Peripheral blood derived cells and angiogenesis in cardiovascular disease

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Peripheral blood derived cells and angiogenesis in cardiovascular disease

Cellen uit het perifeer bloed en angiogenese in cardiovasculaire ziekten
(met een samenvatting in het Nederlands)

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Voor mijn ouders
Voor Eric Jan

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Part I | Chapter 1

1

General introduction

Background

Despite advances in our knowledge of cardiovascular disease, preventive strategies and therapeutic methods, ischemic heart disease is world wide the leading cause of death[1]. Myocardial infarction (MI) is one of the main contributors to ischemic heart disease and is the number one disabling disease in high-income countries[1]. Due to cardiac tissue damage, the contraction force is reduced, which leads to heart failure. MI is usually the consequence of coronary artery atherosclerosis. Due to erosion, destabilization and atherosclerotic plaque rupture acute coronary thrombosis develops, which results in total occlusion of a coronary artery and subsequent infarction of myocardial tissue.
Over the years, various strategies to prevent heart failure after MI have been developed. Early percutaneous coronary intervention (PCI) during acute MI has been of great importance to reduce infarct expansion and improve left ventricular remodeling[2]. However, the abrupt change in oxygen concentration during reperfusion may induce extra damage to the myocardium, so called reperfusion damage[3]. Various cardioprotective agents, including statins, have been investigated in experimental and clinical models to reduce reperfusion damage[4]. Pharmacological therapy might positively influence scar formation in the MI area and subsequent ventricular remodeling[5]. Heart failure might be (in part) reversible after treatment either pharmacological or by device support. This process is called reverse remodeling[6].

Cellular therapy

Over the last few years, cellular therapy aiming at a reduction in infarct area and restoration of dysfunctional cardiac area has extensively been studied[7]. Clinical trials investigating the effect of peripheral blood or bone marrow derived cells infusion into a MI area found moderate improvement in left ventricular function when compared to conventional therapy[8]. Although originally cellular therapy aimed for the infused cells to incorporate in the heart and form new myocytes[9], a paracrine effect of these cells on the ischemic tissue – by inducing angiogenesis, improving cardiac remodeling, reducing inflammation or induction of local progenitor cells – is probably the explanation for the working mechanism of cellular therapy[7;10;11]. This topic is however still highly debated.
The mammalian body reacts to MI by mobilization of various progenitor cell subsets. In addition, cytokines and growth factors levels increase in the peripheral blood[12-15]. Interestingly, evidence exists for the incorporation of endogenous blood derived cells into the heart[16;17]. This systemic response suggests the existence of an extra defense system, which may reduce MI size and positively influence cardiac remodeling. By increasing our knowledge of this phenomenon, we may improve cellular therapy aiming at cardiac regeneration (Figure 1).

Cell trafficking after myocardial infarction

Cell mobilization to the peripheral blood and subsequent migration into the infarcted area after MI is a complex process which is only partially understood. This process, called cell trafficking, is regulated by an orchestra of chemokines, cytokines, growth factors, adhesion molecules and proteolytic proteins and is reviewed in Chapter 2.

Currently, one of the most important regulators of cell trafficking is the SDF-1α / CXCR4 axis. The ischemic myocardium produces high levels of SDF-1α one day post MI, which is able to recruit CXCR4 expressing cells from the peripheral blood[18].

The SDF-1α / CXCR4 axis is known to be negatively regulated by the endopeptidase CD26[19]. CD26 as well as CXCR4 are expressed on many different cell types, including hematopoietic stem cells, lymphocytes and monocytes and regulate their migratory capacities[19;20] (Figure 2).

Outline of this thesis

The thesis consists of 4 parts, each presenting studies on peripheral blood derived cells and angiogenesis, but in different cardiovascular diseases. **Part I** consists of this general introduction and an overview of peripheral blood derived cell trafficking for cardiac regeneration is given in **Chapter 2**. This overview provides the rationale for studies in **Part II** and **Part III**.

In **Part II** clinical studies on patients with acute MI are described. First of all, in **Chapter 3** we present the results from the REPERATOR trial in which we show that early statin treatment after primary percutaneous coronary intervention for acute MI does

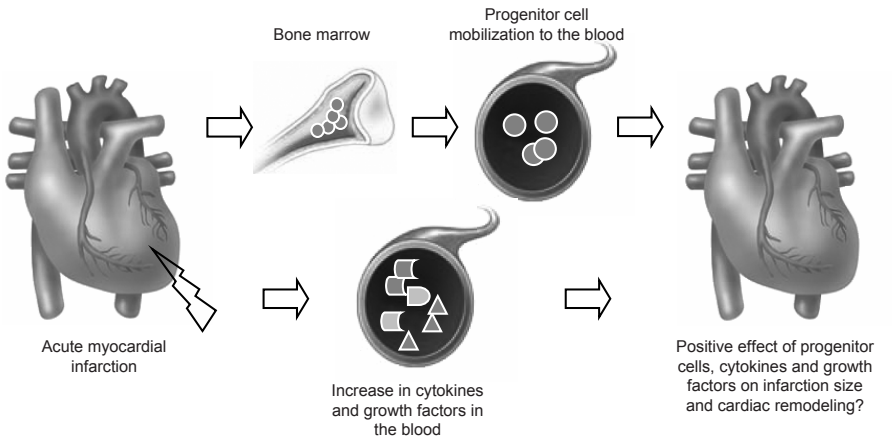

Figure 1
Progenitor cell mobilization, cytokine and growth factor increase after acute myocardial infarction. Modified from[21].

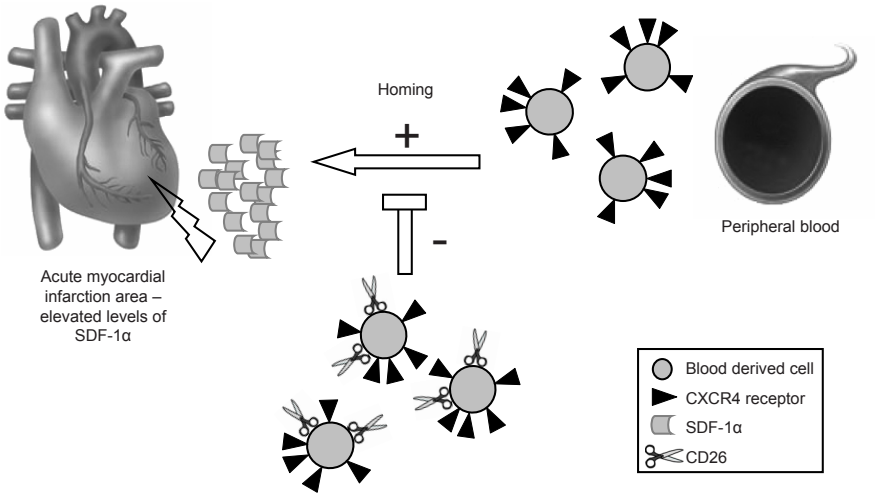

Figure 2
The SDF-1α / CXCR4 axis after acute myocardial infarction and its negative regulation by CD26.

not result in beneficial effects on the left ventricular function or a decrease in infarct size. In **Chapter 4**, the effects of acute MI on peripheral blood derived cells, plasma cytokines and growth factors in time are presented and are compared to patients with stable coronary artery disease. We provide evidence for an increased systemic response to relatively large infarctions and unfavorable hemodynamic conditions. Furthermore, in **Chapter 5**, we studied CD26 expression after acute MI. We show that high CD26 expression on peripheral blood MNCs results in decreased migration capacities to SDF-1α and that high CD26 expression is associated with decreased cardiac function recovery.

Part III consists of two studies on peripheral blood derived cells from HHT-1 patients. In **Chapter 6**, we present evidence for defective vascular repair by HHT-1 peripheral blood derived cells in a mouse MI model. And in **Chapter 7**, we show that HHT-1 MNCs have a decreased homing capacity to SDF-1α due to increased CD26 levels. Inhibition of CD26 normalized in vivo homing to a myocardial infarction area.

In **Part IV**, we present a study on angiopoietins and microvessels in atherosclerotic plaques in **Chapter 8**. We show that in plaques with a high number of microvessels, the associated angiopoietin expression levels may lead to a leaky phenotype and this might result to the development of unstable plaques. Finally, in **Chapter 9**, the results from the previous chapters are discussed.

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Part I | Chapter 2

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Peripheral blood derived cell trafficking for cardiac regeneration

Submitted

Abstract

Systemic available circulating cells play a role in cardiac maintenance and ameliorate cardiac recovery and repair after myocardial infarction. However, only a small number of cells will be incorporated during cardiac damage. Cell mobilization, homing to the ischemic myocardium and engraftment are complex processes depending on many adhesion molecules, proteases, chemokines and their receptors. Physiologic and pathophysiologic circumstances, cytokines, chemokines and certain drugs are able to influence these processes.

For cardiovascular regeneration, understanding how mobilization and homing of blood derived cells is regulated and can be modulated as well as identification of cell populations able to regenerate the heart or reduce damage after myocardial infarction is essential for the development of successful cell based therapies.

Introduction

Ischemic heart disease is world wide the leading cause of death[1]. Primary percutaneous coronary intervention, β-blockers, ACE-inhibitors and many other tools have improved survival of myocardial infarction (MI). However, after infarction, when cardiac tissue is injured and contraction force is reduced, these patients eventually will suffer from heart failure which results in disability and dependence on medical care. The past years, the concept of cellular therapy aiming at restoration of this dysfunctional part of the heart has widely been studied in preclinical and clinical studies[2]. Meta-analyses of clinical trials using peripheral blood or bone marrow derived cells found moderate improvement in left ventricular function compared to conventional therapy[3;4]. These blood or bone marrow derived cells contributing to cardiac regeneration are thought to be progenitor or stem cells, but a positive effect of mature lymphocytes, monocytes/macrophages or circulating endothelial cells can not be ruled out[5-7]. Essential for improved cardiac function - either by tissue regeneration or damage reduction - is recruitment of a sufficient number of cells. Therefore, cells first need to be mobilized from their origin or niche - e.g. the bone marrow, spleen or elsewhere - to the blood. Second, the mobilized cells need to be directed from the blood to the target organ - the heart - a process called homing.

During post infarction cardiac regeneration two processes can be discerned: damage reduction and tissue regeneration. Damage reduction can be achieved by reducing reperfusion injury like cell death, improve vascularization and reduce the formation of a fibrotic scar. To repair the heart (or to rebuild the ventricular wall) new cardiomyocytes, endothelial cells or vascular smooth muscle cell need to be incorporated into the heart. These cells may be either derived from the infused cells, or the infused cells may exert a paracrine effect on the residing cells, promoting local cell proliferation and differentiation, or on the tissue, preventing apoptosis. Both cellular and paracrine processes aim to decrease infarct size, improve cardiac function and eventually improve clinical outcome.

The first hinge that peripheral blood harbors different cell populations that may contribute to cardiac regeneration was demonstrated using sex-mismatched transplantations resulting in cardiac chimerism. Male-derived host cells, harboring a Y chromosome were found in the female heart after X-Y mismatch heart, bone marrow and peripheral blood stem cell transplantations[8-11]. Furthermore, in parabiosis experiments, where the developing circulating systems of a chick and a quail embryo were fused, resulting in free exchange of circulating cells, quail derived endothelial, smooth muscle cells and cardiomyocytes were found in the chick myocardium[12]. However, opposing

results were also reported. For example, Glaser et al only showed cardiac chimerism for smooth muscle cells[13] and a recent parabiosis study with adult APCmin mice, which spontaneously develop adenomas, did not find any contribution of bone marrow derived endothelial progenitors to the tumor vascular endothelium[14].

In this review, we will discuss mobilization and homing of blood derived cells for cardiac regeneration and the influence of health, disease and medicines on both processes.

Peripheral blood derived cell subpopulations for cardiac regeneration

Several peripheral blood derived cell populations that may contribute to cardiac regeneration are known to be mobilized from their original niche where they reside to the peripheral blood and home to an ischemic area. Most of these cells originate from the bone marrow; other niches may include adipose tissue or still unknown locations[15].

Table 1: Human blood derived cells for cardiac regeneration

	Characterization	Isolation method	Clinical trial results
Blood derived MNCs	-	Ficoll density centrifugation[5]	Varying effects on cardiac function[3;4;7;17]
Bone marrow derived MNCs	-	Ficoll density centrifugation[3;4]	Modest improvement of cardiac function[3;4]
HSCs	$CD34^+$ and/or $CD133^+$ and/or c-kit^+[19-21]	FACS	$CD133^+$: improved cardiac function, but increased number of adverse events[28] $CD34^+CXCR4^+$: no significant improvement of cardiac function[29]
EPCs	$CD34^+$ with co-expression of VEGFR2 and/or CD133 or cell culture selected[16]	FACS or culture selection[16]	Improved cardiac function[34]
VSELs	$Lin^-CXCR4^+CD45^-$ $CD133^+CD34^+$ and co-expression of Oct-4, SSEA-4, Nanog and early cardiac differentiation markers[37]	FACS	-
TCSC	$CXCR4^+CD45^-$ $CD133^+CD34^+$[38]	FACS	-
SP	Capacity to efflux Hoechst 33342 dye[41]	FACS	-
MSCs	$CD105^+CD73^+CD90^+$ or cell culture selected[44]	FACS or culture selection	Improved cardiac function[49]

Flow activated cell sorting (FACS).

The different blood derived cells that may contribute to myocardial regeneration can be divided into various groups based on their surface markers, the property to adhere to the cell culture surface or by culturing a certain cell fraction under specific conditions (Table 1)[16].

Blood derived mononuclear cells

Blood derived mononuclear cells (MNCs), which include monocytes/macrophages, lymphocytes, endothelial progenitor cells (EPCs), hematopoietic stem cells (HSC) and other progenitor cells, mobilize from their niche, home to sites of ischemic damage and contribute to cardiac regeneration either by a paracrine mechanism or due to transdifferentiation[5;6] (Chapter 6 and 7). Numerous preclinical studies show both positive and negative results. The MNC cell fraction has already been used in clinical trials to enhance myocardial function after MI however with varying success[3;4;7;17]. The mechanism how MNCs may influence heart repair is not clear. Recently, systemic infusion of irradiated apoptotic blood derived MNCs was shown to reduce the infarct area in a rat model for MI[18]. The reduction in damaged area even was higher when compared to infusion of non-irradiated MNCs. Using apoptotic MNCs, the heart seems to benefit from the delivery of pro-angiogenic factors due to cell homing while negative effects due to local inflammation may be circumvented.

Bone marrow derived mononuclear cells

Clinically, the most widely studied cell fraction is the bone marrow MNC cell population. A systematic review of these clinical studies showed that cellular therapy results in modest improvement of physiologic and anatomic parameters of the heart[3;4]. The bone marrow harbors various stem cell populations such as HSCs, EPCs, very small embryonic like stem cells (VSELs), side population cells (SP) and mesenchymal stem cells (MSCs), which may be mobilized to the peripheral blood. The following paragraphs will describe these cell populations.

Hematopoietic stem cells

One of the first discovered bone marrow / peripheral blood stem cells are the HSC, which are characterized by the expression of CD34 and/or CD133 on their cell surface[19;20]. CD117 (the stem cell factor receptor c-kit) is an other widely used surface marker for HSCs[21]. These cells have extensively been studied by hematologists since they are used to repopulate lethally irradiated bone marrow in, for example, treatment of hematological malignancies[22].

Orlic and coworkers showed in a mouse model that cytokine induced mobilization of bone marrow HSCs after MI resulted in myocardial tissue regeneration[23], however,

similar studies using genetically labeled HSCs did not prove transdifferentiation towards cardiomyocytes[24-26]. A more recent study on bone marrow c-kit$^+$ cells suggested that these cells indeed play a role in cardiac repair, but by initiation of angiogenesis and potentiation of cardiac repair[27]. The improvement of the cardiac function is therefore probably caused by paracrine effects of the cytokines and growth factors within HSCs - either cardioprotective or pro-angiogenic effects.

In a clinical study, intracoronary administration of enriched CD133$^+$ cells after MI was reported to be associated with improved cardiac function; however CD133$^+$ cell infusion was also associated with an increased number of coronary events such as in-stent reocclusion or restenosis[28]. Furthermore, a recent clinical study comparing the infusion of unselected bone marrow cells with infusion of CD34$^+$CXCR4$^+$ cells, reported no significant improvement of the left ventricular ejection fraction and no significant differences between the 2 types of infused cells[29].

Endothelial progenitor cells

One of the first studies investigating regeneration of the vasculature described a population of blood derived cells that could differentiate *in vitro* into cells with endothelial cell-like characteristics and that could be incorporated into the vasculature *in vivo*. These cells were named EPCs[30].

EPCs can be derived from the peripheral blood and from the bone marrow. Currently, no consensus on EPC identification exists, which results in a variety of EPC populations as reviewed by Leone[16]. EPCs are either defined by flow cytometry as CD34$^+$ cells which co-express other surface markers such as VEGFR2 and/or CD133 or by cell growth using a MNC culture in defined medium[16]. Cultured selected EPCs seem to be mostly of monocytic origin[6]. In an experimental model of MI, transplantation of EPCs augmented vascular growth[31]. Furthermore, EPCs have been shown to differentiate into cardiomyocytes *in vitro*[32] and *in vivo*[33]. But this topic remains controversial and these results were not reproduced by other groups. EPC-like cells have already been used in clinical trials, but the major improvements in cardiac function previously found in animal models, are not reproduced in clinical trials using EPC infusion[34;35].

Very small embryonic like stem cells

The existence of a population of VSELs in the mouse bone marrow was reported by Kucia and co-workers[36]. These Sca-1$^+$lin$^-$CD45$^+$ cells express the pluripotent stem cell markers Oct-4 and SSEA-1, resemble undifferentiated embryonic stem cells and are able to differentiate *in vitro* into various cell types including cardiomyocytes. The same group recently described that similar human cells, lin$^-$CXCR4$^+$CD45$^-$CD133$^+$CD34$^+$, are mobilized to the blood of patients with MI[37;38]. These cells also express the

pluripotent stem cell markers Oct-4, SSEA-4 and Nanog and express the early cardiac differentiation markers Nkx-2.5, GATA-4 and Mef2C. VSELs are a subpopulation of the previously described tissue committed stem cells (TCSC) which express the same early cardiac differentiation markers and have similar extracellular characteristics: $CXCR4^{+}CD45^{-}CD133^{+}CD34^{+}$[38;39]. Intramyocardial transplantation of mouse VSELs in a mouse model of MI improved LV function[40], however no clinical studies have been reported with VSELs to date.

Side population

SP cells are characterized by their capacity to efflux Hoechst 33342 dye through ATP-binding cassette transporters[41]. SP cells, originally isolated from the bone marrow, can be derived from various tissues such as the lung or heart. A later study suggested that cardiac SP cells are depleted after MI and are partly reconstituted by bone marrow cells[42]. A mouse experimental study using bone marrow derived SP cells after MI reported cell differentiation to cardiomyocytes and endothelial cells[43].

Mesenchymal stem cells

MSCs can be derived from the bone marrow and a number of other tissues. These cells are known for their differentiation capacity into bone, tendon, cartilage or fat and are defined by culture selection. MSCs have the ability to adhere to tissue culture plastic, positively express CD105, CD73 and CD90 and must be negative for CD45, CD34, CD14 or CD11b, CD79a or CD19, and HLA-DR[44]. However, whether MSCs can be efficiently mobilized into the peripheral blood is still under debate. Peripheral blood MSCs have been reported by some groups to be undetectable under normal circumstances, but others do report low cell numbers in the peripheral blood in response to injury[44;45].
The effects of MSCs on cardiac function are still under debate as reviewed by Karp[44]. I*n vitro* and *in vivo* differention of MSCs towards cardiomyocyte-like cells was reported for murine and pig MSCs respectively[46;47]. Furthermore, in this pig *in vivo* model MI size was reduced and cardiac function enhanced[47]. A different study however, using a porcine ischemia-reperfusion model, showed a reduction in infarction size and improved cardiac function after treatment with MSC conditioned medium[48]. These results strongly suggest a paracrine effect of the MSCs.
MSCs have already been brought to the clinic. A clinical trial using culture expanded MSCs after MI showed that this resulted in an improved left ventricular function[49].

Cardiomyocyte progenitor cells

Various progenitor cell populations have been shown to reside in the heart. Part of these cells can also be detected in the peripheral blood. $Sca\text{-}1^{+}$-like cells, or cardiomyocyte

progenitor cells (CMPCs), are able to differentiate into cardiomyocyte-like cells and improve cardiac function after MI[50;51]. Furthermore, a population of c-kit$^+$ cardiac progenitors has been reported to reside in the heart[52] .

Sca-1$^+$-like cells and c-kit$^+$ may be found at low cell numbers in the peripheral blood, suggesting mobilization from the bone marrow or a different niche[23] (Chapter 4). However MI does not result in significant Sca-1$^+$-like cell mobilization (Chapter 4).

Cell mobilization

To be able to improve cardiac function - either by tissue regeneration or damage reduction -, peripheral blood derived cells need to be mobilized from their origin or niche to the blood. A number of (patho)physiologic conditions are known to stimulate mobilization of different cell populations. Many chemokines, cytokines, growth factors and proteolytic proteins are known to play a role in cell mobilization and affect the adhesion capacities of the cells[22;53] (Table 2). Acute MI for example results in CD34$^+$ cell mobilization from the bone marrow[54].

The concept of regenerating a tissue by cell mobilization is not new. This process is best described for HSC mobilization from the bone marrow[55;56]. For example, in patients with hemato-oncologic diseases mobilized HSCs may be transplanted after myeloablative chemotherapy. These transplanted HSC can repopulate the bone marrow and give rise to cells from all hematopoietic lineages[22]. The mobilization of HSCs may share similarities with mobilization of other cells involved in cardiovascular repair.

Table 2: Chemokines, cytokines and growth factors in cell mobilization

	Affected peripheral blood derived cell	Receptor(s)
SDF-1α (CXCL12)	HSC, EPC[58;63]	CXCR4 and CXCR7
IL-8 (CXCL8)	HSC[66;67]	CXCR1 and CXCR2
G-CSF	HSC, EPC[22;60;65]	G-CSF receptor
GM-CSF	HSC, EPC[22;64]	GM-CSF receptor
Flt-3 ligand	HSC[62]	Flt-3 receptor
Epo	EPC[69]	Erythropoietin receptor
THPO	HSC[71]	c-mpl receptor
SCF (c-kit ligand)	HSC[57;63;70]	c-kit (CD117)
Groβ (CXCL2)	HSC[22;99]	CXCR2
VEGF	HSC, EPC[59;72]	VEGF-R
Ang-1	HSC, EPC[59]	Tie-2
PlGF	HSC[138]	VEGFR1
GH	EPC[73]	GH receptor

Chemokines and cytokines in cell mobilization

Cell mobilization is a complex process which is mediated by a highly regulated crosstalk by many cytokines, growth factors and proteolytic enzymes. Mobilization of MNCs (or progenitor cells) from the bone marrow is mediated by e.g. stromal derived factor-1α (SDF-1α), interleukin-8 (IL8 or CXCL8) and the cytokines granulocyte colony stimulating factor (G-CSF), granulocyte macrophage-colony stimulating factor (GM-CSF), Flt-3 ligand, erythropoietin (Epo), thrombopoietin (THPO) and stem cell factor (SCF)[53;57].

SDF-1α (CXCL12) and its receptor CXCR4 (CD184) - forming the SDF-1α / CXCR4 axis, play a central role in cell mobilization. SDF-1α is a CXC chemokine constitutively produced by stromal cells present in the bone marrow matrix due to the local chronic hypoxic circumstances via hypoxia inducible factor-1α (HIF-1α)[58]. At high stromal SDF-1α levels, most $CXCR4^+$ cells remain in the bone marrow, whereas cells are mobilized from the bone marrow if stromal SDF-1α drops. Interestingly, plasma SDF-1α upregulation by adenovector injection was previously shown to stimulate mobilization of bone marrow HSCs[59].
The SDF-1α / CXCR4 axis is mediated by various cytokines and proteolytic proteins. G-CSF upregulation results in down regulation of CXCR4 and SDF-1α, which results in HSC release from the bone marrow[60]. Furthermore, vascular endothelial growth factor (VEGF) was reported to negatively affect SDF-1α / CXCR4 mediated cell mobilization and Flt-3-ligand was shown to either positively or negatively affect SDF-1α / CXCR4 mediated migration depending on the duration of Flt-3-ligand exposure[61;62].

During HSC mobilization from the bone marrow several adhesion molecules, such as Very Late antigen-4 (VLA4) / vascular cell adhesion molecule (VCAM)-1, SCF / c-kit and SDF-1α / CXCR4 are cleaved. These molecules are degraded by neutrophil elastase, cathepsin G, cathepsin K, cysteine protease, CD26/DPPIV and matrix metalloproteinase 9 (MMP-9) and this process is induced by G-CSF, IL-8 and Groβ [22;57;63], causing a release of the cells from the bone marrow.

G-CSF and GM-CSF are widely used in the clinic for HSC mobilization[22], but are also known to mobilize EPCs[64;65]. Upregulation of IL-8 results in HSC mobilization due to leukocyte function associated antigen-1 (LFA1) - a β2-integrin - detachment and MMP-9 induction[66;67]. And Epo, the stimulant of erythroid precursor mobilization and differentiation[68] which is sometimes abused by atheletes, also stimulates EPC mobilization by increasing the proliferative capacity and reducing apoptosis of bone marrow cells[69]. Furthermore, bone marrow progenitor cell mobilization is dependant on cleavage of stromal cell membrane bound SCF - the ligand for c-kit - by MMP-9,

generating soluble SCF. The increased plasma soluble SCF levels will induce cell migration from the marrow to the circulation[70]. An other cytokine involved in cell mobilization from the bone marrow is THPO. Not only does THPO increase peripheral blood platelet counts, but also bone marrow HSCs are mobilized to the peripheral blood[71]. VEGF, Angiopoietin-1 (Ang-1) and placental growth factor (PlGF) are also involved in HSC mobilization[53]. Adenoviral mediated plasma VEGF upregulation with or without Ang-1 was previously shown to stimulate mobilization of HSCs and EPCs[59]. An important mediator of EPC mobilization is endothelial nitric oxide synthase (eNOS) and VEGF induced EPC mobilization was found to be eNOS dependent[72]. EPCs were also mobilized after growth hormone (GH) treatment, resulting in an increase in NO availability - mediated by insuline-like growth factor (IGF)-1[73] . Furthermore, estrogen mobilizes EPCs from the bone marrow in an eNOS and MMP-9 dependent manner[74].

Peripheral blood derived cell homing

Once being mobilized into the circulation, cells need to be recruited to the ischemic heart to be able to repair the cardiac tissue. For this homing process, cells are recruited from the flowing blood, interact with the vascular endothelial layer, transmigrate and integrate into the target tissue. This complex process may be divided into various steps: rolling, activation by chemo attractants, cell arrest or sticking, transmigration through the endothelium, traversing the basal lamina and migration and invasion of the target tissue[55;56] (Figure 1). Also, the relocated cells should remain at their new location by engraftment. Various chemokines, chemoattractants and matrix degrading proteins are involved (Table 3). This process is best described for HSCs homing to the bone marrow and lymphocyte / monocyte homing to an injured tissue - not all homing steps have been studied for other blood derived cells in detail[55;56] - however, they seem to share similarities.

Rolling

Tethering and rolling of HSCs is mediated by primary adhesion molecules such as vascular selectins (E- and P-selectins) binding with their ligands[75]. They are characterized by fast binding kinetics and high tensile strength, but short bond lifetime[56]. Mouse embryonic EPC homing to tumor microvessels was previously shown to be also mediated by E-, P-selectins and P-selectin glycoprotein ligand[76]. The involvement of E-selectin in EPC homing was confirmed for adult cells in a mouse model for hind limb ischemia[77].

Activation by chemoattractants

The rolling cells are then stimulated by a chemotactic or activating stimulus provided by soluble or surface-bound chemoattractants, which results in arrest of the rolling cells[56].

Many chemotactic proteins have been described to play a role in this process (Table 3). HSCs may be activated by endothelial SDF-1α. This signal is thought to induce a rapid conformational change of the VLA-4 and LFA-1 integrins that results in increased affinity for their ligands. This results in arrest of the rolling cells[78]. The SDF-1α / CXCR4 axis was recently also suggested to play a role in migration of $Sca1^{+}/CD31^{-}$ cardiac SP cells in a mouse myocardial ischemia model[79]. EPC-integrin binding was shown to be activated by monocyte chemoattractant protein 1 (MCP-1), high mobility group box 1 (HMGB1), SDF-1α and VEGF[80;81].

Cell arrest / sticking and transmigration

Cell arrest or sticking is mediated by secondary adhesion molecules, mostly integrins that interact with endothelial ligands of the immunoglobulin superfamily[56]. This process is thought to be initiated by surface protein activation as described previously, increasing affinity for its receptor, and permitting the cells to stop. For example, SDF-1α mediated activation of the integrins VLA-4 and LFA-1 results in firm attachment to VCAM-1 and intracellular adhesion molecule 1 (ICAM-1). This attachment results in arrest of the rolling HSCs[78]. Members of the β2- integrin family, LFA-1, Mac-1 and p150/95, and VLA-4 were previously shown to play an important role in the homing of *ex vivo*-expanded EPCs[80;82].

The arrested blood derived cells then migrate through the endothelial cell layer and basal lamina. For HSC transmigration, this process was shown to be mediated by

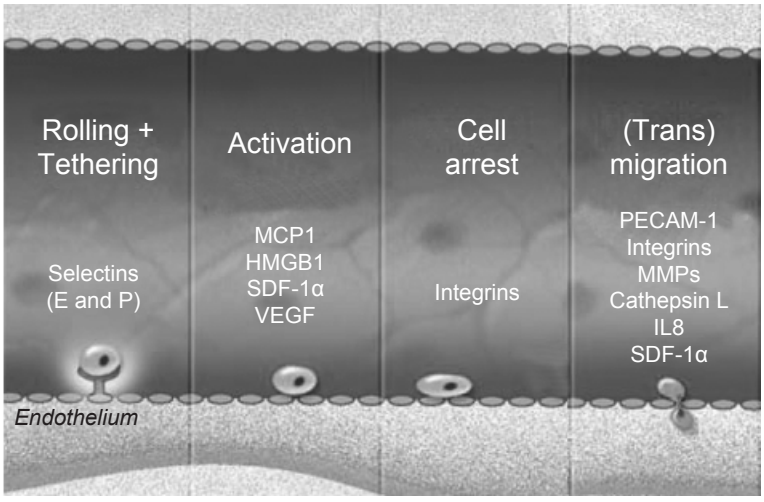

Figure 1
Homing of peripheral blood derived cells. Adapted from[55]

platelet/endothelial cell adhesion molecule-1 (PECAM-1/CD31), CD99 and CD18[83;84]. For EPC transmigration, CD11a (in combination with CD18, LFA-1) and CD49d (in combination with CD29, VLA-4) were shown to be important players[82].

