increase in leukocyte numbers and inflammation via plaque destabilization or growth. However, a study of cholesterol fed swine suggests that the administration of G-CSF causes neither exacerbation nor modification of atherosclerotic lesions.221

The few and small trials do not permit us to draw any meaningful conclusions regarding the safety of G-CSF treatment to chronic ischemic heart disease. Clarification of safety needs studies of more patients with longer follow-up, and preferably the inclusion of a control group.

Does G-CSF reduce myocardial ischemia?
No convincing effect of G-CSF has been described in patient with chronic ischemia.208,212,1 There have been indications of improved subjective measures of efficacy in several trials but objective measures of myocardial perfusion and ischemia were unchanged. We cannot exclude the possibility that the trials have been underpowered and/or endpoint assessment to poor to find a statistical significant difference. The GAIN II trial included 18 patients with chronic ischemic heart disease in a randomized placebo-controlled double-blinded crossover trial of G-CSF using a more accurate primary endpoint (myocardial perfusion by MRI) than SPECT. The trial was presented at ACC 2010222, but remains unpublished. The authors found no effect of G-CSF on the primary endpoint.

Several explanations to the apparent lack of effect of G-CSF in this clinical setting can be suggested.

Angiogenesis or arteriogenesis
Our pretrial hypothesis was that G-CSF and VEGF-A gene therapy would increase angiogenesis in reversible ischemic myocardium by engraftment and differentiation of bone marrow-derived cells. We chose G-CSF since it was a known mobilizer of progenitor/stem cells from the bone marrow, and VEGF-A because it was a major contributor in cell homing (page 23) and angiogenesis (page 11). Further, we included patients with at least one open epicardial artery to the ischemic area since angiogenesis without epicardial blood supply will probably be of little effect. Retrospectively, it is plausible that increasing the number of circulating cells and the tissue expression of VEGF-A is simply ‘too easy’ despite earlier encouraging results. Both vasculogenesis and angiogenesis involve a complex cascade of several cell types and numerous soluble factors – VEGF-A being just one important player.

It should also be considered if arteriogenesis rather that angiogenesis should be the primary aim of neovascularization. Despite an open epicardial artery, blood flow to the capillaries may still be compromised and arteriogenesis would result in large caliber conductance arteries that could more effectively restore blood flow to ischemic myocardium. Recent evidence from a clinical trial of patients with chronic stable coronary artery disease suggest that G-CSF has the capacity to promote coronary collateral growth.223 Fifty-two patients were randomized to a two week period with
G-CSF or placebo every other day. Both ECG signs of ischemia and collateral flow index in a stenotic coronary artery during balloon occlusion improved after G-CSF indicating an improved collateral function.

**Patient population**

It can be speculated whether angiogenic mechanisms to improve blood supply to the ischemic heart are already activated in patients with severe chronic myocardial ischemia, leaving little therapeutic effect for exogenous angiogenic therapy.

We investigated the plasma concentration of factors known to influence angiogenesis, cell mobilization and homing as well as putative stem/progenitor cells in 54 patients with severe chronic ischemic myocardium and 15 healthy controls. Surprisingly, we found that, in general, circulating stem/progenitor cells and plasma concentrations of angiogenic related cytokines were not significantly different from the control group (Table 1).

This result could be influenced by the fact that plasma concentrations of the cytokines are perhaps a poor indicator of the concentrations within the myocardium. A more precise measurement would require cardiac catheterizations of the patients, which is not clinically applicable for patient stratification, and furthermore the procedure could potentially influence the cytokine concentrations as has been shown for pro-b-type natriuretic peptide. In addition, measuring plasma VEGF-A is potentially influenced by release from the platelets during collection and processing of the blood. We have standardized the procedures to diminish this source of error but cannot exclude that this could explain some of the large inter-patient variations observed. However, our results are supported by our finding of unaffected VEGF-A mRNA contents in chronic ischemic myocardial tissue compared to normally perfused myocardium.

We assessed the number of peripheral blood MSC in patients and control subjects using flowcytometry identification of circulating mononuclear cells negative for both the endothelial marker CD34 and the pan-leukocyte marker CD45. This identification procedure has low specificity for MSC since we did not include any positive marker (like CD105, CD73 and CD90), thus our nomenclature in the paper was imprecise since the whole population of CD45+/CD34+ cells should not be referred to as putative stem cells. We focused on the population of CD45-/CD34- circulating cells were several. First, at the time of analyses of the blood no consensus on which surface markers that determined MSC existed. In contrary, different surface markers on MSC where described in an increasing number of publications. Second, very few had published results regarding surface markers identifying PBMSC. And third, we had identified cells within a population of CD45+/CD34+ cells with a mesenchymal-like phenotype after culture. We decided on this basis to focus our attention on the CD45- /CD34+ cells knowing that this would result in an unspecific identification of MSC. We feared that including positive surface markers in the identification process would exclude some of the MSC, since we could find only little consistency regarding surface markers of PBMSC in the literature. We did include a number of surface-markers in the sub classification of the CD45+/CD34+ cells. These were chosen from the literature to include both markers often reported to be expressed by bone marrow-derived MSC (CD105, CD73, CD166), markers found on endothelial cells (CD31, CD144, VEGFR2) and a marker expressed by hematopoietic progenitor cells that has also been found on PBMSC, but not in bone marrow-derived MSC (CD133). We

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=54)</th>
<th>Control subjects (n=15)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-A (10^-12*g/ml)</td>
<td>35.0 (18.6-51.4)</td>
<td>91.7 (8.2-175.1)</td>
<td>0.4</td>
</tr>
<tr>
<td>FGF-2 (10^-12*g/ml)</td>
<td>9.0 (6.2-11.7)</td>
<td>11.3 (2.2-20.3)</td>
<td>0.7</td>
</tr>
<tr>
<td>SDF-1 (10^-10*g/ml)</td>
<td>22.48</td>
<td>20.14 (17.50-22.79)</td>
<td>0.2</td>
</tr>
<tr>
<td>CD34+ (10^3/ml)</td>
<td>2.8 (2.4-3.3)</td>
<td>3.0 (2.1-3.9)</td>
<td>0.6</td>
</tr>
<tr>
<td>CD45+/CD34+ (10^4/ml)</td>
<td>20.8 (17.0-24.6)</td>
<td>21.9 (13.0-31.0)</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Values are mean (95% confidence interval)
observed that the fraction of CD45-/CD34- cells co-
expressing surface markers expressed by bone marrow-
derived MSC (CD105 and CD166) and endothelial cell
markers (CD31, CD144, VEGFR) were higher in ischemic
patients compared to controls (Figure 5) indicating that
ischemia mobilizes both endothelial committed cells and
more undifferentiated cell. We found some evidence for
a relation between the severity of the ischemia and
VEGF-A and FGF-2 concentrations in the patient group.
Due to large inter-individual variations of these
angiogenic factors as reflected by the inability of the
markers to separate healthy from sick, they do not
appear to be suitable as markers for selecting individual
patients for gene or cell therapy.

We performed another trial to test the hypothesis that
germline DNA variations in the VEGF-A promoter and 5´
untranslated region seemed to explain about 30% of the
variation in plasma concentration of VEGF-A, but this
model was identified in a stepwise analysis including
many loci and should thus be interpreted with caution. We
Coronary collaterals can be assessed by several
methods. We could not perform invasive measurements
in these patients and chose to assess collateral flow and
function indirectly using two previously described
angiographic methods. The Rentrop classification
assesses collateral filling of the epicardial artery whereas
the Werner classification assesses the size of
recruitable collaterals following occlusion of an epicardial
artery. The Werner classification has been shown to
closely reflect both invasively determined collateral
resistance and the collateral functional capacity to
preserve ventricular function. We identified an inverse
association between the VEGF-A plasma concentration
and the size of the collaterals as classified by the
Werner classification in patients with chronic
myocardial ischemia. The present results could
suggest that patients with lower concentration of
circulating VEGF-A have decreased coronary collateral
function. This is in concordance with other trials showing
that polymorphism in the VEGF-A promoter is associated
with impaired prognosis in heart failure and affects
diseases such as proliferative diabetic retinopathy and
end-stage renal disease. It can be speculated if the
neutral results of larger clinical trials with VEGF-A
treatment are affected by differences in the VEGF-A
promoter polymorphism leading to a very heterogeneous patient population
regarding VEGF-A plasma concentration. It would be of
conceptual interest to include analysis of size of
coronary collaterals or even hypoxic regulation of VEGF-
A by in-vitro assay in future trials of neovascularization
for cardiovascular disease.

Homing of bone marrow-derived cells into ischemic
myocardium

All studies have demonstrated high concentrations of
putative hematopoietic stem or progenitor cells in the
blood after treatment with G-CSF (Figure 2). This is in concordance with results showing
that VEGF-A plasma concentration seems to increase
shortly after acute myocardial infarction and that
VEGF-A mRNA expression is low in chronic ischemic myocardium but increased in acute ischemia and
reperfused myocardium. A model combining four
polymorphic loci (including two in Hardy-Weinberg
disequilibrium) in the VEGF-A gene promoter and 5´
since the secreted factors are limited to a local area with high concentration. Cell homing is in many aspects a mirror process of bone marrow cell mobilization. Most of the knowledge regarding cell homing and migration comes from studies on hematopoietic stem cells and EPC but it is natural to assume related mechanisms for other cell populations. Local tissue ischemia induces fast increased expression of chemokines, where SDF-1 and VEGF-A in particular has attracted much attention. Homing of cells is a multistep procedure involving interaction with the host endothelium, transmigration through the endothelial barrier and migration into the host tissue. Human CD34+ cells initiate the low affinity rolling phase on E- and P-selectin. A high affinity adhesion results from β2- and β1-integrin interaction with their counter ligands expressed on the endothelial cells (ICAM-1 and VCAM-1). SDF-1 expressed on the endothelia appear crucial in this process of integrin adhesion. The next step of extravasation involves SDF-1 induced cell polarization and degradation of the basal membrane probably by matrix-degrading enzymes, β2-integrin appear important in this process. The next step of migration towards the ischemic area guided by chemokine gradient also involve proteolytic activity e.g. by cathepsin L. The main proposed mechanism of G-CSF therapy to ischemic heart disease is mobilization and subsequent homing of progenitor cells to the ischemic area. The myocardial homing of G-CSF mobilized cells in patients with ischemic cardiomyopathy may be impaired by a number of factors: (1) microarray analysis and real-time PCR could not demonstrate any significant difference in SDF-1 expression between chronic ischemic myocardium and normally perfused myocardium. A study of gene-expression in human limbs following amputation similarly showed a low expression of VEGF-A, SDF-1, and CXCR4 in chronic ischemic tissue compared to non-ischemic tissue. This impaired chemokine responses could potentially reduce cell homing substantially. (2) The angiogenic potency of bone marrow cells have been shown reduced in patients with chronic ischemic heart disease as well as a number of conditions related to ischemic heart disease, such as high cholesterol, high c-reactive protein, aging, renal failure, anemia etc. The angiogenic potential of bone marrow-derived MSC does not seem impaired in patients with chronic ischemic heart disease. (3) Endothelial dysfunction seems to impair neoangiogenesis by reducing tissue nitric oxide synthase expression. (4) The Jak/STAT pathway is a downstream target from CXCR4 on EPC that modulates cell migration. It was recently shown that Jak-2 phosphorylation in response to SDF-1 was reduced in patients with ischemic heart disease indicating a functional impairment of the cells. (5) G-CSF in itself may also affect the bone marrow-derived cells, as impaired migratory capacity following G-CSF has been found. Another study showed that G-CSF mobilized bone marrow-derived mononuclear cells do not engraft in chronic ischemic myocardium. Engraftment was only observed after transplantation of SDF-1 expressing fibroblasts. Finally, G-CSF was shown to reduce expression of adhesion molecules involved in the homing process (CXCR4, β2- and β1-integrin) on circulating CD133+ cells in the RIVIVAL-2 trial. Animal studies have shown that VEGF-A gene transfer combined with G-CSF therapy leads to incorporation of bone marrow-derived cells into ischemic myocardium. However, in animal studies, chronic ischemia will include components of acute and subacute ischemia as well. Most animal studies induce chronic myocardial ischemia, using an ameroid constrictor around the circumflex or anterior descendent artery. Four to five weeks later the myocardium is often called chronic ischemic myocardium. However, the intracellular milieu is probably in many respects not similar to patients’ myocardium suffering from repetitive chronic ischemia for several years. Imaging studies (page 32) are warranted to elucidate the issue regarding homing and engraftment of injected/infused cells in vivo.
G-CSF FOR STEMI

Clinical trials
Encouraging animal and laboratory results led to a quick translation from the bench to patients suffering an acute myocardial infarction and numerous small sample safety and efficacy trials have been conducted and published.250-255

Kuethe et al251 included 14 patients to subcutaneous G-CSF 2 days after primary percutaneous coronary intervention (PCI) and nine patients who refused G-CSF treatment were included as control group. The authors found a non-significantly larger increase in ejection fraction in the G-CSF treated patients when compared with the control group (7.8 vs. 3.2%). A single-blinded, placebo-controlled study with G-CSF treatment 1.5 days after STEMI (n=20) found almost identical results with a non-significant trend towards improvement in ejection fraction252. Leone et al 253 randomized 41 patients 1:2 to unblinded G-CSF or conventional treatment. All patients had anterior STEMI and ejection fraction<50 at inclusion. After 5 months there were a significant improvement in left ventricular ejection fraction (p=0.02) and absence of left ventricular dilatation (p=0.04) when compared to conventional treatment. Remarkably, patients receiving conventional treatment had no change in ejection fraction in the follow-up period (from 38±6% to 38±8%).253 The larger FIRSTLINE-AMI trial (n=50) was a phase 1 randomized, open-label trial of G-CSF treatment initiated within 90 min after primary PCI treated STEMI. The control group did not receive placebo injections. The G-CSF-treated patients had a significant improvement in left ventricular function with enhanced systolic wall thickening in the infarct zone (from 0.3±0.2mm to 1.1±0.3mm) and an improvement in ejection fraction (from 48±4% to 54±8%). In contrast, the control group had less systolic wall thickening (from 0.3±0.3% to 0.6±0.3%) and a decrease in ejection fraction (from 47±5% to 43±5%) measured with echocardiography.256 The finding that patients in the control group did not experience any improvement in left ventricular ejection fraction is remarkable and not consistent with other clinical studies (page 31) and daily clinical experience. Four randomized, double-blinded, placebo-controlled G-CSF trials have been published and all reached similar conclusions despite some differences in study design.4,257-259 The REVIVAL-2 and the STEMMI trials included patients with STEMI treated with PCI within 12 hours after symptom onset (Figure 6). Patients in the STEMMI trial (N=78) received the first G-CSF or placebo injection 10 to 65 hours (with 85% initiated <48 hours after PCI), and five days after the PCI in the REVIVAL-2 trial (N=114). The primary endpoint in the STEMMI trial was change in regional systolic function (systolic wall thickening) and this did not differ significantly between the placebo and G-CSF groups (17±32 versus 17±22 percentage points, Figure 7). IV Left ventricular ejection fraction improved similarly in the two groups measured by both MRI (8.5 versus 8.0; P=0.9) and echocardiography (5.7 versus 3.7; P=0.7). The infarct sizes were unchanged in the 2 groups from baseline to the 6-month follow-up. IV This was probably due to the small sample size, since pooling of all the patients in the STEMMI trial suggested a significant decrease in infarct mass during the first month VI - a result similar to other
MRI studies. The STEMMI trial has some inherent limitations that increase the risk of a false negative result. First, we included 78 patients, and 54 (69%) were available for paired analysis of the primary endpoint – this is a small population even though the pretrial analysis indicated a 90% power to detect a significant change. Second, the trial was designed to include a homogeneous population where early MRI was possible (mean ejection fraction 53% and infarct size 13g); this probably led to exclusion of high-risk patients who would potentially benefit most from the treatment. We intended to increase statistical power by using a paired design with an accurate method (MRI). The primary endpoint of the REVIVAL-2 trial was reduction of left ventricular infarct size according to technetium 99m sestamibi scintigraphy. Between baseline and follow-up, left ventricular infarct size was reduced by a mean (SD) of 6.2% (9.1%) in the G-CSF group and 4.9% (8.9%) in the placebo group (P=0.56). Ejection fraction was improved by 0.5% (3.8%) in the G-CSF group and 2.0% (4.9%) in the placebo group (P=0.14).

Engelmann et al259 included patients (N=44) undergoing late revascularization (6-168 hours) after subacute STEMI. G-CSF was initiated approximately 1½ day after the PCI. Global myocardial function from baseline (1 week after PCI) to 3 months improved in both groups, but G-CSF was not superior to placebo (Δejection fraction 6.2±9.0 vs. 5.3±9.8%, p = 0.77). Ellis et al257 included few patients (N=18) into a pilot dose-escalation randomized trial and found no effect of G-CSF on left ventricular ejection fraction.

In a recent meta-analysis we aimed to evaluate the effect of cell mobilization by G-CSF on myocardial regeneration after acute myocardial infarction. Ten randomized trials, including 445 patients, were included. Compared with placebo, stem cell mobilization by G-CSF did not enhance the improvement of left ventricular ejection fraction at follow-up (Figure 8, mean difference 1.32% [95% confidence interval -1.52 to 4.16; p = 0.36]) or reduction of infarct size (mean difference -0.15 [95% confidence interval -0.38 to 0.07, p = 0.17]).

### Safety of treatment with G-CSF after STEMI

A major issue to consider is the possibility that the neutral outcome of the G-CSF trials is the result of undetected adverse outcomes balancing any benefits of the G-CSF treatment.

In all reported trials G-CSF was generally well tolerated. Only a few patients experienced minor musculoskeletal pain, a well known side-effect of G-CSF.

Safety data from more than 200 patients treated with G-CSF early after STEMI have been published. Four of these severely ill patients died in the follow-up period, one patient had a spleen rupture, and three patients had a sub-acute in-stent thrombosis or re-infarction. IV,211,257 We recently performed a 5-year clinical follow up of the patients included in STEMMI (presented as abstract262). The clinical events were combined into 4 prespecified endpoints: Time to first (1) hospital admittance (all cause), (2) cardiovascular related hospital admittance, (3) major cardiovascular event, (4) Death. Survival analyses in this small cohort showed no differences in the occurrence of any of the 4 prespecified composit endpoints between the two groups (p=0.6; 0.5; 0.8; 0.3). This result must be interpreted with extreme caution due to the low number of both patients and events.

Trials by Kang et al263 and Steinwender et al264 have indicated that G-CSF treatment increase the progression of atherosclerosis and in-stent restenosis if initiated a few days prior to stent implantation, maybe by increased inflammation or blood viscosity. However, both trials have several potentially inflicting issues. In the trial by Steinwender et al264 at the time of cell injection into the infarct related artery, one vessel was occluded, four patients needed additional stents to restore normal antegrade flow, one patient had a guide
wire-induced dissection of the vessel, and only four patients were treated with drug-eluting stents. Therefore, it is more likely that the very high restenosis rate (40%) was procedure and stent-related and not related to the G-CSF treatment. The trial by Kang et al included only few patients, into a clinically irrelevant design with a very late stent revascularization. The trial by Kang et al was published in Lancaster in the middle of the inclusion period of the STEMMI trial. This obviously gave us severe concern regarding the continued inclusion of patients into the trial despite the differences in study design. We chose to do a non-prespecified interim analysis of baseline data, 1-week blood tests, and data from the 5 months invasive follow-up (including intravascular ultrasound) from all patients included at that time (n=41) to evaluate whether it was safe to continue inclusion in the STEMMI trial. The analyses of the intravascular ultrasound and angiograms were performed in a blinded fashion by an independent core laboratory (Bio-Imaging Technologies B.V., Leiden, The Netherlands). In conclusion, we found identical restenosis rates in G-CSF-treated and control groups by quantitative coronary angiography and by intravascular ultrasound and which legitimized continued inclusion. We found it most likely that the differences compared to the trial by Kang et al was caused by significant differences in the timing of G-CSF administration in relation to PCI. Later, our preliminary results were confirmed in the total STEMMI population, the FIRSTLINE-AMI trial, the G-CSF-STEMI trial, the REVIVAL-2 trial, and in a meta-analysis. In conclusion, the treatment with G-CSF following STEMI and PCI seems to be safe. Still, it cannot be totally excluded that G-CSF may have contributed to the serious adverse events reported from the trials leading to an offset of the positive effects observed in animal studies.

Why does G-CSF not improve myocardial function following STEMI?

It is remarkable that early phase clinical trials and in particular the randomized FIRSTLINE-AMI trial including 50 patients suggested a positive effect of G-CSF, whereas all randomized and double-blinded trials using G-CSF for STEMI were neutral in effect. The major differences between the FIRSTLINE-AMI and the later double blinded trials are the lack of placebo treatment in the FIRSTLINE-AMI and thus lack of blinding; and the time of G-CSF administration.

**Time to G-CSF**

G-CSF was administered as early as 89 min (SD 35 min) after reperfusion in the FIRSTLINE-AMI study, which is somewhat earlier than the remaining trials (Figure 9). This could explain some of the differences since there has been some evidence that intramyocardial transplantation of skeletal myoblasts induce ventricular tachyarrhythmia in patients. None of the clinical trials with G-CSF treatment to ischemic heart disease has indicated a pro-arrhythmic effect and an experiment in mice has even indicated a reduced inducibility of ventricular arrhythmias after G-CSF treatment when compared with controls. In conclusion, the treatment with G-CSF following STEMI and PCI seems to be safe. Still, it cannot be totally excluded that G-CSF may have contributed to the serious adverse events reported from the trials leading to an offset of the positive effects observed in animal studies.

**Figure 9.** Recovery of ejection fraction in human trials of G-CSF after STEMI in relation to time to G-CSF. (Adapted from Ripa et al. Exp Hematol 2008 with permission from Elsevier)
experimental evidence in mice suggest a time-sensitive, direct, cardioprotective effect of G-CSF rather than a cell-mediated effect. The study indicated that the anti-apoptotic effect was significantly reduced if treatment was delayed to only 3 days post-myocardial infarction.\textsuperscript{76} It is however important to notice that the G-CSF dose used in the mouse study was 10 to 20 times higher than the dosages used in human trials (up to 100 μg/kg).

The REPAIR-AMI trial revealed a significant interaction between the absolute changes in left ventricular ejection fraction at 4 months and the time from reperfusion therapy to direct intracoronary infusion of bone marrow cell solution or placebo medium, in fact beneficial effect was confined to patients treated later than 4 days after reperfusion.\textsuperscript{50} This corresponds well with results from the STEMMI trial where the peak concentration of CD34\textsuperscript{+} and CD45\textsuperscript{-}/CD34\textsuperscript{-} mononuclear cells, was measured in peripheral blood 4 to 7 days after the initiation of G-CSF treatment.\textsuperscript{7} Some evidence could even suggest a very late time-point of cell therapy as optimal since homing factors involved in myocardial engraftment of mobilized or infused cells (SDF-1) and vascular growth factors (VEGF-A and FGF) only increase slowly during the first weeks after acute myocardial infarction and reach maximum concentrations after 3 weeks.\textsuperscript{32} We have performed a post hoc analysis of the STEMMI trial to address the issue regarding time to treatment in relation to outcome.\textsuperscript{271} There were no indications in this study that the timing of G-CSF treatment in STEMI patients plays a role in the recovery of left ventricular ejection fraction (Figure 10). This result is comparable to a post-hoc analysis of the G-CSF–STEMI trial\textsuperscript{273} concluding that G-CSF after myocardial infarction does not improve myocardial function if the cytokine is given early.

The G-CSF dose

The optimal dose of G-CSF remains unknown. Most clinical trials so far have pragmatically used 10 μg G-CSF/kg per day known from clinical hematology. Only a single clinical trial of patients with myocardial ischemia has addressed this issue. Ellis et al.\textsuperscript{257} randomized 18 patients with STEMI into double blind treatment with placebo (N=6), G-CSF 5 μg/kg per day (N=6), or G-CSF 10 μg/kg per day (N=6). G-CSF treatment led to a 5- to 7-fold increase in CD34\textsuperscript{+} and CD117\textsuperscript{+} cells with no apparent difference in mobilization between the 2 doses of G-CSF.\textsuperscript{257} In contrast, we have found evidence of a dose-dependent cell mobilization in patients with stable ischemic heart disease.\textsuperscript{219} One trial found dose-dependent improvement in regional myocardial function after intracoronary infusion of bone marrow mononuclear cells.\textsuperscript{274} The direct cardioprotective effect of G-CSF seen in mice\textsuperscript{76} could requires an even higher dose of G-CSF since this study used up to 100 μg/kg per day.

**Differential mobilization of cell types**

Another aspect of G-CSF treatment versus direct intracoronary cell infusion is the type of cells used. It remains puzzling that in vivo mobilization of bone marrow–derived cells by G-CSF to the circulation does not result in myocardial recovery comparable to that apparently achieved by ex vivo purification and subsequent intracoronary infusion of bone marrow–derived cells in the REPAIR-AMI trial.\textsuperscript{50} Animal experiments indicate that MSC may be good candidates for cardiac repair,\textsuperscript{163,164,275} whereas the hematopoietic progenitor cells are less likely to improve cardiac function.\textsuperscript{1} One hypothesis could thus be that G-CSF does not mobilize effective cell types (such as MSC) whereas bone marrow aspiration and purification yields these cells. We analyzed peripheral blood cells from the STEMMI trial to investigate this hypothesis.\textsuperscript{7} G-CSF is known to mobilize endothelial and hematopoietic cells from the bone marrow to the peripheral blood (page 9).\textsuperscript{210,247} The mobilization of MSC by G-CSF is more debated. In a much cited paper Pitchford et al. showed that treatment with G-CSF did not increase PBMSC.\textsuperscript{276}
and others have found similar results. However, several other trials have found indications that G-CSF do increase the number of PBMCS. Indirect evidence of bone marrow mobilization and myocardial engraftment of MSC comes from studies of mice receiving bone marrow transplantation with MSC expressing enhanced green fluorescent protein. Following acute myocardial infarction and G-CSF treatment, cells expressing enhanced green fluorescent protein and actinin were identified in the myocardium. More direct evidence come from trials identifying PBMSC (typically CFU-F) in both healthy donors (humans or animals) or following myocardial ischemia. The mechanism of G-CSF induced increase in PBMSC is unknown but the observed differences in the temporal concentrations of MSC, EPC and hematopoietic stem cells in the blood following G-CSF treatment of normal mice could indicate diverse mechanisms. We assessed the number of PBMSC by identification of circulating mononuclear cells negative for both the endothelial marker CD34 and the pan-leukocyte marker CD45 for reasons discussed on page 22. This identification procedure has low specificity for MSC, and to designate the whole population of circulating CD45\(^{-}/\)CD34\(^{-}\) cells as putative MSC in the paper is retrospectively and according to the minimal criteria by The International Society for Cellular Therapy inexact.