2

Migration and invasion of the target tissue

After traversing the endothelial cells, the homed cells need to invade and migrate through the tissue. This process requires breaking down extracellular matrix and secretion of proteolytic enzymes such as MMPs[85]. SDF-1α stimulation of $CD34^+$ cells results in MMP-2 and MMP-9 upregulation by improving their invasion and migration capacity[86]. Furthermore, IL-8, Gro-α and their receptors CXCR1 and CXCR2 are involved in bone marrow derived EPC homing to ischemic myocardial tissue and this may be due to MMP-2 and MMP-9 upregulation[87]. Besides MMPs, the protease cathepsin L is

Table 3:Peripheral blood cell homing

Function	Ligand	Receptor	Investigated cell type
Rolling	ESL-1	E-selectin	HSC, eEPC, EPC[75-77]
	PSGL-1	P-selectin	HSC, eEPC, MSC[75;76;90]
Activation	HMGB1	RAGE, TLR2, TLR4	EPC[81]
	MCP-1 (CCL2)	CCR2	EPC[80]
	SDF-1α	CXCR-4	HSC, EPC[78-80]
	VEGF	VEGF-R	EPC[80]
Cell arrest	ICAM-1	LFA-1 (CD11a/CD18)	HSC, EPC[78;80;82;83]
	ICAM-1	Mac-1 (CD11b/CD18)	HSC, EPC[80;83]
	ICAM-1	P150/95 (CD11c/CD18)	HSC, EPC[80;83]
	VCAM-1	VLA-4 (CD49d/CD29)	HSC, EPC[75;78;82;90]
Transmigration	CD38	PECAM-1 (CD31)	HSC[83]
	CD99	CD99	HSC[84]
	ICAM-1	CD18	HSC, EPC[82;83]
	VCAM-1	VLA-4 (CD49d/CD29)	EPC[82]
Migration	Cathepsin L	-	EPC[88]
	Gro-α	CXCR2	EPC[87]
	IL8	CXCR1, CXCR2	EPC[87]
	MMP-2	-	HSC, EPC[86;87]
	MMP-9	-	HSC, EPC[86;87]
	SDF-1α	CXCR4	HSC[86]

eEPC (embryonic endothelial progenitor cell); PSGL-1 (P-selectin glycoprotein ligand 1; ESL-1 E-selectin ligand 1
RAGE (receptor for advanced glycation end products); TLR2 (Toll like receptor 2); TLR4 (Toll like receptor 4)

involved in EPC homing as mouse EPCs deficient for cathepsin L show dysfunctional homing to an ischemic hindlimb[88].

Although HSC/EPC mobilization is well described, it is unclear whether MSCs actively home to tissues using the mechanisms as described above or just become trapped in capillaries[44]. Especially for *in vitro* expanded MSCs which are enlarged it is likely that they become trapped. However, selectin and integrin mediated homing of MSCs was previously reported[89,90]. Moreover, monocyte chemoattractant protein 3 (MCP-3) was shown to not only positively influence MSC homing to a MI area but also improved cardiac function[91].

Influencing cell mobilization

As previously mentioned, SDF-1α and its receptor CXCR4 play a central role in cell mobilization as described for plasma SDF-1α upregulation by an adenovirus[59]. AMD3100 - a competitive CXCR4 inhibitor - results in rapid mobilization of HSCs into the peripheral blood by disruption of the SDF-1α / CXCR4 balance[92]. Despite its positive effects on cell mobilization, AMD3100 was shown in a mouse model for MI to deteriorate infarction and left ventricular function in macrophage colony stimulating factor treated mice[93]. Many other chemokines, cytokines and growth factors described previously are used in a recombinant form to influence cell mobilization. For example, adenoviral mediated plasma upregulation of VEGF or VEGF and Ang-1 results in mobilization of EPCs and HSCs[59]. Recombinant Epo treatment in a mouse model of MI not only induces EPC mobilization, but also myocardial homing resulting in improved cardiac function[94]. Furthermore, recombinant GH positively influences HSC and EPC mobilization[22,73].
After MI, endogenous G-CSF upregulation correlates with CD34 cell numbers suggesting positive effects on cell mobilization[95]. G-CSF is clinically used by hematologists for HSC mobilization[22]. Furthermore, in a mouse model G-CSF treatment after MI improves cardiac function[96]. However, clinical studies using G-CSF have been disappointing[97]. The lack of positive effect may be explained by an impaired chemotaxis of progenitor cells due to CXCR4 inactivation by N-terminus cleavage after G-CSF treatment[60]. G-CSF was also reported to upregulate CD26 expression on $CD34^+$ cells suggesting increased mobilization capacities, but decreased homing capacities[98]. Furthermore, in a mouse and monkey model, G-CSF induced HSC mobilization was shown to be enhanced by a recombinant form of the chemokine Groβ - SB-251353[99]. A recent experimental study combining G-CSF stem cell mobilization with CD26 inhibition after MI was able to show increased myocardial cell homing and improved cardiac function[100].
Besides these chemokines and growth factors, medicines used for other indications are known to affect cell mobilization. Statins, 3-hydroxy-3-methylglutaryl co-enzyme A

reductase inhibitors, are used in patients with hyperlipidemia and are known to reduce cardiovascular morbidity and mortality and also influence cell mobilization[101]. Statins were previously shown to mobilize EPCs[102;103]. Patients on statin therapy with MI even show an enhanced spontaneous mobilization of peripheral blood progenitor cells[104]. Also intensive statin therapy after MI results in an enhanced EPC mobilization[105].
The ACE inhibitor enalapril was shown to mobilize EPCs in a hindlimb ischemia models. Both enalapril and ischemia increase bone marrow CD26 activity and decreased peripheral blood CD26 activity leading to EPC mobilization[106]. The angiotensin II receptor antagonists olmesartan and ibesartan also resulted in increased peripheral blood EPC counts in patients with type 2 diabetes[107]. Besides anti-hypertensive drugs, drugs used in diabetics such as the PPARγ agonist rosiglitazone and insulin were shown to mobilize EPCs[108;109].

Influencing cell homing

Many studies have recently investigated the SDF-1α / CXCR4 axis and its role in cardiovascular cell homing. Similarly to the hypoxic bone marrow with high SDF-1α levels, in ischemic myocardium high levels of SDF-1α are produced, resulting in the recruitment of CXCR4 expressing cells from the circulation to the ischemic heart[110-113]. Various groups have tried to positively influence cell homing thru manipulation of the SDF-1α / CXCR4 axis. For example SDF-1α gene therapy after MI was shown to enhance HSC recruitment tot the heart[114]. A different approach, using protease-resistant SDF-1α after acute MI, promoted recruitment of $CXCR4^+$/$c\text{-}kit^+$ cells and improved cardiac function[115]. Furthermore, local administration of SDF-1α to wounds of diabetic mice results in improved wound healing[116]. More recently, hypoxic preconditioning of heart derived cardiac progenitor cells was shown to result in increased CXCR4 expression, leading to increased homing to the ischemic myocardium[117].
Besides the CXCR4 receptor, a second receptor for SDF-1α exists - CXCR7. CXCR7 is involved in SDF-1α mediated T-cell and primordial germ cell migration by regulation of CXCR4 activity and SDF-1α internalization[118;119], and may also play a role in cell mediated homing for cardiac regeneration.

The SDF-1α / CXCR4 axis is negatively regulated by CD26, a surface serine dipeptidylpeptidase IV (DPPIV), that cleaves the amino-terminal peptide from SDF-1α, known to interact with the extracellular portion of CXCR4, and can interact and co-internalize with CXCR4[120;121]. CD26 thereby interferes with the SDF-1α / CXCR4 axis, inhibiting the recruitment of cells to SDF-1α. CD26 is expressed on many different cell types, including circulating hematopoietic cells and modulates their migratory capacities[120;122].

Recently, we showed that MNCs from patients with the vascular disease Hereditary Hemorrhagic Telangiectasia type 1 have a dysfunctional homing capacity to an infarcted area as a result of increased CD26 expression[5]. Blocking CD26 with DiprotinA, a selective CD26 antagonist, resulted in improved MNC and CXCR4+ cell homing towards a MI area[100] (Chapter 7). Furthermore, combined treatment of G-CSF and CD26 inhibition with DiprotinA after MI leads to stabilization of myocardial SDF-1α, reduced cardiac remodeling and improved cardiac function[100].
Other CD26 antagonists - sitagliptin and vildagliptin - are currently used as antidiabetic therapies[123]. There seems be a future role for these antagonists in clinical studies investigating cardiovascular cell homing.

Mobilization and homing in disease

Pathophysiologic circumstances are able to influence cell mobilization and cell homing. First of all, acute MI results in the mobilization of various cell populations: $CD34^{+}$[54;124;125], $CD133^{+}$[126], $CD34^{+}/CXCR4^{+}$[38], $CD34^{+}/CD117^{+}$[38], c-met^{+}[38] and VSEL ($lin^{-}CXCR4^{+}CD45^{-}CD133^{+}CD34^{+}$)[37] cells. Furthermore, in the MNC fraction, from which these cells are part off, early cardiac, endothelial and smooth muscle cell characteristics are upregulated after acute MI[38]. On the contrary, peripheral blood $CD45^{-}CD34^{-}$ (MSC-like) cells were reported to be decreased 7 days after MI[127]. Interestingly, mobilization of $CD34^{+}$, $CD117^{+}$, $CXCR4^{+}$ and c-met^{+} progenitor cells is positively correlated with LVEF, which suggests a positive effect of the mobilized progenitor cells on the cardiac function[128].
Second, reduced numbers of EPC were found in patients with risk factors for cardiovascular disease[129]. These cells were also shown to have an impaired migratory response to VEGF. Furthermore, reduced numbers of EPCs in patients with coronary artery disease predict future cardiovascular events[130]. Finally, unstable angina pectoris, heart failure, dilated cardiomyopathy, age, exercise and renal failure affect EPC mobilization as reviewed by Leone and coworkers[16].

Besides effects on cell mobilization, (patho)physiologic circumstances also alter cell homing either positively or negatively. Negative effects were shown for chronic ischemic cardiomyopathy, which decreases HSCs and bone marrow MNCs function; not only *in vitro* migration capacities to SDF-1α were reduced, but also *in vivo* neovascularization capacity in a himdlimb ischemia model was reduced[131]. Similar negative effects were found for EPCs from patients with coronary artery disease. In these EPCs CXCR4 signaling was shown to be disturbed, leading to an impaired neovascularization capacity[132].
Positive effects were shown for peripheral blood $CD133^{+}$ cells, which show an enhanced chemotactic response to VEGF and PlGF after MI[126], suggesting an increased capacity to home to the ischemic heart and may influence cardiac regeneration. However, this

enhanced chemotactic response as well as the total number of mobilized cells was decreased in diabetic patients suffering MI[133].

Manipulation of the SDF-1α / CXCR4 axis for cardiac regeneration in patients with cardiovascular disease will probably also affect atherosclerotic plaques, but currently it is unclear whether this will be a deleterious or beneficial effect. These previous studies suggest that the effects will depend on treatment timing, duration and use of other medications. In a mouse model, it was shown that blocking CXCR4 by AMD3100 resulted in attenuation of atherosclerotic plaque formation by bone marrow derived cells after M-CSF treatment[134]. However, a different study showed that chronic blockade of CXCR4 resulted in increased atherosclerotic plaque formation and that these plaques had an increased neutrophil content with a proinflammatory phenotype[135]. Interestingly, neutrophil depletion by a neutrophil specific antibody in combination with CXCR4 blockade reversed the increased plaque formation. Furthermore, $CD133^+$ cell infusion in a clinical trial was reported to be associated with an increased number of coronary events as in-stent reocclusion or restenosis[28]. It is currently unclear whether these adverse events are affected by interfering in the SDF-1α / CXCR4 axis.

The future: a challenge to facilitate mobilization and homing of peripheral blood derived cells to the heart

Although cellular therapy aiming at restoration of the dysfunctional part of the heart has been widely studied the past years, the optimal therapy for clinical patients still is controversial. Quite importantly, it is currently unclear which cell type is most beneficial for cardiac regeneration. The bone marrow, as well as the heart, harbors various progenitor cells with cardiomyocyte-like characteristics *in vitro* and *in vivo*. However, if the main beneficial effect of cellular therapy is a paracrine effect, differentiation capacities towards a cardiac phenotype do not seem essential.

It is also possible that the physiologic response after MI shows us which cells are most beneficial for cardiac regeneration. However, the (progenitor) cell numbers mobilized after MI are low and result in an insignificant amount of cardiac regeneration. Attempts have been made to enhance the physiologic cell mobilization after MI by cell mobilizing agents. But these mobilizers seem to inhibit cell homing capacities at the same time. Clinical intervention trials circumvene this issue by locally injecting cells, either in the infarct related artery[136] or intra myocardial[137]. This however only results in moderate improvement of the cardiac function[4].

Increasing our knowledge of the physiologic response after MI with respect to the regulated cell movements is essential to improve current cell based therapies for cardiac regeneration. In the future, this may even result in cellular therapy without

previous removal of cells or *in vitro* manipulation. Enhancement of the physiologic cell mobilization and cell homing by use of multiple agents with accurate timing seems to be an attractive method for cardiac regeneration.

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Part II | Chapter 3

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3

Early statin treatment prior to primary PCI for acute myocardial infarction

REPERATOR, a randomized placebo controlled pilot trial

Submitted

Abstract

Aims

In animal studies, early statin therapy reduces reperfusion injury after percutaneous coronary intervention (PCI) for ST-elevated acute myocardial infarction (STEMI). The aim of this pilot study was to determine whether early atorvastatin treatment will reduce left ventricle (LV) remodeling, infarct size and improve microvascular perfusion.

Methods and Results

Forty-two patients who underwent primary PCI for a first STEMI were randomized for pre-treatment with atorvastatin 80 mg (n=20) or placebo (n=22) and continued with the same dosage daily for one week. All patients received atorvastatin 80 mg once daily seven days after primary PCI. LV function and infarct size were measured by MRI within 1 day, at 1 week and 3 months follow up. The primary endpoint was the end-systolic volume index (ESVI) at 3 months. Secondary endpoints were global LV function measurements, myocardial infarct size, biochemical cardiac markers, TIMI flow and ST-T elevation resolution. ESVI three months after STEMI was 25.1 mL/m² in the atorvastatin arm and 25.0 mL/m² in the placebo arm (p=0.74). As well the secondary endpoints did not show any differences between both treatment arms.

Conclusion

Pre-treatment with atorvastatin in STEMI does not result in an improved cardiac function, microvascular perfusion or decreased myocardial infarct size.

Introduction

Early percutaneous coronary intervention (PCI) during acute myocardial infarction (AMI) is of great importance to reduce infarct expansion and improve left ventricular (LV) remodeling[1]. Although reperfusion is required for myocardial tissue salvage, the abrupt reperfusion of ischemic myocardium may add extra damage to the myocardium, leading to reperfusion associated pathologies. Reperfusion injury is associated with four types of cardiac dysfunction: reperfusion induced arrhythmias, myocardial stunning, reversible microvascular injury (no reflow phenomenon) and irreversible cell damage. Irreversible reperfusion cell damage may be decreased by various cardioprotective agents[2,3]. A recent clinical pilot trial showed that cyclosporine treatment during AMI reduced reperfusion injury resulting in a smaller infarct[4].

Statins, 3-hydroxy-3-methylglutaryl co-enzyme A reductase inhibitors, are used in patients with hyperlipidemia and are known to reduce cardiovascular morbidity and mortality[5]. Many experimental animal studies have shown that statin treatment during reperfusion reduces myocardial infarct size by attenuation of reperfusion injury[2,6]. Furthermore, LV remodeling and function improve by statin therapy and is associated with improved survival[7-9]. The effect of early statin treatment on reperfusion injury and LV remodeling in patients who underwent a primary PCI has never been investigated. Therefore, we wanted to determine whether early atorvastatin treatment could reduce LV remodeling, infarct size and improve microvascular perfusion.

Methods

Patient Population

Between March 2006 and November 2007 fifty-five consecutive patients were included in the REPERATOR trial (Prevention of REPERfusion Damage and Late Left Ventricular Remodelling With ATORvastatin Administered Before Reperfusion Therapy)[10]. Patients were included in the St. Antonius Hospital Nieuwegein and University Medical Centre Utrecht, The Netherlands. All patients presented with a first ST-elevated acute myocardial infarction (STEMI) and were treated with PCI. Exclusion criteria were previous myocardial infarction, no sinus rhythm, electrical instability, Killip class 3 or 4 of heart failure, need for intra-aortic balloon counterpulsation therapy, contraindications for magnetic resonance imaging (MRI) investigation, age < 18 years and prior use of statins.

At inclusion, patients were double blind randomized to treatment with atorvastatin 80mg or placebo once daily starting prior to primary PCI. From day eight after PCI all patients were treated with atorvastatin 80mg once daily.

Randomization was done in blocks of 8 and was performed by pulling a sealed envelop containing the study medication assignment (A or B). All procedures were approved by the medical ethics committee of both hospitals. The investigation conforms to the principles outlined in the Declaration of Helsinki. Oral informed consent was obtained prior to primary PCI. Written informed consent was obtained directly after primary PCI. The trial was registered at ClinicalTrials.gov under identification number NCT00286312.

MR Imaging

Early and late LV function and infarction size were assessed by MRI (1.5 T Philips®, Best, The Netherlands), at baseline (within one day), at 7 days and 3 months after AMI. Steady state free precession cine sequences and gadolinium-enhanced images were analyzed using a 12 segment, 6-20 slice model. MRI scans were interpreted by one observer blinded for treatment. Left ventricle end-systolic volume (LVESV), left ventricle end-diastolic volume (LVEDV), left ventricle ejection fraction (EF), cardiac output (CO), percentage enhanced area (PEA), and percentage transmural infarcted area were calculated. The percentage transmural infarcted area was calculated from all slices that were more than 50% gadolinium enhanced.

Angiographic data

Standard emergency PCI was performed to achieve recanalization and revascularization of the infarct related artery. The Thromolysis In Myocardial Infarction (TIMI) angiographic scale and Corrected TIMI frame Count were used to determine the recanalization status after PCI and was assessed visually as described previously[11;12]. Myocardial blush grades were scored as previously described: 0, no myocardial blush; 1, minimal myocardial blush or contrast density; 2, moderate myocardial blush or contrast density; 3, normal myocardial blush or contrast density[13].

ECG analysis

Pre procedural and three hour post procedural 12-lead electrocardiogram (ECG) recordings were assessed by 1 observer blinded for treatment. The sum of ST-segment elevation was measured manually 20ms after the end of the QRS complex from leads I, aVL and V1 through V6 for left anterior descending coronary artery occlusions and leads II, III aVF, V5, V6 and reciprocal ST-segment depressions in V1 and V2 for right coronary artery and left circumflex artery occlusions. Resolution of ST-segment elevation was expressed as a percentage of the initial ST-segment elevation[14;15]. Impaired microvascular reperfusion was defined as less than 70% ST-segment resolution.

Biochemical markers

Creatinin kinase and MB fraction were determined every eight hours after PCI till peak values were reached.

Endpoints

Primary endpoint is end-systolic volume index (ESVI) at three months after AMI. The end-systolic volume index is calculated by dividing the end-systolic volume with the body surface area. Secondary endpoints are global and regional left ventricular function, enhanced area, changes in these measurements between MRI investigations, biochemical markers, TIMI flow, TIMI frame count, blush score and ST-T segment resolution.

Statistics

The sample size was determined before enrolment of any patients[10]. With a power of 0.9 and an alpha error of 0.05, 24 patients per treatment arm had to be enrolled to show a 10% reduction in LVESVI in the treatment arm. Statistical significance was evaluated using the Mann-Whitney U test for comparison between two independent samples, Friedman test for three related samples, Wilcoxon Signed Ranks test for two related samples (Post Hoc analysis with Bonferroni correction after Friedman test) and Pearson Chi-Square test or Fisher's Exact test for comparison of 2 categorized variables. SPSS v16.0 for Windows was used. Results are expressed as means ± standard deviation (SD). A value of $P < 0.05$ was considered statistically significant. All reported P values are two-sided.

Results

Patient characteristics

The study population consisted of forty-two patients (82% male, mean age 61.2 ± 9.8). Thirteen patients out of fifty-five included patients were excluded. Within the excluded patient group, two patients died within one week due to progressive cardiac failure, eight patients withdrew themselves from the study because of claustrophobia, one patient was excluded due to ventricular septal rupture, one patient was excluded because of logistic reasons and one patient was excluded because of previous statin use (Figure 1). The baseline characteristics are summarized in Table 1.

Primary Endpoint

The primary endpoint, LVESVI at three months after AMI is 25.1 mL/m² in the atorvastatin arm and 25.0 mL/m² in the placebo arm (P=0.74, Figure 2, Table 2). The change in ESVI comparing baseline, 1 week and 3 months, is not significantly different between both groups (Table 2).

Table 1: Patient characteristics

	Placebo, n=22	Statin, n=20	Placebo vs. Statin
Age, years	64.6±10.3	57.5±7.7	0.02
Gender (male), n (%)	19(86)	13(65)	0.15
Body Mass Index, kg/m^2	26.4±3.8	28.4±4.2	0.15
Laboratory parameters			
Cholesterol, mmol/L§	4.39±0.68	4.88±0.98	0.10
HDL-cholesterol, mmol/L‖	1.06±0.36	0.84±0.25	0.04
LDL, mmol/L‖	2.87±0.56	3.38±0.80	0.02
Triglycerides, mmol/L‖	1.09±0.49	1.44±0.88	0.07
Glucose, mmol/L*	8.2±2.6	7.8±2.3	0.69
Creatinin, µmol/L	89±17	80±18	0.09
Haemoglobin, mmol/L	8.8±0.92	8.9±1.2	0.95
Leucocytes, G/L	11.7±3.2	12.7±3.9	0.36
Medications before AMI			
ACE-inhibitors, n (%)†	2(11)	2(13)	1.00
Beta-blockers, n (%)†	0(0)	0(0)	N/A
Angiotensin-II-receptor antagonists, n (%)†	1(5)	2(13)	0.58
Calcium antagonists, n (%)†	1(5)	2(13)	0.58
Diuretics, n (%)†	1(5)	4(25)	0.16
Medications after AMI			
Thrombocyte coagulation inhibitors, n (%)	22(100)	20(100)	N/A
Statin, n (%)	22(100)	20(100)	N/A
ACE-inhibitors, n (%)‡	12(55)	11(58)	1.00
Beta-blockers, n (%)‡	20(91)	16(84)	0.65
Angiotensin-II-receptor antagonists, n (%)‡	1(5)	2(11)	0.59
Calcium antagonists, n (%)‡	1(5)	3(16)	0.32
Myocardial infarction			
Anterior infarction, n (%)	7(32)	3(15)	0.28
Total ischemic time, minutes	226±136	217±176	0.37
Risk factors			
Smoking / history of smoking, n (%)	15(68)	17(85)	0.28
Diabetes, n (%)	5(23)	1(5)	0.19
Hypertension, n (%)	5(23)	11(55)	0.06
Hypercholesterolemia, n (%)§	4(19)	6(30)	0.48
Positive family history, n (%)	9(41)	11(55)	0.54

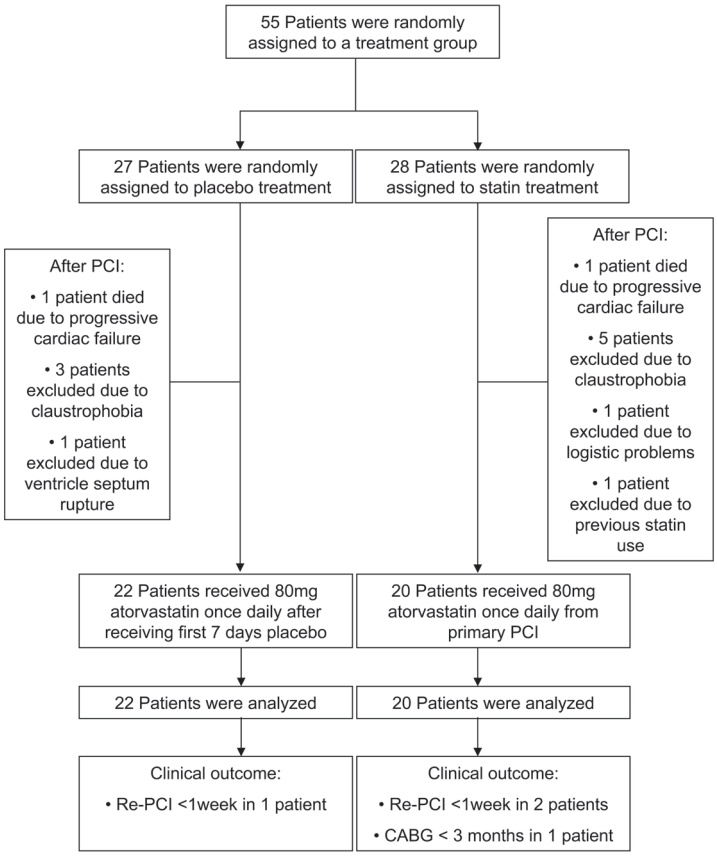

Figure 1
Schematic representation of enrolment, group assignments and follow-up of patients.

Table 1
The placebo treated patients are significantly older than statin treated patients. Baseline HDL- and LDL-cholesterol were significantly lower in first week statin treated patients. All other parameters including medications and risk factors were equally distributed between the patient groups. Data are presented as number (percentage) or mean ± SD. *placebo n=19, statin n=17; †placebo n=19, statin n=16; ‡placebo n=22, statin n=19; §placebo n=21, statin n=20; llplacebo n=20, statin n=20; due to missing data. N/A = not available. AMI=Acute Myocardial Infarction ACE=angiotensin converting enzyme

Early and late cardiac function

No differences between both groups in LVESV, LVEDV, EF, and CO at 3 months follow up compared to baseline are found (Table 2). Within the early statin group, baseline EF is significantly lower than EF at three months (P=0.033, Table 2). Change in CO from baseline to 1 week, is significantly different between placebo and early statin group reflecting a higher increase in the statin group (P=0.037, Table 2).

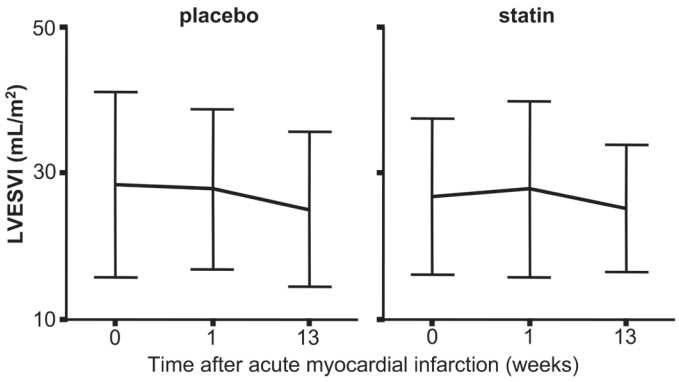

Figure 2
The primary endpoint, LVESVI, was measured by MRI analysis at baseline, 1 week and 13 weeks post-AMI. No significant differences were found between statin or placebo treated patients and between the time points. This suggests that early statin treatment does not result in improved early and late LV remodeling. Points show mean ±SD.

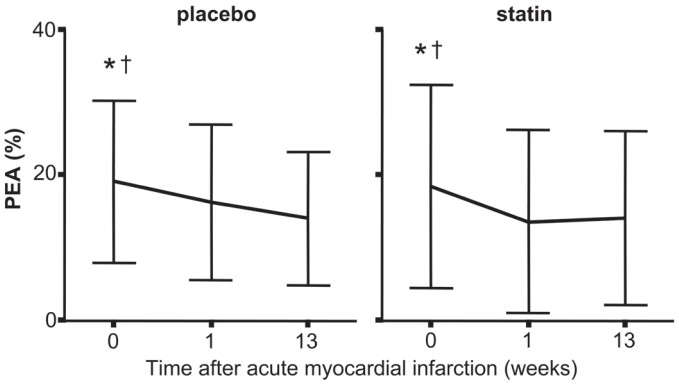

Figure 3
PEA was measured at baseline, 1 week and 13 weeks post-AMI by MRI. No significant differences were found between placebo and early statin treated patients. PEA decreases significantly in time. These data show that early statin treatment does not result in a decreased infarction size. *P<0.01 0 vs 1 week; †P<0.01 0 vs 13 weeks. Points show mean ±SD.

Infarct area

Infarct size measured by PEA and change in PEA is not different between placebo and early statin group (Figure 3, Table 2). Within the placebo and early statin group, PEA shows a significant decrease in time (placebo P<0.001; post hoc analysis: 0 vs 1 week P=0.012; 0 vs 13 weeks P<0.003; statin P=0.001; post hoc analysis: 0 vs 1 week P=0.003; 0 vs 13 weeks P=0.012). Furthermore, the percentage transmural infarcted area does not

Table 2: Cardiac function and infarction size

	Placebo, n=22			Statin, n=20		
	baseline	**1 week after AMI**	**13 weeks after AMI**	**baseline**	**1 week after AMI**	**13 weeks after AMI**
Cardiac function						
LVESVI, mL/m^2	28.4±13	27.8±11	25.0±11	26.8±11	27.8±12.1	25.1±8.8
LVESV, mL	55.9±25	55.1±21	49.8±21	55.2±24	56.2±25	51.8±19
LVEDV, mL	116±33	121±31	117±31	115±30	124±30	119±31
EF, %	53.3±11	55.3±10	58.7±11	52.8±11†	55.3±12	56.9±11
CO, L/min	4.33±0.91	4.12±1.0	4.04±0.82	4.11±0.93	4.46±1.2	4.18±1.1
Infarction area						
PEA (%)	19.0±11*†	16.2±11	14.0±9.1	18.4±14*†	13.6±13	14.1±12
Transmural infarcted heart (%)	11.3±10*	8.4±9.2	7.5±8.3	10.2±11	7.2±11	7.9±11
Biochemical markers						
Peak CK, U/L	1971±1448			2588±3852		
Peak CK-MB, U/L	209±156			272±331		
	Change, time after AMI			**Change, time after AMI**		
	1 vs. 0 weeks	**13 vs. 0 weeks**	**13 vs. 1 weeks**	**1 vs. 0 weeks**	**13 vs. 0 weeks**	**13 vs. 1 weeks**
Cardiac function						
LVESVI, mL/m^2	-1.1±5.7	-3.7±9.1	-2.8±6.2	1.4±8.4	-2.1±11	-2.9±12
LVESV, mL	-2.3±12	-7.2±18	-5.4±12	2.1±17	-4.5±23	-5.1±25
LVEDV, mL	2.8±17	-1.3±22	-4.3±16	11±22	4.6±31	-5.5±32
EF, %	2.5±5.3	5.6±7.7	3.4±6.8	2.2±7.7	5.2±7.2	1.9±8.7
CO, L/min	-0.22±0.9§	-0.30±0.8	-0.08±0.9	0.38±0.9	0.13±0.7	-0.36±0.9
Infarction area						
PEA (%)	-3.0±3.7	-5.3±4.1	-2.2±4.7	-2.9±2.7	-4.9±6.6	-1.7±5.0
Transmural infarcted heart (%)	-2.8±3.6	-3.4±5.8	-0.9±4.8	-2.3±4.3	-2.7±6.5	-0.91±4.4

Overview of parameters and change of cardiac function and PEA measured by MRI and biochemical markers for infarction. Data are presented as mean or percentage ± SD. *P<0.04 baseline versus 1 week after AMI; †P<0.04 baseline versus 13 weeks after AMI; ‡P<0.04 1 week versus 13 weeks after AMI; §P=0.037 placebo versus statin. LVESVI=Left Ventricular End-systolic volume index LVEDV=Left Ventricular End-diastolic volume EF=Ejection Fraction CO=Cardiac Output PEA=Percentage Enhanced Area

show differences between placebo and early statin. Biochemical markers for AMI, peak creatinin kinase and MB fraction, are similar (Table 2).