![Figure 11](image)

**Figure 11.** Ratio of (A) CD34\(^{+}\) cells/1000 leukocytes, and (B) CD45\(^{-}/\)CD34\(^{-}\) cells/1000 leukocytes in the blood during 30 days after myocardial infarction. Full line is G-CSF treatment and bracket line is placebo treatment. (Reproduced from Ripa et al. Circulation 2007 with permission from Wolters Kluwer Health)

It can be hypothesized, that the identified differential G-CSF mobilization of circulating CD34\(^{+}\) and CD45\(^{-}/\)CD34\(^{-}\) cells might in part explain the observed difference in therapeutic effect of G-CSF vs. intracoronary infusion of bone marrow cells (in the STEMMI vs. in the REPAIR-AMI trial). However, the results should be interpreted with caution due to the low number of patients, the exploratory nature of the design, and the low sensitivity and specificity of the surface markers for identifying discrete cell populations.

**Homing of mobilized cells to the myocardium**

Homing of the circulating cells into the ischemic myocardium is a prerequisite for both a paracrine mechanism and a direct incorporation and differentiation of progenitor/stem cells. Patients with ischemic heart disease seem to have impaired homing capacity and additionally G-CSF seems to impair the migratory capacity of the mobilized cells (page 23). We have roughly estimated the number of cells supplied to the ischemic myocardium (and thus available for homing) during the first week after G-CSF/placebo treatment. The purpose of this estimate was to compare our mobilizing approach with the number of cells infused in studies using an intracoronary infusion of bone marrow-derived mononuclear cells, and also to compare the number of cells mobilized in the STEMMI and the FIRSTLINE-AMI trials. For the estimate, we used cell concentrations measured at day 1, day 4, and day 7. The blood flow to ischemic myocardium was approximated at 80 ml/min in all patients based on the method used by Incé et al in the FIRSTLINE-AMI trial. Previously, one trial found a mean flow rate of approximately 60 ml/min through the stented segment following primary PCI and another trial found a mean
flow of 140/118/144 ml/min in the proximal LAD/LCx/RCA and 55/51/64 ml/min in the distal segments of the same arteries. More recently, Erbs et al measured a basal coronary blood flow just below 80 ml/min in the stented infarct related artery 4 days after STEMI in the REPAIR-AMI trial. The estimate has the obvious limitations that differences in volume of ischemic tissue, vascular dilatation, and presence of microvascular obstruction will cause inter-patient differences in the true blood volume supplied to the ischemic area. Compared to FIRSTLINE-AMI we found almost identical numbers of CD34+ cells (2.5x10^10 vs 2.8x10^10). Thus, our G-CSF approach seemed to expose the myocardium to more than 100 times the number of CD34+ cells infused in the REPAIR-AMI trial. We found no association between the estimated total number of CD34+ cells supplied to the postischemic myocardium after myocardial infarction and the subsequent change in left ventricular ejection fraction. An inverse association was found between the estimated number of CD45+/CD34+ cells supplied to the postischemic myocardium and the change in left ventricular ejection fraction (95% CI of regression coefficient -11.4 to -2.2, P=0.004). We found similar results when using the day 7 concentration of CD34+ or CD45+/CD34+ cells rather that the estimated total number of cells to predict recovery of ejection fraction (Figure 12). The association was not reproduced when using systolic wall thickening as dependent variable. This could indicate a statistical type I error. A causality of the association cannot be determined in an observational study, but the results may suggest that a low concentration of the CD45+/CD34+ cells in the blood is due to engraftment of the cells into the myocardium. Thus, patients with a high inert potential for myocardial homing after STEMI will have the highest degree of systolic recovery due to the engrafed cells. Of note, we found a similar inverse association between ejection fraction and CD45+/CD34+ cells in patients with chronic myocardial ischemia. It could alternatively be speculated that CD45+/CD34+ cells are not homing, but potentially reduce the recovery of the myocardial function explaining the inverse association between circulating CD45+/CD34+ cells and changes in global ventricular function. A study in dogs has indicated that intra-coronary infusion of MSC could cause micro-infarctions, probably due to microvascular obstruction by the cells. However, there was no biochemical or electrocardiographic evidence of myocardial ischemia during the G-CSF treatment in the STEMII trial. In addition, circulating mononuclear cells collected after G-CSF treatment and then injected into the infarct related coronary artery in patients with STEMI did not result in any signs of myocardial damage.

Conclusion
There is no convincing evidence that monotherapy with G-CSF early after STEMI improves recovery of left ventricular function, despite the discrepancy in results when compared with previous animal and uncontrolled or unblinded clinical G-CSF trials. We still cannot exclude the possibility that the trials have been underpowered to detect a small difference but the recent meta-analysis makes this unlikely. As for patients with chronic ischemia, it must be speculated if arteriogenesis rather that angiogenesis should be the goal of the therapy. The complex interaction between stem cell mobilization/engraftment and cytokines remains poorly understood, and the results do not exclude the possibility that G-CSF could be part of a treatment strategy combing several cytokines and/or local stem cell delivery in future trials. The MAGIC Cell-5-Combicytokine Trial (clinicaltrial.org, NCT00501917) that was initiated March 2007 to evaluate the efficacy of combination therapy with erythropoietin and intracoronary infusion of G-CSF mobilized peripheral blood stem cells. The SITAGRAMI-Trial initiated in March 2008 aim to test a dual strategy of G-CSF in combination with an inhibition of SDF-1 degradation.
MYOCARDIAL RECOVERY AFTER STEMI

Cardiac MRI is an attractive method for efficacy assessment in early phase clinical trials since the high accuracy and precision allows for inclusion of a minimum of patients (page 16). However, in using MRI-derived endpoints it is important to acknowledge (especially in early trials without randomized control groups) the limited knowledge of the natural course of myocardial recovery following a reperfused acute myocardial infarction with modern guideline treatment. One example is the use of G-CSF for acute myocardial infarction where early non-controlled trials postulated an effect. However, the later randomized trials showed a similar improvement in the placebo treated groups. We therefore performed a study aiming at the investigation of the short-term and long-term effects of current guideline treatment of STEMI, including successful primary PCI, in terms of left ventricular function, morphology, edema, and perfusion using cardiac MRI. Overall, we observed a substantial recovery of all investigated variables primarily within the first month after the reperfusion. Left ventricular ejection fraction increased with more than 8 percentage points (Figure 13), the systolic wall thickening in the infarct area almost doubled (Figure 14), and the perfusion of the infarcted myocardium increased with approximately 50%. These results are potentially biased by the low number of patients with a potential selection bias (n=54) and the post hoc design, but the variance of the means were still acceptable (e.g. 95% CI of the mean ejection fraction change from 4 to 9 percentage points). Furthermore, these patients were all included in a trial and we cannot exclude the possibility that the post AMI care were more careful than daily clinical practice. The results found are comparable to those of a smaller MRI study (N=22) by Baks et al who found an increase in ejection fraction of 7 percentage points. Trials assessing the change in ejection fraction from angiography show minor but still substantial increases (3-6 percentage points). In contrast, one trial including 51 patients with myocardial infarction found no change in ejection fraction. Study design and population can potentially explain some of the differences; first and perhaps most important, the baseline MRI was not performed until 5 days after the infarction, second, only 16 of the patients were treated with angioplasty, 21 with trombolysis and 14 with aspirin alone owing to late admission or diagnosis, and third, the left ventricular volumes were assessed from two long axis cine loops using the modified biplane Simpson’s method whereas we assessed the volumes from short-axis cine loops (usually 10) covering the entire left ventricle. The improvement in wall thickening confirmed a previous trial of 17 patients showing a increase from 22 to 38% in the infarcted area.

Figure 13. Change in ejection fraction. Bold line indicates mean±SE. (Reproduced from Ripa et al. Am Heart J 2007 with permission from Elsevier)

Figure 14. Mean change in systolic wall thickening after STEMI in 3 myocardial areas. (Adapted from Ripa et al. Am Heart J 2007 with permission from Elsevier)
A limitation of our study design is the 30-day time span between the initial and first follow-up examination, which does not allow firm conclusions about the precise timing of the changes observed. However, results from others suggest that the recovery of left ventricular ejection fraction primarily happens within the first week following reperfusion, since these trials with a later baseline MRI observe less recovery of ejection fraction. This spontaneous recovery is probably primarily due to early myocardial stunning following the ischemic event.

The infarct mass was reduced by almost 30% from baseline to 6 months follow-up, a result very similar to the results of others. This is consistent with animal data showing that healing of a myocardial infarct is an ongoing process: After four days central necrosis, hemorrhage and inflammation can be observed. This is followed by infarct resorption, scar formation by tissue composed of fibroblasts in a dense collagen matrix and wall thinning after six weeks. There has been some suggestions that infarct size assessed by MRI in the first days after the infarction overestimates the true infarct size perhaps due to myocardial edema in the adjacent myocardium. However, two methodologically strong studies in dogs by the group of Kim and Judd showed a very close correlation between in vivo and ex vivo infarct size measured with MRI and infarcted regions defined by triphenyltetrazolium chloride staining from 4 hours to 8 weeks after coronary artery occlusion (both with and without reperfusion).

The results of our MRI study underscores the importance of a proper control group in trials including patients after acute myocardial infarction due to the substantial change in all measured parameters during the first month, or at the very least a good knowledge of the natural course of the disease. In addition, it appears of crucial importance that baseline examinations are performed within a narrow time window after the STEMI when comparing several populations.

FROM BED TO BENCH

To date most clinical trials of cells therapy have had a pragmatic design with intracoronary infusion of autologous bone marrow-derived mononuclear cells. Bone marrow-derived mononuclear cells are isolated using density gradient centrifugation following bone marrow aspiration. The bone marrow-derived mononuclear cell suspension primarily comprises nonprogenitor cells and only about 3% hematopoietic stem/progenitor cells or EPC. Cells positive for the hematopoietic surface marker CD34 can also be obtained from the peripheral blood, but an experimental rat study has suggested that bone marrow-derived mononuclear cells may provide an advantage when compared to peripheral blood-derived mononuclear cells. The modest improvement in ejection fraction seen in most clinical trials (3% in a meta-analysis) has shifted the focus towards the use of more specific stem or progenitor cell lines to improve outcomes. One potentially useful cell line is the MSC, which can be isolated from the bone marrow and expanded in culture. Allogeneic MSC has been infused intravenous in a clinical trial in patients after myocardial infarction. It was primarily a safety trial, but the results indicated a positive impact of MSC on ejection fraction by MRI and by echocardiography in anterior wall infarction only. A number of other secondary endpoints (wall thickness, wall motion score index, and 6-min walk test) were unaffected by MSC.

The optimal route of delivery of cells to the heart and the potential mechanism by which cell-based therapy works also remains to be determined. Imaging based cell-tracking can potentially elucidate important mechanistic issues by determining homing, engraftment and growth of cells following transplantation. Furthermore identification of redistribution to other organs where the transplanted cells might lead to side effects is of vital importance.

The ideal imaging technique should allow for serial tracking in humans for a prolonged period of time with high spatial resolution and with the capability of tracking a few cells without affecting the cells or the organ. It is important that the marker remains in the viable cell but is quickly cleared from the tissue upon cell death. Several in vivo imaging techniques are available and currently direct labeling with radionuclides for gamma camera imaging or PET or labeling with iron particles for MRI appear suitable.

Imaging of leukocyte distribution using In-111 is a safe clinical routine procedure. In-111 is commercially available with a half-life of 2.8 days making in vivo tracking up to 2 weeks possible. Experiments by Jin et al and Gholamrezaeezehad et a might suggest that the radioactivity from In-111...
could be toxic to stromal cells, however, we found no indication of radiotoxic effects on viability and/or function after labeling of human MSC with $^{111}$In-tropolone.\textsuperscript{318} Perhaps differences in culture or labeling procedures could explain these differences in results. Previously, several studies have used indium labeling of both MSC and EPC and subsequent in vivo tracking of the radioactivity as a surrogate measure of cell engraftment and migration. These trials have been conducted assuming that radioactivity assesses living and active cells.\textsuperscript{319-322} In one trial $^{111}$In labeled progenitor cells were infused into the coronary artery in patients after acute myocardial infarction ($N=20$).\textsuperscript{319} One hour after infusion of progenitor cells, a mean of 6.9±4.7\% (range, 1\% to 19\%; $n=17$) of total radioactivity was detected in the heart. Radioactivity remained in the heart after 3 to 4 days, indicating homing of progenitor cells to the myocardium.\textsuperscript{319} Several of the experimental trials have confirmed the presence of labeled cells by histology following euthanasia of the animals.\textsuperscript{323-326} We designed a pilot trial to investigate whether the biodistribution and retention of ex-vivo cultured MSC can be determined after direct percutaneous intramyocardial transplantation in a large animal model by $^{111}$In-tropolone radiolabeling of human MSC.\textsuperscript{VII} Labeled MSC were first transplanted into four pigs by trans-endocardial percutaneous injections. The $^{111}$In activity in the heart was 35\% (±11\%) of the total activity in the pig one hour after injection of viable $^{111}$In labeled MSC,\textsuperscript{VII} compared to only 6.9±4.7\% after intracoronary infusion\textsuperscript{319} and from 11.3±3\%\textsuperscript{327} to 20.7±2.3\%\textsuperscript{324} after trans-epicardial injection. SPECT imaging identified the $^{111}$In within the myocardium corresponding to the locations of the intramyocardial injections (Figure 15). Whole body scintigraphy revealed focal indium accumulations in the cardiac region up to 6 days after injection.\textsuperscript{VII} Myocardium with high radioactivity was analyzed by fluorescence in situ hybridization (FISH) and microscopy after euthanization of the animals. No human MSCs were identified with FISH, and microscopy identified widespread necrosis and acute inflammation. Two new pigs were then treated with immunosuppressive therapy to diminish the host-versus-graft reaction observed in the first 4 pigs, but injection of MSC still lead to a similar pattern with focal indium accumulation, and inflammation but no human MSCs could be identified. Two additional pigs were injected with $^{111}$In-tropolone (without cells) to test the tissue response to the gamma radiation from $^{111}$In. In these two pigs radioactive tissue samples, identified using a gamma detection probe, showed normal myocardium without inflammation.\textsuperscript{318} This indicates that neither the gamma radiation nor the intramyocardial injection causes the inflammation and tissue-damage, which is in concordance with our previous experience from intramyocardial injections.\textsuperscript{218} A last pig was injected with dead $^{111}$In labeled cells. The clearance of

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure15.png}
\caption{A, Endocardial NOGA mapping of a pig heart. The dots indicate the injection sites. B, Dual isotope SPECT images of the left ventricle in the short axis showing a hot spot of 111In activity in the anterior wall, corresponding to the injection sites in the NOGA map. (Reproduced from Ripa et al. Int J Cardiovasc Imaging 2010 with permission from the Editor)}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure16.png}
\caption{Relative retention of radioactivity after correction for decay. (Reproduced from Ripa et al. Int J Cardiovasc Imaging 2010 with permission from the Editor)}
\end{figure}
radioactivity of injected dead cells and of $^{111}$In alone appeared faster initially compared to that of viable cells, but retention after injection of viable cells, dead cells and $^{111}$In followed a very similar pattern (Figure 16). The results of this trial were potentially biased by several factors. We only included few animals in a prospective design making this a hypothesis generating trial. However, we did consistently in 6 animals observe intense radioactivity despite disappearance of the cells. In our opinion, a very high specificity (close to 100%) should be demanded of this labeling method, and our results are in conflict with this. Another limitation is the FISH method; we have not quantitatively determined the sensitivity of the method in our setup and thus cannot exclude the possibility that a few of the cells were present despite the negative FISH result. However, based on the radioactivity in the tissue-sample excised for FISH analysis, we would expect $>10^5$ human cells per gram tissue (assessed using our initial mean activity of 1.4 Bq/cell).

In conclusion, the pilot study generates two important hypotheses. First, as radioactivity from $^{111}$In-labeled cells stays in the myocardium for a long time despite the disappearance of transplanted cells, clinical use of $^{111}$In-labeled cells for monitoring of MSC in the human heart seems problematic unless viability can be determined by another method. Second, xenografting of human MSC into a pig leads to an inflammatory response and fast degradation of the cells even under pharmacologic immunosuppression.

Our results indicate that an alternative imaging modality is warranted. Iron-oxide labeling for MRI tracking of injected cells has appeared as a suitable alternative to indium labeling, with the possibility of even longer follow-up. However, recent results very similar to ours using iron labeling were reported. After 3-4 weeks no transplanted cells were detected. Instead a continued enhanced magnetic resonance signal was found from cardiac macrophages that engulfed the labeling particles suggesting that iron-oxide labeling is also an unreliable marker for monitoring cell survival and migration. Reporter gene imaging using clinical PET is another promising modality. The reporter gene is expected to be lost after cell death providing a more specific signal, but the technology still needs further work.

GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

The objective of our investigations was to evaluate G-CSF as a cell-based therapy for ischemic heart disease. We found no effects of combined treatment with G-CSF and VEGF-A-gene therapy in patients with chronic ischemic heart disease in a small scale clinical trial. Concordantly, we must conclude that if G-CSF and VEGF-A have an effect on these patients, the effect is most likely very small. We could not identify any inherent factors in the blood or genetic variations in the VEGF-A gene that could select optimal patients for cell-based therapies.

When randomizing patients with STEMI to G-CSF or placebo we found no clinical effect of G-CSF, but we did observe a substantial recovery of myocardial function following current guideline-based therapy of STEMI. This ‘natural’ effect could potentially explain the apparent effect of G-CSF previously reported in non-controlled trials. Thus, our results did not support our pretrial hypothesis that cell mobilization alone or in combination with modulating molecules would result in clinical effective cardiac regeneration.

Several clinical trials of G-CSF for ischemic heart disease were published and planned prior to the presentation of the STEMII and the RIVIVAL-2 results. The group behind the FIRSTLINE-AMI trial even planned a large-scale multicenter clinical trial of G-CSF after acute myocardial infarction based on their positive results in a non-blinded trial. Our trials brought science past G-CSF as monotherapy for ischemic heart disease despite the negative results.

The heart is a complex organ composed of muscle and non-muscle cells integrated into a three-dimensional structure. Cardiac regeneration will probably require more than simply supplying the right cell to the right tissue, at the right time. So far, clinical trials have had a pragmatic design using the cell types that are readily available. This probably leads to extensive cell death and inadequate integration in a hostile immunoreactive, ischemic or necrotic environment explaining the neutral or small effects observed in clinical trials.

Defining the factors present in the hostile microenvironment of injured myocardium that limit the homing, functional engraftment and survival of transplanted cells will be essential for guiding the development of stem-cell-based therapies.
Unfortunately, no good method for long-term in vivo imaging of transplanted cells exists. To complicate matters even more, the results in clinical trials are potentially biased by a significant change in both morphology, function, and perfusion following PCI treated acute myocardial infarction without cell therapy. Tissue engineering\textsuperscript{331,332} combining cells with artificial or natural scaffolds, or intramyocardial injection of combinations of cell types and/or cytokines may be more effective than a single intracoronary injection of single-cell suspensions. Alternatively, long-time engraftment and survival of transplanted cells may not be necessary if paracrine effects are the main mechanism of cell-based therapies. In that case, identification and administration of the secreted active components could be more appropriate than cell transplantation. As the many remaining questions regarding cardiac regeneration are elucidated, meticulously designed clinical trials should proceed with caution and with a paramount concern for patient safety. Ultimately larger trials are needed to answer the key question if improvement in surrogate endpoints translates into improvement in clinical endpoints such as mortality and morbidity. The publication of both positive and negative trial results provides the research community the important opportunity to progress. Hopefully, the next decade will make the intuitively attractive concept of regenerating the broken heart a reality.


Vores forsøg viste, at subkutan G-CSF sammen med genterapi ikke forbedrer myokardieperfusionen hos patienter med kronisk iskæmi trods en stor stigning i cirkulerende knoglemarvseriverede celler. Ligeledes fandt vi ingen forbedring i angina pectoris eller gangdistance i sammenligning med placebo-behandlede patienter. Vi kunne ikke påvise ændringer i plasmakoncentrationen af hverken angiogene faktorer eller knoglemarvsderiverede celler, som kunne forklare den neutrale effekt af G-CSF.

Derefter undersøgte vi G-CSF behandling som adjuværende terapi umiddelbart efter ST elevations-myokardieinfarkt. Vi fandt ingen effekt hverken på primære effektmål regional myokardiefunktion eller på venstre ventrikels uddrivningsfraktion (sekundært endepunkt) i sammenligning med placebobehandling. I efterfølgende analyser fandt vi forskel på hvilke celletyper, der blev mobiliseret af G-CSF, hvilket kan være en del af forklaring på, hvorfor intrakoronar injektion af knoglemarvs deriverede celler tilsyneladende har bedre effekt end mobilisering med G-CSF.


Endelig fandt vi, at ex vivo mærkning af celler med indium-111 med henblik på in vivo visualisering efter myokardiel injektion er problematisk. En stor del af mærkningen forblev i hjertet selvom cellerne i vores studie var døde. I humane studier er det vanskeligt at afgøre, om cellerne forbliver levende efter injektion, og det vil derfor være vanskeligt at afgøre, om vedvarende indium aktivitet i myokardiet stammer fra viable celler eller ej.

Celle baseret terapi er fortsat i den eksplorative fase, men på baggrund af det store arbejde, der i øjeblikket bliver lagt inden for dette felt, er det forhåbende, at terapiens kliniske relevans inden for en overskuelig fremtid kan vurderes. I sidste ende vil dette kræve gennemførelse af store randomiserede, dobbeltblindede, placebokontrollerede studier med »hårde« kliniske endepunkter som mortalitet og morbiditet.
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Patients With Acute Myocardial Infarction
Stimulating Factor for Mobilization of Bone Marrow Cells in


Intramyocardial injection of vascular endothelial growth factor-A_{165} plasmid followed by granulocyte-colony stimulating factor to induce angiogenesis in patients with severe chronic ischaemic heart disease

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Aims To assess the safety and effects of combined treatment with vascular endothelial growth factor-A_{165} plasmid (VEGF-A_{165}) and granulocyte-colony stimulating factor (G-CSF) mobilization of bone marrow stem cells in patients with severe chronic ischaemic heart disease (IHD).

Methods and results Sixteen patients with severe chronic IHD were treated with intramyocardial injections of VEGF-A_{165} plasmid followed 1 week later by G-CSF (10 μg/kg/day for 6 days). Two control groups included (i) sixteen patients treated with intramyocardial injections of VEGF-A_{165} plasmid and (ii) sixteen patients treated with intramyocardial injections of placebo. In the G-CSF group, circulating CD34+ stem cells increased almost 10-fold compared with the control groups (P < 0.0001). After 3 months, there was no improvement in myocardial perfusion at single photon emission computerized tomography in the VEGF-A_{165} and G-CSF treated group, and clinical symptoms were unchanged. There were no side effects to the gene and G-CSF therapy.

Conclusion Intramyocardial VEGF-A_{165} gene transfer followed by bone marrow stem cell mobilization with G-CSF seemed safe. However, a significant increase in circulating stem cells did not lead to improved myocardial perfusion or clinical effects suggesting a neutral effect of the treatment. To improve homing of stem cells, higher doses of VEGF-A_{165} and/or use of SDF-1 transfer might be considered.

KEYWORDS Cardiovascular diseases; Gene therapy; Stem cell; Angiogenesis; G-CSF

Introduction Several vascular growth factors have the potential to induce angiogenesis in ischaemic tissue.1–3 However, only vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) have been tested in clinical studies of patients with occlusive coronary artery disease (CAD).4–10 Small and unblinded gene therapy studies of intramyocardial delivered genes encoding VEGF-A_{165} or VEGF-A_{121} have been performed in patients with severe CAD and results have been encouraging, demonstrating both clinical improvement and evidence of angiogenesis.5,8–10 However, in the first large double-blind randomized placebo-controlled study, we could not demonstrate significant improvement in myocardial perfusion, when compared with placebo.4

Haematopoietic stem cells from the bone marrow have the potential to induce vasculogenesis in animals with an acute myocardial infarction.11,12 Recent human studies indicate that mononuclear cell solutions aspirated from the bone marrow can induce vasculogenesis both in acute and chronic myocardial ischaemia.13–15 However, it remains unknown, whether the vasculogenesis is induced by the few (2–3%) stem cells within the mononuclear cells suspension17 or by cytokines released from the leucocytes. It has been demonstrated that treatment with granulocyte-colony stimulating factor (G-CSF), in order to mobilize stem cells from the bone marrow, does not induce vasculogenesis in patients with chronic myocardial ischaemia16 or following acute myocardial infarction.22 Animal studies suggest that a combination of treatment with VEGF-A gene transfer followed by G-CSF mobilization of stem cells might be superior to either of the therapies.23

The aim of the present study was, in a clinical phase I safety and efficacy study, to evaluate the safety and clinical
effect of VEGF-A165 gene transfer followed by bone marrow stimulation with G-CSF to induce myocardial vasculogenesis in patients with severe occlusive CAD.

Methods

Population

Patients were eligible in the study if they had (i) significant reversible myocardial ischaemia on an adenosine stress single photon emission computerized tomography (SPECT) as judged by an experienced nuclear physician blinded to all other patient data, (ii) at least one remaining large coronary vessel from which new collateral vessels could be supplied, (iii) age between 18 and 75 years, (iv) stable angina pectoris and Canadian Cardiovascular Society (CCS) class ≥ 3. Excluded were patients with (i) unstable angina pectoris, (ii) acute myocardial infarction within the last 3 months, (iii) diabetes mellitus with proliferative retinopathy, (iv) diagnosed or suspected cancer, (v) chronic inflammatory disease, and (vi) fertile women.

All patients received oral and written information, and signed a written informed consent. The study followed the recommendations of the Helsinki II Declaration and was approved by the Ethics Committee of Copenhagen (KF 01-130/01) and the Danish Drug Agency (2612-1490). The study has been registered in clinicaltrials.gov (NCT00135850).

Study design

We prospectively treated 16 patients with severe chronic CAD and no option for revascularization with open-label VEGF-A165 gene transfer followed by G-CSF bone marrow stem cell mobilization (15 men, 1 woman, mean age 62 years, Table I). Patients were treated with direct intramyocardial injections of the VEGF-A165 plasmid followed one week later by in-hospital daily subcutaneous injection of 10 μg/kg body weight G-CSF (Neupogen®) for 6 days. We (Rigshospitalet, Copenhagen) have previously randomized a Agency (2612-1490). The study has been registered in clinicaltrials.gov (NCT00135850).