Microvascular perfusion

The quality of microvascular (re)perfusion, as assessed by ST-T segment resolution, TIMI flow grade, Corrected TIMI frame Count and Blush score, does not show significant differences between the study groups (Table 3).

Clinical outcome

Two patients died within 1 week after PCI due to progressive cardiac failure related to anterior myocardial infarction. One patient was in the early statin arm, the other patient was in the placebo arm. In one patient treated with placebo, in-stent thrombosis occurred 30 minutes after primary PCI. The thrombus was aspirated and the patient was treated with abciximab resulting in TIMI 3 flow. One other patient treated with early statin, re-PCI was performed within 24 hours due to recurrent complaints and suboptimal result of stenting. One early statin treated patient was referred for CABG within three months after PCI (Figure 1).

Transmural infarctions

Subgroup analysis of 15 placebo and 14 early statin treated patients with a transmural infarction shows similar results compared to all myocardial infarctions (Table 4 and 5).

Table 3: Microvascular (re)perfusion

	Placebo, n=22	Statin, n=20	Placebo vs. Statin, P
ST-T segment resolution, %*	75±25	71±20	0.30
ST-T segment resolution ≥70%, n (%)*	13(62)	11(55)	0.76
TIMI flow 3 after PCI, n (%)*	21 (100)	18(90)	0.23
Corrected TIMI frame count 30, frames†	18,9±6,9	26,8±21,1	0.30
Blush score ≤ 2, n (%)‡	10 (50)	10 (63)	0.45

Microvascular (re)perfusion was assed by angiographic and electrocardiographic measurements. No significant differences were found between the study groups. Data are presented as mean or number ± SD. *placebo n=21, statin n=20; † placebo n=18, statin n=19; ‡ placebo n=20, statin n=16; due to missing data. TIMI = Thrombolysis in Myocardial Infarction

Table 4: Transmural infarction

	Placebo, n=15			Statin, n=14		
	baseline	**1 week after AMI**	**13 weeks after AMI**	**baseline**	**1 week after AMI**	**13 weeks after AMI**
Cardiac function						
LVESVI, mL/m^2	28.6±14	29.0±13	27.2±11	28.8±12	30.4±13	26.7±9.0
LVESV, mL	54.5±26	56.0±24	52.7±22	59±28	61±27	54±19
LVEDV, mL	112±32	120±33	118±33	116±34	130±32	120±30
EF, %	52.9±12.3	54.6±11.2	56.7±11.0	50.1±12	53.7±13	54.9±12
CO, L/min	4.21±0.76	4.14±0.72	3.97±0.83	4.09±1.0*	4.68±1.3‡	4.11±1.2
Infarction area						
PEA (%)	23.9±8.8†	20.5±9.3‡	17.0±8.5	23.8±12*†	19.0±11	18.5±11
Transmural infarcted heart (%)	15.8±8.3*†	11.9±9.1	10.0±8.7	13.9±10	10.6±12	10.9±12
Biochemical markers						
Peak CK, U/L	2323±1496			3498±4318		
Peak CK-MB, U/L	236±158			363±359		
	Change, time after AMI			**Change, time after AMI**		
	1 vs. 0 weeks	**13 vs. 0 weeks**	**13 vs. 1 weeks**	**1 vs. 0 weeks**	**13 vs. 0 weeks**	**13 vs. 1 weeks**
Cardiac function						
LVESVI, mL/m^2	-0.25±6.1	-2.5±10	-1.8±6.8	2.1±9.7	-3.1±13	-4.2±13
LVESV, mL	-0.18±12	-4.4±20	-3.3±12	3.4±19	-7.0±26	-8.2±27
LVEDV, mL	5.2±17	1.8±25	-1.8±14	16±21	2.4±33	-11±28
EF, %	2.0±5.0	4.2±7.3	2.1±6.2	3.4±8.2	6.1±7.9	2.0±9.0
CO, L/min	-0.12±0.70	-0.32±0.81	-0.17±0.81	0.65±0.70	-0.01±-.62	-0.64±0.53
Infarction area						
PEA (%)	-3.3±4.4	-6.9±3.7	-3.5±5.1	-3.7±2.7	-6.2±7.0	-2.6±5.7
Transmural infarcted heart (%)	-3.9±3.8	-5.8±4.8	-1.9±4.7	-3.2±4.8	-3.5±7.3	-1.3±5.2

Analysis of transmural infarcted hearts. Overview of parameters and change in cardiac function and PEA measured by MRI and biochemical markers for infarction. Data are presented as mean or percentage ± SD. *P<0.04 baseline versus 1 week after AMI; †P<0.04 baseline versus 13 weeks after AMI; ‡P<0.04 1 week versus 13 weeks after AMI. §P=0.014 placebo versus statin. Data are presented as mean or percentage ± SD. LVESVI=Left Ventricular End-systolic volume index LVEDV=Left Ventricular End-diastolic volume EF=Ejection Fraction CO=Cardiac Output PEA=Percentage Enhanced Area

Table 5: Microvascular (re)perfusion of transmural infarctions

	Placebo, n=15	Statin, n=14	Placebo vs. Statin, P
ST-T segment resolution, %*	73±14	72±22	0.50
ST-T segment resolution ≥70%, n (%)*	7(50)	8(57)	1.00
TIMI flow 3 after PCI, n (%)	15(100)	13(87)	0.48
Corrected TIMI frame count 30, frames†	19.7±6.7	28.1±22.7	0.40
Blush score ≤ 2, n (%)‡	6 (43)	7 (70)	0.24

Analysis of transmural infarcted hearts. Microvascular (re)perfusion was assed by angiographic and electrocardiographic measurements. No significant differences were found between the study groups. Data are presented as mean or number ± SD. *placebo n=14, statin n=14; †placebo n=12, statin n=13; ‡placebo n=14, statin n=10; due to missing data. TIMI = Thrombolysis in Myocardial Infarction. Data are presented as mean or percentage ± SD.

Discussion

Many experimental and clinical studies have previously been performed with the objective of attenuating reperfusion injury[3]. To our knowledge, this is the first clinical study examining reperfusion injury with early statin treatment prior to PCI during STEMI. The results of this pilot trial indicate that treatment with atorvastatin prior to primary PCI is safe, but does not result in improved LV remodeling and microvascular perfusion or decreased myocardial infarct size.

Animal studies using HMG-CoA reductase inhibitors – statins – to reduce reperfusion damage show favourable results. Not only myocardial infarction size was reduced with statin treatment during reperfusion[2;6], but also LV function was positively influenced[9;16] and the incidence of severe ventricular ischemic arrhythmias was decreased[17]. Statin treatment results in a rapid upregulation of the PI3K/Akt cell-survival pathway, leading to an increase in eNOS and Akt phosphorylation[6]. Activation of this pathway protects the reperfused myocardium. The effects on LV remodeling are thought to be mediated by attenuation of cardiac matrix metalloproteinase (MMP) activity and of collagen production[9;18]. Furthermore, endothelial progenitor cells are mobilized by both statins[19] and acute myocardial infarction[20]. Early statin treatment during acute myocardial infarction could enhance endothelial progenitor cell mobilization and reduce myocardial infarction area by stimulating angiogenesis.
However, major differences between animal models and clinical studies for ischemia-reperfusion injury exist. Clinical studies usually consist of a relative heterogeneous patient group and patients are treated with medication which positively influences the clinical outcome. Furthermore, the duration of myocardial ischemia, follow up and

infarct size are all different in clinical studies when compared to animal models[3].

Many previously performed clinical studies were designed to answer the question whether early statin treatment in acute coronary syndromes (ACS) positively influences clinical outcome. A meta-analysis of randomized controlled trials, including more than 13.000 patients, showed that initiation of statin therapy within 14 days following onset of ACS did not reduce death, myocardial infarction or stroke[21]. However, various observational studies showed positive effects of early statin use in patients with ACS on clinical outcome defined as myocardial infarction and mortality[22-24]. Cahoon et al reviewed the published literature on the effectiveness of pre-procedural statin therapy for the prevention of cardiac events after elective PCI. This review suggests that, in stable patients and in patients with a recent ACS, pre-procedural statin therapy results in reduced post-PCI myocardial necrosis[25]. However, patients with ongoing AMI at the time of PCI were excluded from these studies.

Clinical studies assessing the effect of chronic statin treatment on fibrinolytic therapy or primary PCI in AMI suggest that this results in reduced infarct size and improved perfusion[26-28]. This positive outcome could be influenced by pre-AMI treatment with thrombocyte coagulation inhibitors, ACE-inhibitors or beta-blockers which chronic statin patients were more likely to receive.

Furthermore, a recent randomized clinical trial investigating patients with non-ST-elevation ACS, showed that short term pre-treatment with atorvastatin reduced peri-procedural myocardial infarction in patients undergoing early PCI, as measured by biochemical markers[29]. It is difficult to extrapolate the results of this study to patients with STEMI as intermittent myocardial ischemia may initiate ischemic preconditioning, thereby affecting reperfusion injury.

In our double blind, randomized trial we performed detailed measurements of LV function. Although the study was underpowered for clinical outcome, detailed measurements of cardiac function by MRI have a high interstudy reproducibility[30]. Therefore, even a low sample size is sufficient to show clinically relevant changes in LV function and volume. A major determinant of survival after recovery from AMI is LVESV[31]. We chose the LVESV index as primary endpoint as this is an attractive surrogate endpoint in a trial aiming at post infarction remodelling.

Measurement of the PEA early after AMI with reperfusion may overestimate the infarction area[32]. PEA 3 months after AMI correlated well with PEA at baseline and 1 week after AMI. Therefore, PEA measurements of all time points were included in the data analysis.

In this pilot trial the primary and secondary endpoints did not show any differences

between placebo and early statin treated patients. However, change in CO, baseline versus 1 week, is significantly different between placebo and early statin group. This reflects a relatively high increase in CO in the first week in the early statin group compared to the early placebo group (table 2). CO at 3 months in the early statin group is comparable to the baseline value. As all other cardiac function parameters and change in value show no significant differences, we consider this as a clinical not relevant observation.

Several determinants might be responsible for the lack of left ventricular functional improvement in this study. First of all, our sample size could be too small to show a difference in treatment. Due to the high number of excluded patients, the calculated group size was not reached. However, when extrapolated from the present data it is unlikely that significant differences in primary and secondary endpoints would be found if calculated group sizes were reached. Furthermore, when compared to animal studies which show an infarct reduction of around 50%, our groups are appropriately sized.
Secondly, previous research indicates that cardioprotective agents may be most worthwhile in large infarctions[33]. The patients included in our study have relatively high EF and low PEA. Subgroup analyses of transmural infarctions shows similar results as the main analysis. However, this observation is limited by the small sample size.
Thirdly, the significant difference in age between the study arms could bias our results. It is however unlikely that reperfusion injury is increased in younger patients, resulting in atorvastatin normalized cardiac function, PEA and TIMI flow.
Fourthly, patients were treated with 80 mg atorvastatin as in a large clinical trial was shown that 80 mg atorvastatin was superior to 40 mg pravastatin with respect to major cardiac adverse events after ACS[34]. One could speculate that the 80 mg atorvastatin dosage is too low to result in attenuating reperfusion injury. In humans, atorvastatin 80mg daily results in serum levels of 0.05-0.4 µmol eq/L atorvastatin[35]. Whereas animal experiments showing attenuation of reperfusion injury used atorvastatin concentrations around 25 µmol/L and a decreased response at 5µmol/L[6]. Also the oral administration of atorvastatin prior to PCI could be inefficient to reach high enough serum levels to attenuate reperfusion injury.
Finally, a study using rosuvastatin in mice showed that it attenuated infarction size when rosuvastatin was administered 6 hours before myocardial ischemia. No attenuation of infarction size was found when rosuvastatin was administered 0 or 3 hours before ischemia[36]. The timing of atorvastatin treatment in our study – during ongoing myocardial ischemia – may therefore affect our results.
In conclusion, early statin treatment in humans does not result in major infarct reduction or improvement of LV function as previously found in animal models. It remains to be

investigated whether statin treatment prior to primary PCI in STEMI improves clinical outcome.
Future prospective, randomized trials are warranted to further assess the cardioprotective effect of early statin therapy during acute myocardial infarction.

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Part II | Chapter 4

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4

The physiologic response of peripheral blood derived cells, cytokines and growth factors after acute myocardial infarction and effects of early statin treatment

Insights from the REPERATOR trial, I

In preparation

Abstract

Introduction

The peripheral blood contains cells, cytokines and chemokines that are upregulated after myocardial infarction (MI) and may improve or deteriorate cardiac recovery and repair. Statin therapy was previously shown to mobilize progenitor cells and may also positively affect cardiac function. We therefore studied 1) the effects of MI on circulating (progenitor) cells, cytokines and growth factor levels, 2) its relation to cardiac function and 3) effects of early statin treatment.

Methods and Results

Forty-two patients who underwent primary PCI for a first ST-elevated MI were randomized for treatment with atorvastatin 80 mg (n=20) or placebo (n=22) and continued with the same dosage daily for one week. All patients received atorvastatin 80 mg once daily from day seven after primary PCI. Blood samples were collected and analyzed at primary PCI, 1 week and 3 months after MI. Furthermore, blood samples from 10 patients with stable coronary artery disease were analyzed. Left ventricular function and infarct size of the MI patients were measured by magnetic resonance imaging within 1 day, after 1 week and 3 months follow up. $CD34^+$ and $CD34^+VEGFR2^+$ cell numbers were increased after MI. Furthermore, plasma levels of soluble stromal derived factor-1α (SDF-1α), erythropoietin (Epo) and angiopoietin 2 (Ang-2) were maximal 1 week after MI, whereas hepatocyte growth factor (HGF), transforming growth factor β (TGFβ), soluble endoglin (sEng) and Ang-1 levels showed peak values at baseline. Large infarctions or early worsening of cardiac function were associated with early $CD34^+VEGFR2^+$ cell mobilization, increased HGF, Epo, vascular endothelial growth factor levels and decreased sEng levels. However, no association between increased progenitor cell numbers and cytokine/growth factor levels with improved cardiac function outcome was found. Early statin treatment increased $CD133^+$ progenitor cell mobilization 1 week after MI, when compared to placebo, but no effects were found on cytokine levels/growth factor levels.

Conclusions

Our results show an increased systemic response of progenitor cell mobilization and cytokine/growth factor upregulation to relatively large infarctions and unfavourable hemodynamic conditions. Early statin therapy after MI only affects $CD133^+$ progenitor cell mobilization. As no associations were found between cardiac function outcomes, physiologic progenitor cell mobilization or cytokine/growth factor upregulation within all MI patients, this mobilization/upregulation is probably to low to result in any clinical relevant effects.

Introduction

Myocardial infarction (MI) is the leading cause of death world wide[1]. Despite treatment with beta-blockers, percutaneous coronary intervention (PCI) and statins, MI still has a high morbidity and additional therapies are needed[2]. Early after MI, various circulating (progenitor) cells, cytokines and growth factors were shown to be upregulated[3-6]. In patients with acute MI, the number of mobilized progenitor cells was reported to be positively correlated with left ventricular ejection fraction (LVEF) after MI[7], suggesting that acute ischemia resulting in myocardial necrosis is a stimulus to mobilize progenitor cells which may positively influence left ventricular function. Experimental studies which systemically infused erythropoietin (Epo) or hepatocyte growth factor (HGF) after MI showed to be beneficial for cardiac performance[8;9] – in part this was through an effect on EPC mobilization. Peripheral blood progenitor cell mobilization is known to be further enhanced in patients with MI on statin therapy[10;11]. Furthermore, preclinical mice and rat studies showed statin-induced improvement of endothelial progenitor cell (EPC) mobilization, EPC differentiation, myocardial neovascularisation, left ventricular (LV) function, interstitial fibrosis and survival after experimentally induced MI[12-14].

In two centres in Utrecht and Nieuwegein, we recently conducted the REPERATOR study[15]: Prevention of REPERfusion of damage with ATORvastatin. The REPERATOR study allowed us 1) to study temporal changes in blood derived cells, cytokine and growth factor levels after MI and 2) its relation to cardiac function as measured by MRI. Furthermore, 3) the effects of early statin treatment on progenitor cell mobilization, cytokine and growth factor levels were assessed. We hypothesized that increased progenitor cell mobilization or cytokine/growth factor release positively affects cardiac recovery as measured by MRI. Furthermore, early statin treatment post MI may further augment progenitor cell mobilization and cardiac recovery.

Methods

Patients

Between March 2006 and November 2007 fifty-five consecutive patients were included in the REPERATOR trial[15]. Patients were included in the St. Antonius Hospital Nieuwegein and University Medical Center Utrecht, The Netherlands. All patients presented with a first acute ST-elevation-MI and were treated with PCI. Exclusion criteria were previous myocardial infarction, no sinus rhythm, electrical instability,

Killip class 3 or 4 of heart failure, need for intra-aortic balloon counterpulsation therapy, contraindications for MRI investigation, age < 18 years and prior use of statins.

Study design

At inclusion, patients were randomized to treatment with atorvastatin 80 mg or placebo once daily starting prior to primary PCI. From day eight after PCI all patients were treated with atorvastatin 80mg once daily (for details see Chapter 3). Furthermore, we included ten patients with stable coronary artery disease (CAD). These patients were scheduled for coronary angiography or elective PCI and had a history of documented coronary artery disease. Exclusion criteria were: acute MI within the previous 12 months; acute coronary syndrome within the previous six months; suspected or proven cancer. By including CAD patients as controls for MI patients, we were able to assess the effect of MI on peripheral blood derived cell characteristics, cytokines and growth factor levels. Blood was drawn instantly before emergency coronary artery intervention, at second and third MRI. From the patients with stable CAD blood was drawn at one time point. Blood samples were collected in Potassium/EDTA tubes (Vacuette, Greiner Bio-One, The Netherlands). All procedures were approved by the medical ethics committee of both hospitals. The investigation conforms to the principles outlined in the Declaration of Helsinki. Oral informed consent was obtained prior to primary PCI. Written informed consent was obtained directly after primary PCI. The trial was registered at ClinicalTrials.gov under identification number NCT00286312.

MR Imaging

Early and late LV function and infarct size of REPERATOR patients were assessed by MRI with gadolinium enhancement (1.5 T Philips®, Best, The Netherlands), at baseline (within one day), at 7 days and 3 months after MI. Standard measurements were calculated including left ventricle end-systolic volume (LVESV) and percentage enhanced area (PEA).

Flow cytometry

Flow cytometric analysis was performed using 100µL whole blood. Cells were stained using the following monoclonal mouse-anti-human antibody combinations: anti-CD14-PE (DakoCytomation, Denmark) and anti-Endoglin-Fluorescein (R&D Systems, USA); anti-CD34-FITC (BD Pharmingen, USA) and anti-VEGFR2-PE (R&D Systems, USA); anti-CD133-PE (Miltenyi Biotec, Germany); anti-Sca-1-PE (BD Pharmingen, USA). Isotype-matched fluorochrome-conjugated antibodies were used as controls. After incubation, samples were washed and red blood cells were lysed before measuring fluorescence on a flow cytometer (Cytomics FC500, Beckman Coulter, The Netherlands), Analysis was

performed using CXP software (Beckman Coulter, The Netherlands). The number of positive cells is expressed as absolute cell number per mL of whole blood. The Mean-Fluorescent Intensity (MFI) is presented for cell populations of interest.

Biochemical measurements

Plasma samples were centrifuged twice at 1000g for 15 minutes and were immediately frozen and stored at -80˚C. The concentrations of angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), stromal derived factor -1α (SDF-1α), Epo, HGF, soluble endoglin (sEng) and vascular endothelial growth factor165 (VEGF) were measured using commercially available ELISA kits (R&D Systems, Inc, Minneapolis, USA). In addition, plasma soluble NT-pro-BNP, glucagon like protein-1 (GLP-1) and transforming growth factor-β_1 (TGFβ) were measured using commercially available ELISA kits (Biomedica, Wien, Austria / Alpco Diagnostics, Salem, USA / Bender MedSystems, Vienna, Austria, respectively). Hs-CRP, cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, glucose, creatinin, haemoglobin and leucocytes were measured using routine laboratory tests.

Statistics

Statistical significant difference between placebo and early statin treated patients was determined using the Mann-Whitney U test. If no significant difference was found, we combined the data from both patient groups to determine the effect of MI in time on blood derived cells, cytokines and growth factors. Statistical significance was evaluated with the Friedman test for three related samples, Wilcoxon Signed Ranks test for two related samples (Post hoc analysis with Bonferroni correction after Friedman test), Spearman's rho for correlation calculations and Fisher's Exact test for comparison of 2 categorized variables. Furthermore differences between MI and stable CAD patients were determined using the Mann-Whitney U test for two unrelated samples using SPSS v16.0 for Windows. Results are expressed as median ± interquartile range. A value of $P < 0.05$ was considered statistically significant. All reported P values are two-sided.

Results

Patient characteristics

The study population consisted of forty-two patients with acute MI (82% male, median age 57.0 ± 13.5 years and ten patients with stable coronary artery disease (90% male, median age 66.0 ± 22 years). No significant differences in age or gender were found between the MI patients and stable CAD patients. Thirteen patients out of fifty-five acute MI patients were excluded: two patients died within one week due to progressive cardiac

failure, eight patients withdrew themselves from the study because of claustrophobia, one patient was excluded due to ventricular septal rupture, one patient was excluded because of logistic reasons and one patient was excluded because of previous statin use. The baseline characteristics for MI patients are summarized in Table 1 of Chapter 3.

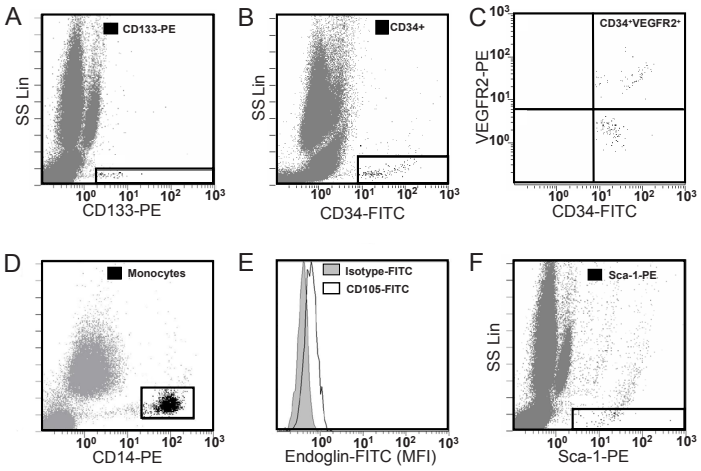

Figure 1
Representative flow cytometry plots for CD133 (A), CD34 (B), CD34VEGFR2 (C), CD14 (D), Eng (E) and Sca-1-like (F) positive cells.

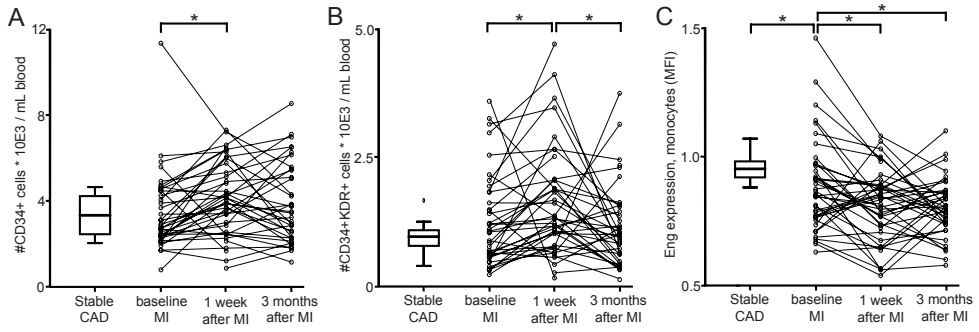

Figure 2
MI leaded to an upregulation of CD34$^+$ and CD34$^+$VEGFR2$^+$ progenitor cell numbers in the peripheral blood at 1 week after MI (A, B; CD34$^+$: baseline vs. 1 week P=0.003; CD34$^+$VEGFR2$^+$: baseline vs. 1 week P=0.003, 1 week vs. 3 months P=0.036). At baseline, monocyte endoglin expression was high and these levels fell 1 week after infarction (C; Eng: baseline vs. 1 week P=0.012, baseline vs. 3 months P=0.045, MI baseline vs. stable CAD P=0.037). Data are expressed as median ± interquartile range (IQR, box), maximal/minimal values within 1.5 IQR (whiskers) and outliers (•) or extremes (°). *P<0.05.

Progenitor cell mobilization after myocardial infarction

Peripheral blood derived cell characteristics were determined by flow cytometry at baseline, 1 week and 3 months after MI (Figure 1). Within the whole MI study group, $CD34^+$ and $CD34^+VEGFR2^+$ progenitor cell numbers were elevated one week after MI when compared to baseline (Figure 2A, B). At baseline, the expression of Endoglin (Eng) on monocytes was high and subsequently decreased 1 week after infarction (Figure 2C). In stable CAD patients monocyte Eng expression was elevated when compared to baseline MI numbers (Figure 2C). No difference was found for Sca-1-like$^+$ and $CD14^+$ cells (data not shown).

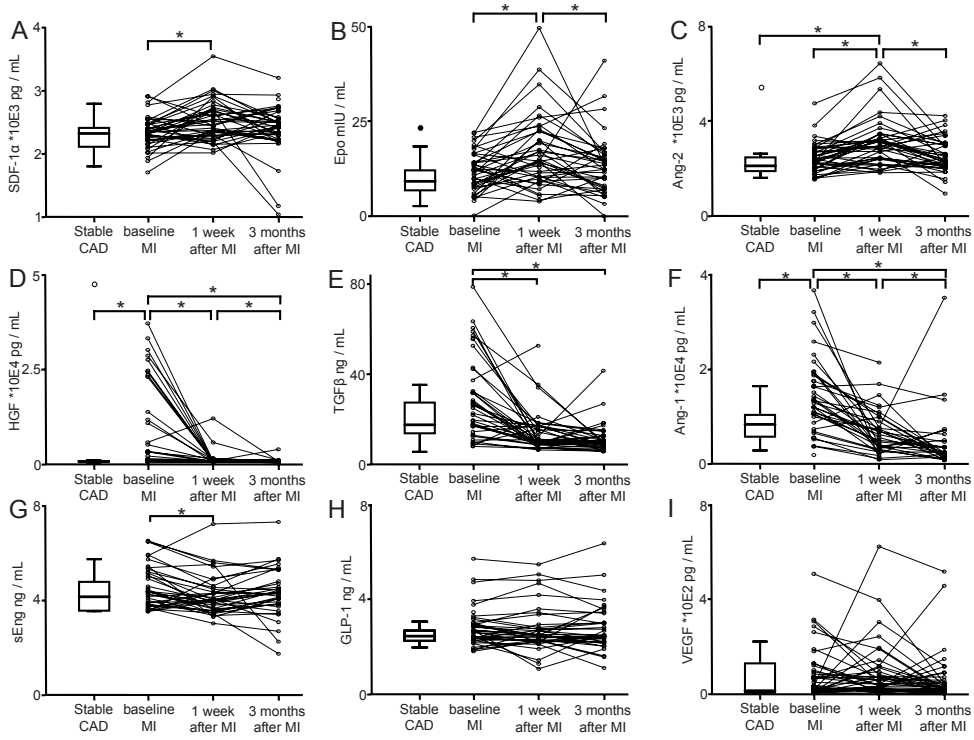

Figure 3

One week after MI, SDF-1α, Epo and Ang-2 blood levels were increased (A, B, C; SDF-1α: baseline vs. 1 week P=0.006, Epo: baseline vs. 1 week P<0.001, 1 week vs. 3 months P=0.03, Ang-2: baseline vs. 1 week P<0.001, 1 week vs. 3 months P=0.006, MI 1 week vs. stable CAD P=0.008). High levels of HGF, TGFβ, Ang-1 and sEng, were found at baseline and these levels decreased from 1 week after MI (D, E, F, G; HGF: baseline vs. 1 week P<0.001, baseline vs. 3 months P<0.001, 1 week vs. 3 months P=0.003, MI baseline vs. stable CAD P=0.025, TGFβ: baseline vs. 1 week P<0.001, baseline vs. 3 months P<0.001, Ang-1: baseline vs. 1 week P<0.001, baseline vs. 3 months P<0.001, 1 week vs. 3 months P=0.03, MI baseline vs. stable CAD P=0.012, sEng: baseline vs. 1 week P=0.003). No differences in time were found for GLP-1 and VEGF levels (H, I). Data are expressed as median ± interquartile range (IQR, box), maximal/minimal values within 1.5 IQR (whiskers) and outliers (•) or extremes (°). *P<0.05.

Effects of MI on plasma cytokines and growth factors

One week after MI, SDF-1α, Epo and Ang-2 blood levels were increased as compared to baseline. Stable CAD patients had lower Ang-2 levels compared to 1 week after MI (Figure 3A, B, C). High levels of HGF, TGFβ, Ang-1 and sEng were found at baseline and these levels decreased 1 week after MI. Furthermore, stable CAD patients had decreased HGF and Ang-1 levels compared to MI (Figure 3D, E, F, G). No significant differences were found for GLP-1 or VEGF levels in time (Figure 3H, I).

Association between MI size, cardiac function and peripheral blood derived cells, cytokines and growth factors

Large infarcts, defined by a high percentage gadolinium enhanced area (PEA) at 3 months post MI, are associated with relatively high $CD34^{+}VEGFR2^{+}$ progenitor cell numbers at baseline (Figure 4A). Early worsening of cardiac function, as expressed by a high difference of ESVI 1 week compared with baseline, is associated with high VEGF (baseline) and high HGF and Epo levels (both at one week) (Figure 4B, C, D). Low sEng at 1 week post MI however, is associated with large infarctions and early worsening of the cardiac function (Figure 4E, F).