Efficacy assessment

The pre-specified efficacy endpoint was changes in perfusion defects at stress SPECT from baseline to 3 months follow-up. Secondary exploratory endpoints were change in (i) CCS angina class, (ii) Seattle Angina Pectoris Questionnaire (SEQ) scores, (iii) frequency of angina attacks, (iv) nitroglycerine consumption, (v) exercise capacity, and (vi) left ventricular volumes measured by SPECT and MRI.

Single photon emission computerized tomography

SPECT studies were performed as a 2-day protocol (500–700 MBq 99mTc-sestamibi at each study) with adenosine infusion over 4–6 min (0.14 mg/kg/min by infusion pump), combined with a submaximal exercise test except in patients with left bundle branch block.24,25 Care was taken to perform the stress tests at the inclusion and at the follow-up studies with identical cumulative adenosine doses and identical submaximal exercise loads. Gated (eight frames) imaging was performed with a two-headed Millennium GE gamma camera, with a Gadolinium interleaved attenuation-scatter correction. A disk with investigations of nine patients belonging to VEGF-A165 and 10 patients to the placebo group was accidentally destroyed and was technically unreadable. Therefore, these data are missing in the comparison analyses.

An independent core laboratory (Bio-Imaging Technologies B.V., Leiden, The Netherlands) performed blinded readings of the SPECT investigations.

NOGA®—electromechanical mapping of left ventricle

Electromechanical evaluation of the left ventricle was performed with the NOGA® system (Bio森 Webserst Webserst Ab/S, Cordis, Johnson & Johnson) as previously described.24,25 The diagnostic NOGA® catheter was introduced percutaneously via the groin into the left ventricle. A sensor at the tip of the catheter in contact with the endocardial surface registered the local myocardial unipolar voltage and regional wall motion/contraction (local shortening) within the left ventricle, and created a three-dimensional colour image of the left ventricle as described previously.4

Intramyocardial injections

The intramyocardial injections were done with the 8-french-sized Myostar® mapping-injection catheter (Bio森 Webserst Webserst Ab/S, Cordis, Johnson & Johnson) inserted into the left ventricle via the groin.4 Ten 0.3 mL injections were given around and within the area with reversible ischaemia with a total dose of 0.5 mg VEGF-A165 or placebo plasmid. The injections were performed slowly (30–40 s) and only to areas with unipolar voltage above 5 mV and with a thickness of the ventricular wall on echocardiography exceeding 6 mm.

Quality control of the injections included (i) the catheter’s tip perpendicular (+30°) to the left ventricular wall in two planes, (i) the loop stability at the same level as during the mapping procedure, if possible <2 mm, and (iii) one or more ectopic extraventricular beats should appear in the exact moment of the protrusion of the injection needle into the myocardium.

Plasmid VEGF-A165 and placebo plasmid

The plasmid contained a cytomegalovirus promotor/enhancer to drive VEGF-A165 expression. The placebo plasmid was identical to
the plasmid VEGF-A165 except for the VEGF-A165 gene that had been cut out, as previously described.4

**Magnetic resonance imaging**

MRI was only performed in the VEGF-A165 + G-CSF group and was not feasible in six patients due to claustrophobia or obesity. Myocardial function and perfusion were examined by MRI before and 3 months after VEGF-A165 and G-CSF treatment. The examinations were performed with 1.5 T (Siemens Vision Magnetom, Siemens AG, Erlangen, Germany) using a standard phased-array chest coil. Left ventricular volumes and systolic function were derived from successive short axis slices. Volumes were determined by planimetry and myocardial mass was determined by applying a density factor of 1.05 g/cm³.

Perfusion estimates were obtained from images acquired in a single position in the true short axis of the left ventricle starting immediately after intravenous injection of gadopentetate dimeglumine (Magnevist®, Schering AG) (0.1 mmol/kg) during intravenous infusion of adenosine (0.14 mg/kg/min), which was allowed to reach steady-state concentration during 2 min initial infusion. Myocardial perfusion was assessed as the change in MR signal intensity as a function of time during the first pass (initial slope).28

The investigations were analysed blinded to all patient-data, using the CMRtools software.

**Statistical analysis**

The sample size of 16 patients in the combined VEGF-A165 gene and G-CSF group was calculated to yield an expected power of 0.8 to detect a difference of 30% from baseline to 3 months follow-up in perfusion defects at stress SPECT, with a two-sided significance level of 0.05 and an assumed standard deviation of 0.04, on the basis of previous results.4 Baseline characteristics were compared using Fisher’s exact test for categorical or one-way ANOVA for continuous variables.

For comparisons between baseline and follow-up data within the groups, we used Wilcoxon signed-ranks test, whereas between group comparisons were done using one-way ANOVA. All data were analysed using SPSS statistical analysis program (SPSS version 12.0, SPSS Inc., Chicago, Il, USA). To account for multiple testing, a difference was only considered statistically significant if two-sided \( P < 0.01 \).

**Results**

The baseline characteristics of the patients are depicted in Table 1. All patients had severe stable chronic angina pectoris and limited exercise capacity. They were all on maximal tolerable anti-angina therapy with short- and long-lasting nitroglycerin, calcium antagonists, beta-blockers, and ACE-inhibitors. All had previously been treated with at least one coronary artery bypass surgery or percutaneous coronary intervention (PCI).

**Safety data**

There were no major side effects during the combined VEGF-A165 gene and G-CSF treatment or in the follow-up period. During G-CSF treatment, two patients had slight muscular discomfort, which disappeared after NSAID treatment. One patient with a previous history of a gall bladder stone abdominal pains developed acute abdominal pains and increase in plasma liver parameters. Symptoms and liver test normalized immediately after cessation of G-CSF treatment. No change was seen in liver function tests in the remaining patients. Five patients in the placebo group had SAEs: one ST-elevation myocardial infarction (STEMI) with third-degree AV-blockade, the patient progressed into cardiogenic shock despite implantation of a pacemaker and subsequently died 2 months after the placebo treatment; two uncomplicated STEMI; one non-STEMI; and one newly developed coronary in-stent stenosis, which needed treatment with PCI. In the plasmid VEGF-A165 group, three patients developed new coronary artery in-stent stenoses with symptoms of unstable angina.

<p>| Table 1 Demographic data for patients treated with placebo, plasmid VEGF-A165, or plasmid VEGF-A165 and G-CSF at baseline |</p>
<table>
<thead>
<tr>
<th>Placebo (n = 16)</th>
<th>VEGF-A165 (n = 16)</th>
<th>VEGF-A165 + G-CSF (n = 16)</th>
<th>P-value</th>
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<tr>
<td>Age (years)</td>
<td>62 ± 9</td>
<td>61 ± 7</td>
<td>62 ± 9</td>
</tr>
<tr>
<td>Gender (m/f)</td>
<td>(14/2)</td>
<td>(15/1)</td>
<td>(14/2)</td>
</tr>
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<td>Body mass index (kg/m²)</td>
<td>29 ± 5</td>
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<td>29 ± 4</td>
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<td>Current smoker, n (%)</td>
<td>1 (6)</td>
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<td>1 (6)</td>
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<td>6 (38)</td>
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<td>7 (44)</td>
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<td>16 (100)</td>
<td>16 (100)</td>
<td>16 (100)</td>
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<td>Previous PCI, n (%)</td>
<td>10 (63)</td>
<td>11 (69)</td>
<td>9 (56)</td>
</tr>
<tr>
<td>Previous STEMI, n (%)</td>
<td>11 (69)</td>
<td>7 (44)</td>
<td>6 (38)</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>53 ± 11</td>
<td>57 ± 9</td>
<td>57 ± 10</td>
</tr>
<tr>
<td>CCS</td>
<td>3.0 ± 0.0</td>
<td>3.1 ± 0.3</td>
<td>3.0 ± 0.0</td>
</tr>
<tr>
<td>Number of patients with two or three-vessels disease, n</td>
<td>2/14</td>
<td>3/13</td>
<td>4/12</td>
</tr>
<tr>
<td>Occluded vessel segments (LM/LAD/LCX/RCA), n</td>
<td>4/12/13/14</td>
<td>2/13/13/12</td>
<td>2/14/13/12</td>
</tr>
</tbody>
</table>

LVEF, left ventricular ejection fraction measured by ventriculography; CABG, coronary artery bypass grafting; CCS, Canadian Cardiovascular Society angina classification; LM, left main artery; LAD, left anterior descending artery; LCX, left circumflex artery; RCA, right coronary artery.

Values are expressed as mean ± SD or n (%).
Single photon emission computerized tomography

The combined VEGF-A165 and G-CSF treated group had no changes in myocardial perfusion at rest and stress between baseline and follow-up, and they had identical summed difference perfusion scores (Table 2). Left ventricular end-diastolic (EDV) and end-systolic volumes (ESV), and ejection fraction showed no significant difference in any of the three groups from baseline to follow-up, and there were no differences between changes in these parameters between groups (Table 3). In addition, regional wall thickening and motion were unchanged from baseline to follow-up in the group treated with VEGF-A165 and G-CSF (Table 4).

**Clinical outcome**

There was no significant difference in changes in CCS classification, angina pectoris attacks, NTG consumption, or exercise time between the three groups (Table 5). This pattern was unchanged, also after classifying patients in the VEGF-A + G-CSF group into CD34+ stem cell responders and non-responders. The SEQ scores demonstrated improvement in physical limitation, angina stability, and angina frequency in all groups, and improvement in treatment satisfaction and disease perception in the VEGF gene transfer group only.

**Magnetic resonance imaging**

Both EDV and ESV increased, and the change in ESV from 40 to 46 mL was statistically significant ($P = 0.009$, Table 6). However, the increase in ESV did not change the left ventricular ejection fraction significantly, and there was no difference in left ventricular mass. There was no significant increase in perfusion in the region treated with gene injections (Table 6). Representative images of the myocardial perfusion are shown in Figure 1.

**Plasma VEGF-A165, SDF-1, and CD34+ progenitor cells**

Circulating CD34+ stem cells from the bone marrow increased significantly during the G-CSF treatment, with
Table 4  Regional wall thickening and motion at rest measured by SPECT

<table>
<thead>
<tr>
<th>Placebo (n = 6)</th>
<th>VEGF-A165 (n = 7)</th>
<th>VEGF-A165 + G-CSF (n = 11)</th>
<th>Difference from baseline to follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Follow-up</td>
<td>P-value</td>
</tr>
<tr>
<td>Apex (%)</td>
<td>37 ± 12</td>
<td>41 ± 11</td>
<td>0.2</td>
</tr>
<tr>
<td>Lateral wall (%)</td>
<td>20 ± 8</td>
<td>26 ± 5</td>
<td>0.7</td>
</tr>
<tr>
<td>Intraventricular septum (%)</td>
<td>22 ± 3</td>
<td>29 ± 6</td>
<td>0.3</td>
</tr>
<tr>
<td>Inferior wall (%)</td>
<td>16 ± 6</td>
<td>25 ± 6</td>
<td>0.8</td>
</tr>
<tr>
<td>Anterior wall (%)</td>
<td>26 ± 3</td>
<td>29 ± 6</td>
<td>0.3</td>
</tr>
<tr>
<td>Regional inner wall motion (0-10 mm range)</td>
<td>Apex (mm)</td>
<td>5.1 ± 1.1</td>
<td>4.8 ± 1.8</td>
</tr>
<tr>
<td>Lateral wall (mm)</td>
<td>7.5 ± 1.6</td>
<td>9.6 ± 0.9</td>
<td>9.5 ± 1.3</td>
</tr>
<tr>
<td>Septal wall (mm)</td>
<td>1.9 ± 1.0</td>
<td>2.1 ± 2.0</td>
<td>2.2 ± 2.3</td>
</tr>
<tr>
<td>Inferior wall (mm)</td>
<td>3.6 ± 0.7</td>
<td>5.6 ± 1.9</td>
<td>5.5 ± 2.2</td>
</tr>
<tr>
<td>Anterior wall (mm)</td>
<td>7.5 ± 1.0</td>
<td>8.3 ± 1.0</td>
<td>8.6 ± 1.1</td>
</tr>
</tbody>
</table>

Mean ± SD.

Table 6  Left ventricular volumes, ejection fraction, and perfusion in patients treated with VEGF-A165 and G-CSF

<table>
<thead>
<tr>
<th>Placebo (n = 16)</th>
<th>VEGF-A165 (n = 16)</th>
<th>VEGF-A165 + G-CSF (n = 16)</th>
<th>Difference from baseline to follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Follow-up</td>
<td>P-value</td>
</tr>
<tr>
<td>ESV (mL)</td>
<td>102 ± 32</td>
<td>109 ± 33</td>
<td>0.12</td>
</tr>
<tr>
<td>EDV (mL)</td>
<td>62 ± 22</td>
<td>74 ± 32</td>
<td>0.16</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>40 ± 20</td>
<td>46 ± 22</td>
<td>0.40</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>63 ± 10</td>
<td>60 ± 10</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Mean ± SD.

The baseline level of plasma VEGF-A165 varied within the groups. The baseline level of plasma VEGF-A165 increased in the two VEGF-A165 groups, but there was no difference between baseline and G-CSF treatment (max CD34+ levels were identical between the three groups. However, four patients were 'poor mobilizers' to the G-CSF treatment (max CD34+ levels were identical between the three groups. However, four patients were 'poor mobilizers' to G-CSF). This phenomenon is well known from clinical haematology.

At baseline, SDF-1 was lower in the control group compared with the two active treated groups (Figure 2A). At all other time points, the SDF-1 level was identical between the two VEGF-A165 treated groups, but there was no difference between baseline levels. Plasma VEGF-A165 increased in the two VEGF-A165 treated groups after 1 week (Figure 2A). At all other time points, the SDF-1 level was identical between the two VEGF-A165 treated groups, but there was no difference between baseline levels. Plasma VEGF-A165 increased in the two VEGF-A165 treated groups, but there was no difference between baseline levels. Plasma VEGF-A165 increased in the two VEGF-A165 treated groups, but there was no difference between baseline levels. Plasma VEGF-A165 increased in the two VEGF-A165 treated groups, but there was no difference between baseline levels. Plasma VEGF-A165 increased in the two VEGF-A165 treated groups, but there was no difference between baseline levels. Plasma VEGF-A165 increased in the two VEGF-A165 treated groups, but there was no difference between baseline levels. Plasma VEGF-A165 increased in the two VEGF-A165 treated groups, but there was no difference between baseline levels.
treated groups and in the placebo group 1 week after the intramyocardial injections (Figure 2C).

Discussion
We present the first, single-centre, clinical safety and efficacy trial, in which patients with chronic reversible myocardial ischaemia have been treated with intramyocardial injections of the gene encoding VEGF-A165, followed by treatment for 6 days with G-CSF stem cell mobilization from the bone marrow. This treatment was well tolerated with no increase in adverse events, when compared with gene transfer or placebo or G-CSF treatment alone.4,20,21 Several small and uncontrolled clinical studies have indicated that growth factor gene transfer might be safe and have the potential to improve myocardial perfusion.5,8–10 In the first large double-blind placebo-controlled study, we could not demonstrate improved myocardial perfusion after VEGF-A165 gene transfer compared with placebo in patients with severe CAD.4 However, the study may have been underpowered since we found a significant improvement within the VEGF-A165 group. Also, in a comparable group of patients, we found that G-CSF mobilization of stem cells from the bone marrow did not improve myocardial perfusion or symptoms.20 Animal studies have suggested that the combination of gene transfer for VEGF-A165 and G-CSF mobilization of stem cells from the bone marrow induce angiogenesis more effectively than gene therapy alone.23

In the present study, we could not demonstrate a significant improvement in myocardial perfusion or symptoms in patients with chronic reversible myocardial ischaemia after the combined therapy with gene transfer of VEGF and subsequent bone marrow stimulation with G-CSF. The discrepancy between animal research and studies in patients, as the present one, might be related to the definition of chronic ischaemia. In animal studies, chronic ischaemia will include components of acute and subacute ischaemia as well. Most animal studies induce chronic myocardial ischaemia, using an ameroid constrictor around the circumflex or anterior descendent artery. Four to five weeks later the myocardium is often called chronic ischemic myocardium. However, the intracellular milieu is probably not equivalent to patients’ myocardium suffering from chronic ischaemia for several years. In patients with acute myocardial infarction, plasma concentrations of the vascular growth factors VEGF and b-FGF, and the stem cell homing factor SDF-1 increase gradually above control levels with maximum ~3 weeks after the infarction. This could indicate that it takes some time to initiate the transcription of the genes for the cytokine production.29

Furthermore, transfection of cells with the VEGF gene after intramyocardial injection is probably similar in chronic human or pig ischemic myocardium. However, the transcription of the transferred VEGF gene and thus the induced VEGF production might be different within the human cells after prolonged ischemia and in animal cells after short-term experimental ischemia.

Recently, it has been speculated if the VEGF production is already increased within chronic ischemic human myocardium, thus attempts to further stimulate angiogenesis via an additional VEGF gene stimulation would potentially be without effect. However, we recently studied biopsies from human chronic ischemic myocardium and found identical quantities of VEGF mRNA in chronic ischemic myocardium compared with non-ischemic normal perfused myocardium in the same patient.30 Thus, it seems that VEGF-A165 gene therapy can potentially increase the local production of the growth factor stimulating the growth of new blood vessels. For safety reasons, however, we chose

Figure 1  MRI perfusion scan at baseline and follow-up in one patient treated with VEGF-A165 gene transfer followed by G-CSF. The recordings were performed 20 s after contrast infusion, demonstrating poor perfusion with no contrast enhancement in the lateral wall (black arrows), moderate perfusion with attenuated enhancement (white arrows) in the inferior wall, and normal perfusion in the anterior wall (transparent arrows).
a low VEGF-A165 plasmid dose. A higher dose should be considered for further studies.

We could not confirm the hypothesis, that the combination therapy would increase local production of VEGF and the number of circulation endothelial progenitor cells homing into the ischaemic myocardium, suggested by experimental animal studies,\textsuperscript{11} in this clinical study of patients suffering from severe, chronic CAD.

SDF-1 has been found essential for stem cell mobilization/homing after arterial injury. In a recent study, it has been demonstrated that SDF-1 gene transfer increased the homing of bone-marrow-derived stem cells in infarcted myocardium but not in normally perfused myocardium, and induced both vasculogenesis and angiogenesis.\textsuperscript{31,32} Moreover, blockade of VEGF prevented all such SDF-1 effects.\textsuperscript{32} We have found that there is no difference between the SDF-1 mRNA levels in normally perfused and chronic ischaemic human myocardium.\textsuperscript{30} Therefore, the missing effect of combined gene therapy and stem cell mobilization might be due to a low SDF-1 level in the chronic ischaemic tissue resulting in poor engraftment of stem cells despite an increased number of circulating stem cells as seen during G-CSF treatment.

The efficacy results of the combined treatment have a potential limitation, as (i) it was not the primary endpoint and (ii) missing data potentially introduced a selection bias. However, the patients with missing data were random (due to a disc error) thus minimizing the risk of selection bias. Still, these results should be interpreted with caution.

In conclusion, combined VEGF-A165 gene transfer and bone marrow stem cell mobilization with G-CSF in patients with chronic myocardial ischaemia is safe but, despite a significant increase in circulating stem cells, there were no signs of clinical effects or improved myocardial perfusion of the ischaemic area. Evaluation of homing of circulating stem cells in the clinical setting needs further studies. Higher VEGF-A165 gene doses and the addition of SDF-1 gene transfer might be considered.

Acknowledgements

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Conflict of interest: none declared.

References

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Circulating angiogenic cytokines and stem cells in patients with severe chronic ischemic heart disease — Indicators of myocardial ischemic burden?

Rasmus Sejersten Ripa, Yongzhong Wang, Jens Peter Goetze, Erik Jørgensen, Hans E. Johnsen, Kristina Tägil, Birger Hesse, Jens Kastrup

Abstract

Background: Angiogenic growth factors and stem cell therapies have demonstrated varying results in patients with chronic coronary artery disease. A reason could be that these mechanisms are already up-regulated due to reduced blood supply to the myocardium. The objective of this study was to examine if plasma concentrations of circulating stem cells and angiogenic cytokines in patients with severe stable chronic coronary artery disease were correlated to the clinical severity of the disease.

Methods: Fifty-four patients with severe coronary artery disease and reversible ischemia at stress myocardial perfusion scintigraphy were prospectively included. The severity of the disease was quantified by an exercise tolerance test, Canadian Cardiovascular Society angina classification, and Seattle Angina Pectoris Questionnaire. Fifteen persons without coronary artery disease served as control subjects.

Results: Plasma concentration of VEGF-A, FGF-2, SDF-1, and circulating CD34+ and CD34−/CD45− cells were similar in the two groups, but early stem cell markers (CD105, CD73, CD166) and endothelial markers (CD31, CD144, VEGFR2) were significantly different between patients and control subjects (p<0.005–0.001). Diabetic patients had higher concentration of SDF-1 (2528 vs. 2150 pg/ml, p=0.004). We found significant correlations between both VEGF-A, FGF-2, and CD34+ to disease severity, including degree of reversible ischemia, angina stability score, and exertional dyspnoea.

Conclusions: Plasma concentrations of circulating stem cells and angiogenic cytokines have large inter-individual variations, which probably exclude them from being useful as indicators of myocardial ischemic burden.

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Keywords: Ischemic heart disease; Angiogenesis; Endothelial progenitor cell; Mesenchymal stem cell; Diabetes

1. Introduction

Acute and chronic coronary artery diseases are the leading causes of morbidity and mortality in cardiology. Numerous patients cannot be treated sufficiently with current therapies, and have a poor quality of life. This has lead to an extensive search for new treatment modalities, including stem cell and growth factor therapies to induce growth of new blood vessels in the ischemic myocardium. So far, therapies with angiogenic vascular endothelial growth factors and bone
marrow suspension of cells have demonstrated both encouraging and more questionable results in phase I–II studies in patients with acute myocardial infarction or chronic coronary artery disease [1–14]. These discrepancies could be due to prior activation of angiogenic mechanisms to improve blood supply to the ischemic heart. Therefore, it is important to know the basic conditions for these factors in the subset of patients intended for treatment, in order to optimize type and timing of the treatment with growth factors and/or stem cells. Currently, there is very limited information about the natural occurrence of angiogenic factors, known to be of importance in the development and growth of new blood vessels in patients with severe chronic coronary artery disease [15], and no information regarding the correlation to the clinical severity of the disease.

The aim of the present study was to determine concentrations of circulating stem cells and angiogenic related cytokines in patients with stable severe chronic coronary artery disease, and evaluate whether concentrations were correlated with the clinical severity of the disease.

2. Materials and methods

2.1. Patients

We prospectively included 54 patients with stable severe occlusive coronary artery disease with evidence of and reversible ischemia as determined by adenosine stress single photon emission computerized tomography (SPECT). Inclusion criteria were: age 20–80 years; Canadian Cardiovascular Society angina classification (CCS) ≥ 3 despite optimal medical treatment; left ventricular ejection fraction ≥ 40%; and coronary angiography with significant coronary artery disease ineligible for revascularization. Exclusion criteria were patients with: unstable angina pectoris, acute myocardial infarction within the last 3 months, diagnosed or suspected malignant disease, chronic inflammatory disease and fertile women. Fifteen

Table 1
Baseline characteristics

<table>
<thead>
<tr>
<th></th>
<th>Controls (n=15)</th>
<th>Patients (n=54)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean±s.d.</td>
<td>Mean±s.d.</td>
</tr>
<tr>
<td>Age, years</td>
<td>54.9±9.3</td>
<td>61.4±8.4</td>
</tr>
<tr>
<td>Males, %</td>
<td>73</td>
<td>87</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>79.9±10.5</td>
<td>83.4±12.7</td>
</tr>
<tr>
<td>Diabetes mellitus, %</td>
<td>6.7</td>
<td>27.8</td>
</tr>
<tr>
<td>ProBNP</td>
<td>5.9±3.8</td>
<td>32.8±25.1</td>
</tr>
<tr>
<td>CCS III/IV, %</td>
<td>N/A</td>
<td>9.3±87.037</td>
</tr>
<tr>
<td>LVEF by angiography, %</td>
<td>N/A</td>
<td>53.4±11.1</td>
</tr>
<tr>
<td>SPECT SDS</td>
<td>N/A</td>
<td>8.8±5.8</td>
</tr>
<tr>
<td>History of myocardial infarction, %</td>
<td>N/A</td>
<td>53</td>
</tr>
<tr>
<td>Exercise time, sec</td>
<td>N/A</td>
<td>507.1±173.4</td>
</tr>
<tr>
<td>Exercise METS</td>
<td>N/A</td>
<td>5.1±1.3</td>
</tr>
<tr>
<td>Nitroglycerin tablets/day</td>
<td>N/A</td>
<td>2.0±2.4</td>
</tr>
<tr>
<td>Angina attacks/day</td>
<td>N/A</td>
<td>1.7±2.4</td>
</tr>
<tr>
<td>Physical limitation *</td>
<td>N/A</td>
<td>44.4±16.2</td>
</tr>
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<td>Angina stability *</td>
<td>N/A</td>
<td>46.1±16.9</td>
</tr>
<tr>
<td>Angina frequency *</td>
<td>N/A</td>
<td>35.2±26.7</td>
</tr>
<tr>
<td>Treatment satisfaction *</td>
<td>N/A</td>
<td>86.6±11.6</td>
</tr>
<tr>
<td>Disease perception quality of life *</td>
<td>N/A</td>
<td>50.5±25.1</td>
</tr>
</tbody>
</table>

CCS=Canadian Cardiovascular Society angina classification, SPECT SDS=single photon emission computerized tomography summed difference stress score, METS=metabolic equivalents, LVEF=left ventricular ejection fraction.

* By Seattle Angina Pectoris Questionnaire.

Table 2
Circulating cell and cytokine concentrations in patients with chronic myocardial ischemia and control subjects

<table>
<thead>
<tr>
<th></th>
<th>Patients (n=54)</th>
<th>Control subjects (n=15)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGF-A (10^-12 g/ml)</td>
<td>35.0 (18.6–51.4)</td>
<td>91.7 (8.2–175.1)</td>
<td>0.38</td>
</tr>
<tr>
<td>FGF-2 (10^-12 g/ml)</td>
<td>9.0 (6.2–11.7)</td>
<td>11.3 (2.2–20.3)</td>
<td>0.67</td>
</tr>
<tr>
<td>SDF-1 (10^-12 g/ml)</td>
<td>22.48 (21.24–23.72)</td>
<td>20.14 (17.50–22.79)</td>
<td>0.15</td>
</tr>
<tr>
<td>CD34+ (10^3/ml)</td>
<td>2.8 (2.4–3.3)</td>
<td>3.0 (2.1–3.9)</td>
<td>0.64</td>
</tr>
<tr>
<td>CD45−/CD34− (10^4/ml)</td>
<td>20.8 (17.0–24.6)</td>
<td>21.9 (13.0–31.0)</td>
<td>0.79</td>
</tr>
</tbody>
</table>

Values are mean (95% confidence interval).