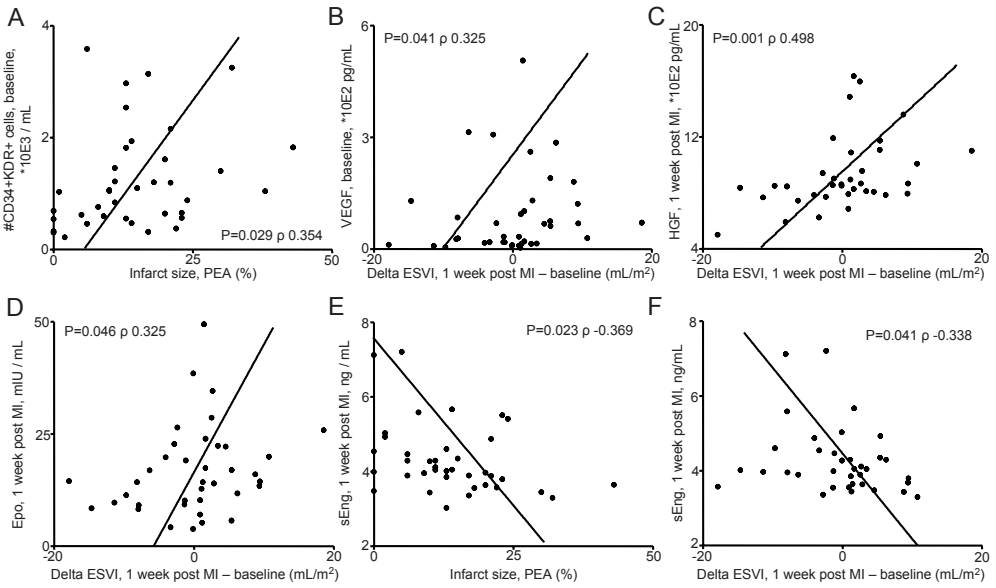

Figure 4
Large infarctions, as expressed by a high PEA, were associated with relatively high $CD34^{+}VEGFR2^{+}$ cell numbers at baseline (A). Early worsening of the cardiac function, as expressed by a high delta ESVI 1 week – baseline, was associated with high HGF, Epo (both at one week) and VEGF (baseline) levels (B, C, D). However, low sEng at 1 week post MI correlated with large infarcts and early worsening of the cardiac function (E, F).

As expected, hsCRP and NT-pro-BNP levels are maximal at one week after MI (Supplementary figures A, B) and early worsening of the cardiac function is associated with high hs-CRP levels at one week after MI (Supplementary figures C).

Statin treatment early after MI results in increased CD133+ progenitor cell mobilization

One week post MI, the number of CD133+ progenitor cells in the early statin treated patients is increased when compared to placebo treatment (Figure 5A). All other cell populations analyzed did not show significant differences between early statin and placebo treated patients (Figure 5B, C, D, E, F).

No differences in plasma cytokines, growth factors, NT-pro-BNP and hsCRP were found between early statin and placebo treated patients. However, we found a significant difference in sEng at baseline and 1 week post MI. We can not explain this finding, as statin treatment started at the time of the first blood drawing.

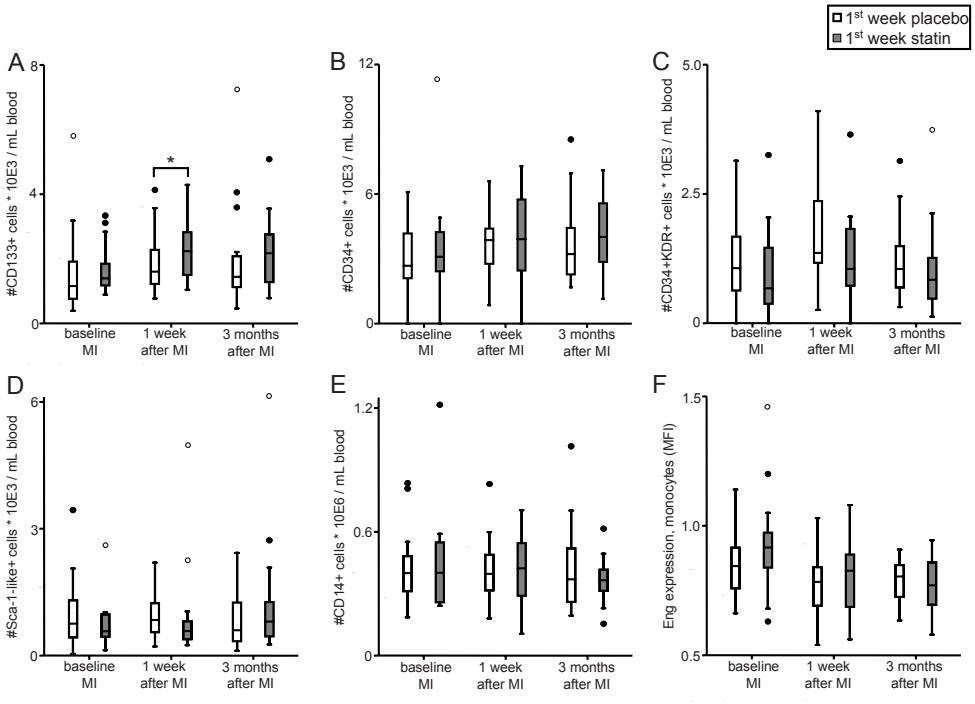

Figure 5
Early statin treatment after MI further increased CD133+ cell mobilization 1 week after MI, when compared to placebo (A, P=0.042). No effects of early statin treatment were found on CD34+, CD34+VEGFR2+, Sca-1-like+, CD14+ cell numbers and monocyte Endoglin expression levels (B, C, D, E, F). Data are expressed as median ± interquartile range (IQR, box), maximal/minimal values within 1.5 IQR (whiskers) and outliers (•) or extremes (°). *P<0.05.

Discussion

Understanding the physiologic response of cell mobilization after MI and identification of factors that may influence cardiac repair are of great importance to improve cell based therapy aiming at cardiac regeneration. In this study, we showed an increased $CD34^+VEGFR2^+$ progenitor cell mobilization and cytokine/growth factor upregulation in patients with relatively large infarctions or unfavorable hemodynamic conditions. Furthermore, early statin treatment after MI was associated with enhanced mobilization of $CD133^+$ progenitor cells. However, within all MI patients, progenitor cell mobilization and cytokine/growth factor upregulation was not associated with overall post MI cardiac recovery.

In peripheral blood post MI we found mobilization of $CD34^+$ and $CD34^+VEGFR2^+$ cells. Previous studies reported post MI mobilization of various cell populations including $CD34^+$ [10;16-18], $CD133^+$ [19], CD34/$CXCR4^+$ [6], CD34/$CD117^+$ [6], c-met^+ [6] and very small embryonic like (VSEL) cells[20]. We found similar mobilization of $CD34^+$ and $CD34^+VEGFR2^+$ progenitor cells. However, MI did not affect the mobilization of Sca-1^+-like cells. It is currently unclear whether blood derived Sca-1^+-like cells may contribute to cardiac recovery.
Interestingly, $CD34^+$, $CD133^+$, $CD117^+$, $CXCR4^+$, c-met^+ and VSEL cell mobilization was shown to be impaired in patients with a decreased LVEF[7;20;21]. Leone and co-workers reported a positive correlation 1 year after MI of $CD34^+$ progenitor cell numbers and LVEF improvement, suggesting a beneficial effect of these cells on a decreased cardiac function. A correlation of CD34+ progenitor cell numbers with LVEF early after MI was however not found in these patients[10].
Contrary to these pervious studies, our results show that relatively large infarcts are associated with an increased $CD34^+VEGFR2^+$ progenitor cell mobilization. Furthermore, we did not find any significant association of blood derived (progenitor) cells with LVEF at 3 months, although LVEF and infarct size are very strong negatively associated.
In the REPERATOR study, only patients with a first acute MI were included, which allowed us to study the effect of progenitor cell mobilization and cytokine/growth factor upregulation in relation to cardiac function recovery after MI in patients from which it is likely that their hearts were previously normal. In the studies performed by Leone and Wojakowski 37-50% of included patients had a history of CAD with possible effects on left ventricular function[7;10;20]. The study by Turan and co-workers also included only patients with a first MI[21], however LVEF and infarction size were assessed by left ventriculography which is a less accurate method than MRI measurement. To our knowledge, the REPERATOR study is the first study which performed detailed measurements of the cardiac function by MRI at three different time points and studied

progenitor cell mobilization. We show relatively large infarcts resulted in an increased $CD34^+VEGFR2^+$ progenitor mobilization, but we did not find any effects on cardiac function improvement.

Previously, we reported that MNCs from endoglin haploinsufficient patients (Hereditary Hemorrhagic Telangiectasia) have an impaired vascular repair capacity due to defective homing[22] (Chapter 7). The increased endoglin expression on monocytes observed directly after MI might suggest that these cells have an increased angiogenic capacity thereby improving cardiac repair. Quite surprisingly, monocyte endoglin expression was even higher in patients with stable CAD, which suggests an initial decrease in endoglin the first 3 months after MI with later endoglin upregulation.

The behaviour of the cytokines and growth factors Epo, SDF-1α, TGFβ, sEng, Ang-1, Ang-2, VEGF and HGF have previously been studied in time post MI. Epo levels were shown to peak 24 hours after admission for MI[3], and already decreased 7 days after MI. We found that the levels of Epo peak 7 days after MI and are decreased at 3 months after MI. Epo was previously shown to stimulate EPC mobilization[23]. We observed however no correlation of Epo levels with circulating EPC numbers (data not shown), but did find increased Epo levels in patients with an early deteriorating cardiac function.
Plasma SDF-1α levels were previously shown to be decreased in patients with unstable angina, while high levels in vitro mediated anti-inflammatory and matrix-stabilizing effects[24]. After MI, plasma SDF-1α levels were shown to increase at day 3, peaking at 21 days after MI, but no correlation of SDF-1 levels with LVEF was found[7,25]. We observed an increase in SDF-1α levels one week after MI, suggesting beneficial circumstances for the infarcted heart by anti-inflammatory and matrix-stabilizing effects. However, there were no correlations with infarct size, improvement of cardiac function or circulating cell numbers (data not shown).
Plasma TGFβ levels have been reported to be unaffected at day 2 and 7 after MI[26]. We show elevated TGFβ levels immediately after MI, which decreased from day 7 after MI. In an experimental mouse MI model, inhibiting TGFβ signalling by blocking the TGFβ type 1 receptor showed to affect cardiac remodeling positively[27]. In this study we found down regulation of circulating TGFβ protein from 1 week after MI, but no effects on cardiac function were appreciated.
sEng was previously described to be downregulated 48 hours after MI and patients who died after MI had a greater decrease in sEng than those who survived[28]. This is in agreement with our results which showed a similar down regulation of sEng post MI and an association between a greater decrease in sEng (1 week / baseline) with large infarctions (Supplementary data D). This suggests that the decreased survival after a

greater decrease in sEng described by Cruz-Gonzalez and co-workers may be related to infarct size, but in this previous study sEng decrease and associated mortality were independent from ejection fraction at entry[28]. Furthermore, sEng is known to be increased in mothers with pre-eclampsia which is characterized by endothelial dysfunction[29] and high sEng induces arteriovenous malformations in VEGF induced angiogenesis[30]. Taken together, these studies suggest that decreased sEng levels are beneficial for normal angiogenesis and therefore cardiac repair after MI. This may explain the decreased sEng levels at 1 week and the correlation of low sEng with large infarcts and early worsening of the cardiac function.

The angiogenic factors Ang-1, Ang-2 and VEGF are found at elevated levels in plasma post MI[4]. Ang-1 was previously shown to peak at 18 weeks, Ang-2 at baseline and 6 weeks, and VEGF at 1 to 6 weeks post MI[4;7;31]. We observed Ang-1 levels to peak at baseline and Ang-2 levels peak at 1 week after MI, without any significant changes in VEGF levels. Blood vessel sprouting is therefore potentially favored 1 week post MI, since Ang-2 and VEGF levels are high[32]. Surprisingly, baseline VEGF levels were associated with early deterioration in cardiac function, suggesting a negative effect of VEGF levels on the cardiac function. However, no association between the 3 months left ventricular function recovery and VEGF levels were observed.

HGF, an other angiogenic factor, is known to be upregulated after MI, with peak levels at 24-48 hours after MI[5;7]. In our patients we found elevated HGF levels at baseline and early worsening of cardiac function – measured by a high change ESVI 1 week compared to baseline – was associated with higher HGF levels at one week. In an experimental mouse MI model, systemic HGF infusion 1 day after MI resulted in increased cardiac function due to reduced fibrosis and apoptosis and increased angiogenesis[8]. Despite increased HGF levels in patients with malfunctioning hearts which may improve cardiac function, we observed no effect on cardiac function improvement.
One of the limitations of this clinical study is that we do not have any information on pre-infarct (progenitor) cell numbers or cytokine/growth factor levels. We are therefore unable to assess the effects of these pre infarct numbers / levels on the subsequent cardiac function outcome.

Statins, 3-hydroxy-3-methylglutaryl co-enzyme A reductase inhibitors, are used for primary and secondary prevention to reduce cardiovascular morbidity and mortality[33]. In rats, statin therapy augmented endothelial progenitor cell (EPC) levels in the early phase post MI and this was associated with improved cardiac function and increased capillary density[13]. In clinical studies, statins were also shown to mobilize EPCs[34].

Hematopoietic progenitor cell ($CD34^+$) mobilization post MI is be further enhanced in patients on statin therapy[10]. Furthermore, intensive statin therapy compared to conventional statin therapy resulted in upregulation of $CD34^+VEGFR2^+$ cell numbers even 4 months after MI[11]. In our study, early statin treatment only resulted in increased mobilization of $CD133^+$ hematopoietic progenitor cells, while statin treatment did not affect $CD34^+$ hematopoietic progenitor cell or $CD34^+VEGFR2^+$ EPC mobilization, nor did we find any improvement in cardiac function as measured by MRI (Chapter 3).

Taken together, we showed that early deterioration of cardiac function or relatively large infarcts resulted in an increased systemic release of HGF, Epo, VEGF, a decrease in sEng and increased $CD34^+VEGFR2^+$ progenitor mobilization, but no effects on cardiac repair were appreciated. Early statin therapy directly after MI resulted in increased mobilization of $CD133^+$ progenitor cells, but no effects on cardiac function were appreciated. Increasing our knowledge of the physiologic response after MI is essential for the development of new therapies for the injured heart using cell mobilizing agents, stimulation of endogenous progenitor cells or cellular transplantation therapies.

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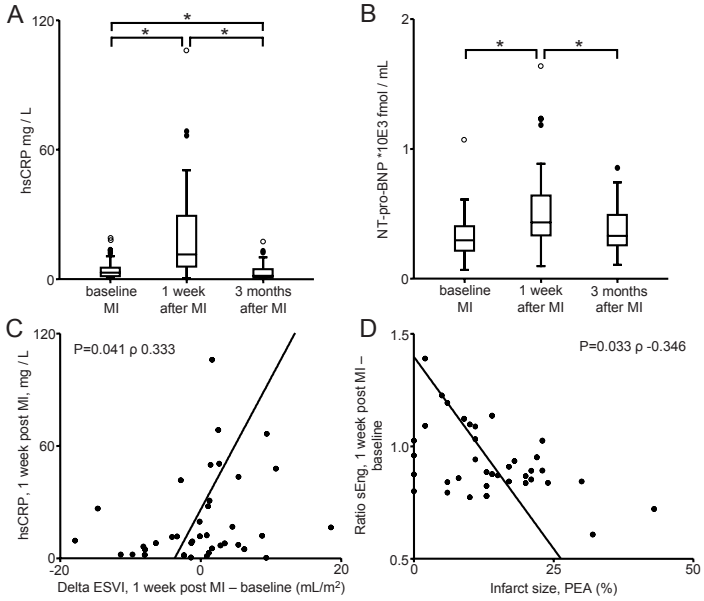
Supplementary data



HsCRP and NT-pro-BNP levels are maximal at one week after MI (A, B). Early worsening of the cardiac function, as expressed by a high delta ESVI 1 week – baseline, is associated with high hs-CRP levels at one week after MI (C). Soluble Eng down regulation post MI, measured by a low sEng ratio – 1 week versus baseline, was associated with large infarctions, measured by PEA (D). Data are expressed as median ± interquartile range (IQR, box), maximal/ minimal values within 1.5 IQR (whiskers) and outliers (•) or extremes (°). *P<0.05.

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Part II | Chapter 5

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5

Reduced CD26 expression is associated with improved cardiac function after acute myocardial infarction

Insights from the REPERATOR trial, II

In preparation

Abstract

Introduction

Peripheral blood mononuclear cells (MNC) enhance cardiac recovery and repair after myocardial infarction (MI). The SDF-1α / CXCR4 axis plays a major role in cell homing to an infarct area and is negatively regulated by CD26. Therefore, we studied the expression of CD26 during MI and its effects on cardiac function.

Materials and Methods

Blood samples from forty-two patients who underwent a primary percutaneous coronary intervention (PCI) for a first ST-elevated MI were collected at primary PCI, 1 week and 3 months after MI. Soluble CD26 (sCD26) and membrane bound CD26 expression on MNCs (mncCD26) were determined. Left ventricular function and infarct size were measured within 1 day, 1 week and 3 months follow up by magnetic resonance imaging.

Results

One week post MI, sCD26 was down-regulated compared to baseline, while mncCD26 was high at baseline and 1 week compared to 3 months. Increased mncCD26 expression at 1 week after MI was associated with decreased overall recovery of left ventricular function as measured by left ventricular end systolic volume index. Furthermore, the *in vitro* migration capacity of MNCs to SDF-1α was decreased 1 week post MI and the migration capacity to SDF-1α was negatively correlated with mncCD26 expression. CD26 inhibition with sitagliptin resulted in improved *in vitro* migration capacities.

Conclusion

Our results suggest that high cellular CD26 expression decreases the migration of MNCs towards SDF-1α and high cellular CD26 expression negatively influences cardiac function post MI. Treating patients shortly post MI with sitagliptin to inhibit CD26 may therefore increase MNC homing to the infarct area and could improve cardiac recovery and repair.

Introduction

Myocardial infarction (MI) is the leading cause of death world wide[1]. Despite treatment with beta-blockers, statins and percutaneous coronary intervention, MI still has a high morbidity. New treatment modalities are therefore urgently needed to prevent remodeling post-MI. Over the years it has become clear that both bone marrow and peripheral blood mononuclear cells (MNCs) improve cardiac recovery and repair. MNCs are currently tested in clinical studies where they are infused intracoronary following successful reperfusion in MI patients[2,3]. Although the mechanism by which MNCs improve cardiac function is not fully understood, it is however important that the cells home to and integrate in the damaged myocardial wall. A key chemokine regulating cellular homing and retention is stromal cell-derived factor-1α (SDF-1α or CXCL12)[4]. SDF-1α is upregulated in the ischemic myocardium shortly after MI, resulting in recruitment of cells expressing the SDF-1α receptor CXCR4 on their surface from the circulation to the ischemic heart[5]. The SDF-1α / CXCR4 homing axis is negatively regulated by the peptidase CD26 (dipeptidylpeptidase IV (DPPIV)) which cleaves the amino-terminal dipeptide from SDF-1α, generating an inactive protein[6,7]. Recently we showed that the dysfunctional homing capacity of MNCs from patients with the vascular disease Hereditary Hemorrhagic Telangietasia type 1 to the infarcted myocardium was due to increased CD26 expression[8] (Chapter 7).

Therefore, we hypothesize that in patients with MI, high CD26 expression is negatively associated with the homing capacity of MNCs to the infarcted area and a subsequent reduced improvement of the cardiac function.

Materials and Methods

Patient Population and study design

Between March 2006 and November 2007 fifty-five patients were included in the REPERATOR trial (Prevention of REPERfusion Damage and Late Left Ventricular Remodeling With ATORvastatin Administered Before Reperfusion Therapy)(Chapter 3)[9]. All patients presented with a first acute ST-elevation-MI and were treated with primary PCI. For inclusion and exclusion criteria see Chapter 3. The investigation was approved by the medical ethics committee of both hospitals and conforms to the principles outlined in the Declaration of Helsinki. Written informed consent was obtained from all patients. The trial was registered at ClinicalTrials.gov under identification number NCT00286312.

Magnetic Resonance Imaging

Early and late left ventricular function and infarct size were assessed by MRI (1.5 T Philips®, Best, The Netherlands), at baseline (within one day), at 7 days and 3 months after MI. Steady state free precession cine sequences and gadolinium-enhanced images were analyzed using a 12 segment, 6-20 slice model. MRI scans were interpreted by one observer blinded for treatment. Percentage enhanced area (PEA) and left ventricle end-systolic volume index (LVESVI) were calculated.

Blood samples

Blood was drawn instantly before PCI, at second and third MRI. Blood samples were collected in Potassium/EDTA tubes (Vacuette, Greiner Bio-One, The Netherlands). Plasma samples were frozen and stored at -80˚C. Peripheral blood MNCs were isolated by density gradient centrifugation using Ficoll Paque Plus (Amersham Biosciences, Sweden), according to the manufacturer's protocol.

Flow cytometry

Flow cytometric analysis was performed using 100µL whole blood or $3*10^{E}5$ MNCs in PBS. Cells were stained according to the manufacturer's instructions using the following monoclonal mouse-anti-human antibody combination: anti-CD14-ECD (Immunotech, Coulter, France), anti-CD26-FITC (Serotec, UK) and anti-CXCR4-PE (BD Pharmingen, USA). Isotype-matched fluorochrome-conjugated antibodies were used as controls. After incubation, samples were washed and, in whole blood samples, red blood cells were lysed before measuring fluorescence on a flow cytometer (Cytomics FC500, Beckman Coulter, The Netherlands). The MNC cell fraction was determined by forward and sideward scatter patterns. Analysis was performed using CXP software (Beckman Coulter, The Netherlands). The number of positive cells is expressed as absolute cell number per mL of whole blood, or as percentage of positive cells within a cell fraction. The Mean-Fluorescent Intensity (MFI) is presented for cell populations of interest.

ELISA

Plasma soluble CD26 (sCD26) and NT-pro-BNP were measured by use of commercially available ELISA kits (R&D Systems, Inc, Minneapolis, USA and Biomedica, Wien, Austria).

MNC migration

The MNC migration capacity from 10 MI patients at the three different time points was assessed in a transwell system using polycarbonate filters with 5µm pores (Corning, The Netherlands). Prior to migration, MNCs were incubated for 1 hour in RPMI 1640

Glutamax medium supplemented with 10% FBS at 37°C. One-hundred thousand MNCs were applied to the upper well. To determine the effect of CD26 inhibition on the migration of MNCs, cells were pre-treated at room temperature for 15 minutes with 1 mM sitaglipine (MSD, Haarlem, The Netherlands) in RPMI 1640 medium supplemented with 10% FBS. In the lower well medium with 0 or 200ng/mL SDF-1α (PeproTech, Rocky Hill, NJ, USA) was added. The cells were allowed to migrate for 3 hours at 37°C. Migration experiments were performed in duplo. After incubation, migrated cells were collected. Subsequently, 75.000 scarlet fluospheres (15µm, Invitrogen, Eugene, Oregon, USA) were added to the cell suspension. The number of MNCs per 10.000 beads was assessed on a flow cytometer. The migration percentage was calculated from the number of cells migrated to SDF-1α compared to the number of cells migrated in the absence of SDF-1α. For the mncCD26 expression - MNC SDF-1α migration correlation experiments, data from 10 patients with stable coronary artery disease were included (Chapter 4).

Statistics

Since no statistical differences were found between early placebo and early statin treated patients using the Mann-Whitney U test, we used the data from both patient groups to determine the effect of MI on CD26 levels and expression in time. Statistical significance was evaluated with the Friedman test for three related samples, Wilcoxon Signed Ranks test for two related samples (Post hoc analysis with Bonferroni correction after Friedman test) and Spearman's rho for correlation calculations using SPSS v16.0 for Windows. Results are expressed as median ± interquartile range. A value of $P < 0.05$ was considered statistically significant. All reported P values are two-sided.

Results

Patient characteristics

The study population consisted of forty-two patients (82% male, median age 57.0 ± 13.5 years). For inclusion and exclusion see Chapter 3. Patient characteristics are shown in Table 1.

CD26 and overall cardiac function improvement

One week post MI, sCD26 levels in plasma were low (Figure 1A). Interestingly, mncCD26 expression was high one week post MI compared to three months (Figure 1B), while no differences in the absolute number of CD26 expressing MNCs were found (Figure 1C). High mncCD26 expression at one week post MI was associated with decreased cardiac function improvement – shown by a low delta ESVI at 3 months compared to baseline (Figure 1D).

MNC post MI migration capacity

To determine the effect of high surface mncCD26 expression on migration towards SDF-1α, we performed an *in vitro* transwell migration assay without or with pre-treatment of the MNCs with sitagliptin, a CD26 inhibitor. The number of migrated MNCs towards

Table 1: Patient characteristics

	MI, n=42
Age, years	57.0 ± 13.5
Gender (male), n (%)	32 (76.2)
Body Mass Index, kg/m^2	27.2 ± 4.0
Laboratory parameters	
Cholesterol, mmol/L§	4.38 ± 1.27
HDL-cholesterol, mmol/L‖	0.82 ± 0.36
LDL, mmol/L‖	2.90 ± 1.30
Triglycerides, mmol/L‖	1.15 ± 0.56
Glucose, mmol/L*	7.5 ± 4.5
Creatinin, µmol/L	80.5 ± 28.5
Hemoglobin, mmol/L	8.90 ± 1.05
Leucocytes, G/L	11.1 ± 4.2
Medications after myocardial infarction	
Thrombocyte coagulation inhibitors, n (%)	42 (100)
Statin, n (%)	42 (100)
ACE-inhibitors, n (%)‡	23 (54.8)
Beta-blockers, n (%)‡	36 (85.7)
Angiotensin-II-receptor antagonists, n (%)‡	3 (7.1)
Calcium antagonists, n (%)‡	4 (9.5)
Myocardial infarction	
Anterior infarction, n (%)	10 (23.8)
Total ischemic time, minutes	168 ± 151
Peak CK, U/L	1198 ± 1596
Risk factors	
Smoking / history of smoking, n (%)	32 (76.2)
Diabetes, n (%)	6 (14.3)
Hypertension, n (%)	16 (38.1)
Hypercholesterolemia, n (%)‡	10 (23.8)
Positive family history, n (%)	20 (47.6)

Data are presented as number (percentage) or median ± IQR. * n=36; † n=35; ‡ n=41; ‖n=40; due to missing data.
ACE=angiotensin converting enzyme

SDF-1α was decreased when isolated 1 week post MI (Figure 2A), while inhibition of CD26 resulted in enhanced migration (Figure 2B). MncCD26 expression correlates negatively with the migration capacity of MNCs to SDF-1α (Figure 2C). However, no relation was found between MNC migration capacity to SDF-1α at 1 week post MI and overall functional improvement – expressed by a low delta ESVI at 3 months compared to baseline (Figure 2D).

Effects of early cardiac failure on CD26 and migration capacities

Soluble CD26 was negatively associated with large infarctions, as determined by PEA (Figure 3A). Also early left ventricular failure – shown by a high delta ESVI at 1 week compared to baseline and high baseline NT-pro-BNP levels – was associated with further reduction of sCD26 (Figure 3B, C). Thus large infarcts and early deterioration of cardiac function were associated with low sCD26 serum levels at 1 week. No correlation

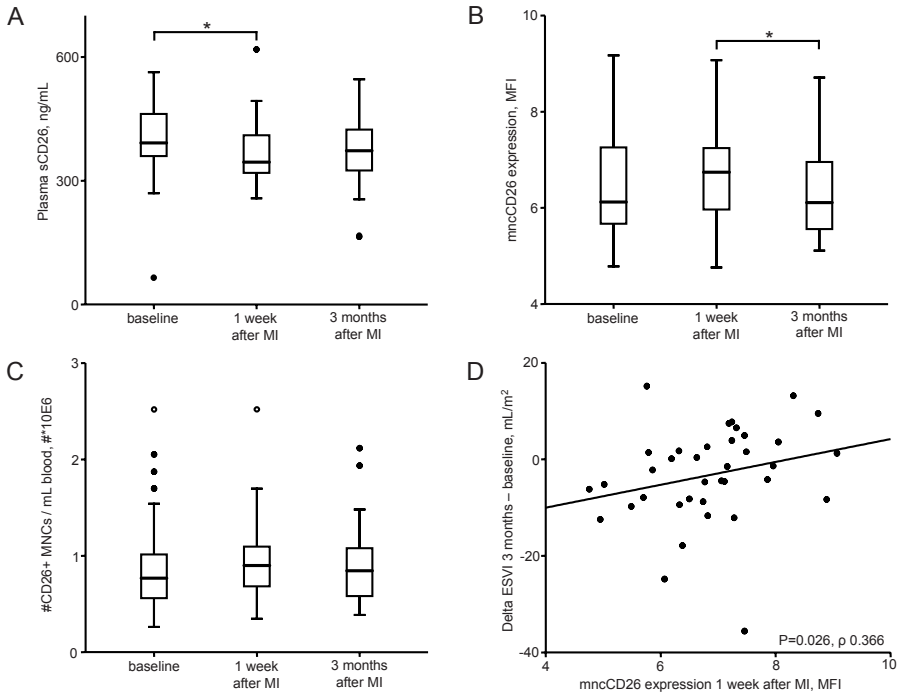

Figure 1

sCD26 levels are decreased 1 week after MI when compared to baseline (P=0.001, A). MncCD26 expression however, measured by MFI for CD26 on $CD26^+$ MNCs, is high at 1 week after MI when compared to 3 months (P=0.015, B). No significant difference in number of $CD26^+$ MNCs is found between the time points (C). High mncCD26 expression is associated with an unfavorable overall change in cardiac function (D). Data are expressed as median ± interquartile range (IQR, box), maximal/minimal values within 1.5 IQR (whiskers) and outliers (•) or extremes (°). *P<0.05.

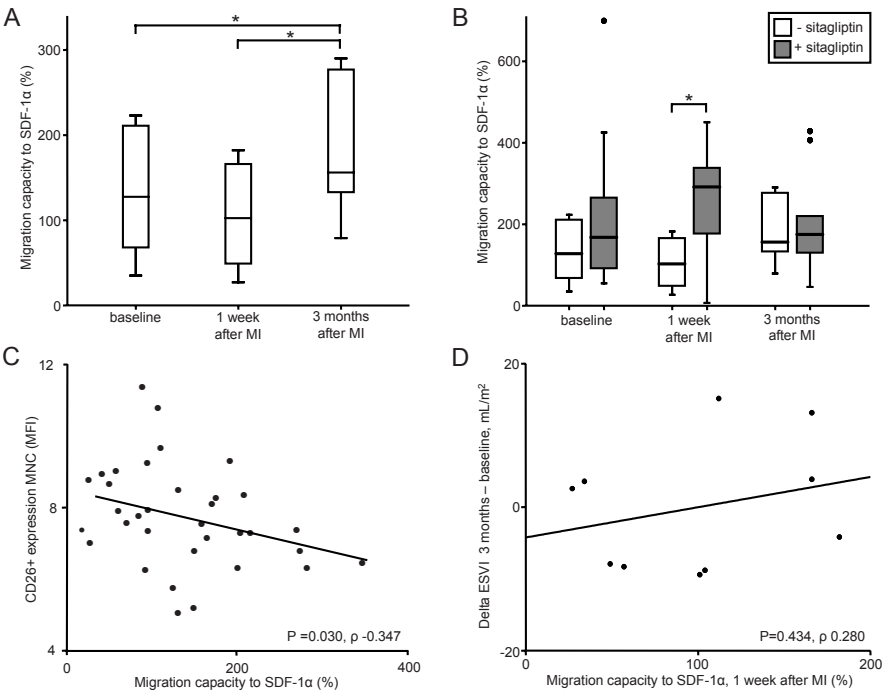

Figure 2
MNCs show a decreased *in vitro* migration capacity to SDF-1α 1 week after MI (baseline versus 3 months P=0.024, 1 week versus 3 months P=0.033, A). CD26 inhibition with sitagliptin resulted in improved *in vitro* migration one week post MI (P=0.022, B). MncCD26 expression is negatively associated with MNC migration capacity towards SDF-1α (C). No association is found for overall change in cardiac function and migration capacities towards SDF-1α at 1 week (D). Data are expressed as median ± interquartile range (IQR, box), maximal/minimal values within 1.5 IQR (whiskers) and outliers (•) or extremes (°). *P<0.05.

was found for early left ventricular failure – expressed by a high delta ESVI 1 week vs. baseline – and mncCD26 expression (Figure 3D). No correlations of baseline sCD26 and mncCD26 expression with infarct size or change in ESVI were found (data not shown). Furthermore, early left ventricular failure – expressed by a high delta ESVI 1 week versus baseline – was associated with increased migration at 1 week post MI (Figure 3E).
These associations suggest that large infarctions or early left ventricular function deterioration results in an adapted systemic response to maximize homing to the infarcted myocardium.

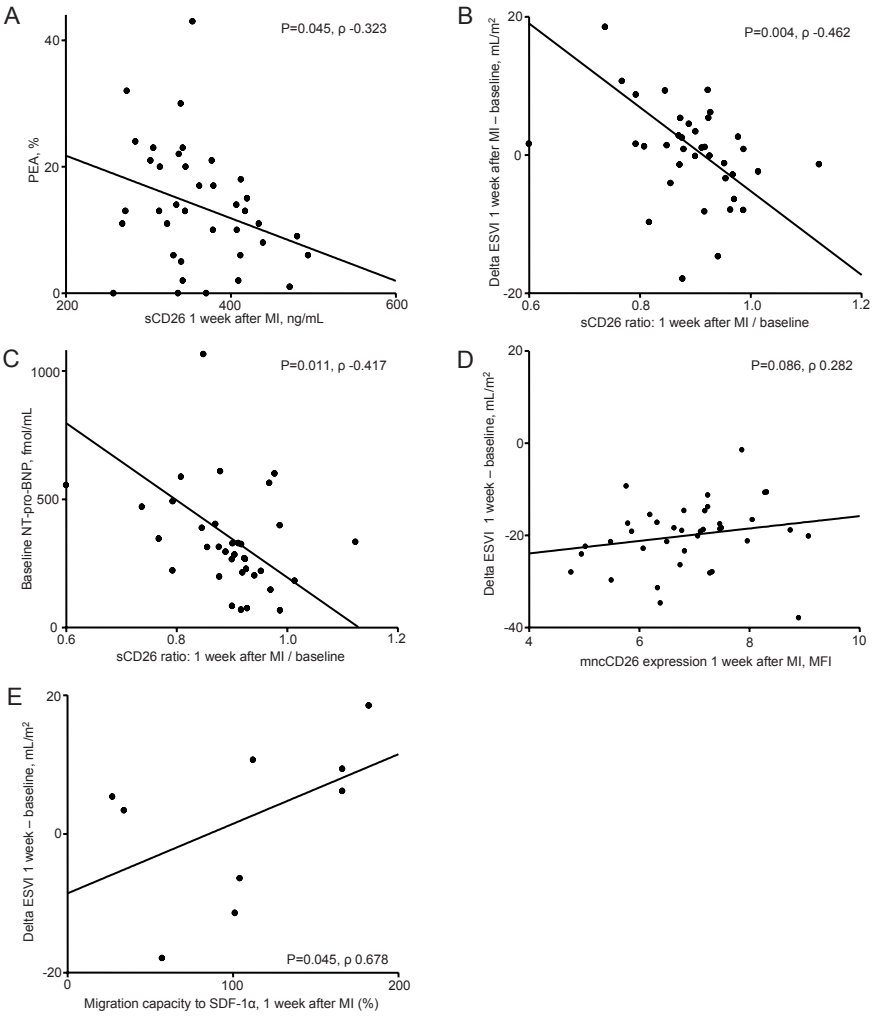

Figure 3
Large infarctions, measured by PEA, correlate with low sCD26 concentrations one week post MI (A). An early unfavourable change in cardiac function, represented by a high delta ESVI (1 week – baseline) and high NT-pro-BNP, are associated with higher sCD26 down regulation (ratio 1 week/baseline) (B, C). No significant correlation exists between mncCD26 expression at 1 week and early LV malfunctioning (D). Early LV malfunctioning is associated with increased migration capacities towards SDF-1α at 1 week (E).

Discussion

Understanding the pathophysiologic responses after MI, its effects on cell trafficking and identification of factors that influence cell homing and retention are of great importance to improve cell based therapy aiming at cardiac regeneration. In the past years the SDF-1α / CXCR4 axis has gained much attention. This axis was shown to be important for hematopoietic stem cell mobilization and homing[10], HIV infection[6,7] and is a central regulator to guide cells towards the infarcted myocardium[4]. The SDF-1α / CXCR4 axis is negatively regulated by the dipeptidylpeptidase CD26, which is expressed on the surface of several cell types and can be found in a soluble form in plasma[11-13].
Previous studies suggest that low plasma levels of sCD26 result in increased homing of $CXCR4^+$ cells towards area's with high SDF-1α expression[11,14]. Zaruba and coworkers recently showed in a murine myocardial infarction model that systemic CD26 inhibition post MI results in improved homing of $CXCR4^+$ cells towards the infarct area, reduced cardiac remodeling and improved cardiac function[14]. In patients with Sézary syndrome – a cutaneous T-cell lymphoma – reduced activity of sCD26 was found, which suggests increased homing capacities of tumour cells (all CD26 negative) towards the high SDF-1α levels in the skin[11]. Here we show that low plasma sCD26 levels are associated with early LV malfunctioning and large MIs. These results suggest that the physiologic response to create favorable circumstances for cell homing to an infarct area is depending on the gravity of infarction. Further down regulation of plasma sCD26 may positively influence cell migration to the myocardium by enhancing the SDF-1α homing gradient and improve cardiac function. CD26 inhibition in patients can be achieved by treatment with a CD26 inhibitor such as sitagliptin or vildagliptin, which are currently used as antidiabetic therapies[15].
CD26 is not only involved in cell homing, but also in mobilization of cells from the bone marrow into the circulation. Both G-CSF and GM-CSF, known to be upregulated after MI and mobilize progenitor cells from the bone marrow, induce cellular CD26 expression and activity on $CD34^+CD38^-$ human cord blood cells, decreasing their migration capacities towards SDF-1α present in the bone marrow[16]. Interestingly, we found that mncCD26 expression 1 week after MI is relatively high, while MNC migration towards SDF-1α is decreased at this time point. And mncCD26 expression is negatively correlated with MNC migration towards SDF-1α. Furthermore, high mncCD26 expression at 1 week after MI is associated with decreased overall recovery of left ventricular function as measured by left ventricular end systolic volume index. Therefore, cellular CD26 expression not only affects the migration of MNCs towards SDF-1α, but these results suggest that it also affects functional improvement after MI. Thus inhibition of CD26 using e.g. sitagliptin may positively influence MNC homing and cardiac function after MI.

The presence of the CXCR4 on MNCs is essential for migration towards SDF-1α[17;18]. One week post MI, the number of $CXCR4^+$ cells is reduced, while the expression level of CXCR4, as measured by mean fluorescent intensity (MFI), is not changed (Supplementary Figure 1) as also observed by Wojakowski and coworkers[19], and no relation with infarct size or cardiac function could be found (data not shown). The combination of high cellular CD26 expression and the lower numbers of CXCR4 bearing cells generates an unfavourable combination for SDF-1α mediated homing.

In conclusion, our results suggest that high cellular CD26 expression decreases the migration of MNCs towards SDF-1α and high cellular CD26 expression negatively influences cardiac function post MI. Treating patients shortly post MI with sitagliptin to inhibit CD26 may therefore increase MNC homing to the infarct area and could improve cardiac recovery and repair.

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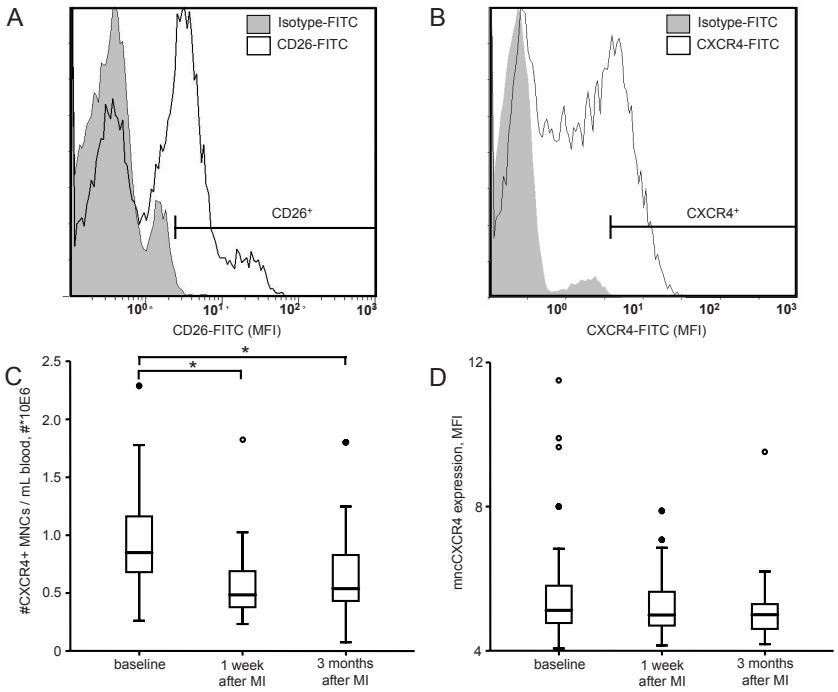
Supplementary data



Supplemental figure 1
Representative flow cytometry plot for CD26 and CXCR4 (A, B). The number of CXCR4 expressing cells was decreased 1 week and 3 months after infarction, compared to baseline (baseline versus 1 week P<0.001; baseline versus 3 months P=0.006, C). No significant difference was found for mncCXCR4 expression between the time points (D). Data are expressed as median ± interquartile range (IQR, box), maximal/minimal values within 1.5 IQR (whiskers) and outliers (•) or extremes (°).

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Part III | Chapter 6

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6

Endoglin has a crucial role in blood cell-mediated vascular repair

Based on: Endoglin has a crucial role in blood cell-mediated vascular repair

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Abstract

Background

Endoglin is an accessory receptor for transforming growth factor-β in vascular endothelial cells and is essential for angiogenesis during mouse development. Mutations in the human gene cause hereditary hemorrhagic telangiectasia type 1 (HHT1), a disease characterized by vascular malformations that increase with age. Although haploinsufficiency is the underlying cause of the disease, HHT1 individuals show great heterogeneity in age of onset, clinical manifestations and severity.

Methods and Results

In situ hybridization and immunohistochemical analysis of mouse and human hearts revealed that endoglin is upregulated in neoangiogenic vessels formed after myocardial infarction (MI). Microvascularity within the infarct zone was strikingly lower in mice with reduced levels of endoglin (Eng+/-) compared to wild-type mice, which resulted in a greater deterioration in cardiac function as measured by Magnetic Resonance Imaging (MRI). This did not appear to be due to a defect in the host inflammatory cell numbers in the infarct zone, which accumulated to a similar extent in wild-type and heterozygous mice. However, defects in vessel formation and heart function in Eng+/- mice were rescued by injection of mononuclear cells (MNCs) from healthy human donors but not by MNCs from HHT1 patients.

Conclusions

These results establish defective vascular repair as a significant component of the etiology of HHT1. Since vascular damage or inflammation occurs randomly, they may also explain disease heterogeneity. More generally, the efficiency of vascular repair may vary between individuals because of intrinsic differences in their MNCs.

Introduction

Revascularization of injured, ischemic and regenerating organs is essential to restore organ function. Neovascularisation results from the proliferation, migration and remodeling of terminally differentiated endothelial cells (ECs) from pre-existing blood vessels, a process referred to as angiogenesis[1]. Increasing evidence suggests that mononuclear cells (MNCs), which include endothelial progenitor cells (EPCs), circulating endothelial cells (CEC) and bone marrow monocytic lineages home to sites of ischemic damage and contribute to the formation of new blood vessels through vasculogenesis, transdifferentiation into ECs, and by secretion of growth factors and cytokines that stimulate neoangiogenesis[2, 3].

Hereditary hemorrhagic telangiectasia (HHT, also known as Rendu-Osler-Weber syndrome) is an autosomal dominant disorder with a low prevalence, estimated to be 1 in 10,000[4]. It is characterized primarily by epistaxis, telangiectases and multiorgan vascular dysplasia[4, 5]. Two variants of HHT, HHT1 and HHT2, have been described that are linked to mutations in the ENG (endoglin) and ACVRL1 (activin receptor-like kinase 1 or ALK1) genes, respectively[6, 7]. Recently, two more genes have been implicated: MADH4[8], and an unidentified HHT3 gene linked to chromosome 5[9]. Endoglin is highly expressed by active ECs from most types of blood vessels[10]. Deletion of endoglin in mice revealed its critical role during cardiovascular development. Mutant endoglin (Eng-/-) mice die at embryonic day (E)10.5 as a result of defects in vessel and heart development. Vasculogenesis in the Eng-/- mice is normal but angiogenesis is impaired along with the remodeling of the primary vascular plexus[11, 12]. Endoglin is an accessory receptor for transforming growth factor-β (TGF-β)[13, 14], a multifunctional cytokine that controls proliferation, migration, adhesion and apoptosis of many cell types[15]. The activated form of TGF-β bind to type II receptors (TβRII) which recruit distinct TGF-β type I serine/threonine kinase receptors to the complex and propagate signals from the cell surface to the nucleus by phosphorylating intracellular effectors termed Smads[16, 17]. Two type I receptors are known to mediate TGF-β signaling: ALK1 whose expression is mainly restricted to endothelium and ALK5, which is widely expressed in most cell types. In ECs, TGF-β/ALK5 signaling via Smad2/3 leads to inhibition of cell migration and proliferation, whereas TGF-β/ALK1 signaling via Smad1/5/8 promotes cell migration and proliferation[18]. Endoglin and ALK1 are thought to act in a common pathway and to inhibit TGF-β/ALK5 signaling indirectly. The combined effect promotes the activation phase of angiogenesis during which vascular permeability increases, the basement membrane is degraded and ECs proliferate and migrate[19, 20].

Several studies have implied haploinsufficiency as the mechanism responsible for HHT and have indicated that disease heterogeneity is not related to the position or type

of mutation[21, 22]. Thus, all mutations in the ENG gene result in significant reductions in functional cell surface protein and to deregulation of TGF-β signaling pathways[4,23]. However, individuals with HHT1 show great variability in age of onset of the disease, clinical manifestations and severity, both within and between families with the same mutation. In addition, epigenetic factors, including exposure to UV light and local inflammation, have been implied as contributing to its diversity, suggesting that HHT is a complex genetic disorder[24].

Here, we have used experimental myocardial infarction (MI) in wild-type and endoglin heterozygous mice to investigate the effects of the HHT1 mutation on angiogenesis and vasculogenesis which are integral components of the remodeling that occurs after MI[25]. Thus, experimental MI represents a useful model for studying these processes in normal and mutant adult mice. In addition, since cardiac biopsies are available from adult humans after MI, there are opportunities to translate the findings in mice to human disease.

6

Methods

Mice and coronary artery ligation

Endoglin mice containing a β-galactosidase reporter cassette in the disrupted locus have been described[11]. Analyses were carried out on wild-type (Eng+/+) and endoglin heterozygous transgenic mice (Eng+/-) from a C57Bl/6J genetic background. Balb/c mice were also used for injection of human MNCs via the tail vein, as described previously[26]. Myocardial infarction (MI) was induced as described before[27]. Briefly, adult mice weighing 20-35 g were intubated and ventilated with 2% isoflurane/98% oxygen. The left coronary artery was exposed via left thoracotomy and opening of the pericardium and occluded just below the inferior border of the left auricle using a 7-0 Prolene ligature. Sham-operated mice underwent the same operation without ligation of the coronary artery. All procedures with experimental animals were approved by the Institute Animal Care Committee.

Isolation, culture and injection of human MNCs

Blood samples from healthy human volunteers or HHT1 patients were collected in Potassium/EDTA tubes (Monovette, Sarsted). Peripheral blood mononuclear cells (MNCs) were isolated by density gradient centrifugation using Ficoll Paque (Amersham Biosciences), according to the manufacturer's protocol. MNCs were harvested and washed twice with PBS supplemented with 2 mM EDTA. For MNC injection, mice underwent CA ligation as described above. One to three hours after MI, 5 x 10^6 human MNCs in 40

µL PBS were administered via tail vein injection and mice were immunosuppressed with tacrolimus 5 mg/kg/day subcutaneously. Mice were randomized and divided into groups for follow-up of 4, 7, 14 or 30 days as indicated in results, to assess homing of human MNCs to the infarct area, angiogenesis and cardiac function.

For EPC culture, 6-well plates were fibronectin-coated and 10^7 cells per well were cultured in EGM-2 medium (Cambrex, Walkersville, USA) supplemented with 20% FCS, 100ng/mL $VEGF_{165}$ (R&D Systems) and penicillin/streptomycin. Cells were incubated in 5% CO_2 at 37˚C. After eight days of culturing, the adherent cells were detached by trypsin/EDTA and the number of cells was determined with an automatic cell counter (Cellcyn). To assess the effect of Alk-5 kinase inhibition on EPC attachment, SB431542 0.01µM (Sigma-Aldrich, USA) was added to the culture medium. The number of attached EPCs to a fibronectin coated coverslip was determined by DAPI staining and subsequent microscopy cell counting after fixation with 4% paraformaldehyde in PBS.

All procedures were approved by the medical ethics committee of the St. Antonius Hospital Nieuwegein, the Netherlands. The investigation conforms to the principles in the Declaration of Helsinki.

Analyses of myocardial function by MRI

Cardiac and respiratory triggered cine MR Images were acquired on a 9.4-T scanner (Bruker Biospin GmbH, Rheinstetten, Germany) using a birdcage RF coil. A gradient echo pulse sequence was used to acquire data with repetition time = 9.8 ms, echotime = 1.9 ms, matrix of 256 x 256, field of view of 3.0 x 3.0 cm, slice thickness of 1 mm, flip angle 18°, and four signal averages. The number of phases was 11-13 depending on the heart rate. Seven to eight short-axis slices were needed to image the entire left ventricle. Images were processed with dedicated imaging software (CAAS-MRV, Pie Medical Imaging BV, Maastricht, The Netherlands).

Isolation and processing of hearts

The hearts were dissected from euthanized mice 7, 14 or 30 days post-MI, fixed overnight (o/n) in 4% paraformaldehyde in (PFA) PBS, washed twice in 0.83% NaCl, washed once in 0.42% NaCl, in 50% EtOH and in 70% EtOH, all o/n at 4°C. After embedding in paraffin wax, hearts were sectioned (6 µm) onto coated slides (Klinipath) and stored at 4°C. For MNC homing experiments, hearts were isolated 4 days post-MI and processed for cryosections as described[28]. Human fetal hearts were collected after elective abortion and with informed consent as previously[29]. Biopsies from adult human cardiac tissue were obtained from the Pathology Department after autopsy. Formalin fixed samples were embedded in paraffin and 6 µm sections were used for immunohistochemical analysis.

RNA isolation and Real-time RT-PCR

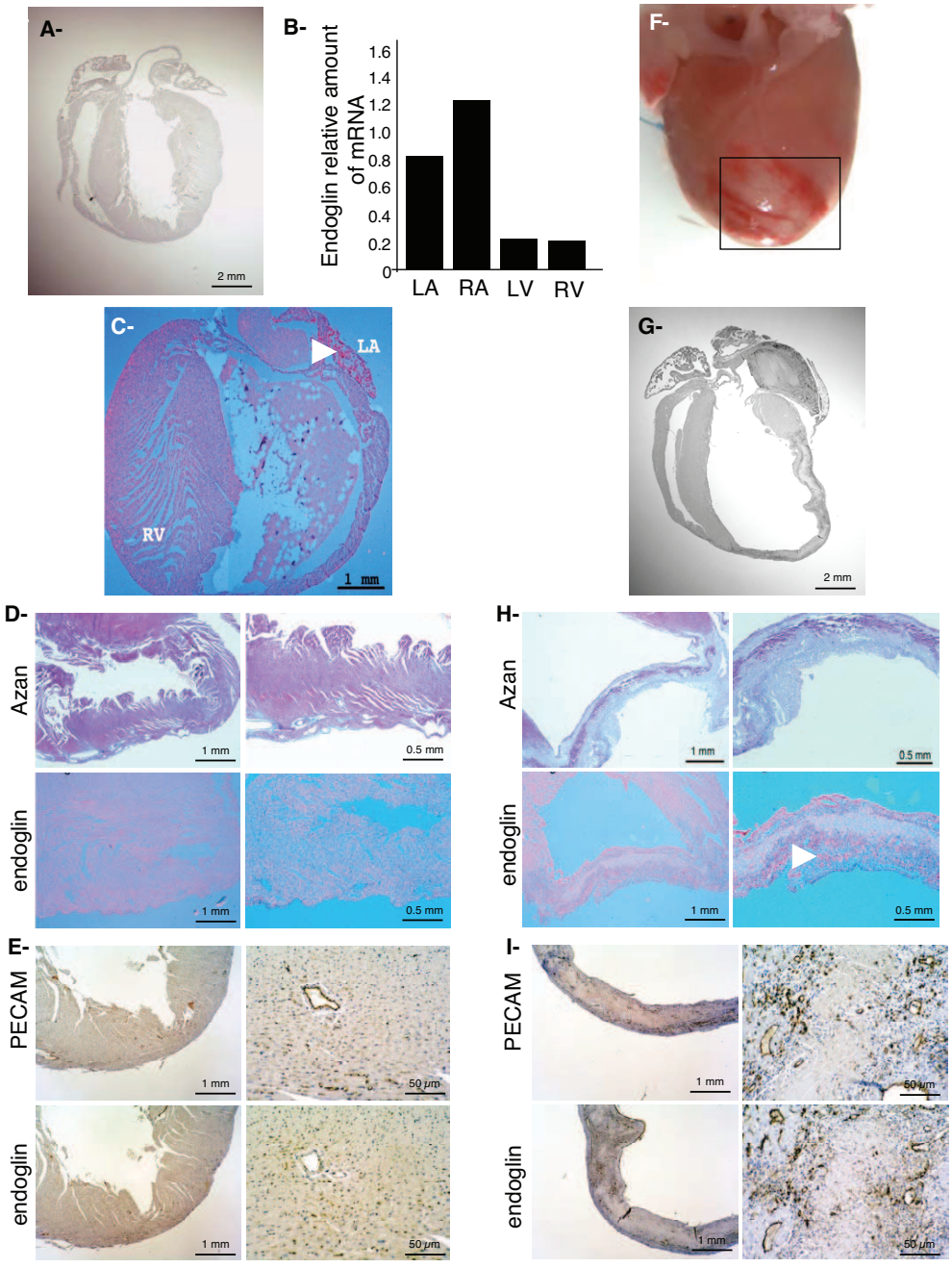
Total RNA from dissected atria and ventricles were isolated using TRIzol® Reagent (Invitrogen) according to the manufacturer's instruction. Samples were DNAse I treated to eliminate genomic DNA and 1µg RNA was reversed transcribed. Real-time PCR was performed in a MyiQTM single-color real time detection system (Bio-RAD). Samples were normalized using GAPDH.

Immunohistochemistry and immunofluorescence on sections

Paraffin sections were stained using Tyramide Signal Amplification (TSA) Biotin System (Perkin Elmer, Life Science). Briefly, sections were treated with 0.25% trypsin in 9mM $CaCl_2$ and 50mM Tris-HCl, pH 7.8 for 30 minutes at room temperature (RT) for antigen retrieval. Primary antibodies were rat anti-mouse PECAM (Clone MEC 13.3, dilution 1:100, BD Biosciences), rat anti-mouse endoglin (Clone 2Q1707, dilution 1:100, USBiological), rat anti-mouse CD68/macrosialin (Clone FA-11, dilution 1:100, Serotec Ltd), rat anti-mouse Mac-3 (Clone M3/40, dilution 1:100, BD Biosciences-Pharmingen), and rat anti-CD45 (Clone 30F11.1, dilution 1:100, Biosciences-Pharmingen), incubation o/n at 4°C. Biotin-conjugated goat anti-rat IgG (DAKO, 1:250) was used as a secondary antibody, incubation for 1 hour at RT. Peroxidase activity was detected using 3,3'-diaminobenzidine tablet set (Fast DAB, Sigma), according to manufacturer's instructions.

Paraffin sections used for Ki67 immunohistochemistry were treated as described previously[30]. Primary antibody used was mouse anti-Ki67 (Clone MM1, dilution 1:500, Monosan) incubation o/n at 4°C. Goat anti-mouse PowerVision™ Poly-HRPMS Conjugates (ImmunoVision Technologies) was used as secondary antibody, incubation for 30 minutes at RT. Peroxidase activity was detected using 3,3'- diaminobenzidine tablet set (Fast DAB, Sigma), according to manufacturer's instructions. Sections were counterstained with hematoxylin, dehydrated and mounted in DePex. Cryosections of

Figure 1
Upregulation of endoglin expression following myocardial infarction. (A-E) Endoglin expression in normal (sham operated) adult mouse heart. (A) Paraffin section of mouse heart. (B) Real-time PCR for endoglin using left atrium (LA), right atrium (RA), left ventricle (LV), and right ventricle (RV) cDNA. Samples were normalized using GAPDH primers. Note the relatively low expression of endoglin in the LV and RV. (C) Radioactive in situ hybridization for endoglin showing enhanced endoglin expression in atrial tissue (arrow). (D-E) High magnification of the LV. (D, upper) Azan-Mallory staining to illustrate viable tissue (red/magenta); (D, lower) radio-active in situ hybridization (endoglin mRNA in red) and (E) immunohistochemistry for endoglin showing that endoglin is not detectable in the LV of adult heart. (F-I) Endoglin expression is upregulated in the infarcted zone after MI. (F) Microscopic view of a one-week post-MI heart. (G) Paraffin section showing the dilation of the LV in a one-week post-MI heart. (H, upper) Azan-Mallory staining showing infarcted area (blue); (H, lower) Radioactive in situ hybridization and (I) immunohistochemistry increased endoglin expression (arrow) in the infarcted zone of MI adult heart one- week post-MI. (E, I) PECAM staining was used as a marker for capillaries and blood vessels.


A-
2 mm
B-
Endoglin relative amount of mRNA
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
LA
RA
LV
RV
F-
C-
LA
RV
1 mm
G-
2 mm
D-
Azan
endoglin
1 mm
0.5 mm
H-
Azan
endoglin
1 mm
0.5 mm
E-
PECAM
endoglin
1 mm
50 µm
I-
PECAM
endoglin
1 mm
50 µm

hearts containing human MNC-derived ECs were fixed in acetone for 10 minutes at 4°C, dried for 30 minutes at RT, permeabilized for 5 minutes with 0.2% Triton X-100 in PBS and blocked with 2% BSA in PBS at RT for 1 hour. The slides were then incubated with rat anti-mouse PECAM antibody (Clone MEC 13.3, dilution 1:100, BD Biosciences) o/n at 4°C, washed four times in PBS and incubated 1 hour simultaneously with goat anti-rat Cy3 (Jackson ImmunoResearch Laboratories) and Ulex europaeus agglutinin-1 (UEA-1) lectin coupled to FITC (1:100 dilution from 1 mg/ml stock, Sigma) diluted in 2% BSA in PBS. Cryosections of human fetal hearts were incubated with UEA-1 and with goat anti-human PECAM antibody (Clone M- 20, dilution 1:100, Santa-Cruz). The slides were then washed four times in PBS and mounted in Mowiol before confocal laser microscope analysis.

In situ hybridization

The endoglin probe was generated from a BamHI fragment (266-1039bp) from the mouse full length endoglin (clone pCDNA1-7/18) cloned into Bluescript and linearised with XmaI. The anti-sense RNA probe was generated by transcription of the T7 RNA polymerase in the presence of [α^{35}S]-UTP (Amersham). Autoradiography was performed using Ilford photo emulsion. The slides were exposed for 1-2 weeks at 4°C. Photography consisted of combining bright-field (blue filter) and dark-field (red filter) images.

Statistics

Statistical significance was evaluated using Mann-Whitney U test for comparison between two groups, Wilcoxon Signed Ranks Test for two paired groups, and the Median test for multiple group comparisons using SPSS v11.5 for Windows. Results are expressed as medians ± interquartile range. A value of $p< 0.05$ or $p< 0.01$ denoted statistical significance.