Fig. 1. Subclassification of CD45−/CD34− cells.

Fig. 2. Mean concentrations of cells and cytokines with 95% confidence interval in patients with or without diabetes.
healthy subjects with a similar age and gender distribution served as a control group. Venous blood samples for stem cell and cytokine analyses were obtained after 15 min in resting supine position. All patients received oral and written information, and provided written consent. The study was approved by the Ethical Committee of Copenhagen (KF02-078/00).

2.2. Clinical and SPECT data

All patients underwent a maximal bicycle exercise tolerance test. Angina pectoris was described using the CCS classification, Seattle Angina Pectoris Questionnaire (SAQ), frequency of angina attacks and nitroglycerine consumption per week. Dyspnoea was graded as moderate to severe if present during light exercise or at rest.

SPECT studies were performed as a 2-day protocol (500–700 MBq 99 mTc-sestamibi at each study) with adenosine infusion over 4–6 min (0.14 mg/kg/min by infusion pump), combined with a sub-maximal exercise test, except in patients with left bundle branch block [16]. Gated (8 frames) imaging was performed with a two-headed Millennium GE gamma camera, with a Gadolinium interleaved attenuation-scatter correction. Blinded, visual analysis according to a 17 segment model of the SPECT images (myocardial slices in three planes) was performed as consensus readings by 2 experienced nuclear medicine specialists, using an eNTEGRA working station (GE Medical). Summed stress scores were calculated by the Echo-Tool Box software program.

2.3. Quantification and characterization of stem cells

The concentration of circulating CD34 positive (CD34+) cells and circulating putative mesenchymal stem cells (MSC) in the blood, quantified as CD45 and CD34 negative (CD45–/CD34–) cells, was measured by multiparametric flow cytometry as previously described [17]. A panel of monoclonal and polyclonal antibodies were used to further characterize MSCs, including anti-CD45 (H330 clone, IgG1; Becton Dickinson (BD)) and anti-CD34 (BG12 clone, IgG1; BD), as well as anti-CD105 (Endoglin, N1-3A1, clone, IgG1; Ancell), anti-CD31 (PECAM-1, platelet endothelial cell adhesion molecule, WM59 clone, IgG1; BD), anti-CD133 (haematopoietic stem cell antigen, AC133 clone, IgG1; Miltenyi Biotec), anti-VEGF (PI-linked; AD2 clone, IgG1; BD), anti-CD166 (ALCAM, activated leukocyte adhesion molecule, 3A6 clone, IgG1; BD), anti-VEGF A (VEGF-A), fibroblast growth factor 2 (FGF-2), stromal derived factor 1 (SDF-1) were measured in plasma concentrations of vascular endothelial growth factor A (VEGF-A), fibroblast growth factor 2 (FGF-2), and stromal derived factor 1 (SDF-1) measured in plasma concentrations of vascular endothelial growth factor A (VEGF-A), fibroblast growth factor 2 (FGF-2), and stromal derived factor 1 (SDF-1).

2.4. Quantification of plasma cytokines

Plasma concentrations of vascular endothelial growth factor A (VEGF-A), fibroblast growth factor 2 (FGF-2), and stromal derived factor 1 (SDF-1) were measured in plasma concentrations of vascular endothelial growth factor A (VEGF-A), fibroblast growth factor 2 (FGF-2), and stromal derived factor 1 (SDF-1) measured in plasma concentrations of vascular endothelial growth factor A (VEGF-A), fibroblast growth factor 2 (FGF-2), and stromal derived factor 1 (SDF-1).
CD34 cell (MSC) population is heterogeneous and sub-characterizing by co-expression of early stem cell markers (CD105, CD73, CD166) and endothelial markers (CD31, CD144, VEGFR2) revealed highly significant differences between patients and control subjects (Fig. 1). Patients with chronic ischemic heart disease had significantly higher levels of MSCs with both early stem cell markers (CD105 and CD166), and endothelial progenitor cell markers (CD144 and VEGFR2), whereas cells with the haematopoietic marker CD133 were significantly lower in patients compared to control subjects, suggesting an important functional difference.

In a subgroup analysis of patients with diabetes, we found significantly higher plasma concentrations of SDF-1 compared to non-diabetic patients (Fig. 2). The positive correlation between VEGF-A and FGF-2 appears to be explained by a marked association within the patient group.

The bivariate correlation between clinical severity of the disease and the angiogenic factors are shown in Tables 4 and 5. Plasma FGF-2, circulating CD34+, and CD45− were found to correlate with the clinical severity of the ischemic heart disease. The independent, clinical predictors for circulating angiogenic factors analyzed by multivariate linear regression are outlined in Table 6.

4. Discussion

To evaluate if angiogenic factors within the myocardium are activated in patients with stable chronic myocardial ischemia and whether such activation is reflected by altered concentrations in the circulation, we examined a patient population with severe chronic clinical myocardial ischemia and stress induced myocardial perfusion defects documented by SPECT. We found that, in general, circulating stem cells and plasma concentrations of angiogenic related cytokines were not significantly different from the control group. Nevertheless, some association between different measures of the severity of the disease was demonstrated, but a large inter-individual variation will probably limit the clinical applicability of these measures.

Since we expected a substantial difference between the plasma concentration of stem cells and angiogenic factors after a short-term acute ischemic period compared to sustained or repeated periods of ischemia in our population, this study did not include patients with acute myocardial infarction. We have recently found that angiogenic factors (VEGF-A, FGF-2 and SDF-1) in the month following an acute myocardial infarction increase substantially above the concentrations found in the present study. Conversely, CD34 positive and mesenchymal stem cells were in the same range as in the present study [19]. These variations in concentrations of angiogenic factors and stem cells are important since patients with acute myocardial infarction are included in several angiogenic trials, however, whether these differences are of clinical importance, remains to be elucidated. One could speculate that therapy with exogenous angiogenic

### Table 6

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Independent predictors</th>
<th>β</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-A</td>
<td>Dyspnoea</td>
<td>−66.2</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>Angina stability</td>
<td>−1.0</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Angina attacks</td>
<td>5.9</td>
<td>0.04</td>
</tr>
<tr>
<td>FGF-2</td>
<td>Dyspnoea</td>
<td>−1.1</td>
<td>0.04</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Diabetes mellitus</td>
<td>388.4</td>
<td>0.004</td>
</tr>
<tr>
<td>CD34+</td>
<td>SPECT summed difference score</td>
<td>0.14</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Left ventricular ejection fraction</td>
<td>−3.6</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Independent predictors included in the analysis: gender, age, weight, history of myocardial infarction, diabetes mellitus, left ventricular ejection fraction, dyspnoea, CCS class, METS, mean number of short acting nitroglycerine, mean number of angina attacks, physical limitation according to Seattle Angina Pectoris Questionnaire (SAQ), angina stability according to SAQ, treatment satisfaction according to SAQ, disease perception quality of life according to SAQ, SPECT summed difference score.
induced ischemia shown by SPECT (Table 4).

4.1. VEGF-A

VEGF-A is a major mediator of neovascularization in physiological and pathophysiological conditions, with a key role in regulation of hypoxia-induced tissue angiogenesis. In patients with acute myocardial ischemia and infarction, elevated contents of VEGF-A mRNA in myocardial tissues have been reported, suggesting VEGF-A to be an important local response to oxygen deprivation [20]. We found a trend towards lower concentrations in patients as compared to control subjects, but with very large interindividual variations in both groups. In addition, we found an inverse relation to CCS class and to anginal stability (Table 6). These observations are surprising, since we expected that the patient population with documented frequent myocardial ischemia during stress, would have an induction of VEGF production as previously found in 20 patients with stable angina pectoris [15]. However, the finding may be in accordance with our recent finding of unaffected VEGF mRNA contents in chronic ischemic myocardial tissue compared to normally perfused myocardium, in patients undergoing coronary artery by-pass surgery [21].

The association with CCS class should be evaluated with extreme caution due to the small numbers of patients in classes II and IV. However, one might speculate that patients with innate low level of VEGF-A will form fewer coronary collaterals and thus will be more prone to symptomatic myocardial ischemia. This hypothesis is supported by the work of Schultz et al. [22], showing that monocytes from patients with ischemic heart disease and few collaterals have reduced hypoxic induction of VEGF-A. In addition, a recent work by Lambiase et al. [23] showed a non-significant trend towards the same results.

4.2. FGF-2

FGF-2 is a potent stimulant of angiogenesis like VEGF-A [24], and we established an anticipated association between those two factors. Moreover, we detected an increase in plasma FGF-2 in association with increasing degree of reported dyspnoea in both univariate and multivariate analysis (Tables 5 and 6). To our knowledge, this has not been reported previously.

It should be noted that patients with heart failure were not intentionally included in our trial and all had ejection fraction above 40%. Hence, it appears likely that the differences in dyspnoea reflect an angina equivalent rather than the degree of heart failure. This hypothesis is further strengthened by the correlation between FGF-2 and stress induced ischemia shown by SPECT (Table 4).

4.3. SDF-1

SDF-1 is a chemokine ligand involved in numerous biological processes including vasculogenesis, cardiogenesis, and haematopoiesis. SDF-1 mediates its function through the CXCR4 transmembrane receptor [25]. SDF-1 has been suggested to augment recruitment of endothelial progenitor cells into ischemic tissue and thus enhance ischemic neovascularization [26].

We found similar concentrations of plasma SDF-1 in patients and healthy controls. This is in contrast to a recent study by Damás et al. [27] who reported a significantly higher concentrations in control subjects compared to patients with stable angina. In a previous in vitro study, SDF-1 induced secretion of VEGF from lymphohaematopoietic cells. This is in concordance with our finding of a trend towards an association between the plasma concentration of SDF-1 and VEGF-A ($p=0.07$).

Interestingly, we found a highly significant, positive correlation between circulating SDF-1 and presence of diabetes by both univariate and multivariate analyses. To our knowledge, this has never been described before. However, the result is supported by a very recent reporting that the concentration of circulating SDF-1 is correlated to the severity of diabetic retinopathy in patients [28].

SDF-1 is produced in almost all organs, but most abundantly in the pancreas [29]. The association between high concentrations of plasma SDF-1 and diabetes could thus be due to a high endocrine drive on the diabetic pancreas leading to an excess production of SDF-1. Recent experiments in mice suggest that intravitreal SDF-1 is a key mediator of diabetic proliferative retinopathy [30] and it can be speculated if the increased concentrations of SDF-1 in the blood is instrumental to pathological formation of new blood vessels in other vascular beds in diabetes. Since this was a subgroup analysis and not the primary objective of our trial, it would be interesting to determine if our finding can be reproduced in diabetic patients without concomitant ischemic heart disease.

4.4. CD34 positive cells

Endothelial progenitor cells have been isolated from circulating CD34+ mononuclear cells and are thought to participate in vasculogenesis. The number of circulating CD34+ cells have been found both to increase and to be unchanged in the period after an acute myocardial infarction treated with primary percutaneous coronary intervention and stenting [19,31]. Valgimigli et al. [32] recently showed that CD34+ cells and EPC mobilization occurs in heart failure and displays a biphasic response with elevation and depression in the early and advanced phases, respectively. It thus seems plausible that CD34+ cells are also related to the severity of ischemic heart disease. We detected a week association to self-reported angina stability and to the amount of reversible ischemia determined by SPECT. These
results could indicate that patients with a large myocardial area with reversible ischemia have an augmented mobilization of CD34+ cells into the circulation. However, the cell concentrations are close to the detection limit of the method used, and thus the results should be evaluated with caution.

Recently, Eizawa et al. [33] found that patients with stable angina and diabetes had decreased CD34+ cells compared to control subjects. These results were not confirmed in our study. This may be due to differences in patient selection; our trial appears to have included a population with more severe myocardial ischemia.

4.5. CD45−/CD34− mesenchymal stem cells

The MSC can differentiate into endothelia, bone, cartilage and even myoblasts if appropriately stimulated. MSC are characterized by the non-expression of hematopoietic markers CD34 and CD45. Several trials using MSC therapy for ischemic heart disease are currently being planned, and it is therefore important to determine the baseline concentration for ischemic heart disease that mobilization of CD45−/CD34− cells with early stem cell and endothelial markers (CD133, CD105, CD166) and cells with endothelial markers (CD144, CD31, VEGF-receptor) were increased, whereas another mesenchymal stem cell marker (CD73) was unaffected. In contrast, the fraction of cells with the hematopoietic marker (CD133) was decreased. These results could indicate that both early and endothelial differentiated mesenchymal stem cells are mobilized and are potentially involved in vasculogenesis in ischemic heart disease. Furthermore, we observed a significant negative association between CD45−/CD34− cells and left ventricular ejection fraction. This could indicate that mobilization of CD45−/CD34− cells is more affected by left ventricular dysfunction than ischemia, and it would be interesting to examine this relationship in patients with ejection fraction below 40%.

5. Conclusion

The plasma concentrations of VEGF-A, FGF-2, circulating CD34+ and mesenchymal stem cells were not different from controls. However, they seemed related to the severity of the disease in patients with severe stable coronary artery disease, but with a very large inter-individual variation. Mesenchymal stem cells with early stem cell and endothelial cell markers were more pronounced in cardiac patients. The plasma concentration of SDF-1 was increased in diabetic patients, which might influence the progression of the vascular disease. Due to the large inter-individual variations of these angiogenic factors, they do not appear to be suitable for use as clinical indicators of myocardial ischemic burden.

Acknowledgments

We appreciate the skilful and enthusiastic laboratory assistance of Steen Mortensen. We are grateful to Dr. C. Burton for help with the preparation of the manuscript.

References


ORIGINAL ARTICLE

The influence of genotype on vascular endothelial growth factor and regulation of myocardial collateral blood flow in patients with acute and chronic coronary heart disease

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Abstract

Objective: To test the hypothesis that mutations in the vascular endothelial growth factor (VEGF) gene are associated with plasma concentration of VEGF and subsequently the ability to influence coronary collateral arteries in patients with coronary heart disease (CHD).

Methods: Blood samples from patients with chronic ischemic heart disease (n=53) and acute coronary syndrome (n=61) were analysed. Coronary collaterals were scored from diagnostic biplane coronary angiograms.

Results: The plasma concentration of VEGF was increased in patients with acute compared to chronic CHD (p=0.01). The genotype frequencies differed significantly from Hardy-Weinberg equilibrium in three of 15 examined loci. Four new mutations in addition to the already described were identified. The VEGF haplotype did not seem to predict plasma VEGF concentration (p=0.5). There was an association between the genotype in locus VEGF-1154 and coronary collateral size (p=0.03) and a significant association between the VEGF plasma concentration and the collateral size (p=0.03).

Conclusion: VEGF plasma concentration seems related to coronary collateral function in patients with CHD. The results did not support the hypothesis that polymorphisms in the untranslated region of the VEGF gene were associated with the concentration of circulating VEGF. Increased understanding of VEGF in the regulation of myocardial collateral flow may lead to new therapies in CHD.

Key Words: Coronary collaterals, geno-phenotype, ischemic heart disease, VEGF

Introduction

The opening and growth of preexistent collateral coronary arterioles are innate mechanisms in response to acute and chronic coronary heart disease (CHD) [1,2]. Within minutes to hours after symptom onset, in patients with ST-elevation infarction, collaterals have been shown in angiograms. On the other hand, it is known that collateral arteries may take up to 3 months to become fully developed [3,4]. The development of collaterals is a complex process involving growth modulators (promoters and inhibitors), growth factor receptors, different cell types, and proteolytic enzymes [1]. Recently, it was shown that monocyte transcription profile influence coronary collateral vessel growth [5]. Vascular endothelial growth factor (VEGF) as collateral growth promoter has received much attention [6–9] and evidence suggests that the genotype of the VEGF promoter and 5’ untranslated region can influence the induction of VEGF [10–14].

The angiogenic cytokines VEGF and fibroblast growth factor (FGF) have been tested in clinical studies of patients with mainly chronic occlusive coronary artery disease [15–20]. However, in the first larger double-blind randomized placebo-controlled VEGF gene therapy study we could not demonstrate significant improvement in collateral flow and myocardial perfusion, when compared to placebo [15].

The aim of the present study was to test the hypothesis that germline DNA variations in the VEGF promoter and 5’ untranslated region was associated with the plasma concentration of VEGF,
and that these DNA variations as well as the VEGF plasma concentration influences the ability to open coronary collateral arteries in patients with acute and chronic obstructive CHD.

**Material and methods**

We included patients with acute CHD (n=61) or stable chronic CHD (n=53). Patients with acute CHD had either ST elevation myocardial infarction [21] or non-ST elevation myocardial infarction [22]. Patients with chronic CHD had: (i) Canadian Cardiac Society (CCS) class ≥3 angina and significant reversible myocardial ischemia on an adenosine stress single photon emission computed tomography (SPECT), (ii) at least one remaining larger coronary vessel from which new collaterals/vessels could be supplied, (iii) preserved left ventricular ejection fraction (>40), and (iv) been without unstable angina or acute infarction within the last three months.

Demographic and clinical data were recorded and blood samples for plasma VEGF analysis were obtained. All patients underwent diagnostic coronary angiography (ST-elevation myocardial infarction patients immediately before the primary PCI). All patients received oral and written information about the study and signed an informed consent before inclusion in the study. The study followed the recommendations of the Helsinki II Declaration and was approved by the Ethics Committee of Copenhagen.

**VEGF SNP genotyping**

DNA was extracted from peripheral blood. A total of 15 previously published SNPs in the VEGF promoter or 5' untranslated region were genotyped in all patients using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) using the Assay-by-Design Service from Applied Biosystems (VEGF-7, -634, 1001, -1455, -1498, -1952, -2155, -2488) or direct sequencing using an ABI 310 Sequencer (a single fragment covering VEGF-1075, -1154, -1179, -1190, -1198, -1203, -1210). Sequences of all polymerase chain reaction primers and reporter probes are available from the authors. The human DNA reference sequence AL136131.15, GI:10045276 was used.

**Collection and analysis of peripheral blood**

Blood samples were collected from the antecubital vein in vacutainer tubes containing EDTA as an anticoagulant. Samples were kept on ice until processing 15 to 30 minutes after collection. Plasma was obtained by 10-minute centrifugation at 3000g. The plasma closest to the cells was not used. Plasma for VEGF analysis were immediately frozen and stored at −80°C.

Plasma concentrations of VEGF-A were measured by a colorimetric ELISA kit (R&D Systems, Minneapolis, Minn). The lower limits of detection were 9 pg/mL for VEGF-A. To account for day-to-day variation in the concentration of VEGF-A, a mean concentration of two samples was used for analysis. The first VEGF sample was drawn 24–48 hours after the angiogram and the next sample 30 days later.

**Grading of coronary collaterals**

Angiograms were assessed by two experienced invasive cardiologists blinded to all patient data. The final collateral grading was a consensus agreement between the two readers. Coronary collateral filling was graded according to the method described by Rentrop et al. [23]: 0=no filling of any collateral vessels, 1=filling of side branches of the artery by collateral vessels without visualization of the epicardial segment, 2=partial filling of the epicardial artery by collateral vessel, 3=complete filling of the epicardial artery by collateral vessels.

In addition, the visible diameter of the collateral connection was graded as described by Werner et al. [24]: CC0=no continuous visible connection between donor and recipient branch, CC1=continuous threadlike connection, or CC2=continuous small sidebranch–like connection. Patients with thrombolysis in myocardial infarction (TIMI) flow 3 in the culprit artery were excluded from scoring of collaterals. If multiple coronary collaterals were present, then the most prominent was used for grading.

**Statistical analysis**

Analyses were performed using SPSS (version 12.0, SPSS Inc, Chicago, Ill). The level of statistical significance was set at p<0.05. Continuous variables in the disease groups were compared using the Mann-Whitney U test and correlation coefficients were tested using Spearman’s Rank Correlation. Univariate geno-phenotype associations were analysed using Mann-Whitney U test. Association between the VEGF haplotypes and VEGF plasma concentrations were tested using the non-parametric Kruskal-Wallis test. Stepwise ANCOVA with genotype as independent factors and VEGF plasma concentration as outcome, type of ischemic disease and age was included as covariate was employed to identify a model associated with plasma VEGF concentration. Association between genotype and coronary collaterals were tested using Fisher’s exact test, whereas VEGF plasma concentration and coronary collaterals were tested using multinominal logistic regression with collateral score as outcome.

**Results**

Patients included in the two disease groups (acute and chronic CHD) had typical cardiac risk factors as
We found no mutations in two examined loci (VEGF-1001, -1210), and very rarely mutations in four other loci (VEGF-1198, -1203, -1952, -2155). Mutations in VEGF-1498 very often segregated with mutations in VEGF-2488. In addition to the already described mutations and polymorphisms we identified four new mutations within the fragment analyzed by direct sequencing. Four patients were heterozygote for an identical 2bp deletion (VEGF-1283 to -1284delAG). Three single base mutations (VEGF-1111G, -1142C, -1277G) were identified in 3 other patients. The identified mutations resulted in 27 different haplotypes in the included patients, 13 of these haplotypes were identified in only one individual per haplotype and three haplotypes were identified in only two individuals per haplotype.

Geno- pheno-type association

The association between VEGF plasma concentration and genotype is shown in Table III. Only one marker (VEGF-1154) showed a significant difference in VEGF concentration by univariate analysis. We found no indication of an effect of the VEGF haplotype on the VEGF plasma concentration (p=0.5 using the Kruskal-Wallis test and only including 11 haplotypes identified in three or more individuals). Stepwise analysis of the loci identified a model combining VEGF-7, -1075, -1154, and -1498 with plasma VEGF concentration as outcome with a $r^2$ of 0.30. The relation was not affected when including type of ischemic disease and/or age into the model.

Coronary angiography

An analysable coronary angiogram was available in 97 (85%) of the patients. As expected, coronary collaterals were mainly distinct in patients with chronic CHD (Table I). The correlation between the collateral filling (Rentrop grade) and collateral size (Werner classification) was highly significant in both patients with chronic CHD ($r=0.4$, $p=0.003$) and acute CHD ($r=0.7$, $p<0.001$).
There was an association between the genotype in locus VEGF-7 and coronary collaterals graded by the Werner score ($p = 0.03$), but no association with the Rentrop grading ($p = 0.3$). All remaining genotypes tested did not predict the coronary collaterals.

There was a significant association between the VEGF plasma concentration and the collateral size as classified by the Werner classification ($p = 0.03$) when controlling for type of disease (acute or chronic CHD) (Figure 2). From Figure 2 it is apparent that patients with chronic CHD are primarily in CC1 or CC2 with an inverse relation to plasma VEGF plasma concentration, whereas collateral size in patients with acute CHD is directly related to the VEGF plasma concentration. Excluding the three cases with chronic CHD and CC0 and the seven cases with acute CHD and CC2 from analysis do not change the results significantly. The interaction between type of disease and VEGF concentration in predicting Werner classification is not statistically significant ($p = 0.6$).

**Discussion**

Patients with CHD have large variations in plasma VEGF concentrations. The VEGF phenotype was found to be related to the size of the coronary collaterals. It is well known that the size and number of coronary collaterals is a major determinant of symptoms in patients with CHD. It would thus be of clinical significance to identify patients with inert potential for development of coronary collaterals. Previously, VEGF polymorphisms have been reported to affect several diseases such as proliferative diabetic retinopathy [25] and end-stage renal disease [26]. The genotype of the VEGF promoter and 5' untranslated region has been shown to influence the induction of predicting Werner classification is not statistically significant ($p = 0.6$).

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Data are $n$ (%).

HWE, Hardy-Weinberg equilibrium.

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Median (interquartile range).

*p = 0.01.*
Recent results showing that VEGF plasma concentration of VEGF differed between patients with acute versus chronic CHD, and this is in concordance with the plasma concentration of VEGF in future trials. The plasma concentration of VEGF in a future trial. The plasma concentration of VEGF is known to be large and it can be speculated if these neutral results are – at least in part – caused by a very heterogeneous patient-population regarding VEGF plasma concentration. It would be of conceptual interest to include analysis of coronary collateral size or even hypoxic regulation of VEGF by in-vitro assay in future trials to diminish this source of error but cannot totally exclude that this could explain some of the large inter-patient variation observed.

Recent larger clinical trials with VEGF treatment in chronic CHD have been disappointing [15,18], and it can be speculated if these neutral results are – at least in part – caused by a very heterogeneous patient-population regarding VEGF plasma concentration. It would be of conceptual interest to include analysis of coronary collateral size or even hypoxic regulation of VEGF by in-vitro assay in future trials of neovascularization for cardiovascular disease.

We did not find results in support of the hypothesis that the VEGF genotype was associated with the concentration of plasma VEGF. The results could suggest that patients with lower concentration of circulating VEGF have decreased coronary collateral function. This is of potential interest to future clinical trials for induction of neovascularization.

**Acknowledgements**

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Copenhagen University. The study was supported by grants from the Danish Heart Foundation (No. 0442B18-A1322141); Danish Stem Cell Research Doctoral School; and the Faculty of Health Sciences, Copenhagen University.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

References


Stem Cell Mobilization Induced by Subcutaneous Granulocyte-Colony Stimulating Factor to Improve Cardiac Regeneration After Acute ST-Elevation Myocardial Infarction

Result of the Double-Blind, Randomized, Placebo-Controlled Stem Cells in Myocardial Infarction (STEMMI) Trial

Rasmus Sejersten Ripa, MD; Erik Jørgensen, MD; Yongzhong Wang, MD; Jens Jakob Thune, MD; Jens Christian Nilsson, MD; Lars Søndergaard, MD; Hans Erik Johnsen, MD; Lars Køber, MD; Peer Grande, MD; Jens Kastrup, MD

Background—Phase 1 clinical trials of granulocyte-colony stimulating factor (G-CSF) treatment after myocardial infarction have indicated that G-CSF treatment is safe and may improve left ventricular function. This randomized, double-blind, placebo-controlled trial aimed to assess the efficacy of subcutaneous G-CSF injections on left ventricular function in patients with ST-elevation myocardial infarction.