Results

We examined endoglin expression by *in situ* hybridization and immunohistochemistry in hearts of normal adult mice (Fig.1A-E) and one week after coronary artery ligation that induced myocardial infarction (MI) (Fig. 1F-I). Endoglin mRNA was predominantly expressed in the atria but was detectable at low levels, in the ventricles of normal hearts (Fig.1B-C). One week after MI, expression was strongly increased in the infarcted area compared to healthy tissue in sham-operated hearts (Fig. 1D, H). Staining of comparable sections from sham-operated and MI hearts with anti-PECAM and anti-endoglin antibodies one week post operatively revealed that their protein expression

overlapped, indicating that endoglin is only expressed in ECs in the infarct zone and not in myofibroblasts (Fig. 1I), as previously described[30], or in vessels in the non-infarcted zone (Fig.1E). Neoangiogenesis within the scar is an integral component of the remodeling process that occurs after MI[31, 32]. To compare the prevalence of activated blood vessels in hearts from sham-operated mice, the healthy non-infarcted zones and the corresponding infarcted zones of adult mice one-week post-MI, sections were stained with antibodies against Ki67, a marker for cycling cells, PECAM and endoglin. In both the sham operated hearts and the non-infarcted zone one-week post-MI, 5-8% of blood vessels were Ki67 (+). By contrast, approximately 50% of blood vessels were Ki67 (+) in the infarct zone (Fig.2A). Staining of sections from biopsies of human hearts with MI taken from within and outside the infarct zone showed similar differences in the prevalence of activated vessels (Fig. 2B). Because our data indicated that endoglin expression was highly associated with sites of active neoangiogenesis in both mice and humans, we used endoglin heterozygous (Eng+/-) mice to investigate its function in adult neovascularization. Eng+/- mice survive into adulthood but have reduced endoglin levels and can develop HHT symptoms[33]. Hearts from Eng+/+ and Eng+/- mice were stained for endoglin after MI. This revealed that its expression was increased in both groups in the infarct zone but at higher levels in Eng+/+ mice as previously reported (Fig. 3A)[34]. We also characterized the inflammatory cell accumulation in the infarct zones of these mice one-week post-MI by staining with antibodies against CD68 and Mac-3 for macrophages or with anti-CD45 for polymorphonuclear leukocytes (Fig. 3B). This revealed no significant differences in the number of immunoreactive cells in the infarct zones of Eng+/- mice compared with Eng+/+ mice (Fig. 3C-E). In contrast, although we found a slight increase in the basal number of vessels in Eng+/- mice (Supplementary Fig. 1A), the number of vessels in the infarct zone of Eng+/- mice was significantly lower than in Eng+/+ mice one-week post-MI (657 ± 39 versus 1138 ± 86 vessels/mm2) (Fig. 3F), suggesting that defects in angiogenesis occur during cardiac remodeling as a result of reduced endoglin levels. We next analyzed heart function in these mice by MRI. There were no differences between Eng+/+ and Eng+/- mice before MI or one-week post-MI (Fig.4B and supplementary data Fig. 1B-C). The survival curves of the Eng+/+ and Eng+/- mice were also identical (Supplementary data Fig. 2A. 65% versus 67%, respectively, p=0.979). However, MRI analysis one-month post-MI showed that stroke volume index (SVI), cardiac index (CI) and the ejection fraction (EF) were significantly lower in Eng+/- mice compared to Eng+/+ littermates (Fig. 4A-E. 0.914±0.286 versus 1.348±0.556 ml/kg, p=0.001; 0.426±0.097 versus 0.7±0.219 l/min/kg, p=0.001; 16.7%±14.6 versus 35.3%±16.0, p=0.001, respectively). The number of vessels was also lower in Eng+/- compared to Eng+/+ mice (Fig. 4F. 260±153 versus 476.7±75.2 vessels/mm^2, p=0.004), indicating that neoangiogenesis defects in Eng+/- mice were

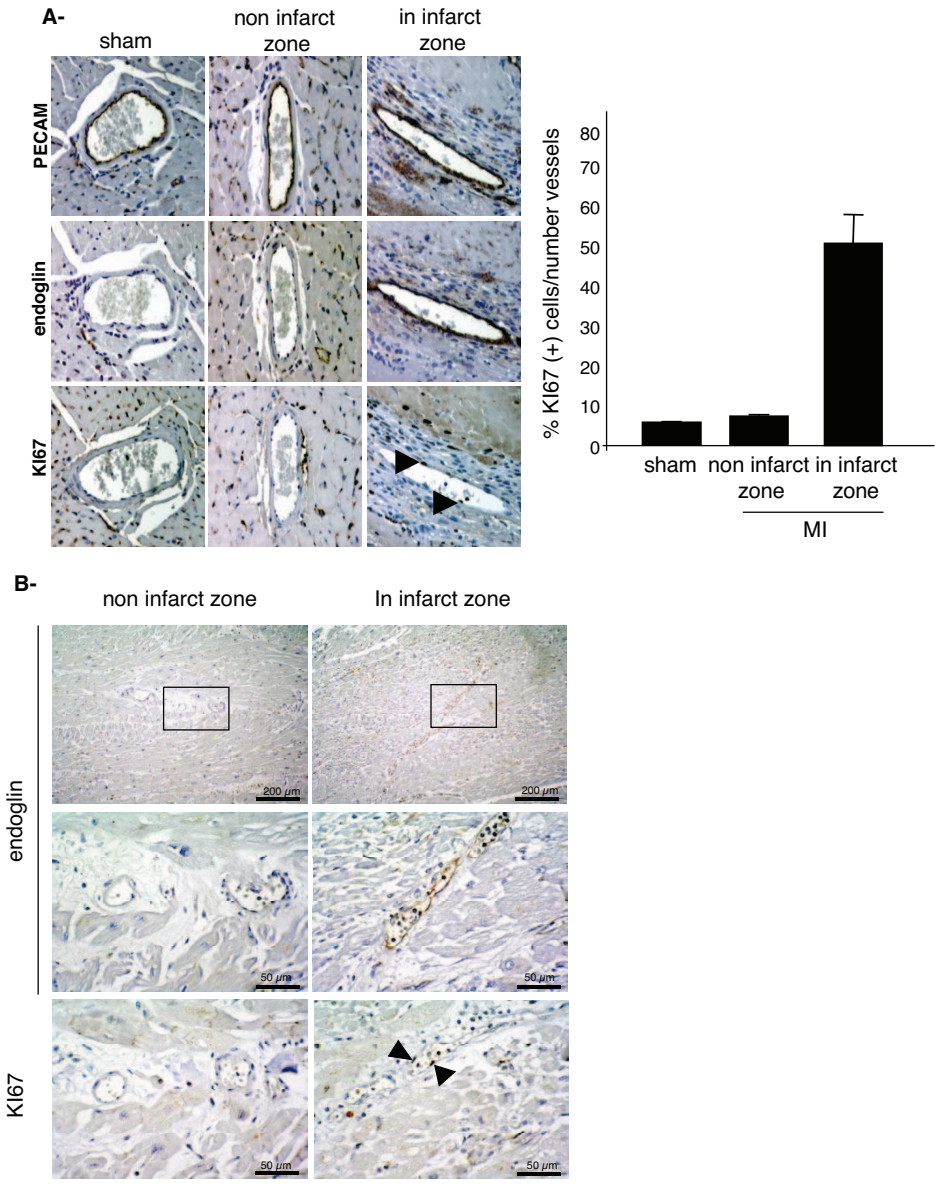


Figure 2
Endoglin is upregulated in active endothelium. (A) Upregulation of endoglin is associated with neoangiogenesis following MI. PECAM is expressed in all vessels (upper panels), whereas endoglin is specifically expressed in the infarct zone (right panels). Ki67 (+) ECs were associated with endoglin (+) vessels of the infarct zone (lower panels). (B) Endoglin and Ki67 immunohistochemistry of human infarcted hearts shows that upregulation of endoglin expression is associated with neoangiogenesis. Black arrows show Ki67 (+) cells.

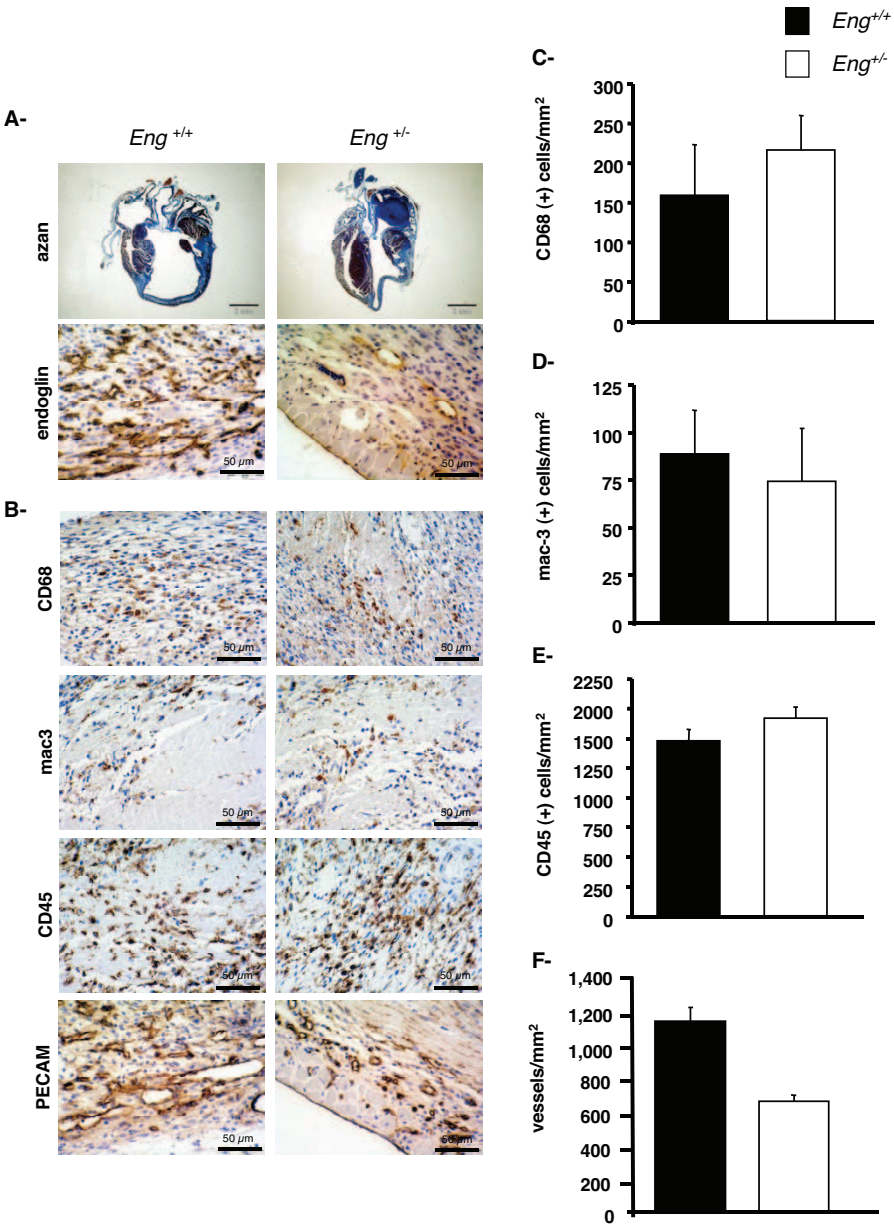
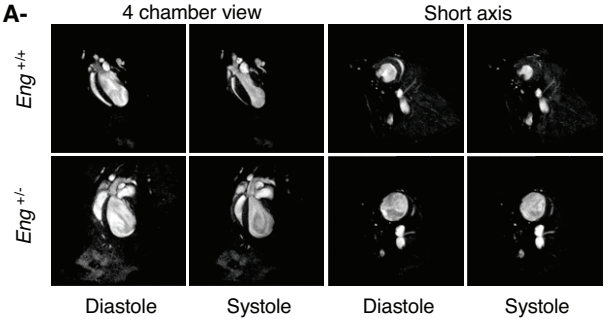


Figure 3
Inflammatory cell accumulation and angiogenesis in Eng+/- mice. (A) Representative examples of AZAN-Mallory and endoglin staining of Eng+/+ (left) and Eng+/- (right) adult mouse heart one-week post-MI. Endoglin protein expression was reduced in Eng+/- hearts. (B) Paraffin sections were immunostained for PECAM, CD68, Mac-3 and CD45. Accumulation of immunoreactive cells was quantified as either number cells/mm^2 and did not significantly change between wild-type and Eng+/- hearts (C, D, E). The total number of vessels/mm^2 was reduced in the Eng+/- compared to Eng+/+ hearts one-week post-MI in the infarct area (blue area of the azan staining).

A-



B-

	Non MI		4 weeks post-MI				
	Eng+/+ (n=2)	Eng+/+ (n=2)	Eng+/+	Eng+/-	Eng+/- + Healthy MNCs	Eng+/- + HHT1 MNCs	P values (corrected for multiple group comparisons)
Age (weeks)	18.5	18.5	12.5 ± 9.7	12.0 ± 9.0	8.0 ± 13.0	15.0 ±14.0	0.402
Weight (g)	23.50	23.55	24.4 ± 4.1	25.2 ± 4.1	28.5 ± 5.1	28.3 ±3.9	0.128
HR (bpm)	542.5	550.0	455.5 ± 85.8	442.7 ± 54.0	500.0 ± 90.0	484.0 ±115.0	0.174
EDV (ml)	0.0371	0.0369	0.100 ± 0.102	0.124 ± 0.046	0.067 ± 0.082	0.125 ± 0.070	0.827
ESV (ml)	0.0116	0.0114	0.062 ± 0.089	0.104 ± 0.052	0.045 ± 0.065	0.105 ± 0.062	0.364
EDVI (ml/kg)	1.598	1.571	3.485 ± 3.770	4.800 ± 1.458	2.367 ± 3.626	4.432 ± 1.779	0.073
ESVI (ml/kg)	0.500	0.486	2.335 ± 3.748	4.126 ± 1.597	1.595 ± 2.818	3.697 ± 1.843	0.364
SV (ml)	0.026	0.026	0.036 ± 0.019	0.022 ± 0.006	0.022 ± 0.021	0.022 ± 0.008	0.066
SVI (ml/kg)	1.097	1.084	1.348 ± 0.556	0.914 ± 0.286	0.771 ± 0.805	0.872 ± 0.189	0.002
CO (ml/min)	0.014	0.014	0.018 ± 0.008	0.011 ± 0.002	0.012 ± 0.007	0.012 ± 0.003	0.008
CI (l/min/kg)	0.596	0.600	0.700 ± 0.219	0.426 ± 0.097	0.423 ± 0.384	0.421 ± 0.058	0.005
MM (g)	0.072	0.073	0.106 ± 0.035	0.094 ± 0.015	0.094 ± 0.036	0.114 ± 0.038	0.106
EF (%)	68.9	69.0	35.3 ± 16.0	16.7 ± 14.6	34.9 ± 3.4	18.8 ± 10.8	0.005

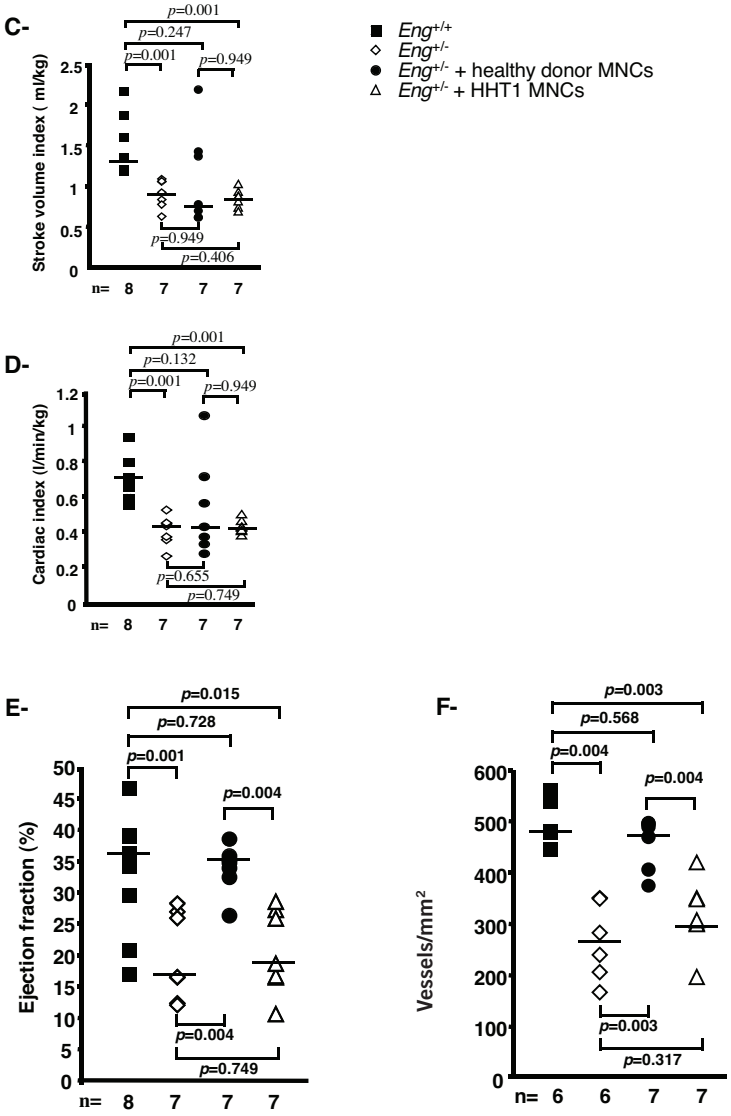
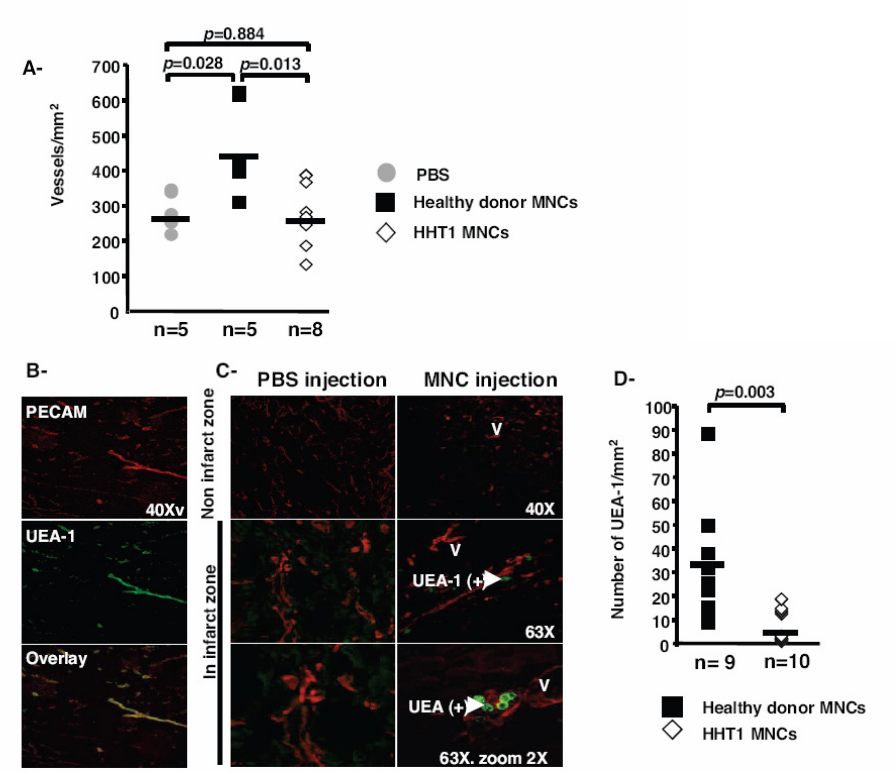
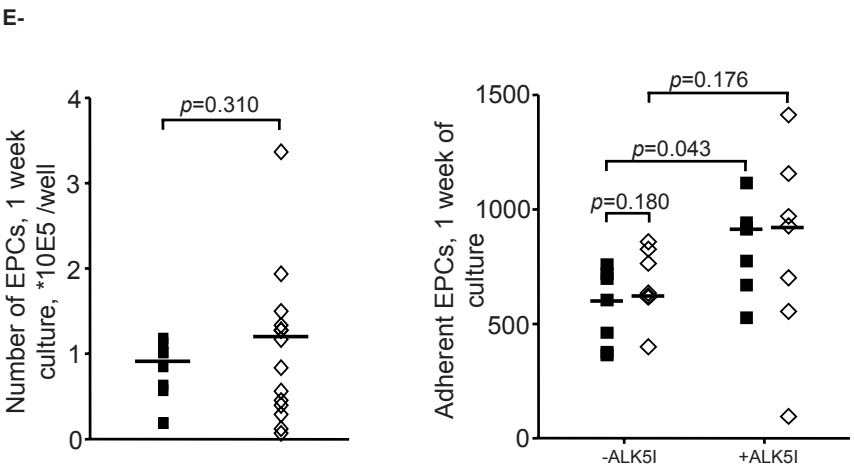


Figure 4
Injection of healthy donor, but not HHT1 MNCs into Eng+/- mice with acute myocardial infarction rescues neoangiogenesis and deterioration in cardiac function associated with reduced endoglin expression. (A) MRI analysis of Eng+/+ and Eng+/- mice four weeks post-MI. (B) Table shows detailed analysis of heart function of Eng+/+ and Eng+/- mice before and one month post-MI. (C-E) Cardiac functions of Eng+/- mice are reduced compared to Eng+/+ mice. (E-F) After healthy donor but not HHT1 MNC injection, (E) ejection fraction is improved and (F) neoangiogenesis is rescued in Eng+/- mice. HR: heart rate; EDV: end diastolic volume; ESV: end systolic volume; EDVI: end diastolic volume index; ESVI: end systolic volume index; SV: stroke volume; SVI: stroke volume index; CO: cardiac output; CI: cardiac index; MM: myocardial mass; EF: ejection fraction. Gender. No significant difference (Fisher's exact test p=1.000) Age. No significant difference (Mann-Whitney U test. HHT1 patients: 35.30 ± 16.80; Healthy donors: 29.20 ± 6.40; p=0.461).


A-
p=0.884
p=0.028
p=0.013
Vessels/mm²
700
600
500
400
300
200
100
0
n=5
n=5
n=8
PBS
Healthy donor MNCs
HHT1 MNCs
B-
PECAM
40Xv
UEA-1
Overlay
C-
PBS injection
MNC injection
Non infarct zone
In infarct zone
V
40X
UEA-1 (+)
63X
UEA (+)
63X. zoom 2X
D-
p=0.003
Number of UEA-1/mm²
100
90
80
70
60
50
40
30
20
10
0
n= 9
n=10
Healthy donor MNCs
HHT1 MNCs


E-
Number of EPCs, 1 week culture, *10E5 /well
p=0.310
4
3
2
1
0
Adherent EPCs, 1 week of culture
p=0.176
p=0.043
p=0.180
1500
1000
500
0
-ALK5I
+ALK5I

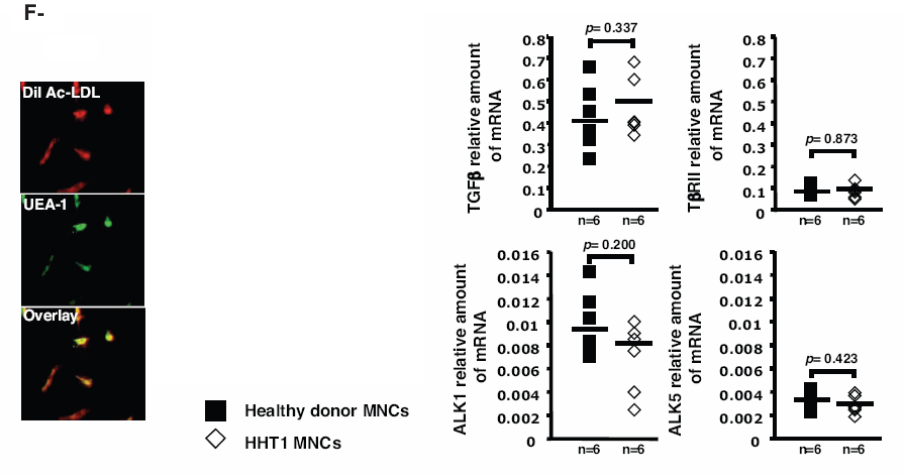


Figure 5
MNCs isolated from HHT1 patients fail to stimulate neoangiogenesis. (A) Number of vessels in the infarcted zone of hearts of Balb/c mice 14 days post-MI was determined by counting PECAM (+) vessels after immunohistochemical staining. (B) Ulex europaeus agglutinin-1 (UEA-1) lectin coupled to FITC stains EC of human fetal heart specifically and not other cell types. (C) MNC-derived ECs were found only in the infarcted zone. (D) MNCs isolated from HHT1 patients are impaired in their ability to home to infarcted mouse hearts. The number of MNC-derived ECs per mm^2 in hearts of Balb/c mice 4 days post-MI was determined by counting UEA-1 (+) cells. (E) MNCs from healthy donors and HHT1 patients showed no differences in the number of cultured EPCs after 1 week of culturing. Unlike healthy donor EPCs, HHT1 EPCs did not show an increased capacity to attach to fibronectin when simulated with Alk 5 kinase inhibitor (Alk5I). (F) Expression levels of the components of the TGF-β signaling pathway in the HHT1 MNC fractions were comparable to those in healthy donor MNCs.

associated with a markedly greater deterioration in cardiac function post-MI.
In the light of evidence that MNCs contribute to the formation of new blood vessels, we investigated whether MNCs might contribute to these angiogenesis defects by injecting MNCs from healthy human donors and MNCs from HHT1 patients into the tail veins of Eng+/- mice post-MI. Details of the ENG mutations in the patients studied are in supplementary table 1 and 2. Previous analysis of endoglin protein levels in affected patients strongly supported haploinsufficiency and associated reduced levels of functional protein as the underlying cause of HHT1[4]. This had suggested that disease heterogeneity cannot be explained by the position and type of mutations[21-22]. The survival curves of the injected groups were identical to those of the non-injected groups (Supplementary table 2A). Whilst MRI analysis one month later revealed that MNCs from healthy human donors significantly improved heart function of Eng+/- mice (Fig. 4B and 4E. EF: 16.7%±14.6 versus 34.9%±3.4, p=0.004) and also stimulated neoangiogenesis (Fig. 4F: 260±153 versus 460±89.5 vessels/mm^2, p=0.003), MNCs from HHT1 patients failed to

improve these parameters (Fig. 4B and 4E. EF: 16.7%±14.6 versus 18.8%±10.8, p=0.749 and Fig.4F: 260±153 versus 305.8±55.0 vessels/mm^2, p=0.317, respectively). To confirm the difference in the ability of MNCs from healthy donors and from HHT1 patients to contribute to vascular repair, we next injected MNCs intravenously into wild-type mice 1-3 hours post-MI. 75% of mice receiving PBS vehicle alone, 89% receiving healthy donor MNCs and 81% receiving HHT1 MNCs, recovered normally from the procedure (Supplementary Fig. 2B). The number of vessels in the infarct zone was determined 14 days post-MI. Vessel formation was efficiently stimulated by intravenous injection of MNCs from healthy donors (271.8±106.5 versus 418±265.8 vessels/mm^2, p=0.028) (Fig. 5A). However, MNC from HHT1 patients showed a consistently impaired ability to stimulate neoangiogenesis (271.8±106.5 versus 269.8±181.2 vessels/mm^2; p=0.884) (Fig. 5A).

Since endoglin is expressed in vascular and various hematopoietic lineages, MNC-derived ECs homing to the infarct zone were traced by co-staining with mouse specific anti-PECAM antibody and human Ulex europaeus agglutinin-1 (UEA-1) lectin, a marker commonly used for human cells with endothelial characteristics (Fig. 5B)[35]. MNCs homed only to the infarcted zone, as previously reported (Fig. 5C)[25, 26]. The number of UEA-1 (+) cells that accumulated in the infarct zone of mice receiving cells from HHT1 patients was significantly lower than mice receiving cells from healthy donors (4.01±12.33 versus 32.4±26.7 MNC-derived ECs/mm^2, p=0.003) (Fig. 5D).

Analysis of MNCs from HHT1 patients and healthy controls showed that the number of EPCs cultured from 10^7 MNCs after eight days of growth (121000±110000 versus 88000±68750 adherent cells; p=0,310) were similar (Figure 5E). Nor was there a difference in the ability to attach as determined in vitro (651±211 versus 615±344; p=0.180)(Figure 5E). Treatment with an ALK5 inhibitor (ALK5I) resulted in an increased number of adherent EPCs from healthy controls (924±272; p=0.043). However, no difference was found for adherent HHT1 patient derived EPCs (944±602; p=0.176) (Figure 5E). Flow cytometry analysis showed no difference in the number of circulating CD34$^+$VEGFR2$^+$ cells. Interestingly, the number of CD34$^+$ cells in HHT1 patients was increased compared to healthy control patients (Chapter 7). In addition, we had no evidence of changes in expression of any of the major components of the TGF-β signaling pathway (Fig. 5F).

Discussion

Neovascularization is a normal component of the remodeling process that occurs after MI. Its promotion has been proposed as an important target for therapeutic improvement of heart function[36]. However, the mechanisms underlying neovascularization following MI are still not fully understood although signaling pathways activated by TGF-β, vascular endothelial growth factor (VEGF) and insulin-like growth factor (IGF) are thought to be involved[37]. Here, we examined the function of endoglin, a TGF-β receptor involved in angiogenesis during development. We demonstrated that endoglin has a crucial role in the normal remodeling process post- MI that coincides with upregulation of its major ligand, TGF-β, in myofibroblasts in the infarct zone[38]. Specifically, upregulation of endoglin in ECs, possibly stimulated by local TGF-β[39], correlates with neoangiogenesis. Further, Eng+/- mice showed impaired angiogenesis post-MI, which resulted in an enhanced deterioration in cardiac function; this confirmed recent findings using an alternative experimental model of ischemic hindlimb injury[34]. Our results are consistent with reports demonstrating that (1) endoglin is a marker of angiogenesis[40]; (2) endoglin activates TGF-β/ALK1 signalling and inhibits TGF-β/ALK5 signalling to promote EC proliferation[20]; (3) down regulation of endoglin expression induces EC apoptosis[41]. More generally, impaired angiogenesis in Eng+/- mice may at least in part be mediated by EC defects.

Compelling evidence indicates that recruitment of circulating vascular as well as hematopoietic cells contributes to the revascularization of ischemic tissues[42]. Because endoglin is expressed in various cell lineages that comprise the mononuclear cell fraction, we have investigated the effects of a reduction of endoglin expression in these circulating cells using experimental MI in mice as a model system for study. With respect to the disease HHT1 specifically, our study represents the first evidence that MNCs derived from patients are impaired in their capacity to stimulate vessel formation. Moreover, injection of healthy MNCs in Eng+/- mice was sufficient to restore vessel formation and to improve heart function defects associated with reduced levels of endoglin but MNCs from HHT1 patients were not, demonstrating that the etiology of HHT1 is possibly associated with a defect in the ability of MNCs to repair local vessel damage.

It is unclear which cell populations are affected by the decrease of endoglin expression. Previous studies have shown that (1) blood outgrowth ECs from HHT have abnormalities that would be compatible with a role in vascular lesions[43]; (2) endoglin functions to support lineage-specific hematopoietic development from Flk-1 precursors[44], and defines Long Term Repopulating hematopoietic population[45, 46]; (3) endoglin is expressed in activated monocytes[47, 48]. In our study, we demonstrated that MNC-derived

ECs from HHT1 patients have a reduced ability to accumulate to the infarct zone in vivo and to stimulate vessel formation in mice that had undergone experimental myocardial infarction. One possible explanation is that there are differences in the MNC populations between patients and controls. However, we found no differences in the number of MNCs to attach and differentiate to EPCs, nor did we find differences in the number of $CD34^+/VEGFR2^+$ cells. Despite equal ALK5 expression in vitro ALK5 inhibition of MNCs under EPC differentiating conditions only increased healthy donor cell attachment and not HHT1 cell attachment. This may be the net result of the relatively low ALK1 levels in HHT1 cells, leading to a decreased capacity to attach to fibronectin.

Therefore, the differential behavior in the heart rescue experiments may be due to defective homing, transdifferentiation, proliferation or secretion of angiogenic factors, all of which require further investigation in future studies. Although we found no differences in TGF-β, TβRII, ALK1 or ALK5 expression in HHT1 MNCs in vitro, we have shown previously that in mouse development, ECs lacking endoglin entirely are defective in their ability to process and secrete active TGF-β[49], which in turn affects their ability to differentiate and recruit smooth muscle cells to the vessel wall. Defective processing of TGF-β or other cytokines could play a role in patients and also directly or indirectly affect neoangiogenesis.

In conclusion, since vessel damage may occur randomly in HHT patients as a result of trauma, UV exposure, inflammation or multiple individual pathological or physiological differences, this defect in normal repair may contribute to explaining why families or even individual family members with the same mutation present with highly variable symptoms of the disease. Secondly, and more generally, in the light of ongoing trials investigating the use of autologous bone marrow in vascular repair after MI, the results suggest that HHT-1 patients and others with intrinsic defects in their capacity to form endothelial progenitors may derive greater benefit from transplantation with matched heterologous bone marrow.

Acknowledgements

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Supplementary data

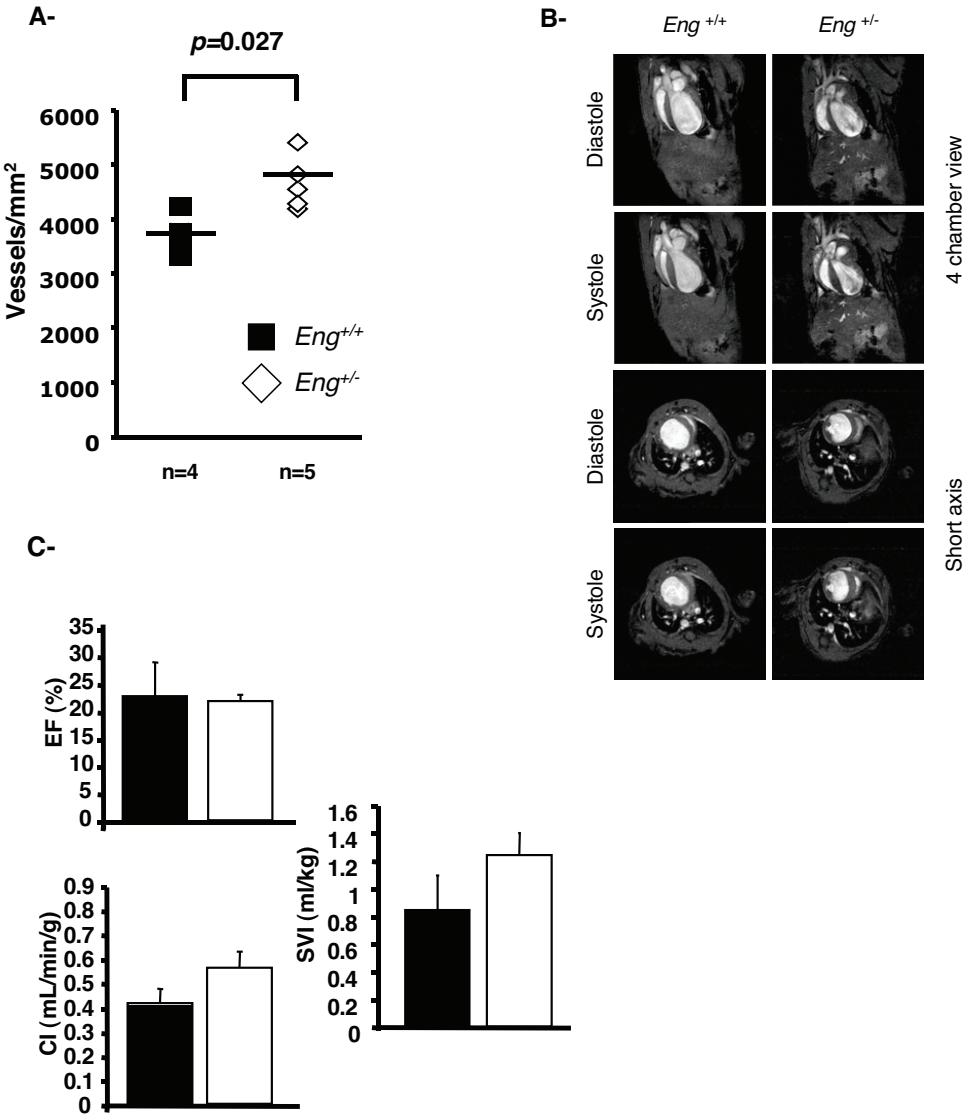

Figure 1
(A) Vessel density is slightly increased in sham operated heart of Eng+/- mice compared to Eng+/+ mice. (B-C): No differences in heart function of Eng+/- and Eng+/+ mice one week post-MI as determined by MRI. SVI: Stroke Volume Index; CI: Cardiac Index; EF: Ejection Fraction.

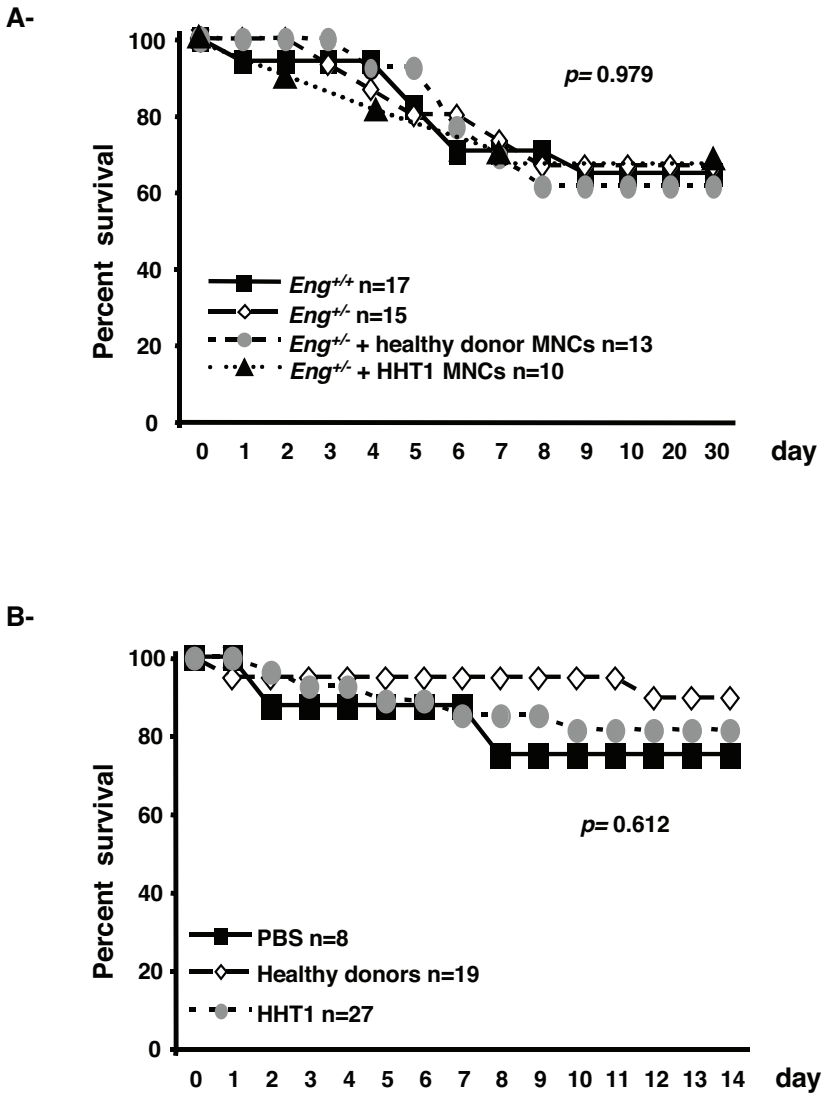

Figure 2
(A) Peri- and post-operative mortality for experimental MI is identical in Eng+/+ , Eng+/- and Eng+/- mice receiving healthy donor or HHT1MNCs. (B) No significant difference in peri- and post-operative mortality of Balb/c mice receiving PBS, MNCs from healthy donors or from HHT1 patients after MI.

Table 1: Characteristics of HHT1 patients used in the homing experiments (Fig.5D). Statistical analysis has been performed by comparison with the healthy donor control group.

Mutation type	Exon	DNA	Protein	Gender	Age
Nonsense	2	c157C>A	pC53X	W	36
	3	c247C>T	pQ83X	W	39
Deletion/insertion	7	c887-918del; c919-920ins		M	55
		del22bpIns11bp		M	55
		CAAGCTCCCAG			
	8	c.1083AA		W	57
Splice site	1	c.1A>G	p.M1V	W	30
	9b	c.1310delG		W	19
Missense	7	c.991G>A	p.G331S	W	32
	7	c.991G>A	p.G331S	M	60

Gender. No significant difference (Fisher's exact test p=1.000)
Age. No significant difference (Mann-Whitney U test. HHT1 patients: 42.56 ± 14.59; Healthy donors: 35.80 ± 10.49; p=0.278).

Table 2: Characteristics of HHT1 patients used to measure the effect of the injected MNCs on neoangiogenesis and heart function associated with MI (Fig.4 and Fig.5A). Statistical analysis has been performed by comparison with the healthy donor control group.

Mutations Type	Exon	DNA	Protein	Gender	Age
Nonsense	2	c.157C>A	pC53X	W	36
	3	c.247C>T	pQ83X	W	39
	3	c.247C>T	pQ83X	W	44
	3	c.247C>T	pQ83X	W	62
	3	c.247C>T	pQ83X	M	36
Deletion/insertion	7	c887-918del; c919-920ins		M	28
		del22bpIns11bp			
		CAAGCTCCCAG			
	8	c.1117_1118insT	p.K373fs	M	48
	8	c.1117_1118insT	p.K373fs	M	50
	8	c.1117_1118insT	p.K373fs	W	15
Splice site	9b	c.1311G>AAGCGGggag	p.R437R	M	20
	9b	c.1310delG		W	19
Missense	7	c.991G>A	p.G331S	M	60

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Part III | Chapter 7

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7

Impaired recruitment of HHT-1 mononuclear cells to the ischemic heart is due to an altered CXCR4/CD26 balance

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Abstract

Aim

Mononuclear cells (MNCs) from patients with hereditary hemorrhagic telangiectasia type 1 (HHT1), a genetic disorder caused by mutations in endoglin, show a reduced ability to home to infarcted mouse myocardium. Stromal-cell derived factor-1α (SDF-1α) and the chemokine receptor CXCR4 are crucial for homing, and negatively influenced by CD26. The aim of this study was to gain insight into the impaired homing of HHT1-MNCs.

Methods

CXCR4 and CD26 expression on MNCs was determined by flow cytometry. Transwell migration to SDF-1α was used to analyze *in vitro* migration. Experimentally induced myocardial infarction in mice, followed by tail vein injection of MNCs, was applied to study homing *in vivo*.

Results

HHT1-MNCs expressed elevated levels of CXCR4, but this was counterbalanced by high levels of CD26, resulting in decreased migration towards an SDF-1α gradient *in vitro*. Migration was enhanced by inhibiting CD26 with Diprotin-A. While MNCs from healthy controls responded to TGFβ stimulation by increasing CXCR4 and lowering CD26 expression levels, HHT1-MNCs did not react as efficiently: in particular CD26 expression remained high. Inhibiting CD26 on MNCs increased the homing of human cells into the infarcted mouse heart. Interestingly, the defect in homing of HHT1-MNCs was restored by pre-incubating the HHT1-MNCs with Diprotin-A before injection into the tail vein.

Conclusions

We show that a decreased homing of HHT1-MNCs is caused by an impaired ability of the cells to respond to SDF-1α. Our results suggest that modulating CD26 levels using inhibitors like Diprotin-A can restore homing in cases where increased expression of CD26 contributes to the underlying pathological mechanism.

Introduction

Hereditary hemorrhagic telangiectasia (HHT, also known as Rendu-Osler-Weber disease) is an autosomal dominant vascular disorder with an estimated prevalence of approximately 1 in 10.000. Several different types of HHT have been described,but characteristically they are all associated with mutations in components of the transforming growth factor-beta (TGFβ) signaling pathway. The underlying cause of HHT type 1 (HHT1) are mutations in endoglin (CD105), an accessory TGFβ type III receptor. Endoglin is primarily expressed on proliferating endothelial cells *in vitro* and angiogenic blood vessels *in vivo* but also on other cells types, like circulating blood mononuclear cells (MNCs) although to a lesser extent. Clinically, HHT1 is characterized by telangiectasias and epistaxis[1,2]. With age, the incidence and severity of bleedings increases. Because endoglin is crucial for the formation of new vessels[3], the higher number of hemorrhages in the endoglin haplo-insufficient HHT1 patients may result from a reduced ability to restore the injured vasculature. Vascular repair is mediated by activation of endothelial cells lining the vessel wall to replace the damaged cells, but it has become evident that circulating MNCs also have the ability to restore damaged vessels[3]. Vessel repair and the influx of MNCs including monocytes and lymphocytes, are important in the restoration of the injured heart, for example after a myocardial infarction (MI). MNCs promote healing of the damaged heart via stimulation of myofibroblast proliferation, deposition of collagen and the stimulation of angiogenesis[4]. Recently, we demonstrated that the recruitment of human MNCs to the infarcted murine heart and subsequent vessel formation is severely impaired when using HHT1-derived MNCs compared to healthy MNCs[5].

Homing and trafficking of cells is regulated to a large extent by the chemokine stromal cell-derived factor-1α (SDF-1α or CXCL12) and its receptor CXCR4. SDF-1α is upregulated in ischemic myocardium shortly after MI, resulting in the recruitment of CXCR4 expressing cells from the circulation to the damaged area[6-8]. Besides SDF-1α, the expression of transforming growth factor β (TGFβ) is increased after MI[9]. TGFβ is a growth factor that controls the proliferation, adhesion, apoptosis, homing and migration of many cell types[10]. TGFβ was shown to positively influence the SDF-1α/CXCR4 axis by increasing the expression of CXCR4 on circulating blood cells and different cancer cells[11-14]. Furthermore, TGFβ reduced the levels of the homing-inhibiting peptidase CD26 on these cell types[11-14].

CD26 is a serine di-peptidylpeptidase (also known as DPP-IV) that cleaves the amino-terminal dipeptide from SDF-1α, known to interact with the extracellular portion of CXCR4. Additionally CD26 can co-internalize with CXCR4[15,16]. CD26 therefore interferes

with the SDF-1α/CXCR4 axis by preventing the recruitment of cells to SDF-1α. Many different cell types carry CD26 on their surface, thereby modulating their migratory capacity[16,17]. Inhibiting CD26 using Diprotin-A has been shown to increase homing and migration of cells *in vitro*[18], and *in vivo*[19,20], and enhanced their engraftment in the bone marrow of lethally irradiated recipient mice[21].
Since all mutations in the endoglin gene reported to date result in reduced functional cell surface protein levels and deregulation of TGFβ signaling, we hypothesized that this mutation alters the balance between CXCR4 and CD26 on HHT1-MNCs. This would lead to an impaired ability of HHT1-MNCs to respond to elevated SDF-1α levels *in vitro* and *in vivo*.

Materials and methods

Patients and blood samples

Venous blood samples from age and gender matched healthy human volunteers and HHT1 patients were collected in potassium/EDTA tubes (Vacuette, Greiner Bio-One, the Netherlands). Peripheral blood MNCs were isolated by density gradient centrifugation using Ficoll Paque Plus (Amersham Biosciences, Sweden), according to the manufacturer's protocol. Isolated MNCs were washed twice with PBS supplemented with 2 mM EDTA, and counted on a hemocytometer. All procedures were approved by the medical ethics committee of the St. Antonius Hospital Nieuwegein, the Netherlands. The investigation conforms to the principles outlined in the Declaration of Helsinki.

Flow cytometry

Flow cytometric analysis was performed using 100µl of whole blood or $3*10^5$ MNCs in PBS. Whole blood was stained with anti-CD14-PE (DakoCytomation, Denmark), anti-Endoglin-Fluorescein (R&D Systems, USA), anti-CD34-FITC (BD Pharmingen, USA) and anti-VEGFR2-PE (R&D Systems, USA). MNCs were stained with anti-CD14-ECD (Immunotech, Coulter, France), anti-CD26-FITC (Serotec, UK) and anti-CXCR4-PE (BD Pharmingen, USA). Isotype-matched fluorochrome-conjugated antibodies were used as controls. Red blood cells were lyzed before measuring fluorescence on a flow cytometer (Cytomics FC500, Beckman Coulter, the Netherlands). Analysis was performed using CXP software (Beckman Coulter, the Netherlands). The number of positive cells is expressed as absolute cell number per ml of whole blood, or as percentage of positive cells within a cell fraction. The Mean-Fluorescent Intensity (MFI) is presented for cell populations of interest.

MNC migration

Migration of freshly isolated MNCs was assessed in a transwell system using polycarbonate filters with 5µm pores (Corning, the Netherlands). Prior to migration, MNCs were incubated for 1 hour in RPMI 1640 Glutamax medium, supplemented with 10% FBS at 37°C. If applicable, MNCs were stained with Calcein AM (Invitrogen, Karlsruhe, Germany). MNCs were pretreated at room temperature for 15 minutes with 5 mM Diprotin-A (Sigma-Alderich, St Louis, USA) to inhibit CD26 or for 30 minutes at room temperature with 5µg/mL AMD3100 (Sigma, Saint Louis, USA) to block CXCR4. One-hundred thousand MNCs were applied to the upper well, and in the lower well medium, without or with 200ng/ml SDF-1α (PeproTech, Rocky Hill, NJ, USA), was added. The cells were allowed to migrate for 3 hours at 37°C.

After migration, cells were collected and 75.000 PeakFlow^{tm} carmine flow cytometry reference beads (6µm, Invitrogen, Eugene, Oregon, USA) were added. The number of MNCs per 10.000 beads was assessed by flow cytometry. The migration percentage was calculated from the number of cells migrated to SDF-1α compared to the number of cells migrated in the absence of SDF-1α.

MNC stimulation

Freshly isolated MNCs were stimulated for 24 hours at 37°C with 2ng/ml TGFβ_1 (PeproTech, Rocky Hill, NJ, USA) in RPMI medium supplemented with 1% FBS, and pretreated for 15 minutes at room temperature with 5 mM Diprotin-A (Sigma-Alderich, St Louis, USA) to inhibit CD26. Stimulated MNCs were stained with anti-CD14-ECD, anti-CD26-Fluorescein and anti-CXCR4-PE. Expression levels of CXCR4 and CD26 were determined by measuring MFI. Expression changes were calculated by dividing the MFI after by the MFI before stimulation and presented as fold induction.

Induction of myocardial infarction in mice

All experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and with prior approval by the Animal Ethical Experimentation Committee, Utrecht University. In 28 BALB/cOlaHsd mice (Harlan, the Netherlands), aged 10-12 weeks, a myocardial infarction (MI) was induced as described previously[22]. Briefly, mice were intubated and ventilated with 2% isoflurane/98% oxygen. A left thoracotomy was performed and the left anterior descending coronary artery (LAD) was visualized. The LAD was permanently occluded by placing a 7-0 prolene suture.

Intravenous injection of human MNCs

One day after induction of MI, BALB/c mice received $5*10^6$ human MNCs (HHT1 or control, n=7 per group) via tail vein injection. Mice were immunosuppressed by subcutaneous injection of tacrolimus (5 mg/kg/day) for four days.

Tissue collection

Five days after MI, surviving mice were euthanized (CTL-: n=5, CTL+Dip: n=6, HHT-: n=7, HHT+dip: n=5). The hearts were flushed with 5 ml of PBS via the right ventricle and dissected. The tissue was processed for cryosectioning in OCT compound (Sakura, the Netherlands).

Immunohistochemistry

Frozen longitudinal 7µm thick sections of the whole ventricle were stained using a mouse anti-human nuclei antibody (Chemicon, Temecula, CA, USA) for the identification of human cells. Briefly, sections were fixed in acetone, air dried and rehydrated in PBS. Endogenous peroxidase activity was blocked, followed by incubation with avidin and biotin respectively. The tissue was permeabilized in 0.2% Triton X-100 in PBS and blocked with 3% BSA in PBS for 30 minutes. During this incubation, the anti-human nuclei antibody was biotinylated 1:50 using the Dako-ARK for mouse primary antibodies (Dako, the Netherlands) according to the manufacturer's protocol. Sections were incubated with α-human nuclei, PBS or mouse IgG_1 (Dako, the Netherlands) as an isotype control overnight at 4°C. After washing, slides were incubated with ABC peroxidase complex (DAKOcytomation, Dako, the Netherlands) at room temperature for 30 minutes. Peroxidase activity was detected using AEC substrate. After counterstaining with hematoxylin sections were mounted in gelatin/glycerin.

Semi-quantitative polymerase chain reaction for human Alu sequences

Genomic DNA was isolated by pooling 10-50 crysections (10µm) followed by incubation in 500 µl lysis buffer (0.1 M Tris-HCl pH 8.5, 5 mM EDTA, 0.2% SDS, 0.2 M NaCl). Genomic DNA was extracted using phenol-chloroform, and precipitated with ethanol. The polymerase chain reaction (PCR) primers were described previously and are specific for human Alu sequences, resulting in a product of 224 basepairs[23]. PCR was carried out using 50 ng of total DNA under the following conditions: 95°C for 10 minutes, followed by the appropriate number of cycles of 95°C for 30 seconds, 58°C for 45 seconds and 72°C for 45 seconds; and 72°C for 10 minutes. Amplification of DNA isolated from mice receiving Diprotin-A treated or untreated MNCs was optimized at 23 and 27 cycles, respectively. The samples were run on a 10% polyacrylamide gel and quantified using Quantity One software (Biorad, the Netherlands).

Statistics

Statistical significance was evaluated using the Mann-Whitney U test for comparison between two independent samples, Wilcoxon Signed Ranks test for two related samples, Spearman's rho for correlation calculations and Fisher's Exact test for comparison of 2 categorized variables, using SPSS v11.0 for Windows. Results are expressed as mean ± standard error of the mean (SEM). A value of $P < 0.05$ was considered statistically significant.

Results

Circulating cell populations

To establish whether the attenuated response of HHT1 to ischemic injury[5] is due to fewer circulating cells capable of participating in restoration of vascular damage, we compared peripheral blood of HHT1 patients to age and gender matched healthy controls (see supplementary Tables 1 and 2 for patient characteristics). Monocytes (defined as $CD14^+$) are circulating cells that are able to home to damaged tissue and activated monocytes are known to express endoglin[24].We found no difference in the number of circulating $CD14^+$ cells (Figure 1A,B), and observed low levels of endoglin expression on circulating $CD14^+$ monocytes that decreased in HHT1 patients (Figure 1C,D).

Other cells that can contribute to vascular repair are those within the CD34 expressing cell fraction. Endothelial progenitor cells (EPCs) are part of this $CD34^+$ population, and one way to identify EPCs may be as $CD34^+$-$VEGFR2^+$ cells. In contrast to our expectation, HHT1 patients show a significantly higher number of $CD34^+$ cells (Figure 1E,F), but the number of $CD34^+$-$VEGFR2^+$ cells is comparable between HHT1 patients and controls (Figure 1G,H).

Distribution of CXCR4 and CD26

The CXCR4/SDF-1α axis is particularly important for cell homing, chemotaxis, engraftment and retention in ischemic tissues. Analyzing the distribution of CXCR4 on MNCs (Figure 2A) showed that, although the number of cells in the MNC population expressing CXCR4 was not different between groups (Figure 2B), the mean expression level of CXCR4 on the cell surface (represented by MFI) was significantly higher on HHT1-MNCs (Figure 2C). CD26 is a negative regulator of the SDF1/CXCR4 axis. The HHT1-MNC population showed a significant increase in the expression level of CD26 but no difference in the total number of CD26 positive cells (Figure 2E,F). Additionally, within the MNC subpopulations lymphocytes and monocytes demonstrated similar patterns in the expression of CXCR4 and CD26 (Supplementary Figure 1A,B).

HHT1-MNCs show impaired migratory response *in vitro*

CD26 can regulate SDF-1α/CXCR4 mediated chemotaxis; therefore we performed a migration assay using SDF-1α as a chemoattractant. HHT1-MNCs exhibited decreased migration towards SDF-1α compared to controls (Figure 3A). Interestingly, while pretreatment with the CXCR4 inhibitor AMD3100 completely blocked the migration of both control and HHT1-MNCs, pretreatment with the CD26 inhibitor Diprotin-A only significantly improved the migration of the HHT1-MNCs compared to untreated MNCs (Figure 3B, C). Analyzing the CXCR4 and CD26 expression on migrated cells revealed that the number of CXCR4$^+$ MNCs positively correlated with the migration capacity for both control as well as HHT1-MNCs (Figure 3D, F). Interestingly, the mean CD26 expression on HHT1-MNCs was negatively correlated with their migratory capacity (Figure 3G), while the migration of control MNC did not correlate with CD26 levels (Figure 3E).

MNC response to TGFβ stimulation

TGFβ can play a role in the homing and migration of cells to damaged tissue. After MI, TGFβ levels are increased in the ischemic area. Since in HHT1 patients TGFβ signaling

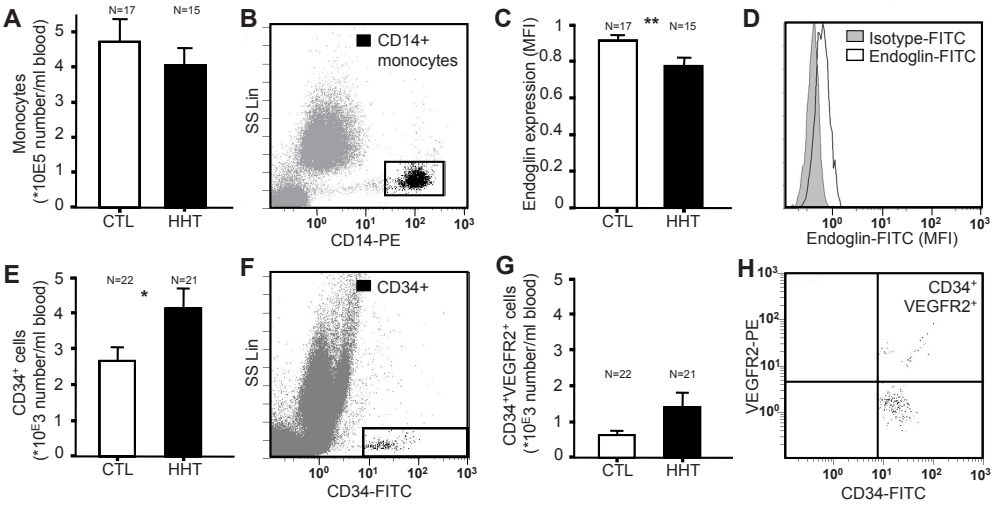

Figure 1.
Composition of the circulating cells in peripheral blood. Flow cytometric analysis of CD14 expression in peripheral blood cells showed no significant difference in the number of CD14+ monocytes obtained from HHT1 patients (HHT) or controls (CTL) (A). (C) The mean fluorescent intensity (MFI) of endoglin expression on CD14+ monocytes shows a reduced surface expression on HHT1-MNCs compared to controls (CTL n=17; HHT1 n=15). (E) HHT1 patients have an increased number of CD34+ cells compared to controls. (G) There is no significant difference in CD34+VEGFR2+ EPCs in HHT1 patients (CTL, n= 22; HHT1, n=21). Representative flow cytometry plots are shown in B, D, F and H. #P<0.05; *P<0.03; **P<0.005. Bars show mean ± SEM.

is disturbed[25] and their homing capacity is abrogated, we investigated the effect TGFβ stimulation has on the CXCR4 and CD26 expression levels on MNCs. Although flow cytometric analysis of TGFβ stimulated MNCs revealed a pronounced increase in total percentage of CXCR4 expressing cells as well as the mean CXCR4 expression on both HHT1 and control MNCs (Figure 4A, B), the relative induction of receptor levels was less on HHT1-MNCs than on control MNCs (3.4 fold for HHT1 versus 5.1 fold for control MNCs, Figure 4C). After TGFβ stimulation, the total number of CD26 expressing cells, as well as the mean expression level, was reduced in both control and HHT1-MNCs (Figure 4D, E). Importantly, the percentage of CD26 positive cells was higher within HHT1-MNCs than within control MNCs, even after TGFβ stimulation (Figure 4D, E). Since CD14$^+$ monocytes might have angiogenic activity[26] we analyzed the effect TGFβ has on these cells. CD14$^+$-monocytes showed similar TGFβ induced CXCR4 expression (supplementary data, figure 1D), and reduced CD26 expression (supplementary data, figure 1E). These experiments show that endoglin haploinsufficiency results in decreased TGFβ responsiveness. Although HHT1-MNCs are able to respond to TGFβ stimulation by increasing CXCR4 and down regulating CD26 expression, this occurred

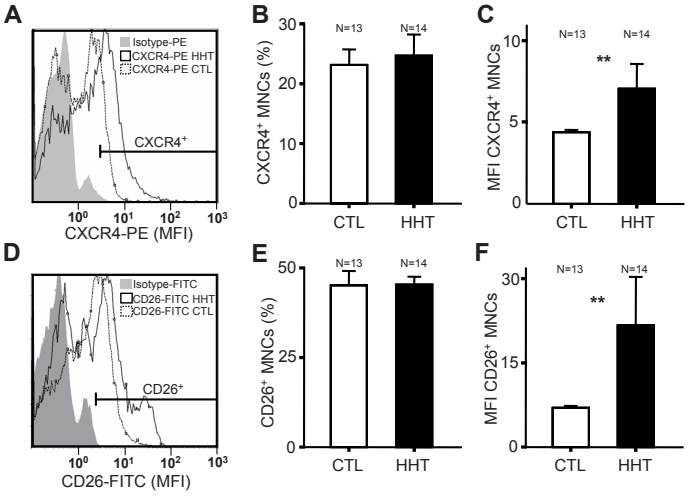

Figure 2.
CXCR4 and CD26 expression on circulating cells. CXCR4 cell surface expression was measured by multivariant flow cytometry. (A) Representative flow cytometry plot for CXCR4 and isotype control. (B) There is no difference in the number of CXCR4$^+$ cells between HHT1 and control MNCs. (C) However, the mean surface expression of CXCR4 within CXCR4$^+$ population is higher on HHT1-MNCs. (D) Representative graph of MFI analysis showing the CD26 levels versus the isotype control. The HHT1-MNC population contains the same number of CD26$^+$ cells (E). The mean surface expression of CD26 is significantly higher in HHT1 CD26+ MNCs (F). CTL, n=13; HHT1, n=14. #P<0.05; *P<0.03; **P<0.005. Bars show mean ± SEM.

to a lesser extent, and the net result is a decreased migratory capacity to SDF-1α compared to controls.

In vivo MNC homing to myocardial infarction

To evaluate the importance of the increased expression of CD26 on MNCs in HHT1 patients for homing and migration to damaged tissue, we used the mouse MI model. Mice underwent a permanent ligation of the LAD. Since SDF-1α levels reach a maximum 24 hours post-MI, one day after MI the mice received an intravenous injection of human MNCs, which were pretreated or not with the CD26 inhibitor Diprotin-A. Mice were sacrificed five days after MI to assess MNC homing.