Methods and Results—Seventy-eight patients (62 men; average age, 56 years) with ST-elevation myocardial infarction were included after successful primary percutaneous coronary stent intervention 12 hours after symptom onset. Patients were randomized to double-blind treatment with G-CSF (10 μg/kg of body weight) or placebo for 6 days. The primary end point was change in systolic wall thickening from baseline to 6 months determined by cardiac magnetic resonance imaging (MRI). An independent core laboratory analyzed all MRI examinations. Systolic wall thickening improved 17% in the infarct area in the G-CSF group and 17% in the placebo group (P=1.0). Comparable results were found in infarct border and noninfarcted myocardium. Left ventricular ejection fraction improved similarly in the 2 groups measured by both MRI (8.5 versus 8.0; P=0.9) and echocardiography (5.7 versus 3.7; P=0.7). The risk of severe clinical adverse events was not increased by G-CSF. In addition, in-stent late lumen loss and target vessel revascularization rate in the follow-up period were similar in the 2 groups.

Conclusions—Bone marrow stem cell mobilization with subcutaneous G-CSF is safe but did not lead to further improvement in ventricular function after acute myocardial infarction compared with the recovery observed in the placebo group. (Circulation. 2006;113:1983-1992.)

Key Words: angiogenesis ■ heart failure ■ magnetic resonance imaging ■ myocardial infarction ■ stem cells

In patients with acute ST-elevation myocardial infarction (STEMI), treatment with acute percutaneous coronary stent intervention (PCI) is the method of choice to reestablish coronary blood flow. Although normal epicardial blood flow is reestablished within a few hours after symptom onset, myocardial damage is usually unavoidable and may result in heart failure caused by adverse left ventricular remodeling.1

Animal studies indicate that treatment with bone marrow–derived adult stem cells after STEMI may regenerate myocardium by inducing myogenesis and vasculogenesis and improve left ventricular function.2 Most clinical studies have used intracoronary infusion of bone marrow mononuclear cells during cardiac catheterization after acute STEMI.3–5 However, the results are not conclusive, and the optimal route of stem cell administration remains to be determined.

Prolonged pharmacological mobilization of bone marrow stem cells with granulocyte colony stimulating factor (G-CSF) is an attractive alternative because the treatment is noninvasive and well known from clinical hematology.6 Phase 1 clinical trials after myocardial infarction have indi-
The STEMMI trial was a prospective, double-blind, randomized, placebo-controlled study, the efficacy of subcutaneous G-CSF injections on the regional and global left ventricular myocardial function in patients with STEMI and successful primary PCI.

Methods

Study Population

Seventy-eight patients with STEMI (62 men, 16 women; age, 56 ± 8 years, mean ± SD) who had been treated successfully with primary PCI within 12 hours after the onset of symptoms were included in the study. STEMI was diagnosed from typical chest pain at rest lasting >30 minutes, the presence of cumulative ST-elevations ≥2 contiguous leads on a standard 12-lead ECG, and a significant rise in serum markers of myocardial infarction. Only patients who were between 20 and 70 years of age with a culprit lesion located in the proximal section of a large coronary artery branch, plasma creatine kinase-MB >100 µg/L, or development of significant Q waves in the ECG were included. Exclusion criteria were prior myocardial infarction, significant stenosis in a nonculprit coronary vessel, ventricular arrhythmia after PCI requiring treatment, pregnancy, unprotected left main stem lesion, diagnosed or suspected cancer, New York Heart Association class 3 to 4 heart failure symptoms, or known severe claustrophobia.

The study was approved by the local ethical committee (KF 01–239/02) and the Danish Medicines Agency (2612–2225). All patients received oral and written information about the study and signed an informed consent before inclusion in the study.

Study Design

The STEMMI trial was a prospective, double-blind, randomized, placebo-controlled study allocating patients with acute STEMI in a 1:1 ratio after the primary PCI to G-CSF or placebo as a supplement to treatment according to guidelines. Randomization was done in blocks of 4 patients through the use of sequentially numbered, sealed envelopes. Patients received double-blind treatment with either G-CSF (Neupogen, Amgen Europe BV, Breda, The Netherlands; 10 µg/kg body weight) or a similar volume of placebo (isotonic sodium-chloride) as a subcutaneous injection once daily for 6 days. The treatment was initiated from 1 to 2 days after the STEMI, and 78 patients were included from June 2003 to January 2005, as illustrated in Figure 1. Four patients withdrew consent before completion of G-CSF/placebo treatment as a result of severe claustrophobia during the baseline magnetic resonance imaging (MRI). MRI was not feasible in another 16 patients because of claustrophobia or obesity; these patients remained in the trial and were followed up per protocol with echocardiography and clinical and invasive examinations. Three patients refused follow-up examinations.

The authors had full access to the data and take full responsibility for its integrity. All authors have read and agree to the manuscript as written.

End Points

The prespecified primary end point was change in regional systolic wall thickening from day 1 to 6 months evaluated with cardiac MRI. Secondary end points were (1) change in ejection fraction, end-systolic and end-diastolic volumes, and infarct size by MRI and (2) change in ejection fraction and end-systolic and end-diastolic volumes by echocardiography. Safety end points were (1) death of any cause, reinfarction, and new revascularization; (2) other adverse events; (3) in-stent restenosis; and (4) changes in inflammatory parameters (C-reactive protein and erythrocyte sedimentation rate). The 30-day clinical safety data have been published previously.12

Cardiac MRI

MRI was performed before and 1 and 6 months after inclusion. A detailed description of the method is available in the online supplement.1 In short, cine images and late contrast-enhanced images were obtained with a 1.5-T clinical scanner (Siemens Vision Magnetom, Siemens AG, Erlangen, Germany).

The independent core laboratory (Bio-Imaging Technologies BV, Leiden, the Netherlands) analyzed all examinations using the MRI-MASS version 6.1 (MEDIS Medical Imaging Systems, Leiden, the Netherlands). The core laboratory was blinded to all patient data and to the order of the follow-up examinations. The analyses of cardiac MRI have a low interobserver variability (3% to 6%); to reduce this variability even more, each analysis was quality controlled by a second MRI technician before final approval. Regional left ventricular function was assessed by systolic wall thickening in the infarct region, the border region, and normal myocardium. Relative systolic wall thickening was calculated as the difference between diastolic and systolic divided by the diastolic wall thickness.

Left ventricular end-diastolic volumes, end-systolic volumes, and myocardial mass were automatically calculated by the software. Infarct mass was quantified by selecting the signal intensity threshold of the hyperenhanced area.
Echocardiography

Two-dimensional echocardiography was performed in 55 patients at baseline and after 6 months of follow-up. All patients were examined in the left recumbent position with a Vivid7 scanner (GE Medical Systems, Horten, Norway). Left ventricular volumes at end systole and end diastole were assessed by Simpson’s biplane method. Left ventricular ejection fraction was assessed in multiples of 5% by visual assessment by 1 experienced echocardiogram reader blinded to all patient data.

Quantitative Coronary Angiography

All patients were scheduled to undergo coronary angiography and, if required, repeated PCI before the 6-month MRI follow-up. All coronary angiograms for quantitative coronary angiography (QCA) analysis were acquired after intracoronary injection of 0.2 mg glyceryl nitrate according to guidelines. The independent core laboratory (Bio-Imaging Technologies BV) performed the QCA analysis using the QCA-CMS version 5.3 software (MEDIS Medical Imaging Systems). Before analysis, the core laboratory conducted

### TABLE 1. Baseline Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=39)</th>
<th>G-CSF (n=39)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>54.7±8.1</td>
<td>57.4±8.6</td>
</tr>
<tr>
<td>Male gender, n (%)</td>
<td>34 (87)</td>
<td>28 (72)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>28.1±3.9</td>
<td>27.4±4.4</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>31 (80)</td>
<td>22 (66)</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>4 (10)</td>
<td>3 (8)</td>
</tr>
<tr>
<td>Known hypertension, n (%)</td>
<td>10 (26)</td>
<td>13 (33)</td>
</tr>
<tr>
<td>Family history of ischemic heart disease, n (%)</td>
<td>14 (37)</td>
<td>15 (40)</td>
</tr>
<tr>
<td>Median time from symptom debut to primary PCI (quartiles), h</td>
<td>4:23 (3:32–6:44)</td>
<td>3:47 (2:43–5:25)</td>
</tr>
<tr>
<td>Index infarct-related artery, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LAD</td>
<td>13 (33)</td>
<td>21 (54)</td>
</tr>
<tr>
<td>LCx</td>
<td>7 (18)</td>
<td>5 (13)</td>
</tr>
<tr>
<td>RCA</td>
<td>19 (49)</td>
<td>13 (33)</td>
</tr>
<tr>
<td>TIMI flow grade 0 or 1 before primary PCI</td>
<td>34 (87)</td>
<td>33 (85)</td>
</tr>
<tr>
<td>TIMI flow grade 3 after primary PCI</td>
<td>39 (100)</td>
<td>36 (92)</td>
</tr>
<tr>
<td>Type of stent, n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No stent</td>
<td>2 (5)</td>
<td>1 (3)</td>
</tr>
<tr>
<td>Bare metal</td>
<td>26 (67)</td>
<td>25 (64)</td>
</tr>
<tr>
<td>Drug eluting</td>
<td>11 (28)</td>
<td>13 (33)</td>
</tr>
<tr>
<td>Platelet glycoprotein IIb/IIIa inhibitors, n (%)</td>
<td>18 (46)</td>
<td>14 (36)</td>
</tr>
<tr>
<td>PCI of second vessel during acute procedure, n (%)</td>
<td>4 (10)</td>
<td>9 (23)</td>
</tr>
<tr>
<td>Median maximum serum CK-MB concentration (quartiles), µg/L</td>
<td>274 (141–441)</td>
<td>320 (194–412)</td>
</tr>
<tr>
<td>Discharge medication, † n (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>38 (100)</td>
<td>39 (100)</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>37 (97)</td>
<td>39 (100)</td>
</tr>
<tr>
<td>β-Blocker</td>
<td>33 (87)</td>
<td>33 (85)</td>
</tr>
<tr>
<td>Statin</td>
<td>38 (100)</td>
<td>39 (100)</td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>15 (40)</td>
<td>20 (51)</td>
</tr>
<tr>
<td>Medication at 6-mo‡ follow-up</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>33 (97)</td>
<td>37 (100)</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>33 (97)</td>
<td>37 (100)</td>
</tr>
<tr>
<td>β-Blocker</td>
<td>27 (79)</td>
<td>31 (84)</td>
</tr>
<tr>
<td>Statin</td>
<td>34 (100)</td>
<td>37 (97)</td>
</tr>
<tr>
<td>ACE inhibitor</td>
<td>21 (62)</td>
<td>24 (65)</td>
</tr>
</tbody>
</table>

LAD indicates left anterior descending artery; LCx, left circumflex artery; RCA, right coronary artery; and CK-MB, creatine kinase myocardial band. Data are mean±SD when appropriate.

*Normal upper limit=5.
†One in-hospital death in the placebo group.
‡Five and 2 patients lost to follow-up, respectively.
the standardized frame selection on the angiograms according to the worst view. The selected frames were end diastolic, showed minimal foreshortening, had no overlap, and had good contrast. All frame selections and QCA analyses were conducted according to established standard operating procedures. After each analysis, a second QCA technician performed the quality control before final approval. The QCA technicians were fully blinded to all patient data.

**Analyses of Peripheral Blood**

Samples of venous blood were obtained before the G-CSF/placebo treatment (baseline), at days 4 and 7, and at 1 month. The concentrations of CD34+ cells, CD45−/CD34−, and subpopulations in the peripheral blood were measured by flow cytometry as previously described. Plasma concentrations of vascular endothelial growth factor A (VEGF-A) and stromal cell-derived factor 1 (SDF-1) concentrations were measured in duplicate by a colorimetric ELISA kit (R&D Systems, Minneapolis, Minn). The lower limits of detection were 10 pg/mL for VEGF-A and 18 pg/mL for SDF-1.

**Statistical Analysis**

The pretrial power calculation showed that a sample size of 50 would yield an expected power of >90% to detect a difference of 15 percentage points between the treated and the placebo groups, with a 2-sided significance level of 0.05, and an assumed standard deviation of 15 percentage points for the systolic wall thickening change from baseline to 6 months in both groups. To allow for 33% dropout and claustrophobia or difficulties with breathholding during the very early baseline MRI, we decided on a sample size of 78 patients.

Analyzes were performed on an intention to treat basis. The effects of the G-CSF treatment were analyzed in a 2-factor ANOVA with repeated measures as a within-subject factor and treatment group as a between-subjects factor or Student’s t test comparing changes from baseline to 6 months follow-up in the G-CSF and placebo groups.

Subgroup analyses were not prespecified but based on recent results from the Reinfusion of Enriched Progenitor cells And Infarct...
Remodeling in Acute Myocardial Infarction (REPAIR-AMI) trial. The level of statistical significance was set at \(P<0.05\), except for the subgroup analyses (see online-only Data Supplement Table), for which significance was set at \(P<0.01\) to account for multiple testing.

**Results**

The clinical and angiographic characteristics of the study population are shown in Table 1. The groups were homogeneous with a trend toward more smokers and slightly longer time from symptom onset to primary PCI in the placebo group. All patients received optimal medical treatment in the follow-up period (Table 1). Subcutaneous G-CSF or placebo was initiated 10 to 65 hours (with 85% initiated \(<48\) hours) after the primary PCI, resulting in a rapid parallel mobilization of leukocytes and CD34+ cells into the peripheral circulation in the G-CSF group (Figure 2A), whereas the cells remained unchanged or decreased slightly in the placebo group. In addition, treatment with G-CSF resulted in mobilization of a mesenchymal stem cell line CD45−/CD34− cells expressing the SDF-1 receptor CXCR-4 and the VEGF receptor 2 (Table 2).

The plasma concentration of SDF-1 increased rapidly and significantly within the first days after the STEMI in the placebo group and remained elevated in the first month (Figure 2B). In contrast, SDF-1 was unchanged during G-CSF treatment and increased significantly at the 1-month follow-up. VEGF-A fluctuated nonsignificantly without differences between the placebo and G-CSF groups (Figure 2C).

**Treatment Effect of G-CSF on Left Ventricular Function**

Change in systolic wall thickening in the infarct area from baseline to the 6-month follow-up did not differ significantly between the placebo and G-CSF groups (17±32 versus 17±22 percentage points; Figure 3A and 3D). The corresponding changes in systolic wall thickening in the infarct border zone (Figure 3B and 3D) and in noninfarcted normal myocardium (Figure 3C and 3D) tended to be lower in the G-CSF group (8±23 and −2±30 percentage points) compared with the placebo group (23±23 and 19±38 percentage points). However, these differences can be attributed to differences in the baseline values because the values were similar in the 2 groups at the 6-month follow-up.

No significant differences were identified between the 2 groups in terms of end-diastolic volume, end-systolic volume, left ventricular mass, and left ventricular ejection fraction derived from the MRI examinations (Figure 4). These results are further confirmed by similar changes in the placebo and G-CSF groups measured with echocardiography (Figure 5). In addition, baseline measures of global left ventricular morphology and function were very similar in the 2 groups (Table 3). The homogeneity of left ventricular ejection fraction changes measured with MRI between the placebo and G-CSF groups was consistent in a number of subgroups analyzed (Data Supplement Table).

The initial infarct size measured by late contrast enhancement MRI was similar in the G-CSF (median, 8 g; range, 1 to 31 g) and placebo (median, 9 g; range, 1 to 37 g) groups. The infarct sizes were unchanged in the 2 groups from baseline to the 6-month follow-up (Figure 6A). Similar results were found for infarct size in percent of left ventricular mass (Figure 6B).

**Safety of Treatment With G-CSF Soon After STEMI**

G-CSF treatment was well tolerated, with a trend toward more patients in the G-CSF group reporting mild to moderate musculoskeletal pain (Table 4) during the treatment. No patients withdrew consent because of this side effect. C-reactive protein and erythrocyte sedimentation rates were analyzed as markers of inflammation. C-reactive protein was elevated similarly at baseline in the 2 groups (median, 17 and 19 mg/L) and subsequently normalized in the placebo group, whereas the concentration increased slightly during G-CSF treatment (median at day 4, 34 mg/L; at day 7, 22 mg/L) but had normalized at the 1-month follow-up (G-CSF treatment effect, \(P=0.07\)). Blood sedimentation rates showed a similar pattern in the G-CSF and placebo groups \(P=0.2\).
As previously reported, there were 2 in-hospital major adverse cardiac events (Table 4). One patient in the placebo group progressed into cardiogenic shock after the primary PCI and died 2.5 days later, despite aggressive treatment (intra-aortic balloon pump, dialysis, and ventilator therapy). At the time of death, the patient had received a total of 2 injections of placebo.

One patient in the G-CSF group had subacute stent thrombosis 2 days after the primary PCI. The patient initially had a thrombotic total occlusion of the distal right coronary artery, which was treated with a bare metal stent (18×4 mm), resulting in TIMI grade 3 flow. Forty-eight hours later, the patient had recurrent chest pain and reelevation of the ST-segment on the ECG but no recurrent increase in biochemical markers. An acute angiogram showed thrombotic occlusion at the proximal edge of the stent. This occlusion was treated with a new stent implantation. TIMI grade 3 flow was restored, and there was complete ST resolution. The subacute stent thrombosis occurred 6 hours after the first subcutaneous injection of G-CSF.

No sustained ventricular arrhythmias were detected during in-hospital telemetric monitoring. No additional death, reinfarction, or stent thrombosis occurred in the follow-up period (Table 4). However, 2 patients in the placebo group were referred for heart surgery. One patient had mitral valve repair, and 1 patient underwent coronary artery bypass surgery as a result of progressive symptoms of coronary artery disease.

Coronary angiograms were obtained in 31 placebo (80%) and 35 G-CSF (90%) patients on average 5 months after the initial PCI. Target vessel revascularization was performed in 8 patients (12%) in relation to the angiographic follow-up (4 patients in the placebo group, 4 in the G-CSF group; P=1.0), whereas non–target vessel revascularization was performed in 4 (13%) patients in the placebo group and 2 (6%) in the G-CSF group (P=0.4) (Table 4). The results of the quantitative coronary angiography are shown in Table 5; 5 angiograms were not analyzable for technical reasons. There were no differences between groups at baseline and at follow-up.
Discussion
This first double-blind, randomized, placebo-controlled trial demonstrated that subcutaneous G-CSF mobilization of bone marrow stem cells had no effect on left ventricular myocardial function or infarct size after STEMI treated with primary PCI. However, treatment with G-CSF seemed safe and well tolerated.

G-CSF is a potent hematopoietic cytokine that increases the production of granulocytes and is involved in mobilization of granulocytes and stem and progenitor cells from the bone marrow into the blood circulation. The mobilization process is not fully understood but is mediated through enzyme release, leading to digestion of adhesion molecules, and through trophic chemokines; SDF-1 and its receptor CXCR-4 seem of paramount importance. Animal studies and phase 1 clinical trials have suggested a beneficial effect of G-CSF on left ventricular function after myocardial infarction. Orlic et al. reported favorable results after stem cell mobilization with G-CSF and stem cell factor in mice with acute myocardial infarction. A recent experiment with G-CSF after reperfused myocardial infarction in rabbits showed an improvement in left ventricular ejection fraction and reduced remodeling.

Kuethe et al. compared 14 patients treated with G-CSF 2 days after STEMI with 9 patients who refused G-CSF treatment. The treated group had a nonsignificantly higher increase in ejection fraction compared with the control group.

Similar results were found in a single-blinded, placebo-controlled study including 20 patients 1.5 days after STEMI. The Front-Integrated Revascularization and Stem Cell Liberation in Evolving Acute Myocardial Infarction by Granulocyte Colony Stimulating Factor (FIRSTLINE-AMI) trial was a phase 1 randomized, open-label trial of 50 patients. G-CSF treatment was initiated 1.5 hours after STEMI. The control group did not receive placebo. The trial suggested improvement in left ventricular function with enhanced wall thickening, improvement in ejection fraction, and no change in end-diastolic diameter. The control group had less systolic wall thickening, decreased ejection fraction, and increased end-diastolic diameter. Thus, several studies support the efficacy of G-CSF treatment after STEMI, suggesting an

<table>
<thead>
<tr>
<th>Placebo</th>
<th>G-CSF</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MRI, n</td>
<td>29</td>
<td>28</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>55.7±9.8</td>
<td>51.2±15.4</td>
</tr>
<tr>
<td>End-diastolic volume, mL</td>
<td>127.4±28.3</td>
<td>125.5±36.5</td>
</tr>
<tr>
<td>End-systolic volume, mL</td>
<td>57.5±22.5</td>
<td>64.0±36.5</td>
</tr>
<tr>
<td>Left ventricular mass, g</td>
<td>186.2±39.0</td>
<td>181.8±46.8</td>
</tr>
</tbody>
</table>

| Echocardiography, n | 26 | 29 |
| Ejection fraction, % | 48.9±10.6 | 49.9±10.8 | 0.7 |
| End-diastolic volume, mL | 100.1±28.8 | 90.3±26.8 | 0.2 |
| End-systolic volume, mL | 49.7±17.9 | 45.4±18.9 | 0.4 |

Data are mean±SD.
improvement in ejection fraction of 6% to 8%, improved systolic wall thickening in the infarct zone, and unchanged end-diastolic volume. When our results, analyzed by a blinded, independent core laboratory, are reviewed, it is remarkable that the changes in our G-CSF group are comparable to those of the MRI groups. The changes in global left ventricular function in the placebo group we report are comparable to those of the MRI groups. The changes in global left ventricular function in the treatment of STEMI. These findings emphasize the need for caution in the interpretation of positive results of phase 1 G-CSF trials.

Possible explanations for the absence of an additional improvement in left ventricular function, despite a very significant G-CSF mobilization of CD34+ and CD45−/CD34− mononuclear cells with the potential for homing to the necrotic areas, are lack of homing signals from the myocardium, too low a dose of G-CSF, wrong timing of the treatment, mobilization of inactive subsets of stem cell populations, and use of inappropriate end points.

Plasma concentration of stromal cell–derived factor-1 (SDF-1) is known to increase rapidly during the first week after a myocardial infarction and to reach a maximum concentration after 3 weeks, which is in concordance with our findings in the placebo group. The SDF-1 expression is thought to play a crucial role in induction of stem cell engraftment to ischemic tissue. However, we found an unchanged concentration of SDF-1 during the G-CSF treatment. This could be due to an inhibition of production of homing signals from the myocardium or to the consumption of SDF-1 by cells that engrafted to the infarcted myocardium. In addition, the quantity of membrane-bound SDF-1, which may be the key mediator of homing, was unknown.

Our trial was not designed to investigate either the optimal dose or the optimal time point for G-CSF treatment. The treatment regimen resulted in a maximum concentration of CD34+ mononuclear cells 4 to 7 days after the STEMI, which corresponds well to the recent results from the REPAIR-AMI trial that indicated that the optimal time for intracoronary infusion of bone marrow mononuclear cells is day 5 to 6 after the infarction. However, considering that plasma SDF-1 and VEGF-A reach a maximum 3 weeks after the infarction, one can speculate whether G-CSF treatment should have been postponed until this period. In contrast, a recent study in mice has indicated that G-CSF inhibits apoptosis of cardiomyocytes by directly affecting the cells rather than through mobilization of bone marrow cells. The study further indicated that the antiapoptotic effect of G-CSF was significantly reduced if treatment was delayed to 3 days after the infarction. This concept is interesting because the FIRSTLINE-AMI treated patients 1.5 hours after the STEMI and found an improvement in left ventricular function. We can only speculate if the difference in time to G-CSF can account for some of the difference in outcome.

### TABLE 4. Cumulative Clinical Events in the 6-Month Follow-Up Period

<table>
<thead>
<tr>
<th>In-hospital events</th>
<th>Placebo (n=39), n</th>
<th>G-CSF (n=39), n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Death</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Reinfarction</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stent thrombosis</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Documented ventricular arrhythmia</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Musculoskeletal discomfort*</td>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>6-Month follow-up (cumulative)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Death</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Reinfarction</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Stent thrombosis</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Thoracic surgery</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Target vessel revascularization</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Non-target vessel revascularization</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Documented ventricular arrhythmia</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Cumulative death or reinfarction</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Cumulative death, reinfarction, or target vessel revascularization</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

*P=0.2

### TABLE 5. Angiographic Core Laboratory Results

<table>
<thead>
<tr>
<th>In-Lesion Zone</th>
<th>In-Stent Zone</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>G-CSF</strong> (n=32)</td>
<td><strong>Placebo</strong> (n=30)</td>
</tr>
<tr>
<td>Reference vessel diameter, mm</td>
<td>3.04±0.60</td>
</tr>
<tr>
<td>Minimal lumen diameter, mm</td>
<td>2.66±0.57</td>
</tr>
<tr>
<td>After primary procedure</td>
<td>2.14±0.72</td>
</tr>
<tr>
<td>Follow-up</td>
<td></td>
</tr>
<tr>
<td>Diameter stenosis, %</td>
<td>12.4±14.1</td>
</tr>
<tr>
<td>After primary procedure</td>
<td>28.0±22.2</td>
</tr>
<tr>
<td>Follow-up</td>
<td></td>
</tr>
<tr>
<td>Late lumen loss, mm</td>
<td>0.43±0.59</td>
</tr>
<tr>
<td>Binary (&gt;50%) restenosis, n (%)</td>
<td>3 (10)</td>
</tr>
</tbody>
</table>

Data are mean±SD when appropriate.
We studied a homogenous patient population without numerous factors that would tend to confound the results, but this has probably also led to exclusion of high-risk patients who would potentially benefit most from the treatment. Only patients with a significant rise in biochemical markers or an ECG indicating a large myocardial infarction were included. In addition, we had an upper age limit of 70 years because several previous trials have indicated that the mobilization of CD34+ cells decreases with increasing age.21 To further increase the power of our study, we chose regional myocardial function rather than global myocardial function as a primary end point. Thus, we consider the present findings very “robust” despite the discrepancy in results when compared with previous uncontrolled or unblinded phase I G-CSF trials, but we cannot exclude the possibility that the trial has been underpowered to detect a very small difference. The complex interaction between stem cell mobilization and cytokines remains poorly understood, and the results do not exclude the possibility that G-CSF could be part of a treatment strategy combining several cytokines and/or local stem cell delivery in future trials.

Safety

The trial indicates good short-term safety with G-CSF treatment. The subcutaneous injections were well tolerated, with few patients experiencing mild musculoskeletal pain. There were no indications of excessively increased progression of atherosclerosis in the G-CSF group by angiography and no deaths or myocardial infarctions in patients treated with G-CSF. We found no indications of clinical significant change in blood viscosity caused by the increase in white blood cell count. This is in concordance with the unaltered late lumen loss in the G-CSF and placebo groups was low and compared with previous uncontrolled or unblinded phase I G-CSF trials, but we cannot exclude the possibility that the trial has been underpowered to detect a very small difference. The complex interaction between stem cell mobilization and cytokines remains poorly understood, and the results do not exclude the possibility that G-CSF could be part of a treatment strategy combining several cytokines and/or local stem cell delivery in future trials.

Disclosures

None.

Acknowledgments

The STEMMI trial has been funded with grants from the Danish Heart Foundation (No. 0442B18-A1322141); Danish Stem Cell Research Doctoral School; Faculty of Health Science, Copenhagen University; Research Foundation of Rigshospitalet; Raimond and Dagmar Ringgård-Bohn’s Foundation; Aase og Ejnar Danielsens Foundation; Erik og Martha Scheibels Legat; and Arvid Nilssons Foundation.

References

Previous research indicated that mobilization of bone marrow–derived stem cells with granulocyte-colony stimulating factor (G-CSF) could help regenerate cardiac cells after acute myocardial infarction by increased vascularization, regeneration of cardiomyocytes, and/or a direct antiapoptotic effect. Accordingly, we performed a randomized, placebo-controlled, double-blind trial to test whether patients with an ST-segment elevation myocardial infarction treated with primary percutaneous coronary stent intervention (PCI) derive therapeutic benefit from addition of G-CSF to modern conventional therapy. Disappointingly, there was no evidence of additional benefit of G-CSF after 6 months follow-up.

Systolic wall thickening in the infarct area had increased 17% in the G-CSF group and 17% in the placebo group. Left ventricular ejection fraction increased at nearly identical rates in the 2 groups measured by both magnetic resonance imaging and echocardiography. Rates of death or myocardial infarction, new revascularization, and restenosis 6 months with primary percutaneous coronary intervention (PCI) were also similar in the G-CSF and placebo groups. Thus, subcutaneous G-CSF after PCI for ST-elevation infarction was safe, but did not lead to further improvements of left ventricular function when compared to placebo. It remains to be determined if G-CSF treatment could be an effective part of a treatment strategy combining several cytokines and/or local stem cell delivery. However, this trial underscores the need for binding and placebo controls in the evaluation.

ONLINE SUPPLEMENT 1

Cardiac MRI

Myocardial function and infarct size was examined by MRI before, 1 and 6 months after the G-CSF or placebo treatment. All MRI images were obtained with a 1.5-T clinical scanner (Siemens Vision Magnetom, Siemens AG, Erlangen, Germany) using a phased array chest coil. Patients were examined in the supine position with care taken to ensure identical position in the scanner at all three exams. All images were acquired using ECG gating and breath-hold technique.

Cine images were acquired with a fast low angle shot (FLASH) cinematographic pulse sequence. The entire left ventricle was covered with a stack of short axis images. Slice thickness was 8 mm with 2 mm inter-slice gaps.

A bolus of gadopentetate dimeglumine (Magnevist, Schering), 0.15 mmol per kilogram of bodyweight was injected intravenously. After a 15 minutes break with the patient positioned in the scanner, the inversion time was set to null normal myocardium, using a segmented inverse recovery turbo-FLASH sequence. Using this optimized inversion time the total infarct area was covered with consecutive short axis slices with slice thickness of 8 mm and 2 mm inter-slice gaps.

All MRI exams were analyzed by an independent core laboratory (Bio-Imaging Technologies B.V., Leiden, The Netherlands) using the MRI-MASS® version 6.1 software (MEDIS Medical Imaging Systems, Leiden, The Netherlands). The core laboratory was blinded to all patient-data and to the order of the two follow-up exams. All analyses were conducted according to established standard operating procedures.

Endocardial and epicardial borders were manually traced in all end-diastolic and end-systolic short-axis slices by one technician using the automated contour detection as starting point. The analyses of cardiac MRI have a low interobserver variability (3-6%). After each analysis a second MRI technician performed a quality control before final approval to diminish the variability even more. Regional left-ventricular function was assessed by determining systolic wall thickening in the infarct region, the border region, and normal myocardium. The systolic and diastolic thicknesses were measured along 100 chords on each slice that were equally distributed along the circumference of the LV. To correct for rotational transformation during systole, we used the internal posterior junction of the right ventricular wall with the interventricular septum as landmark for cord number 1. The mean thickness in each region was determined by averaging the measurements of the chords within the regions. Relative systolic wall thickening was calculated as the difference between diastolic and systolic wall thickness divided by the diastolic wall thickness. The position of the three regions was determined using the baseline contrast enhanced images, where areas adjacent to the region with delayed contrast enhancement were defined as the border regions.

Left ventricular end-diastolic volumes and left-ventricular end-systolic volumes were automatically calculated by the software using the planimetrically measured areas of the cavities multiplied by slice thickness plus inter-slice gap. The left-ventricular myocardial mass generated by the software is a direct multiplication of the myocardial volume (ml) times a density factor of 1.05 g/ml, including the papillary muscles as part of the myocardium.

For assessment of infarct volumes, late contrast enhancement was quantified. Infarct size on each of the short-axis images was assessed by selecting the signal intensity threshold of the hyperenhanced area (i.e. the pixels between the endo- and epi-cardial borders considered to be late enhanced). The infarct size in each patient was calculated as total infarct area multiplied by the section thickness. After determining the total mass of enhanced tissue, the percentages of enhanced tissue for total LV myocardium was calculated by dividing with the total mass of LV myocardium and multiply with 100.

References

## Online Supplemental Table 1. G-CSF treatment effect on left ventricular ejection fraction changes from baseline to 6 months follow-up by MRI in different subgroups.

<table>
<thead>
<tr>
<th></th>
<th>G-CSF treatment effect*</th>
<th>P-value ‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males (n=43)</td>
<td>1.6 (-5.1 to 8.2)</td>
<td>0.6</td>
</tr>
<tr>
<td>Females (n=11)</td>
<td>-3.9 (-17.9 to 10.0)</td>
<td>0.5</td>
</tr>
<tr>
<td>Age &lt; 58 years (n=25)†</td>
<td>-5.4 (-11.8 to 1.0)</td>
<td>0.1</td>
</tr>
<tr>
<td>Age ≥ 58 years (n=29)†</td>
<td>4.5 (-5.3 to 14.3)</td>
<td>0.4</td>
</tr>
<tr>
<td>Time from symptom onset to G-CSF</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;34:30 hours (n=24) †</td>
<td>3.5 (-2.9 to 9.9)</td>
<td>0.3</td>
</tr>
<tr>
<td>≥34:30 hours (n=30) †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline ejection fraction &lt;56 (n=26) †</td>
<td>6.5 (-2.9 to 15.8)</td>
<td>0.2</td>
</tr>
<tr>
<td>Baseline ejection fraction ≥56 (n=28) †</td>
<td>-6.2 (-11.8 to -0.6)</td>
<td>0.03</td>
</tr>
<tr>
<td>Infarct size &lt;8.5% (n=26) †</td>
<td>-4.1 (-12.0 to 3.9)</td>
<td>0.3</td>
</tr>
<tr>
<td>Infarct size ≥8.5% (n=26) †</td>
<td>2.6 (-5.8 to 11)</td>
<td>0.5</td>
</tr>
</tbody>
</table>

* Mean difference (95% confidence intervals) between placebo and G-CSF group in percentage points; positive numbers indicates G-CSF better than placebo.  
† Median values of the total study population are used as cut-off.  
‡ Significance level set at p<0.01 to 0.05.
Bone Marrow–Derived Mesenchymal Cell Mobilization by Granulocyte-Colony Stimulating Factor After Acute Myocardial Infarction

Results From the Stem Cells in Myocardial Infarction (STEMMI) Trial

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Erik Jørgensen, MD; Steen Mortensen, Tech; Lene Bindslev, MSc, PhD;
Tina Friis, MSc, PhD; Jens Kastrup, MD, DMSc

Background—Granulocyte-colony stimulating factor (G-CSF) after myocardial infarction does not affect systolic function when compared with placebo. In contrast, intracoronary infusion of bone marrow cells appears to improve ejection fraction. We aimed to evaluate the G-CSF mobilization of subsets of stem cells.

Methods and Results—We included 78 patients (62 men; 56±8 years) with ST-elevation myocardial infarction treated with primary percutaneous intervention <12 hours after symptom onset. Patients were randomized to double-blind G-CSF (10 μg/kg/d) or placebo. Over 7 days, the myocardium was exposed to 25×10⁶ G-CSF mobilized CD34⁺ cells, compared with 3×10⁸ cells in placebo patients (P<0.001); and to 4.9×10¹³ mesenchymal stem cells, compared with 2.0×10¹³ in the placebo group (P<0.001). The fraction of CD34⁺ cells/leukocyte increased during G-CSF treatment (from 0.3±0.2 to 1.1±0.9 ×10⁻³, P<0.001 when compared with placebo), whereas the fraction of putative mesenchymal stem cells/leukocyte decreased (from 22±17 to 14±11 ×10⁻³, P=0.01 when compared with placebo). An inverse association between number of circulating mesenchymal stem cells and change in ejection fraction was found (regression coefficient −6.8, P=0.004), however none of the mesenchymal cell subtypes analyzed, were independent predictors of systolic recovery.

Conclusions—The dissociated pattern for circulating CD34⁺ and mesenchymal stem cells could be attributable to reduced mesenchymal stem cell mobilization from the bone marrow by G-CSF, or increased homing of mesenchymal stem cells to the infarcted myocardium. The inverse association between circulating mesenchymal stem cells and systolic recovery may be of clinical importance and should be explored further. (Circulation. 2007;116[suppl 1]:I-24–I-30.)

Key Words: stem cells ▪ angiogenesis ▪ heart failure ▪ magnetic resonance imaging ▪ myocardial infarction

Granulocyte-colony stimulating factor (G-CSF) therapy, with the mobilization of bone marrow stem cells soon after ST-elevation acute myocardial infarction in the Stem Cells in Myocardial Infarction (STEMMI) trial, did not improve left ventricular systolic function when compared with placebo.¹ ² These results have been confirmed by the REVIVAL-2 trial³ and the G-CSF-STEMI trial.⁴ G-CSF was hypothesized to have beneficial effects on the myocardium both indirectly through the mobilization of bone-marrow stem cells into the peripheral circulation, and also perhaps directly by inhibiting myocardial apoptosis.⁵ Direct infusion of bone marrow mononuclear cells into the coronary arteries after an acute myocardial infarction has been investigated in several medium sized clinical trials,⁶–⁸ but only ²⁹¹⁰ are randomized, double-blind, and placebo-controlled. The REPAIR-AMI trial suggested a significant improvement of left ventricular ejection fraction,⁹ whereas only regional systolic function appeared to improve after intracoronary infusion of bone marrow mononuclear cells in the trial by Janssens et al.¹⁰ The apparently contradictory results of direct intracoronary infusion of bone marrow–aspirated cells versus pharmacological mobilization of bone marrow–derived stem cells are puzzling. G-CSF is known to mobilize cells from the hematopoietic cell line,¹¹ and recent evidence suggests that bone marrow–derived mesenchymal stem cells, in particular, have cardiac reparative properties.¹²

The primary end point of the published STEMMI trial was change in regional systolic wall thickening from day 1 to 6 months as evaluated by cardiac magnetic resonance imaging (MRI). Changes in ejection fraction by MRI were a secondary end point.¹

The objective of this substudy was to describe (1) the cells mobilized by G-CSF with special emphasis on mesenchymal...
stem cells, and (2) the association between the plasma concentration of the cells and subsequent changes in left ventricular systolic function.

Methods

Study Design

All 78 patients from the STEMMI trial were included for analyses. Details of the study design and inclusion criteria have been published previously. Briefly, patients were included if they had a first-time ST-elevation myocardial infarction (STEMI) successfully treated with primary percutaneous coronary intervention within 12 hours after the onset of symptoms. Patients were randomized to double-blind treatment with subcutaneous G-CSF (Neupogen, Amgen Europe BV, Breda, The Netherlands; 10 μg/kg body weight) or a similar volume of placebo (isotonic sodium-chloride) once daily for 6 days. The study was approved by the local ethical committee (KF 01 to 239/02), the Danish Medicines Agency (2612–2225), and was registered in clinicaltrials.gov (NCT00135928). All patients received verbal and written information about the study, and gave their signed consent before inclusion.

Quantification and Characterization of Stem Cells

The concentration of circulating CD34-positive (CD34<sup>+</sup>) cells and circulating putative mesenchymal stem cells in the blood quantified as CD45<sup>-</sup> and CD34<sup>-</sup> double-negative (CD45<sup>-</sup>/CD34<sup>-</sup>) cells was measured by multiparametric flow cytometry using anti-CD45 (Becton Dickinson) and anti-CD34 (BD), as previously described. A panel of monoclonal and polyclonal antibodies was used to further characterize mesenchymal stem cells, including anti-CD105 (Endoglin; Ancell), anti-CD133 (hematopoietic stem cell antigen; Miltenyi Biotec), anti-CD73 (BD), anti-CD166 (activated leukocyte adhesion molecule; BD), anti-CD133 (hematopoietic stem cell antigen; Miltenyi Biotec), anti-CD144 (VE- cadherin; BenderMed). Analytic gates were used to enumerate the total number and subsets of circulating CD45<sup>-</sup>/H11002/CD34<sup>-</sup>/H11002 cells. Cell suspensions were evaluated by a FACS Calibur (BD). At least 100,000 cells per sample were acquired. Analyses were considered informative when adequate numbers of CD45<sup>-</sup>/CD34<sup>-</sup> events (300–400) were collected in the analytic gate.

Plasma Cytokines

Plasma concentrations of vascular endothelial growth factor A (VEGF-A) and stromal cell-derived factor 1 (SDF-1) concentrations were measured in duplicate by a colorimetric ELISA kit (R&D Systems). The lower limits of detection were 10 pg/mL for VEGF-A and 18 pg/mL for SDF-1, respectively.

Cardiac MRI

MRI was performed at baseline and 6 months after inclusion as previously described in details. In short, cine and late contrast-enhancement images were obtained with a 1.5-T scanner (Siemens Vision Magnetom, Siemens AG). The examinations were analyzed by an independent core laboratory (Bio-Imaging Technologies B.V.) using the MRI-MASS v6.1 (MEDIS Medical Imaging Systems). The core laboratory was blinded to all patient-data. MRI was feasible in 28 and 31 patients in the placebo and the G-CSF group, respectively.

Statistical Analyses

Data were analyzed using SPSS 13.0. The numbers of cells in the 2 treatment groups were compared using Mann-Whitney U test. The effects of the G-CSF treatment on cell, SDF-1, and VEGF-A concentrations were analyzed in a 2-factor ANOVA with repeated measures as a within-subject factor and treatment group as a between-subjects factor after logarithmic transformation to assume normal distribution if appropriate. Associations between number of cell supplied to the postischemic myocardium and change in left ventricular systolic function were determined using linear regression models with type of treatment included as covariate in all analyses. All data are expressed as mean±SD unless otherwise stated. All tests were 2-sided, and statistical level of significance was set at P<0.05.

The authors had full access to the data and take responsibility for its integrity. All authors have read and agree to the manuscript as written.

Results

Patients

Main baseline characteristics are shown in Table 1, a detailed description has been published previously. We included 39 patients in each group. In the placebo group, 3 patients withdrew consent and 1 patient died, and 1 patient in the G-CSF group withdrew consent before completion of the study treatment. Thus, cell data pertaining to the placebo and G-CSF were available from 35 and 38 patients, respectively.

Table 1. Baseline Characteristics

<table>
<thead>
<tr>
<th></th>
<th>Placebo (n=39)</th>
<th>G-CSF (n=39)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>54.7±8.1</td>
<td>57.4±8.6</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>34 (87)</td>
<td>28 (72)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>28.1±3.9</td>
<td>27.4±4.4</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>4 (10)</td>
<td>3 (8)</td>
</tr>
<tr>
<td>Known hypertension, n (%)</td>
<td>10 (26)</td>
<td>13 (33)</td>
</tr>
<tr>
<td>Current smoker, n (%)</td>
<td>31 (80)</td>
<td>22 (56)</td>
</tr>
<tr>
<td>Median maximum serum CK-MB concentration (quartiles), μg/L</td>
<td>274 (141–441)</td>
<td>320 (194–412)</td>
</tr>
</tbody>
</table>

Mobilized Cells

The G-CSF treatment led to a substantial increase in the plasma concentration of leukocytes (from 8.3±2.2 to 51.2±18.0 ×10³/mL) and CD34<sup>+</sup> cells (supplemental Table 1, available online at http://circ.ahajournals.org), with a peak
value 7 days after initiation of G-CSF therapy and normalization after 30 days. Thus, over 7 days, the postischemic myocardium was exposed to approximately $25\pm20\times10^9$ circulating CD34$^+$ cells, compared with approximately $3.2\pm1.4\times10^9$ cells in the patients receiving placebo ($P<0.001$).

The number of circulating putative mesenchymal stem cells (CD45$^-$/CD34$^+$) increased approximately 4-fold (supplemental Table I) during G-CSF treatment, resulting in myocardial exposure to $5.0\pm3.7\times10^9$ cells after G-CSF treatment compared with $2.0\pm1.2\times10^9$ in the placebo group ($P<0.001$).

Figure 1 shows the number of CD34$^+$ cells (panel A) and CD45$^-$/CD34$^+$ (panel B) relative to the leukocytes. The fraction of CD34$^+$ cells increased during G-CSF treatment, whereas the fraction of CD45$^-$/CD34$^+$ cells surprisingly decreased during the treatment. The fraction of mononuclear cells in the blood without the CD45 marker but with the CD34 marker (CD45$^-$/CD34$^+$) was unaffected by the G-CSF treatment ($P=0.1$: $0.14\pm0.09$ versus $0.19\pm0.14$ per 1000 leukocytes at day 7 in the placebo and G-CSF groups, respectively).

The CD45$^-$/CD34$^+$ mesenchymal stem cells were characterized by early stem cell markers (CD105, CD73, CD166, CXCR4), endothelial markers (CD31, CD144, VEGFR2), or a hematopoietic marker (CD133). Figure 2 shows the relative change from baseline to day 7 in both the placebo and the G-CSF groups. Most of the mesenchymal stem cells subfractions were increased (had a value above 1) in the placebo group and reflect the natural course after a myocardial infarction. However, the fraction of CD45$^-$/CD34$^+$ cells with the early stem cell marker CD73 had a significantly lower increase in the G-CSF group compared with the increase in the placebo group; and the fraction with endothelial marker CD31 was significantly decreased in the G-CSF treated group. As expected, cells with the hematopoietic marker CD133 increased significantly more in the G-CSF group than in the placebo group. Furthermore, it is apparent from Figure 3 that most of the sub-cell fractions tended to decrease during G-CSF treatment.

**Change in Left Ventricular Systolic Function and Stem Cell Mobilization**

There was no association between the total number of CD34$^+$ cells supplied to the postischemic myocardium after myocardial infarction and the subsequent change in left ventricular ejection fraction (Figure 4A; 95% CI of regression coefficient $-8.5$ to $1.5$, $P=0.2$).

An inverse association was found between the number of CD45$^-$/CD34$^+$ cells supplied to the postischemic myocardium and the change in left ventricular ejection fraction (Figure 4B) (95% CI of regression coefficient $-11.4$ to $-2.2$, $P=0.01$).
This association remained significant when controlling for infarct size, plasma concentration of SDF-1, plasma concentration of VEGF, and leukocyte concentration. The association was not reproduced when using systolic wall thickening in the infarct area (regression coefficient $-0.08$, $P=0.2$), the infarct border area (regression coefficient $-0.08$, $P=0.1$), or infarct size (regression coefficient $2.04$, $P=0.3$) as outcome. There was no significant interaction between type of treatment and number of CD45^-CD34^- cells indicating that the association was not significantly affected by the G-CSF treatment.

Next, we examined the association between the subtypes of CD45^-CD34^- cells and changes in left ventricular ejection fraction (Table 2). None of the subtypes were independent predictors when controlling for treatment type and the total number of CD45^-CD34^- cells. Only cells with the hematopoietic marker CD133 tended to have a positive association with the systolic improvement ($P=0.07$).

### Change in SDF-1 and VEGF-A Plasma Concentrations

The individual time course of homing factor SDF-1 and the vascular growth factor VEGF-A plasma concentrations are shown in supplemental Figure I. As previously shown, the SDF-1 time course differed significantly between the G-CSF and placebo groups ($P<0.001$), whereas the VEGF-A time course were similar ($P=0.4$). Classic cardiovascular risk factors (diabetes mellitus, smoking, age, and gender) as well as time to reperfusion and infarct size did not relate to the plasma concentration of SDF-1 by linear regression analysis, but high VEGF concentration at day 4 and 7 were signifi-

![Figure 3. Subtypes of CD45^-CD34^- cells during 30 days after myocardial infarction. Compared using repeated measures ANOVA.](image)

![Figure 4. Association between changes in left ventricular ejection fraction during 6 months and (A) CD34^- and (B) CD45^-CD34^- cells. Regression line with 95% confidence interval. *Abscissa in logarithmic scale.](image)

<table>
<thead>
<tr>
<th>Cells in 10^9 per ml</th>
<th>Regression Coefficient*</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45^-/34^-/105^-</td>
<td>$-0.15$</td>
<td>$-0.38$ to $0.38$</td>
</tr>
<tr>
<td>CD45^-/34^-/73^-</td>
<td>$-1.13$</td>
<td>$-3.45$ to $1.18$</td>
</tr>
<tr>
<td>CD45^-/34^-/166^-</td>
<td>$0.50$</td>
<td>$-0.30$ to $1.29$</td>
</tr>
<tr>
<td>CD45^-/34^-/CXCR4^-</td>
<td>$-0.09$</td>
<td>$-0.25$ to $0.8$</td>
</tr>
<tr>
<td>CD45^-/34^-/31^-</td>
<td>$0.01$</td>
<td>$-0.04$ to $0.07$</td>
</tr>
<tr>
<td>CD45^-/34^-/144^-</td>
<td>$-0.03$</td>
<td>$-0.20$ to $0.14$</td>
</tr>
<tr>
<td>CD45^-/34^-/VEGF-R^-</td>
<td>$0.21$</td>
<td>$-0.22$ to $0.63$</td>
</tr>
<tr>
<td>CD45^-/34^-/133^-</td>
<td>$1.23$</td>
<td>$-0.10$ to $2.57$</td>
</tr>
</tbody>
</table>

*Type of treatment and total number of CD45^-/CD34^- are included as covariates in all models.
cantly related to long time to reperfusion (regression coefficient 43.2, P=0.002).

There were no apparent association between either CD45+/CD34− or CD34+ and VEGF-A (supplemental Table II), whereas both cell types were negatively associated with SDF-1 on all 3 days in the G-CSF group. Only CD34+ seemed to be negatively associated with SDF-1 in the placebo group, whereas CD45+/CD34− was not.

Neither SDF-1 nor VEGF-A concentrations in the week after the STEMI predicted the recovery of ejection fraction (Figure 5). In addition, there was no demonstrable association between leukocyte concentration and recovery of ejection fraction (P=0.4).

**Discussion**

This trial demonstrated that G-CSF treatment induced a dissociated pattern in circulating CD34−/CD45+ mononuclear cells and CD34+/CD45− mononuclear cells (mesenchymal) with higher concentration per leukocyte of CD34+, compared with CD45+/CD34− cells. In addition, treatment with G-CSF causes a shift in the subtypes of mesenchymal stem cells in the peripheral blood. Finally, the numbers of circulating mesenchymal stem cells appeared to predict the change in left ventricular ejection fraction after STEMI.

The initial optimism regarding the application of stem cell therapy to ischemic heart disease after the preclinical and early clinical studies has been dampened recently by larger clinical trials designed to investigate efficacy. Inotropic effects of ex vivo purified bone marrow mononuclear cells might have effects on left ventricular recovery after STEMI, even though results are still scattered.9,10 G-CSF therapy to mobilize bone marrow stem cells, however, has failed to improve left ventricular restoration in 3 double-blind placebo-controlled trials using several end points.1,3,4

It seems paradoxical that in vivo mobilization of bone marrow–derived cells to the circulation does not result in myocardial recovery comparable to that achieved by ex vivo purification and subsequent intracoronary infusion of bone marrow–derived cells. There may be several reasons for this. For example, G-CSF may not mobilize effective cell types. This hypothesis would be difficult to test in humans, however, because (1) the cells infused intracoronary are very heterogeneous15 and the cell(s) with potential for cardiac repair have not been established; and (2) G-CSF mobilization is difficult to assess because the measured concentration of circulating stem cells is dependent on both the mobilization and the homing of cells to the infarcted myocardium. Recent animal experiments indicate that mesenchymal stem cells may be good candidates for cardiac repair.12,16 whereas the hematopoietic cells (CD34+) are less likely to improve cardiac function.17 The information presented here supports a dissociated G-CSF mobilization of CD34+ cells compared with mesenchymal stem cells which may indicate a different therapeutic potential of G-CSF stem cell mobilization versus intracoronary purified cell infusions.

Furthermore, if cells are not directly responsible for the treatment effect after intracoronary infusion of bone marrow solution, but rather it is substances such as paracrine factors secreted by the cells,18,19 this might also explain some of the differences when compared with G-CSF treatment. Also, recent evidence suggests that G-CSF treatment impairs the migratory response to SDF-1 of endothelial progenitor cells.20 It is thus of importance to include measures of functional capacities in future cell trials.

The hypothesis of the STEMMI trial was that G-CSF mobilized CD34+ or CD45+/CD34− would home to and engraft into infarcted myocardium and participate in neovascularization and perhaps cardiomyocyte regeneration. This study demonstrated that there was no statistical significant association with the calculated total number of CD34+ cells, and an inverse association with the calculated CD45+/CD34− cells delivered to the myocardial perfusion during G-CSF treatment and the subsequent improvement in left ventricular ejection fraction. These results may indicate that a low concentration of the mesenchymal stem cells in the blood is attributable to engraftment of the cells into the myocardium. Thus, patients with a high inert potential for myocardial homing of the mesenchymal stem cells after STEMI will have the highest degree of systolic recovery attributable to the engrafted stem cells. We measured the plasma concentration of cytokines SDF-1 and VEGF as indicators of inert homing potential. Neither of these cytokines appeared to influence the recovery of ejection fraction, but a high concentration of SDF-1 was associated with a low concentration of cells indicating that SDF-1 may increase homing of the cells. However, plasma concentrations of the cytokines are proba-
as poor indicators of the concentrations within the myocardium, but a more precise measurement would require repeated catheterizations of the patients. Recent evidence suggests that acute myocardial ischemia does not upregulate SDF-1 gene expression in human heart shortly after ischemia and reperfusion, whereas VEGF-A gene expression seemed upregulated after reperfusion.23 It would be very interesting to label mesenchymal stem cells within the bone marrow, and then follow their potential engraftment within the myocardium during G-CSF treatment.

If mobilization of CD34+ cells does not contribute to myocardial recovery after STEMI, and if circulating CD45+ / CD34+ cells are not homing but potentially reduce the recovery of the myocardial function, this may also explain the inverse association between circulating mesenchymal stem cells and changes in ventricular function. However, the result could also be a random finding, because we could not demonstrate any correlation to changes in regional systolic function, and the calculated total amount of cells during G-CSF treatment is an estimate of cardiac exposure to stem cells based on several assumptions.

Previously, a study in dogs has indicated that intracoronary infusion of mesenchymal stem cells could cause microinfarctions, probably attributable to microvascular obstruction by the cells.22 However, circulating mononuclear cells collected after G-CSF treatment and then injected into the infarct related coronary artery in patients with STEMI did not result in any signs of myocardial damage.23 In addition, there was no biochemical or electrocardiographic evidence of myocardial ischemia during the G-CSF treatment in the present trial (data not shown). These issues are of importance considering several ongoing trials with intravenous delivery of mesenchymal stem cells in the treatment of STEMI, such as the Provacel Clinical trial (ClinicalTrials.gov: NCT00114452). One study previously observed higher rates of restenosis in patients treated with G-CSF when injected before primary percutaneous coronary intervention.24 In contrast, several recent clinical trials report occurrences of restenosis within the expected range,3,4,25 and intravascular ultrasound demonstrated no increased neointima formation after G-CSF treatment.

A potential limitation of this study is the exploratory nature of the analyses, which warrants for caution in the interpretation. Especially the lack of statistical significant association between CD34+ cells and systolic recovery could be attributable to low power. Also, it is important to recognize that differences in patient characteristics (eg, age, level of inflammation, infarct mass, and success of revascularization) between patients included in G-CSF trial and patients included in trials with intracoronary infusion could contribute to the differences in results.

Nevertheless, human data regarding stem cell treatment of ischemic heart disease are sparse and potentially clinically important aspects should be identified for further human or laboratory exploration. In addition, detailed descriptions of the cells used for therapy should be supplied in published trials to promote interstudy comparisons.

The identified dissociated G-CSF mobilization pattern for circulating CD34+ and mesenchymal stem cells might explain the neutral clinical effect of G-CSF treatment soon after a STEMI. The inverse association between circulating putative mesenchymal stem cells and subsequent recovery of left ventricular ejection fraction is of potential importance for ongoing and future trials with mesenchymal stem cells and should be investigated further.

Sources of Funding

The STEMMI trial has been funded with grants from the Danish Heart Foundation (No. 0442B18-A1322141); Danish Stem Cell Research Doctoral School; Faculty of Health Sciences, Copenhagen University; Lundbeck Foundation, Raimond og Dagmar Ringgaard-Bohn’s Fund; Aase og Ejnar Danielsens Fond; Erik og Martha Scheibels Legat; Fonden af 17.12.1981; and Arvid Nilsson’s Fond.

Disclosures

None.

References


Data Supplement Figure

**Legend:**
Individual time course of SDF-1 (upper panel) and VEGF-A (lower panel). Bold line indicate mean and standard deviation. Ordinate in logarithmic scale.

### Data Supplement Table 1. G-CSF induced cell mobilization*

<table>
<thead>
<tr>
<th></th>
<th>Placebo (N=33)</th>
<th>G-CSF (N=37)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CD34⁺, /µl</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>3.9±1.7 [1-9]</td>
<td>3.6±2.1 [0-9]</td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>4.0±2.1 [2-12]</td>
<td>34.2±23.7 [4-105]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Day 7</td>
<td>3.7±1.6 [2-8]</td>
<td>55.1±53.3 [6-259]</td>
<td></td>
</tr>
<tr>
<td>Day 28</td>
<td>3.1±1.9 [0-8]</td>
<td>2.5±1.4 [0-7]</td>
<td></td>
</tr>
<tr>
<td><strong>CD45⁻/CD34⁺, /µl</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>246±164 [28-963]</td>
<td>253±205 [28-963]</td>
<td></td>
</tr>
<tr>
<td>Day 4</td>
<td>229±174 [49-828]</td>
<td>587±480 [150-2196]</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Day 7</td>
<td>260±202 [58-770]</td>
<td>1015±1114 [68-4805]</td>
<td></td>
</tr>
</tbody>
</table>

Data are mean values±SD [range], statistical comparison by repeated measures ANOVA after logarithmic transformation.

*Data are previously published in Ripa et al. Circulation 2006;113:1983-1992

### Data Supplement Table 2. Linear regression between cell cytokine concentrations.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>CD34⁺</th>
<th>CD45⁻/CD34⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>VEGF-A</strong></td>
<td>Regression coefficient (95% CI)</td>
<td>Regression coefficient (95% CI)</td>
</tr>
<tr>
<td>Baseline</td>
<td>83 (47 to 120)*</td>
<td>0.1 (-0.1 to 0.4)</td>
</tr>
<tr>
<td>Day 4</td>
<td>0.9 (-2.6 to 4.4)</td>
<td>1.0 (0.5 to 1.5)*</td>
</tr>
<tr>
<td>Day 7</td>
<td>0.05 (-1.4 to 1.5)</td>
<td>0.02 (-0.05 to 0.09)</td>
</tr>
<tr>
<td><strong>SDF-1</strong></td>
<td>Regression coefficient (95% CI)</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>-81 (-134 to -29)</td>
<td>-0.7 (-1.3 to -0.2)</td>
</tr>
<tr>
<td>Day 4</td>
<td>-6 (-12 to -0.4)</td>
<td>0.8 (-0.09 to 1.6)*</td>
</tr>
<tr>
<td>Day 7</td>
<td>-4 (-7 to -1)</td>
<td>0.8 (0 to 1.6)*</td>
</tr>
</tbody>
</table>

* Significant interaction with treatment group, indicating a statistical significant difference in association between cells and cytokines in the two groups.
Short- and long-term changes in myocardial function, morphology, edema, and infarct mass after ST-segment elevation myocardial infarction evaluated by serial magnetic resonance imaging

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Copenhagen and Hvidovre, Denmark

Background Knowledge of the natural course after an ST-elevation myocardial infarction (STEMI) treated according to guidelines is limited because comprehensive serial magnetic resonance imaging (MRI) of systolic left ventricular function, edema, perfusion, and infarct size after STEMI has not been undertaken. The aim of this study was to evaluate effects of therapy for STEMI on left ventricular function and perfusion and to test the hypothesis that myocardial perfusion by MRI predicts recovery of left ventricular function.

Methods Cine MRI, edema, first-pass perfusion, and late enhancement imaging were performed in 58 patients at day 2 and at 1 and 6 months after successful primary percutaneous coronary stent intervention for STEMI.

Results Ejection fraction increased 6.3% during the first month (P < .001) and 1.9% from 1 to 6 months (P < .06), indicating a maximal recovery very early after the infarction. The systolic wall thickening in the infarct area almost doubled (P < .001), the perfusion of infarcted myocardium increased approximately 50% (P = .02), and perfusion improved in 72% of patients. Edema decreased with a mean of 2 segments (P < .001) during the first month and another 2.5 segments from 1 to 6 months (P < .001). Infarct size decreased to 1 month (P = .01) and was unchanged from 1 to 6 months (P = .5). Baseline perfusion did not predict improvement in ejection fraction (r = 0.2, P = .2) but did predict regional systolic function (P = .03).

Conclusions Left ventricular function, perfusion, and infarct mass recovered substantially after STEMI, with the main part of the change within the first month. First-pass perfusion at rest appeared to predict regional ventricular recovery. (Am Heart J 2007;154:929-36.)

Acute percutaneous coronary stent intervention (PCI) is the method of choice to treat patients with acute ST-elevation myocardial infarction (STEMI). However, although normal epicardial blood flow is reestablished within hours after onset of symptoms, transient and permanent myocardial damage is usually unavoidable and may result in impaired cardiac function and eventually heart failure due to adverse left ventricular remodeling.

Several authors1–6 have shown that cardiac magnetic resonance imaging (MRI) allows for analysis of left ventricular recovery, but information using different MRI imaging modalities at several time points is very limited. Magnetic resonance imaging measures are often used as end points in clinical trials of ischemic heart disease due to high accuracy and precision and low risk, allowing for inclusion of a minimum of patients. However, several early human trials after STEMI have used MRI results as end points without including a control or placebo group. This constitutes a potential bias because a comprehensive analysis of systolic left ventricular function, edema, perfusion, and infarct mass after STEMI using serial MRI has not been undertaken and the knowledge of the natural course of these variables after a STEMI treated according to guidelines is thus limited. In addition, in most previous studies, baseline MRI is performed around

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a week after the acute event and thereby potentially introducing important bias due to recovery of hibernating myocardium.

The objective of this study was (1) to investigate the short-term and long-term effects of current guideline treatment of STEMI, including successful primary PCI in terms of left ventricular function, morphology, edema, and perfusion using cardiac MRI and (2) to test the hypothesis that measures of myocardial perfusion by MRI shortly after STEMI predicts recovery of left ventricular function.

Methods

Patient population

We included all patients from the Stem Cells in Myocardial Infarction (STEMMI) trial, with a baseline and at least 1 follow-up MRI examination. Detailed inclusion criteria of the STEMMI trial have been published previously; briefly, patients with ages between 20 and 70, who had been treated successfully with primary PCI within 12 hours after the onset of symptoms, were included in the study. The culprit lesion was located in the proximal section of a large coronary artery branch, and plasma creatine kinase myocardial band was greater than 100 μg/L. The exclusion criteria included prior myocardial infarction, significant stenosis in a nonculprit coronary vessel, ventricular arrhythmia after PCI requiring treatment, pregnancy, unproctected left main stem lesion, diagnosed or suspected cancer, treated with intracoronary stenting to achieve optimal epicardial flow during the MRI examinations.

The magnetic resonance imaging was performed as soon as possible after inclusion and 1 and 6 months after inclusion. All examinations. The analyses of cardiac MRI have a low interobserver variability (3%-6%); to reduce this variability even more, each analysis was independently performed by 2 blinded MRI technologists. The location of the slice was in the middle of the infarct area as defined by cine and STIR MRI. The identical anatomical position was used at the follow-up examinations. A bolus of gadopentetate dimeglumine (Magnevist, Schering), 0.1 mmol/kg of bodyweight, was injected immediately after the perfusion scan (without scanning), adding up to a total of 0.15 mmol/kg gadopentetate dimeglumine for late enhancement imaging. After 15 minutes, the inversion time was again set to null normal myocardium using a segmented inversion-recovery turbo fast low-angle shot pulse sequence. Using this inversion time, the total infarct area was covered with consecutive short axis slices.

Cardiac MRI protocol

Magnetic resonance imaging analyses

The independent core laboratory (Bio-Imaging Technologies B.V., Leiden, The Netherlands) analyzed all examinations, except the STIR images, using the MRI-MASS v6.1 (MEDIS Medical Imaging Systems, Leiden, The Netherlands). The core laboratory was blinded to all patients' data and to the order of the follow-up examinations. The analyses of cardiac MRI have a low interobserver variability (3%-6%); to reduce this variability even more, each analysis was quality-controlled by a second blinded MRI technician before final approval. Left ventricular end-diastolic volumes, end-systolic volumes, and myocardial mass were automatically calculated by the software. Regional left ventricular function was assessed by systolic wall thickening (SWT) in the infarct region, the border region, and normal myocardium by the centerline method. The position of the 3 regions was determined using the baseline contrast-enhanced images, where areas adjacent to the region with delayed contrast enhancement were defined as the border regions. Relative SWT was calculated as the difference between diastolic and systolic divided by the diastolic wall thickness.

Myocardial perfusion was assessed in the middle of the infarct and in the border area of the infarct as the change in MRI signal intensity as a function of time during the first pass (initial slope). The imaging protocol was identical at all 3 time points and consisted of 4 parts:

1. For myocardial volumes, cine true short axis images encompassing the entire left ventricle were acquired using a cinematographic gradient echo pulse sequence with a temporal resolution of 50 milliseconds. Slice thickness was 8 mm with 2-mm interslice gaps.

2. Images for visualizing myocardial edema covered the entire left ventricle using the same views as those used for cine MRI. The images were acquired in the diastole using a T2-weighted short-inversion-time, inversion-recovery (STIR) breath-hold MRI pulse sequence with segmented data acquisition.

3. One short axis view was chosen for perfusion imaging because 1 slice would cover infarcted, noninfarcted, and border zone myocardium. The location of the slice was in the middle of the infarct area as defined by cine and STIR MRI. The identical anatomical position was used at the follow-up examinations. A bolus of gadopentetate dimeglumine (Magnevist, Schering), 0.1 mmol/kg of bodyweight, was injected immediately after the perfusion scan (without scanning), adding up to a total of 0.15 mmol/kg gadopentetate dimeglumine for late enhancement imaging. After 15 minutes, the inversion time was again set to null normal myocardium using a segmented inversion-recovery turbo fast low-angle shot pulse sequence. Using this inversion time, the total infarct area was covered with consecutive short axis slices.

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Myocardial perfusion was assessed in the middle of the infarct and in the border area of the infarct as the change in MRI signal intensity as a function of time during the first pass (initial slope). The assessments were normalized by the initial slope in normal noninfarcted myocardium.
Infarct mass was quantified planimetrically by outlining the late enhanced area and using a density factor of 1.05 g/cm³. The average value of the signal intensity of the left ventricle blood pool in all slices was used as starting point for the threshold, the next step involved the fine-tuning of the threshold by manually adapting it (max. 10% below or above the calculated threshold) until the outlined region matched the late enhanced area as identified by visual assessment. A black area in the center of the enhanced region at the baseline imaging was defined as microvascular obstruction.

Myocardial edema was assessed independently by 2 investigators (R.S.R., J.C.N.) blinded to all patient data and time sequence of the examinations using the 17-segment model of the left ventricle. Each segment was semiquantitatively assigned a score of 0 (total absence of edema) or 1 (presence of edema). The segment scores for the entire left ventricle were finally summated. Interobserver disagreement in the score of a single segment in an examination was adjudicated by taking the mean of the 2 final scores. All other interobserver disagreements were solved by adjudication after review of the examination. The 2 observers reached exact agreement in 89% of the segments with a κ of 0.78 (good strength of agreement).

Statistics

All data regardless of treatment with G-CSF or placebo were pooled because we and others have found no effect of G-CSF on the tested variables. However, the treatment group (G-CSF or placebo) was entered as covariate in all regression and analysis of variance models to account for any minor difference between the groups. This covariate was not significantly related to any of the outcomes.

Data are given as mean ± SD unless otherwise stated. The effects of time were analyzed in a repeated measures analysis of variance model. Post hoc analyses of change from baseline to 6 months, from baseline to 1 month, and from 1 month to 6 months were performed using paired sample t test without correction for 2 comparisons. Associations between functional data were described using linear regression analysis and Pearson correlation coefficient. All available results were included in analyses (eg, if a patient refused MRI at 6 months, then only baseline and 1 month results were included in analyses).

Analyses were performed with SPSS (version 12.0, SPSS Inc, Chicago, IL). The level of statistical significance was set at P < .05.

Results

Fifty-eight patients went through a baseline and at least 1 follow-up MRI examination and were included in this study. 55 (95%) of these patients had all 3 examinations (3 patients refused MRI follow-up at 6 months). There were 5% to 15% of the data points missing (except for infarct mass at baseline) (Table I). Approximately 50% of these were rejected by the core laboratory due to poor quality (eg, massive breathing artifacts or wrong electrocardiographic triggering), and a part of the MRI examination was not performed in the remaining cases (eg, due to severe patient discomfort or technical problems during the scanning). The perfusion part of the scanning required longer breath-hold and was performed late in the examination, thus more perfusion examinations were rejected or not performed (Table I). A substantial part of the baseline infarct mass results were missing (Table I). The first 6 patients included did not have baseline infarct mass results because they by mistake were scanned using a nonsegmented scanner pulse sequence, and the next 10 patients were rejected for analysis by the core laboratory.
due to poor image quality. The baseline MRI examination was performed early at a median of 51 hours (interquartile range: 26-58) after the primary PCI.

The clinical and angiographic characteristics of the study population are shown in Table II. Patients received medical treatment in accordance with guidelines in the follow-up period. Angiotensin-converting enzyme inhibitors were administered if the patient had ejection fraction below 0.40 on an echocardiogram, clinical symptoms of heart failure, or high risk of new ischemic event (eg, diabetes, hypertension, and peripheral artery disease).

Global volumes

The individual time courses of left ventricular ejection fraction are shown in Figure 1. We found a highly significant increase in ejection fraction during the 6 months after an acutely reperfused STEMI ($P < .001$). This covers a mean increase of 6.3 percentage points during the first month (95% confidence interval [CI] 3.4-9.2, $P < .001$) and a trend toward a small increase of 1.9 percentage points from 1 to 6 months (95% CI $-0.1$ to $3.9$, $P < .06$), indicating a maximal recovery very early after the infarction. The recovery was not statistically significantly affected by the use of angiotensin-converting enzyme inhibitor ($P = .7$).

The mean values of end-diastolic volume, end-systolic volume, and mass are shown in Table III. It is evident that the primary change occurs within the first month after the infarction and that the observed increase in ejection fraction is caused by both an increase in end-diastolic volume and a decrease in the end-systolic volume.

Regional left ventricular function

The SWT was measured in the center of the infarct, in the border zone of the infarct, and in the noninfarcted (normal) myocardium. Mean data are shown in Table IV, and again it is apparent that the main change occurs within the first month and primarily in the infarcted area, whereas the increase in the noninfarcted myocardium only barely reaches statistical significance.

Myocardial edema

All patients had at least 3 segments with myocardial edema at baseline MRI (mean 9.3 ± 2.6 segments), and only 3 patients (6%) were without any edema 6 months after the infarction. The amount of edema decreased significantly over time (Figure 2) ($P < .001$) with a mean decrease of 2 segments (95% CI of difference 1.1-2.8, $P < .001$) during the first month and another 2.5 segments from 1 to 6 months (95% CI of difference 1.6-3.4, $P < .001$). There was no association between the initial amount of myocardial edema and the subsequent increase in ejection fraction.

Infarct mass

Infarct mass was assessed by late enhancement imaging. Central microvascular obstruction on late enhancement images was present at the baseline examination in 16 (42%) patients. We observed a significant decrease in infarct mass from baseline (13.2 ± 10.9 g) to 1 month (95% CI of difference $-0.8$ to $-6.0$ g, $P = .01$) and unchanged mass from 1 month (11.4 ± 9.4 g) to 6 months (95% CI of difference 2.1 to $-1.0$ g, $P = .5$).

We found a very close correlation ($r = 0.9$) between the logarithmic transformed initial and final infarct mass, with an approximately 30% reduction in infarct mass from

![Figure 1](image-url)
baseline to 6 months follow-up (Figure 3). In addition, the initial infarct mass predicted the ejection fraction after 6 months ($r = 0.67$, $P < .001$).

**Myocardial perfusion**

The perfusion improved from baseline to 6 months follow-up in 72% of the patients. We found a mean increase of 28 percentage points (95% CI 15-41, $P < .001$) in the infarcted area and of 18 percentage points (95% CI 8-28, $P = .001$) in the border zone of the infarct. The observed increase in perfusion occurred primarily in the first month (Table IV).

Surprisingly, we found no correlation ($P = .3$) between the baseline perfusion measured with first-pass perfusion and the amount of initial central microvascular obstruction as determined with late enhancement MRI. In addition, baseline perfusion in the infarct area did not predict the improvement in ejection fraction ($r = 0.2$, $P = .2$). However, there was a trend toward improved recovery of SWT in patients without baseline microvascular obstruction as determined with late enhancement MRI compared with patients with microvascular obstruction (19.8 ± 28.3 vs 8.1 ± 21.3, $P = .2$). This was even more evident when comparing the baseline perfusion of the infarcted area with the recovery of SWT (Figure 4, A). This association was primarily driven by a very low recovery of SWT in patients in the quartile with the poorest baseline perfusion (Figure 4, B).

**Discussion**

The results of this trial represent a comprehensive MRI description of the “natural course” of left ventricular myocardial recovery after a STEMI reperfused by primary PCI within 12 hours using 4 MRI imaging modalities at 3 time points with a very early baseline measurement. The following are the main findings: (1) substantial recovery of all investigated variables; (2) the main part of the changes occurs within the first month after the reperfusion; and (3) impaired perfusion very early after the STEMI seems to predict subsequent low improvement in regional myocardial function.

The population in this trial is homogenous but with some differences that could potentially influence the result (eg, culprit artery, thrombosis in myocardial infarction flow grade before PCI, and time to PCI). This trial do not have statistical power to properly control for these factors; however, they were tried to be minimized by only including patients with proximal coronary lesions and enzyme rise indicative of larger infarctions.

The MRI modality is especially appealing for repeated measurements because of lack of radiation exposure to the patient. In addition, the high spatial resolutions allow for

### Table IV. Change over time in systolic wall thickening and perfusion

<table>
<thead>
<tr>
<th></th>
<th>Baseline, mean (SD)</th>
<th>1 mo, mean (SD)</th>
<th>6 mo, mean (SD)</th>
<th>$P^*$</th>
<th>Change from baseline to 6 mo, mean (SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SWT$^y$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infarcted myocardium</td>
<td>19.5 (16.1)</td>
<td>35.4 (30.8)</td>
<td>35.1 (27.8)</td>
<td>&lt;.001</td>
<td>16.8 (3.7)</td>
</tr>
<tr>
<td>Border myocardium</td>
<td>24.8 (17.5)</td>
<td>37.3 (24.6)</td>
<td>39.8 (26.4)</td>
<td>&lt;.001</td>
<td>15.8 (3.3)</td>
</tr>
<tr>
<td>Normal myocardium</td>
<td>47.2 (32.9)</td>
<td>58.2 (36.9)</td>
<td>56.3 (42.1)</td>
<td>.04</td>
<td>9.0 (4.9)</td>
</tr>
<tr>
<td><strong>Perfusion$^z$</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Infarcted myocardium</td>
<td>53.4 (39.5)</td>
<td>82.7 (46.2)</td>
<td>79.6 (44.4)</td>
<td>.02</td>
<td>27.8 (6.5)</td>
</tr>
<tr>
<td>Border myocardium</td>
<td>74.6 (32.1)</td>
<td>90.5 (35.3)</td>
<td>91.8 (33.9)</td>
<td>.04</td>
<td>18.0 (5.6)</td>
</tr>
</tbody>
</table>

*$^*$Repeate measures analysis of variance.  
$^y$In percentage of the diastolic wall thickness.  
$^z$In percentage of perfusion in noninfarcted myocardium.
precise and accurate measurement of regional morphology, function, and perfusion compared with other imaging modalities such as echocardiography and nuclear imaging. The drawbacks are primarily the low temporal resolution as well as low accessibility in many countries. The present MRI results are in good agreement with previous findings using other imaging modalities, but MRI has a better precision and accuracy. Magnetic resonance imaging-derived measures have been used for end point assessment in several recent clinical trials of stem cell therapy for acute and chronic ischemic heart disease.⁴,¹⁵

Left ventricular remodeling and infarct healing have been studied extensively with echocardiography and nuclear imaging. More recently, MRI⁴,¹⁶,¹⁷ and computerized tomography¹⁸ have offered a wide range of high-resolution imaging modalities. However, in using MRI-derived end points, it is important to acknowledge (especially in early trials without randomized control groups) the limited knowledge of the natural course of myocardial recovery after a reperfused acute myocardial infarction. A striking example is the use of G-CSF after an acute myocardial infarction. Several early trials reported very positive results,¹⁹,²⁰ whereas 2 trials designed to test efficacy were neutral because the recovery in the placebo group was substantial.⁸,¹¹

Most trials with stem cell therapy for ischemic heart disease have used global or regional systolic function as end points. In our trial, myocardial perfusion was additionally assessed because poor microvascular perfusion is related to worse outcome²¹ and neoangiogenesis is a proposed mechanism of stem cell therapy. We thus hypothesized that baseline perfusion would predict the extent of systolic recovery. First-pass perfusion at rest by MRI was used to assess change in regional microvascular perfusion as previously described.²² This method is not widely validated (eg, it can be speculated if the replacement of dead tissue with fibrous tissue in the infarct area may lead to a change in paramagnetization characteristics); however, we and others¹ found a strong
correlation between the baseline perfusion and subsequent recovery of regional left ventricular function. At the same time though, baseline perfusion did not predict the recovery of global systolic function (ejection fraction). In addition, we found postinfarction myocardial edema and an almost 30% shrinkage of the infarct mass, a result very important to recognize. In addition, we observed an approximately 50%. Thus, the effects of primary PCI, pharmacological therapy, and salvaging with MRI; however, the method needs validation.

The present results could serve as preliminary data for designing future drug-related interventional trials or even as “controls” in the interpretation of future trials without a control group. It is thus remarkable that left ventricular ejection fraction increased with more than 8 percentage points, the SWT in the infarct area almost doubled, and the perfusion of the infarcted myocardium increased with approximately 50%. Thus, the effects of primary PCI, pharmacological therapy, and ‘nature’ are substantial and important to recognize. In addition, we observed an almost 30% shrinkage of the infarct mass, a result very similar to the recent results of others.2,23 Another essential observation is the fact that the changes almost exclusively occurred within the first month after the infarction. Thus, baseline examinations should be performed as soon as possible after the STEMI to observe these changes.

The inherent limitation of this study design is the 3 time points used for MRI imaging, which do not allow to draw firm conclusions about the precise timing of the changes observed or if further changes occur after 6 months. The results were potentially biased by the missing data points especially the infarct masses; however, the missing data points seemed to be randomly distributed between the patients, and statistical analyses were based on repeated measures where most of the patients had at least 2 data points.

These results are also of interest when assessing patients in daily clinical practice. However, it is important to have in mind that these objective measures do not necessarily correlate with the subjective symptoms of the patients.

We appreciate the help from Professor C. Thomsen and staff at the Section of Magnetic Resonance Imaging, Rigshospitalet, Denmark.

References


Serial in vivo imaging of the porcine heart after percutaneous, intramyocardially injected $^{111}$In-labeled human mesenchymal stromal cells

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Abstract This pilot trial aimed to investigate the utilization of $^{111}$In-labeling of mesenchymal stromal cells (MSC) for in vivo tracking after intramyocardial transplantation in a xenotransplantation model with gender mismatched cells. Human male MSC were expanded ex vivo and labeled with $^{111}$In-tropolone. Ten female pigs were included. The labeled cells were transplanted intramyocardially using a percutaneous injection system. The $^{111}$In activity was determined using gamma camera imaging. Excised hearts were analyzed by fluorescence in situ hybridization (FISH) and microscopy. Gamma camera imaging revealed focal cardiac $^{111}$In accumulations up to 6 days after injection ($N = 4$). No MSC could be identified with FISH, and microscopy identified widespread acute inflammation. Focal $^{111}$In accumulation, inflammation but no human MSC were similarly seen in pigs ($N = 2$) after immunosuppression. A comparable retention of $^{111}$In activity was observed after intramyocardial injection of $^{111}$In-tropolone (without cells) ($N = 2$), but without sign of myocardial inflammation. Injection of labeled non-viable cells ($N = 1$) also led to high focal $^{111}$In activity up to 6 days after intramyocardial injection. As a positive control of the FISH method, we identified labeled cells both in culture and immediately after cell injection in one pig. This pilot trial suggests that after intramyocardial injection $^{111}$In stays in the myocardium despite possible disappearance of labeled cells. This questions the clinical use of $^{111}$In-labeled cells for tracking. The results further suggest that xenografting of human MSC into porcine hearts leads to inflammation contradicting previous studies implying a special immunoprivileged status for MSC.

Stig Lyngbæk and Rasmus S. Ripa contributed equally to this work.

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DOI 10.1007/s10554-009-9532-4
Keywords  Imaging · Stem cell tracking · Ischemic heart disease · Indium labeling

Introduction

Utilization of stem cells has emerged as a potential treatment modality for ischemic heart disease [1–3]. Tracking of these cells in vivo is pivotal for the future implementation of stem cell therapy by determining their fate in the patient. Imaging based cell-tracking can potentially elucidate issues regarding homing, engraftment and growth of cells following transplantation. Furthermore, identification of cell redistribution to other organs where potential side effects may take place is important. Several in vivo imaging techniques are available and currently direct labeling with radionuclides for gamma camera imaging or positron emission tomography or labeling with iron particles for magnetic resonance imaging appear suitable [4]. Imaging of leukocyte distribution by gamma camera imaging using $^{111}$In-tropolone or -oxine is a safe clinical routine procedure. $^{111}$In is commercially available with a half-life of 2.8 days making in vivo tracking up to 2 weeks possible.

Several clinical studies regarding treatment of ischemic heart disease with bone marrow mononuclear cell solutions have been conducted with conflicting results. A potential disadvantage of utilizing mononuclear cell solutions is the low fraction of stem cells that are believed to be therapeutically useful. This has shifted the focus towards the use of more specific cells lines. One candidate is multipotent mesenchymal stromal cells (MSC), which can be isolated in low numbers from the bone marrow. However, MSC can be expanded in culture and stimulated to differentiate into different cell types such as an endothelial or cardiac phenotype before injection [5–7]. Labeling of human MSC with $^{111}$In-tropolone does not appear to affect viability or differentiation capacity [8]. MSC are currently used in several ongoing clinical trials of ischemic heart disease.

The objective of this hypothesis-generating pilot trial was to investigate whether the retention of ex-vivo cultured MSC can be determined after direct percutaneous intramyocardial transplantation in non-ischemic tissue in a large animal model by $^{111}$In-tropolone radiolabeling of human MSC and use of gamma camera imaging.

Materials and methods

Experimental design

Human cells were transplanted into a xenogeneic porcine model. Percutaneous intramyocardial injections were performed on day 0. Gamma camera imaging was performed approximately $\frac{1}{2}$, 24 and 48 h after the cell injections.

The design is outlined in Fig. 1. In detail: pigs no. 1–2 were sacrificed following the 48 h scan whereas the remaining pigs underwent an additional fourth scintigraphy on day 4 or 6 just prior to euthanasia (Fig. 1). The first 4 pigs were injected with sex-mismatched $^{111}$In-labeled human MSC. Pigs no. 5 and 6 were immunosuppressed by treatment with cyclosporine (5 mg/kg per day) from 3 days before injection of $^{111}$In-labeled sex-mismatched human MSC. Immunosuppression was sustained until the pigs were sacrificed.

Four pigs were used as controls: Two (no. 7 and 8) were injected with $^{111}$In-tropolone (without cells) to test the clearance of $^{111}$In-tropolone from the myocardium and to test the tissue response to the gamma radiation from $^{111}$In. Pig no. 9 was injected with $^{111}$In-labeled human MSC killed by heating in 60°C just prior to injection to test the clearance of $^{111}$In from dead cells. The last pig was injected with $^{111}$In-labeled human MSC immediately post-mortem to serve as positive control of the histological identification of the cells.

Fig. 1 Experimental protocol
Animal protocol

Ten female domestic pigs weighing 35–40 kg were included. Animal handling and care followed the principles stated in the federal law on animal experiments and the national animal research committee approved the protocol. The animals were premedicated with midazolam. Anaesthesia was induced and maintained with intramuscular tiletamine, zolazepam, xylazine, ketamine and methadone.

Cardiac catheterization was performed via an arterial sheath in the right femoral artery. We did not induce myocardial infarction since this would potentially lead to migration of the cells and thus difficulty in post-mortem cell identification. The pigs were heparinised (100 IU bolus/kg and 50 IU/kg per ½ h). Pigs no. 6 and 7 (immunosuppressed) were treated with prophylactic antibiotic (dihydrostreptomycin 25 mg/kg and benzylpenicillinprocaine 20,000 IU/kg) at the day of cell injection and the following 2 days.

Bone marrow cell preparation

Bone marrow cells were obtained from the iliac crest of 1 healthy male human volunteer by needle aspiration under local anaesthesia. A total of 30 ml of bone marrow aspirate was immediately combined with 6 ml 1000 IE heparin/ml (Hospital pharmacy, Copenhagen, Denmark). The marrow sample was diluted 1:1 with phosphate-buffered saline (PBS) minus Ca²⁺ and Mg²⁺ (Hospital pharmacy, Copenhagen, Denmark). The diluted marrow was processed as previously described [8]. In short, the mononuclear cells (MNC) were isolated with a density gradient centrifugation method and collected using Lympho-prep (Medinor, Denmark). The MNC were cultured with GMP accepted EMEA-medium (Gibco, Invitrogen GmbH, Lofer, Austria) supplemented with 10% EMEA-FBS (PAA Laboratories, by, Austria), and 1% penicillin/streptomycin (GIBCO, Invitrogen GmbH, Lofer, Austria). The cells were incubated and the medium was changed every 3 or 4 days. When the adherent MSC were confluent, the cells were harvested and frozen in 95% EMEA-FBS + 5% dimethylsulfoxide (DMSO, Wak-Chemie Medical GmbH, Steinbach, Germany) in liquid nitrogen until 5 days prior to transplantation where the cells were thawed, plated in 25 cm² flasks in EMEA-medium and grown to confluence.

¹¹¹In-tropolone radiolabeling

Cells were labeled in 25 cm² flasks (approximately 1 × 10⁶ cells/flask), as previously described [8]. In short, the adherent cells were washed with PBS and incubated with 2.5 MBq ¹¹¹In-tropolone per flask at 37°C for 15 min. Cells were washed twice with PBS buffer and harvested by incubation with 3 ml Tryple Select (animal origin free (GIBCO, Invitrogen, Taastrup, Denmark) for 10 min at 37°C. To inactivate the Tryple Select, 7 ml of the culture medium was added and the cell-suspension was centrifuged. Before injection, cells were resuspended in 2 ml of PBS + 1% HSA.

Labeling efficiency was measured with a dose calibrator (CRC120 Capintec, Capintec Instruments Inc, USA). Cell viability was determined by Nigrosin staining after labeling.

From 1.5 to 3.3 × 10⁶ cells were available for injection in each pig with a mean activity of 1.4 ± 0.3 Bq/cell and with a mean viability of 96.7% (range 94–98). Pig no. 7 and 8 were injected with respectively 4.6 and 4.4 MBq ¹¹¹In-tropolone without cells.

NOGA-mapping and cell injection

Electromechanical evaluation of the left ventricle via the groin was performed with the NOGA XPl® system (Biosense Webster A/S, Cordis, Johnson & Johnson) [9]. The intramyocardial injections were done with the 8-french-sized MYOSTAR® mapping-injection catheter (Biosense Webster A/S, Cordis, Johnson & Johnson) inserted into the left ventricle via the groin. Ten 0.3 ml injections were given within a predefined arbitrary chosen area. The injections were performed slowly (30–40 s). Retained radioactivity in the catheter and syringe was determined after delivery.

In pig no. 10 we injected cells in suspension into small excised tissue-samples from the myocardium (1–3 g) using a syringe and a needle immediately prior to formaldehyde fixation since the objective of pig no. 10 was to serve as a positive control of the FISH method (and not as a control of the injection method).
Gamma camera image acquisition

All animals had a whole-body scanning performed half an hour after injection of the $^{111}$In-labeled cells (day 0). Scanning was repeated 1, 2, and in pigs no. 3–9 also 4 or 6 days after injection using the same dual-headed gamma camera with identical settings (GE Millenium, with two energy peaks 170 and 245 keV ± 10%, medium energy, general purpose collimator). On day 1 or 2 $^{99m}$Tc-sestamibi (100–150 MBq) was injected intravenously, and myocardial SPECT was acquired 30 min later with the same camera and collimator. The center of the lower energy peak was changed from the usual 140 keV for Tc-99m to 155 keV, in order to reduce the high count rate from the $^{99m}$Tc-activity in the left ventricular myocardium. With this dual isotope image acquisition and window setting, the left ventricle could be outlined as a “background image” for the much lower count rates derived from the focal, myocardial $^{111}$In accumulations in the 245 keV window representing the injected cells. With the combined SPECT images (Fig. 2) the exact localization of $^{111}$In activity in the left ventricular walls could be well defined. Whole body scintigraphy of pig no. 1 after 24 h was performed after $^{99m}$Tc-sestamibi injection, making quantification of $^{111}$In retention at this day impossible. As consequence, we injected $^{99m}$Tc-sestamibi after the whole body scintigraphy in all subsequent pigs.

The myocardial retention of $^{111}$In at follow up was determined by the decrease in count rates within

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**Fig. 2** a Endocardial NOGA mapping of a pig heart. The dots indicate the injection sites. b Dual isotope SPECT images of the left ventricle in the short axis showing a hot spot of $^{111}$In activity in the anterior wall, corresponding to the injection sites in the NOGA map
regions of interest (ROI) as applied on the scintigraphy from day 0 (anterior view) after correction for physical decay and background activity (a ROI outside the pig). The size and shape of the ROI was manually drawn to include the focal $^{111}\text{In}$ accumulation in the heart on the baseline image. This ROI was copied onto the following examinations using the imaging processing software. The position was manually adjusted to cover the same part of the heart potentially introducing a bias in the count-numbers. However, this bias appeared minimal since the tissue surrounding the focal $^{111}\text{In}$ accumulation had very low count-numbers compared to the focal accumulation (as it is apparent from Fig. 3) and the size of the ROI was set to include a minimum of surrounding tissue.

Tissue harvesting

All animals were euthanized after the final imaging session. Tissue-samples were collected from lungs, liver, spleen and kidney (three samples per organ). All samples were weighed (1–2 g), and the count rates were measured together with an $^{111}\text{In}$ standard with known activity/ml in a lead-shielded well counter (Packard COBRAII model 5003, Canberra Packard Canada, Mississauga, Ontario, Canada) to calculate the specific count rate per gram tissue sample after correction for radioactive decay.

Sites of $^{111}\text{In}$-labeled cell injections in the left ventricle were identified using a gamma detector probe (Neo2000, Neoprobe, Dublin, OH, USA) and 3 transmural tissue-blocks (1–3 g) containing the injection sites were fixed in formaldehyde for histology and fluorescence in situ hybridization (FISH). In addition, 3 tissue-blocks from injection sites and 3 blocks from remote myocardium were excised for measurement of radioactivity in the well counter.

Histology and FISH

Formalin fixed transmural specimens from the heart identified by the gamma detector probe were embedded in paraffin from which serial sections were cut to obtain full coverage of the tissue. Samples were stained with haematoxylin-eosin.

FISH was applied using probes for the X- and Y-chromosomes to distinguish transplanted human male cells from female porcine cells. Bicolor FISH was performed on 1–2 mm thick paraffin sections using specific $\alpha$-satellite DNA-probes for the X-chromosome (CEPHX (DXZ1, $\alpha$-satellite) and Spectrum-Green™ (Vysis, Downers Grove, IL, USA)), and the Y-chromosome (CEPHY (DYZ3, $\alpha$-satellite) and Spectrum-Orange™ (Vysis)). The standard protocol was recommended by DakoCytomation (Copenhagen, Denmark), using heating in pre-treatment-solution followed by proteolytic digestion by cold ready to-use-pepsin at room temperature. Denaturation was performed for 1 min at 82°C and hybridization took place over night at 42°C. $^{111}\text{In}$-labeled human MSC (identical to the ones injected in the pigs) in culture were used as positive control of the Y-chromosome probe. In addition, one pig was sacrificed immediately prior to cell injection and used as positive control of the cell identification method (pig no. 10).
Statistics

Data are presented as mean ± SD. No statistical significance testing was done in this exploratory trial due to the few animals (1–2) in each regimen. All count rates were corrected for both background activity and for decay. Time-activity curves (Fig. 4) were quantified by curve-fitting to assess clearance half-lives using GraphPad Prism 5.02 (GraphPad Software Inc, CA, USA). Retention following cell injection (viable or dead) was best described using two phase exponential fitting whereas one phase exponential fitting described the clearance of In-tropolone without cells (pig no. 7 and 8).

Results

111In labeling

Human MSC from a single male donor were cultured and labeled with 111In-tropolene in vitro. From 1.5 to 3.3 × 10⁶ cells were available for injection in each pig with a mean activity of 1.4 ± 0.3 Bq/cell and with a mean viability of 96.7% (range 94–98). We have previously published results indicating that this labeling does not affect cell viability or differentiation using the same protocol [8]. The cells injected were CD34 and CD45 negative, whereas the majority of cells were positive for CD13 (99%), CD73 (98%), CD90 (99%), and CD105 (97%). Approximately 27% of the radioactivity was retained in the catheter system and syringes after delivery.

111In-labeled cells injected into pig no. 9 were killed by heating (60°C) resulting in 33% viability immediately following heating and 0% viability 12 h later as determined by Nigrosin staining.

Gamma camera imaging of the pigs

The 111In activity in the heart was 35% (±11%) of the total activity in the pig 1 h after injection of viable 111In-labeled MSC and 30% in the pig injected with dead cells. The corresponding initial activity in the 2 pigs injected with 111In-tropolone alone was only 11 and 16% respectively.

SPECT imaging qualitatively identified the 111In within the myocardium corresponding to the locations of intramyocardial injections as identified on the NOGA mapping system (Fig. 2). After 6 days a focal accumulation in the cardiac region was still readily identified on whole body scintigraphy (Fig. 3). The relative retention of radioactivity in relation to the initial, decay corrected, activity following intramyocardial injection of 111In-labeled cells is shown in Fig. 4 with an estimated half life of 0.6 days in the initial rapid phase and 10 days in the slow phase. Animals treated with immunosuppressive therapy seemed to have higher retention after 6 days (rapid half life 0.7 and slow phase 35 days). Less retention was found following injection of dead cells (pig no. 9, rapid half life 0.3 and slow phase 7 days) or 111In alone (pig no. 7–8, half life 0.9). This was most pronounced initially, but retention after injection of viable cells, dead cells and 111In followed a similar pattern (Fig. 4).

Tissue distribution of 111In activity

The animals were sacrificed at various time points following intramyocardial injection (Fig. 1) and the count rates per gram tissue were measured in different tissues (Table 1). Consistent with the in vivo imaging, the injected myocardium had the highest specific count rate in all animals. Injection of living cells (pigs no. 1–6) lead to activities in the lungs after both 2 and 6 days, whereas the kidneys showed a high initial activity and then a decline. The total radioactivity after 6 days was lower in all organs (8% of delivered radioactivity) in the animal treated

![Fig. 4](https://example.com/fig4.png) Relative retention of radioactivity after correction for decay. Numbers on the graph corresponds to the pig number on Fig. 1. Data from pig no. 1 were only available on days 0 and 2 and are indicated by black dots (see text)
with dead cells (pig no. 9) compared to those receiving living cells (13%). Injection of $^{111}$In alone without cells (pigs no. 7–8) led to a higher accumulation of radioactivity in kidneys, liver, and spleen, whereas the counts in non-injected (normal) myocardium and lungs were lower when compared to animals receiving labeled cells.

Detection of MSC by FISH and histology

Human male MSC were readily identified in cell culture using FISH (Fig. 5a). The cells injected after euthanization of pig no. 10 were identified by FISH without unspecific labeling of the pig myocardium (Fig. 5b).

Samples of myocardium with high count rates with the gamma probe were analyzed by FISH and microscopy to verify the presence of human MSC 2–6 days after injection (pig no. 1–4). However, we were unable to identify any human MSC with Y-chromosomes despite intensive search by an experienced histopathologist and a technician skilled in FISH. During section of the heart we observed macroscopic changes on the endocardium at the site of the injections (Fig. 6). The microscopy of haematoxylin-eosin stained sections from the first biopsies (2 days) identified widespread necrosis bordered by acute inflammatory cells in several cases clearly outlining the needle shaped traces after the injection. Later biopsies (6 days) showed increasing numbers of mononuclear cells often with macrophages forming multinuclear cells and early granulation tissue formation. The lesions included the endocardium and reached deep myocardium (Fig. 7a, b). In the two animals (pigs no. 5–6) treated with immunosuppression we could still not identify any human cells by FISH despite high count rates in the tissue samples. Inflammation as described above was still evident with light microscopy. Two pigs (no. 7–8) were injected with $^{111}$In-tropolone without cells. In these two pigs radioactive tissue samples, identified by a gamma detection probe, showed normal myocardium without inflammation (Fig. 7c, d).

Discussion

This descriptive pilot study generates two important hypotheses. First, as radioactivity from $^{111}$In-labeled cells stays in the myocardium for a long time despite the disappearance of transplanted cells, clinical use of $^{111}$In-labeled cells for monitoring of MSC in the human heart seems problematic. Second, our results do not support the hypothesis that xenografting of human MSC into a pig does not lead to an inflammatory response and fast degradation of the cells even under pharmacologic immunosuppression.

Imaging techniques and labeling

The ideal imaging technique should allow for serial tracking in humans for a prolonged period of time

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Table 1

<table>
<thead>
<tr>
<th></th>
<th>Injected myocardium$^a$</th>
<th>Normal myocardium</th>
<th>Kidney</th>
<th>Hepar</th>
<th>Spleen</th>
<th>Lungs</th>
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<tr>
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<td></td>
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<tr>
<td>Pig 1</td>
<td>63,155</td>
<td>385</td>
<td>1,234</td>
<td>929</td>
<td>209</td>
<td>6,827</td>
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<tr>
<td>Pig 2</td>
<td>357$^a$</td>
<td>242</td>
<td>898</td>
<td>376</td>
<td>98</td>
<td>1,192</td>
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<tr>
<td>At day 6</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Pig 3</td>
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<td>N/A</td>
<td>N/A</td>
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<tr>
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<td>718</td>
<td>1,522</td>
<td>371</td>
<td>1,029</td>
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<td>11,748</td>
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<td>1,404</td>
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<td></td>
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<tr>
<td>Pig 9</td>
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<td>22</td>
<td>123</td>
<td>353</td>
<td>20</td>
<td>897</td>
</tr>
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</table>

$^a$ Identified using a gamma detector probe, except for pig number 2
with high spatial resolution and with the capability of tracking a few cells without affecting the cells or the organ. It is important that the marker remains in the viable cell but is quickly cleared from the tissue upon cell death.

A concern of radioactive labeling is the potential radiation damage to the cell and determining the effect of labeling on proliferation and differentiation is imperative prior to clinical application to ensure safety. We have previously shown that $^{111}$In labeling of human MSC (with 30 Bq/cell) or freezing did not affect viability or differentiation capacity in vitro [8, 10]. Similarly, Aicher et al. [11] found that $^{111}$In-oxine labeling (15 MBq) of cultivated circulating endothelial progenitor cells did not significantly affect viability, proliferation, or migration. The same group [12] found different results when labeling less

Fig. 5 Fluorescence in situ hybridization. Y-chromosome with green label (white arrow) and X-chromosome with orange label (black arrow). 

a $^{111}$In-labeled human mesenchymal stromal cells in culture. 
b Paraffin embedded myocardium from one pig (no. 10) sacrificed immediately prior to cell injection ($\times$1,000)

Fig. 6 Macroscopic inflammation after cell injection
differentiated circulating hematopoietic progenitor cells with higher dose of radioactivity (30 MBq). Experiments by Jin et al. [13] and Gholamrezanezhad et al. [14] might suggest that the doses used in our trial could be toxic to stromal cells and that the lethal effect is not manifest until 48 h after labeling. We previously tested viability and differentiation capacity up to 1 week after labeling using the exact same culture and labeling procedure as in the present study with no indication of radiotoxic effects on viability and/or function [8]. We can only speculate if differences in culture or labeling procedures compared to other trials could explain the differences in results.

Cell labeling with $^{111}\text{In}$ requires a chelator to mediate the transport of $^{111}\text{In}$ into the cell where $^{111}\text{In}$ binds firmly to macromolecules [15]. Both tropolone and oxine can be used as chelator. We found no difference in labeling efficiency or viability of MSC with oxine compared to tropolone as chelator (unpublished data).

An optimal route for cell delivery has not been established. We used trans-endocardial injection since evidence exists that intramyocardial injection leads to higher retention 1 h after injection compared to intracoronary infusion [16]. This was confirmed by our findings that 1/3 of the total radioactivity in the injected pigs ½ h after injection was still located in the heart, compared to only $6.9 \pm 4.7\%$ after intracoronary infusion [17] and from $11.3 \pm 3\%$ [16] to $20.7 \pm 2.3\%$ [18] after trans-epicardial injection. Most of the initial loss after intramyocardial injection might be caused by leakage trough the needle channel and clearance through the capillary network of the myocardium.

Xenografting of human MSC

When designing this study evidence existed that MSC were immunoprivileged and both allografting [19–21] and xenografting [22–25] of MSC would be possible. In this context it was unexpected that we found extensive inflammation in all cardiac biopsies after injection of cells and were unable to detect any transplanted cells by FISH even after only 2 days. Two pigs were treated with immunosuppressive therapy to diminish host-versus-graft reaction, but injection of MSC still led to intense inflammation and

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**Fig. 7** Haematoxylin-eosin staining of paraffin embedded myocardium. **a, b.** Pig injected with human $^{111}\text{In}$-labeled MSC, show intense focal inflammation with central necrosis. **c, d.** Pig injected with $^{111}\text{In}$-tropolone without cells show normal myocardium. Magnification is $\times 25$ in **a, c** and $\times 100$ in **b, d**.
cell degradation. Therefore, two experiments were conducted that confirmed that $^{111}$In-tropolone labeling did not interfere with the FISH method: $^{111}$In-tropolone labeled cells were identified by FISH in culture, and immediately following intramyocardial injection (Fig. 5). Obviously, we cannot exclude the possibility that a few viable cells were present despite the negative histological result; however, based on the intense radioactivity in the tissue-sample excised for FISH analysis, we would expect $10^5$ human cells per gram tissue (assessed using our mean activity of 1.4 Bq/cell). The myocardial tissue for the FISH analysis was identified using a gamma detector probe ensuring that cardiac tissue with $^{111}$In activity was studied; in addition, the needle track was visible in several samples. It seems unlikely that the cells would have released the radioactivity after transplantation and migrated into the myocardium especially since no myocardial ischemia was induced. Two pigs injected with $^{111}$In-tropolone without cells showed no microscopic inflammation suggesting that neither the gamma radiation from the $^{111}$In nor the transendocardial injection causes inflammation. We speculate that the clearance of transplanted cells is related to an immunological reaction invoked. This hypothesis is supported by the observation that immunosuppressed pigs seem to have higher retention of the radioactivity (Fig. 4). Our data supplement recent evidence suggesting that MSC are not immunoprivileged and will therefore lead to a cellular response after both allogenic and xenogenic transplantation [26–28].

In vivo cell tracking

Previously, several studies have used $^{111}$In labeling of both MSC and endothelial progenitor cells and subsequent in vivo tracking of the radioactivity as a surrogate measure of cell engraftment and migration [17, 29–31]. However, we have consistently in 6 of 6 pigs found high retention of radioactivity despite cell death. This is probably because $^{111}$In remains firmly bound to macromolecules from within the cells.

In our opinion this makes $^{111}$In labeling problematic for in vivo cell tracking in humans, since the viability of the cells then must be determined in vivo by another method, which is very difficult in humans. It could be of future interest to repeat our labeling studies with autologous MSC injections into the pig and to compare the intramyocardial injection method with intracoronary cell infusion.

Iron-oxide labeling for magnetic resonance imaging tracking of injected cells has appeared as a suitable alternative to $^{111}$In labeling, with the possibility of even longer follow-up. However, recently results very similar to ours using iron labeling were found [32, 33]. After 3–4 weeks transplanted cells could not be detected though a continued enhanced magnetic resonance signal existed representing engulfed labeling particles in cardiac macrophages suggesting that iron-oxide labeling is also an unreliable marker for monitoring cell survival and migration [32, 33].

Another method for in vivo cell tracking has emerged recently. Reporter gene imaging using clinical positron emission tomography is a promising modality [34], but the method still needs further validation.

Limitations

We did not induce myocardial infarction in the pigs since the objective was to evaluate the labeling method and not engraftment, migration and functional improvement. This probably led to impaired engraftment and especially migration. We intended to succeed with infarcted animals to address serial cell tracking had the method proven efficient for human use. We find it unlikely that a prior myocardial infarction would have changed the conclusions of the present trial, since the conclusions relates to efficacy of the labeling method and not cell engraftment or migration.

Our results indicate a fast immunological reaction and degradation of human MSC following transplantation into the pig heart. Results by other groups have indicated that the radioactivity from the labeling could have led to cell death [13, 14]. However, this controversy does not in any way alter the primary conclusion that cell death (caused by immunological reaction or radioactivity) is not followed by fast clearance of $^{111}$In. Thus labeling with $^{111}$In can potentially lead to false conclusions in cell tracking experiments.

We have no results showing the $^{111}$In retention in the myocardium with viable cells. However, results by others [35] suggest that the retention we observe
with dead cells is comparable to the one seen with viable cells.

**Conclusion**

Our results suggest that $^{111}$In may remain in the myocardial tissue for a prolonged period after cell death. This makes $^{111}$In labeling of MSC unsuitable for human use unless in vivo cell viability after transplantation can be determined by another method.

The results further suggest that transplantation of human MSC into porcine heart results in a fast inflammatory response and cell degradation. Therefore, this xenogenic model seems rather unsuitable for pre-clinical trials. Due to the small number of animals examined the results and hypotheses generated need confirmation, but call for caution in interpretation of in vivo cell tracking studies.

**Conflicts of interest**

The authors declare that they have no conflict of interest.

**References**