Immunohistochemical staining using an anti-human nuclei antibody revealed human

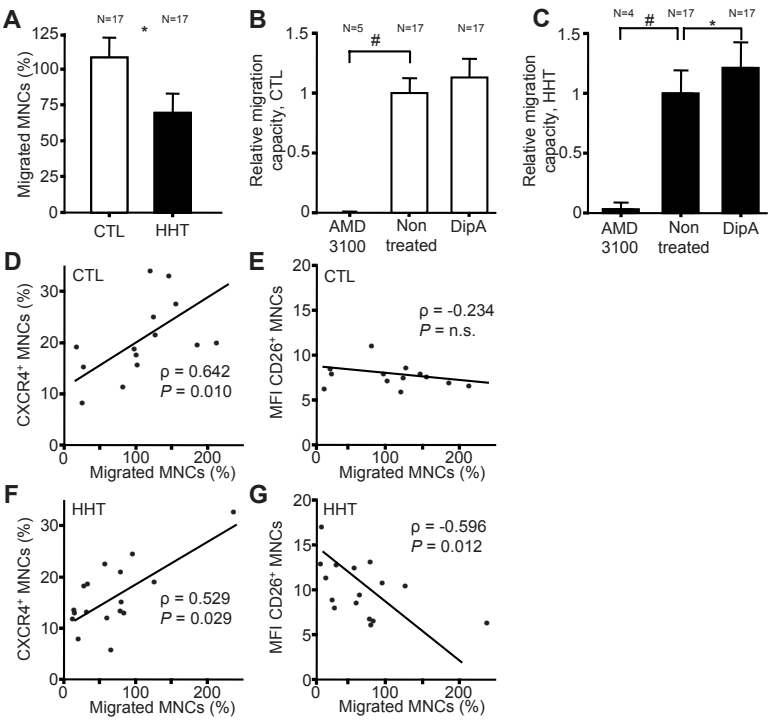

Figure 3.
Migration of MNCs *in vitro*. The potential of MNCs to migrate to SDF-1α was determined in a transwell migration system. (A) HHT1-MNCs have a decreased capacity to migrate to SDF-1α, compared to healthy controls. (B and C) The effect of pretreatment of MNCs with AMD3100 or Diprotin-A on migration. Depicted is the relative migration capacity towards SDF-1α compared to untreated cells. MNC migration positively correlates with the number of $CXCR4^+$ cells shown by a positive Spearman's rho (D and F). However, only HHT1-MNC migration negatively correlates with CD26 expression levels (E and G). CTL, n=17; HHT1, n=17; AMD3100 CTL, n=5; HHT1, n=4; Correlations CTL, n=15; HHT1, n=17. #P<0.05; *P<0.03; **P<0.005. Bars show Mean ± SEM.

cells scattered throughout the injured myocardium five days after infarction (Figure 5A-C). The number of human cells found in the myocardium was quantified by semi-quantitative PCR for human specific Alu repeats (Figure 5D). Five days post-MI, a significantly lower amount of human DNA was found in the myocardium of mice injected with HHT1-MNCs as compared to those receiving untreated control MNCs (Figure 5E, 0.15 ± 0.05 and 0.29 ± 0.06 arbitrary units respectively, see supplementary table 3). Strikingly, when human MNCs were pre-treated with Diprotin-A, there was no longer a difference in the homing and engraftment into the infarcted heart between HHT1 patients and controls (Figure 5F, 0.64 ± 0.18 versus 0.55 ± 0.22 for HHT1 and controls respectively, see supplementary table 3). These results underline the importance of CD26 expression for the homing and migratory capacity of MNCs.

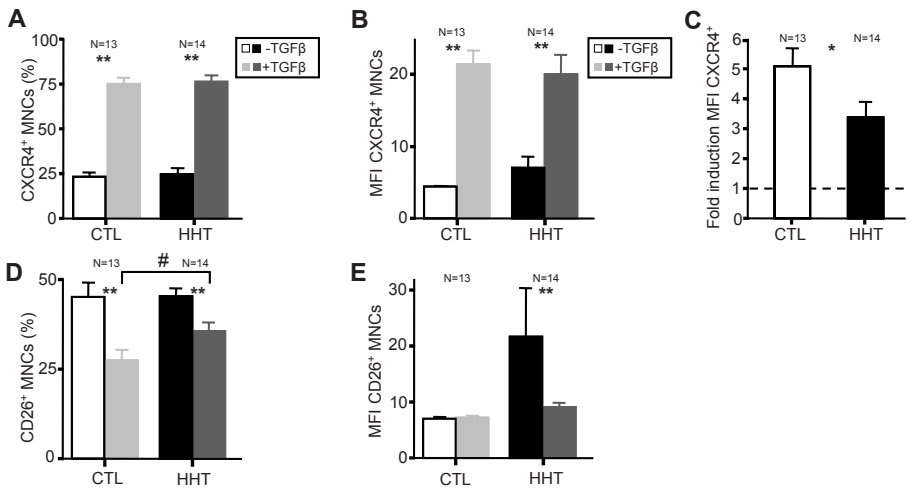

Figure 4.
CXCR4 and CD26 expression before and after TGFβ stimulation Expression of CXCR4 and CD26 was compared by flow cytometry. (A and B) Both HHT1 and control MNCs are capable of CXCR4 upregulation after TGFβ stimulation. (C) However, the relative induction of the receptor was significantly lower on HHT1-MNCs compared to controls. (D and E) Similarly CD26 is downregulated in response to TGFβ stimulation on HHT1 and control MNCs. (D) The number of CD26⁺ cells within HHT1-MNCs after TGFβ stimulation is significantly higher compared to controls. CTL: n=13; HHT1: n=14. #P<0.05; *P<0.03; **P<0.005. Bars show mean ± SEM.

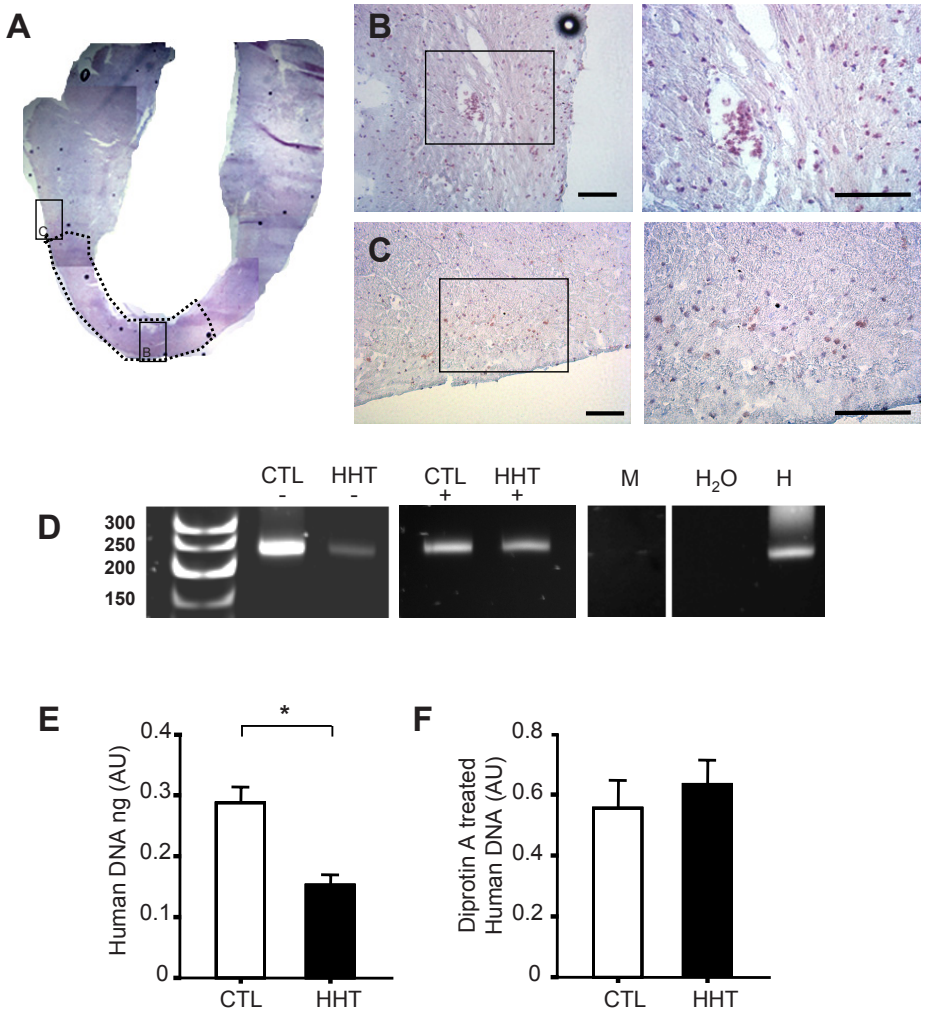

Figure 5.
Improved homing of MNCs *in vivo* by CD26 inhibition. Mice were injected intravenously with 5*10E6 human MNCs one day post MI. Five days after infarction, mice were sacrificed and human cells were stained using an anti-human nuclei antibody. (A) Cross-sectional view of the heart reconstructed from multiple images. Infarcted region is demarcated with dotted line. (B) Overview of infarct region. Red-stained human cells are found throughout the infarct, scale bar represents 100 µm. (C) Overview of peri-infarct region. Red-stained human cells are found scattered throughout the infarct border zone, scale bar represents 100 µm. (D) The amount of human DNA was determined by semi-quantitative PCR. Polyacrylamide gel showing an example of the Human Alu repeats PCR resulting in a product of 224 base pairs. CTL- and HHT-: DNA samples of mice receiving untreated MNCs from controls and HHT1 patients respectively, CTL+ and HHT+: DNA samples of mice receiving MNCs from controls and HHT1 patients after DipA treatment. M: wildtype mouse DNA; H: human genomic DNA; -: H2O: water control. (E) Quantification of DNA isolated from hearts of mice injected with control MNCs (n=5) or HHT1 MNCs (n=7). (F) Quantified DNA isolated from hearts of mice injected with Diprotin-A pretreated control (n=6) or HHT1 (n=5) MNCs. #P<0.05; *P<0.03; **P<0.005. Bars show mean ± SEM.

Discussion

One of the hallmarks of HHT1 is an increasing frequency of hemorrhages with age, which can be the result of impaired vascular repair. Restoration of vasculature is initiated by a local rapid increase in chemotactic chemokines followed by homing of cells to the site of injury. Previously, we have shown that the HHT1-MNC population had a reduced ability to accumulate and induce vessel formation in the infarcted region of the heart[5]. Here we demonstrate that this observed defect in homing can not be explained by changes in the numbers of angiogenic cells known to participate in tissue repair within the heterogeneous MNC population. Analysis of the MNC composition using flow cytometry did not show a significant difference in the number of circulating $CD14^+$-monocytes or $CD34^+VEGFR2^+$ cells. HHT1 patients did have a significant higher number of $CD34^+$ cells, which may be the result of diffuse vascular damage in HHT-1 patients, resulting in increased $CD34^+$ progenitor cell mobilization. Similarly, increases in $CD34^+$ cell numbers were found in patients after myocardial infarction[27]. Progenitor cell numbers do not seem to be the cause of the HHT1-MNC dysfunction. Therefore, an alternative explanation for the reduced cell numbers in the ischemic heart[5,25] may be an impaired chemotactic response of the circulating HHT1-MNCs rather than reduced circulating MNC numbers.

Myocardial wound healing is a tightly controlled process and can been divided into distinct phases[28]. One recognizable phase is the infiltration of MNCs into the infarct area, inducing blood vessels growth, myofibroblast proliferation and extracellular matrix production. An important mechanism for cell recruitment to ischemic areas is the formation of an SDF-1α gradient, resulting in the mobilization of circulating cells expressing its cognate receptor CXCR4. Binding of SDF-1α to CXCR4 was shown to be essential for mobilization and migration of different cell types e.g. hematopoietic stem cell (HSC)[19,29], monocytes/mesothelial cells[30] and tumor metastasis[20,31] . Since SDF-1α expression is increased as early as 1 hour after induction of hypoxia in the myocardium, it is believed to play a role in the initiation of tissue repair. More importantly, modulating the SDF-1α/CXCR4 axis, either positively by delivering a protease-resistant SDF-1α[32] or negatively, by inhibiting SDF-1α binding to its receptor using the CXCR4 antagonist AMD3100, was shown to influence the recruitment and engraftment of cells in infarcted myocardium as well as myocardial repair[7]. Furthermore, the expression of SDF-1α serves as a retention signal and is crucial for the engraftment and maintenance of pro-angiogenic CXCR4 expressing cells within the tissue[33]. The lower number of HHT1-MNC within the infarcted myocardium can be explained by reduced numbers of $CXCR4^+$ cells or reduced CXCR4 expression per cell. However, we found no difference between

HHT1 patients and controls. In HHT1 patients, the mean expression of CXCR4 on the cell-surface was even higher than on control MNCs. Therefore, the reduced number of HHT1- MNCs found in the injured heart cannot be explained by a reduction in CXCR4 levels.

Although CXCR4 expression levels are known to be important for migration, our data imply that this alone does not predict the migratory behavior of cells to SDF-1α. SDF-1α mediated chemotaxis is also regulated by the cell surface peptidase CD26, which cleaves the amino-terminus of SDF-1α[17]. This N-terminal cleavage will block its binding to CXCR4 and limit the effectiveness of SDF-1α as a chemoattractant in the inflammatory environment of infarcted myocardium. CD26 has been reported to be expressed by several cell types within the MNC fraction, including $CD14^+$ and $CD34^+$ cells[34]. Analysis of CD26 indicated that the HHT1-MNCs have higher expression levels of CD26 per cell. These increased CD26 levels suggest that although the high CXCR4 levels on HHT1-MNCs would imply increased recruitment of cells to ischemic tissue, their homing capacity is negatively influenced by the high CD26 expression levels. Using a transwell migration assay, we indeed found a decreased chemotactic response of the HHT1- MNCs towards SDF-1α as compared to healthy controls. Furthermore, the migration of HHT1 cells to SDF-1α was significantly improved when the activity of CD26 was blocked by Diprotin-A; a tri-peptide which was previously shown to increase to homing of embryonic stem cells[35] and HSCs to SDF-1α[19,36]. Additionally, we show that the effect of CD26 on cell migration is negatively correlated with its expression level. The inability of Diprotin-A to influence the migration of control MNCs is probably due to low CD26 levels on these cells that did not correlate with their migratory behavior capacity. Our results clearly demonstrate that the balance of CD26 levels in relation to CXCR4 expression levels is of great importance to predict the chemotactic response of MNCs. The increased number of high CD26 expressing cells in the HHT1 population, causing a misbalance between CD26 and CXCR4, may have a major impact on their total homing and retention capacity.

As a consequence of the endoglin mutations underlying HHT1, TGFβ signaling is impaired[2,25]. TGFβ has previously been reported to influence the SDF-1α/CXCR4 axis, by increasing the expression of CXCR4 and decreasing the CD26 levels on tumor and mesothelial cells[14,30]. We investigated the response of the MNCs to TGFβ stimulation. TGFβ stimulation increased the surface expression of CXCR4 on control MNCs, whereas HHT1-MNCs show impaired induction of CXCR4 expression and impaired reduction in CD26 levels. These observations suggest that the imbalance in the CXCR4/CD26 axis we observe in these cells could be caused by an impaired response of HHT1-MNCs to TGFβ stimulation. Therefore, when HHT1-MNCs are exposed to stress signals after myocardial infarction, like TGFβ, their capacity to shift the balance between CXCR4 and

CD26 is disturbed, thereby rendering the cells less capable for migration and homing. The relatively high CXCR4 expression observed on unstimulated HHT1-MNCs might be a compensatory mechanism to deal with the high CD26 expression on these cells.
Since *in vitro* analysis of the HHT1-MNCs indicated CD26 imbalance as an explanation for impaired homing, we interfered with its expression. Previous studies have shown that pretreatment of HSCs with Diprotin-A greatly enhanced their homing capacity towards the bone marrow[21,36]. Therefore, we investigated whether altering the CXCR4/CD26 balance, by inhibiting CD26 with Diprotin-A, also increased their homing *in vivo*. When untreated MNCs were injected one day after MI, we found significantly fewer HHT1-MNCs in the infarcted area 5 days after MI, compared to healthy controls. This confirmed the impaired homing capacity we reported previously[5], as well as, the *in vitro* data presented in this study. Strikingly, pretreatment with Diprotin-A completely normalized the homing of HHT1-MNCs to the infarcted heart, indicating the significance of CD26 for homing not only for cells towards the bone marrow but also for MNCs to ischemic tissue.
In conclusion, the SDF-1α/CXCR4 axis and its negative regulator CD26 are crucial for the homing of MNCs to the ischemic myocardium. In HHT1 patients, the balance of CXCR4 and CD26 on HHT1-MNCs is skewed. Restoring the balance in this axis, via CD26 inhibition, resulted in "normal" homing capacities *in vitro* and *in vivo*, providing an explanation for the dysfunctional homing of HHT1-MNCs. Interestingly, CD26 inhibition may not only be of importance for HHT, but also for other diseases that aim at regeneration of ischemic areas by improving homing and engraftment of circulating cells into the injured organ.

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Supplementary data

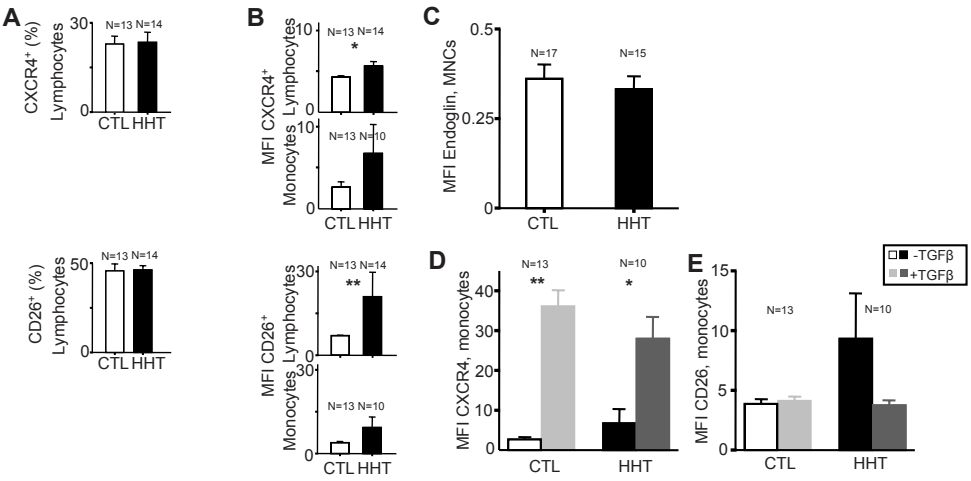

Figure 1.
CXCR4 and CD26 expression profiles of HHT1 lymphocytes and monocytes show similar trends in expression as control MNCs (A) Percentages of CXCR4 and CD26 positive cells within lymphocyte and monocytes cell-fraction. 100% of monocytes were CXCR4 and CD26 positive (graphs not shown). (B) MFI of CXCR4 and CD26 positive lymphocytes and monocytes: similar expression profiles as MNC fraction. (C) Endoglin expression profile of MNC fraction. No significant difference between HHT1 and control. (D) HHT1 and control monocytes are capable of CXCR4 upregulation after TGFβ stimulation. (E) After TGFβ stimulation CD26 is not significantly downregulated on control and HHT1 monocytes. Monocytes: CTL: n=13; HHT1: n=10. Lymfocytes: CTL n=13; HHT1: n=14 #P<0.05; *P<0.03; **P<0.005. Bars show mean ± SEM.

Table 1

Exon	Mutation	Gender	Age
3	Gln77stop	M	23
3	Gln77stop	F	48
3	Gln77stop	M	64
3	Glu83stop	F	65
3	Glu83stop	M	15
3	Glu83stop	F	50
3	Gln83stop	M	39
3	Gln83stop	F	42
3	Glu83stop	M	15
6	trp261Arg	M	37
7	887-918delins870-880	M	56
7	887ins18bp	F	36
7	887ins18bp	M	28
7	Gly331Ser	F	32
7	Gly331Ser	M	60
8	1083delAA	F	57
8	1117insT	M	48
8	1117insT	F	16
8	1117insT	M	50
8	1122delAG	M	70
9	1255delA	M	55
9	1255delA	F	53
9	1310delG	F	19
9	Arg437Arg	M	20
9	Arg437Arg	F	42
9	Arg437Arg	M	36
9	Arg437Arg	M	51
9	Arg437Arg	M	23
9	Arg437Arg	F	63
9	Arg437Arg	F	23
9	Gly413Val	F	36
10	1346delCT	M	35

Characteristics of HHT1 patients used in all experiments. Statistical analysis has been performed by comparison with the control group.

Table 2

	Flow cytometry analysis CD14, Endoglin	Flow cytometry analysis CD34, VEGFR2	Flow cytometry analysis CXCR4, CD26	In vitro migration	Mouse in vivo
Age, Mann-Whitney (p)	0.663	0.798	0.837	0.756	0.200
Gender, Fisher exact (p)	1.000	0.543	0.453	1.000	0.567

Investigated HHT1 patients and controls showed no significant difference in age en gender. The table shows P-values for comparison of age and gender in all subgroups of HHT1 patients and controls used for the different experiments. For comparison of age Mann-Whitney U tests were performed. For comparison of gender Fisher's Exact tests were used.

Table 3

Mouse ID	DipA	PCR cycles	Relative Intensity
Control MNC1	-	27	0.372
Control MNC2	-	27	0.252
Control MNC3	-	27	0.231
Control MNC4	-	27	0.261
Control MNC5	-	27	0.319
HHT-1 MNC1	-	27	0.110
HHT-1 MNC2	-	27	0.236
HHT-1 MNC3	-	27	0.106
HHT-1 MNC4	-	27	0.132
HHT-1 MNC5	-	27	0.149
HHT-1 MNC6	-	27	0.141
HHT-1 MNC7	-	27	0.190
Control MNC6	+	23	0.718
Control MNC7	+	23	0.795
Control MNC8	+	23	0.667
Control MNC9	+	23	0.530
Control MNC10	+	23	0.434
Control MNC11	+	23	0.184
HHT-1 MNC8	+	23	0.674
HHT-1 MNC9	+	23	0.625
HHT-1 MNC10	+	23	0.845
HHT-1 MNC11	+	23	0.347
HHT-1 MNC12	+	23	0.709

Overview of PCR quantification per individual mouse per group
Listed is the mouse ID, which indicates what type of human cells were injected (HHT or CTL). In the next column is indicated if human cells were pretreated with Diprotin A or not. The column cycles represents the number of PCR cycles that were applied to multiply human DNA. The relative intensity shown the calculation of the intensity of PCR bands

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Part IV | Chapter 8

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8

The balance between angiopoietin-1 and angiopoietin-2 is in favor of angiopoietin-2 in atherosclerotic plaque with high microvessel density

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Abstract

Introduction

Atherosclerotic plaque microvessels are associated with plaque hemorrhage and rupture. The mechanisms underlying plaque angiogenesis are largely unknown. Angiopoietin-1 (Ang-1) and Angiopoietin-2 (Ang-2) are ligands of the endothelial receptor Tie-2. Ang-1 induces formation of stable vessels, whereas Ang-2 destabilizes the interaction between endothelial cells and their support cells. We studied the expression patterns of Ang-1 and -2 in relation to plaque microvessels.

Methods and results

Carotid endarterectomy specimens were studied (n=100). Microvessel density was correlated with presence of macrophages and with a (fibro)atheromatous plaque phenotype. A negative correlation was observed between expression of Ang-1 and microvessel density. A positive correlation was observed between the ratio of Ang2/Ang1 and microvessel density. Ang-2 expression was correlated with matrix metalloproteinase-2 (MMP-2) activity. Immunohistochemical staining of Ang-1 was observed in smooth muscle cells, whereas Ang-2 was detected in endothelial cells, smooth muscle cells and macrophages.

Conclusions

In plaques with high microvessel density, the local balance between Ang-1 and Ang-2 is in favor of Ang-2. Plaque Ang-2 levels are associated with MMP-2 activity. Ang-2 induced MMP-2 activity might play a role in the development of (unstable) plaque microvessels.

Introduction

Plaque neovascularization is a frequently observed phenomenon in atherosclerotic lesions. Plaque microvessels originate from adventitial vasa vasorum and less frequently from the arterial lumen. Plaque neovessels have long been proposed to play a role in intimal hemorrhage and coronary thrombosis[1,2]. More recently, it has been demonstrated that plaque hemorrhage and plaque rupture are associated with an increased microvessel density[3-5]. In addition, microvessel related intra plaque hemorrhage has been associated with extracellular lipid core expansion through the accumulation of erythrocyte membrane derived cholesterol[6]. Leakiness or disruption of plaque microvessels and subsequent intra plaque hemorrhage is therefore considered an important contributor to plaque progression. Thus far, however, the molecular regulators of plaque neovascularization are largely unknown.

The angiogenic factor angiopoietin-1 (Ang-1) is an agonist ligand of the endothelial receptor tyrosine kinase Tie-2. In adults, the Ang-1/Tie-2 signaling system is essential for the maturation of vessels. Experiments in transgenic mice have revealed that vascular endothelial growth factor (VEGF) induces leaky and hemorrhagic vessels, whereas Ang-1 induces leakage-resistant vessels[7]. Ang-1 stabilizes vessels by maximizing the interactions between endothelial cells and their surrounding support cells and matrix[7]. Angiopoietin-2 (Ang-2) is the natural antagonist of Ang-1[8]. By inhibition of Tie-2 signaling, Ang-2 leads to a loosening of cell-matrix and cell-cell interactions[9-11]. This antagonistic effect is thought to be a requirement for sensitivity of endothelial cells to other angiogenic factors such as VEGF. In the absence of angiogenic growth or survival signals, Ang-2 action results in destabilization and finally regression of vessels[9].

Because leakiness or disruption of plaque microvessels is a frequently observed phenomenon, we hypothesized that the local balance between Ang-1 and Ang-2 is in favor of Ang-2 in atherosclerotic plaque. We studied the expression patterns of Ang-1 and -2 in carotid endarterectomy specimens in relation to plaque neovascularization. We report that the local balance between Ang-1 and Ang-2 is in favor of Ang-2 in plaques with high microvessel density. In addition we show an association between plaque Ang-2 levels and matrix metalloproteinase 2 (MMP-2) activity.

Methods

Human endarterectomy specimens

Patients are included in the ATHEROsclerotic plaque EXPRESSion study (ATHERO-EXPRESS), which is an ongoing longitudinal multi center cohort study in The

Netherlands[12]. This study was approved by the Medical Ethical Committees of the participating hospitals and written informed consent was obtained from each patient. Carotid endarterectomy specimens were studied from 100 randomly selected patients undergoing carotid surgery. During the operation, the endarterectomy specimen was brought to the laboratory without delay and dissected in parts of 0.5 cm. The culprit lesion was fixed in formalin, paraffin embedded and used for (immuno)histochemistry. Adjacent segments were immediately frozen in liquid nitrogen and used for protein isolation. In this study, we assumed that the plaque characteristics in the formalin fixed segment are associated with protein levels in the adjacent segment[12].

Plaque Phenotyping

Formalin-fixed endarterectomy segments were embedded in paraffin and 5-µm sections were cut for histological (immuno) staining. The following stainings were performed to characterize the plaque: hematoxylin and eosin, elastin von Gieson's (internal elastic lamina), picro-Sirius red (collagen), CD68 (macrophages), α-actin (smooth muscle cells) and CD34 (endothelial cells).

Two independent observers microscopically scored all stainings. Plaques were categorized into 3 groups based on their overall appearance (fibrous, fibroatheromatous or atheromatous) as described previously[13]. A plaque was considered atheromatous when it contained a large atheroma and when it lacked collagen and smooth muscle cells. The more fibrous lesions typically lacked an athermoma and revealed strong staining for collagen and smooth muscle cells. The CD68 staining was analyzed as follows: absent or minor staining with negative or few scattered cells and moderate or heavy staining with clusters of cells with >10 cells present. Using a 100x magnification, CD34 positive microvessels were counted in 3 areas of the plaque with the highest microvessel density. Subsequently, the average microvessel density per mm^2 of these areas was calculated for each plaque.

Immunohistochemistry

The CD68 and α-actin stainings were performed as described previously[13]. Ang-1 was detected using a mouse anti-human angiopoietin-1 monoclonal antibody (R&D Systems, Minneapolis, Minnesota) at a dilution of 1:25. To visualize Ang-2, sections were stained with a mouse anti-human angiopoietin-2 monoclonal antibody (R&D Systems) at a dilution of 1:25. To make the Ang-2 epitope accessible for the antibody, sections were pretreated with EDTA. Next, sections were incubated with poly-HRP anti-Mouse IgG (ImmunoVision, Brisbane, CA). Endothelial cells of microvessels were detected using a mouse anti-human CD34 antibody (1:400 dilution; Immunotech, Marseille, France) followed by incubation with biotinylated horse anti-mouse IgG

(Vector Laboratories, Burlingame, CA) and peroxidase labeled streptavidin. All signals were visualized with diaminobenzidine. Sections were counter stained with haematoxylin.

Western Blotting and zymography

Protein concentrations were measured using DC protein Assay (Biorad, Hercules, California). Non-reduced protein samples (20 µg/lane) were separated on a 6% SDS-polyacrylamide gel (angiopoietin-1) or on an 8% SDS-polyacrylamide gel (angiopoietin-2) and blotted on to a Hybond-P membrane (Amersham Biosciences, Piscataway, New Jersey). Blocking and incubation steps were done in 5% nonfat dry milk in PBS/0.1% Tween[20]. Blots were incubated with mouse anti-human angiopoietin-1 (1:100; R&D Systems) or angiopoietin-2 antibody (1:500; R&D Systems) and rabbit anti-mouse peroxidase (1:2000; DakoCytomation, Glostrup, Denmark), respectively. Visualization and analysis of all blots was done using chemiluminescence substrate (Sigma, St. Louis, Missouri) and the ChemiDoc XRS system (Biorad). Expression levels were expressed as arbitrary units. Blots that were incubated with an irrelevant antibody of the same isotype (IgG2b) served as negative controls. As a positive control, recombinant Ang-1 (R&D systems) or Ang-2 (R&D systems) was loaded on each gel. A Ponceau S staining was used to test for equal loading.

To determine pro- and active MMP-2 and MMP-9 levels in a subset of 38 plaques, zymography was performed and analyzed as described previously[14]. After equalizing the protein levels for all samples, each sample was loaded on an 8% SDS-polyacrylamide gel containing 1% gelatin. After running, the gel was rinsed twice in a 2.5% Triton solution for 15 minutes each and then incubated (o/n at 37°C) with a Tris–HCl buffer (50 mM, pH 7.4) containing 0.05% Brij and 10 mM CaCl2. The o/n incubation was followed by staining the gel with Coomassie blue stain and additional destaining of the gel resulting in clear white bands against a blue background. The white bands were quantified using the ChemiDoc XRS system (Biorad) and expressed in arbitrary units. The white bands for (pro and active) MMP-2 and MMP-9 were identified on the basis of their molecular size and by taking into account the recombinant MMP-2 and MMP-9 proteins on zymography gel.

Statistics

Data are presented as median and inter-quartile range. A Mann-Whitney U test was used to compare continuous variables between two groups. A Kruskal Wallis test was used to compare continuous variables between four groups. A Spearman's rank correlation test was used to test the correlation between two variables.

Results

Plaque phenotype

One hundred carotid endarterectomy specimens were studied: 34 fibrous plaques, 33 fibro-atheromatous plaques and 33 atheromatous plaques. The average microvessel density was 5.8±3.7 (range 0-19) / mm^2; median (inter-quartile range) 5.4 (3.0-7.7) / mm^2. Microvessel density was higher in (fibro)atheromatous plaques than in fibrous plaques, microvessel density 6.0 (3.5-8.7) versus 4.0 (2.3-7.0) /mm^2, respectively (P= 0.030). Moderate or heavy macrophage staining was observed in 58/100 (58%) plaques. Microvessel density was higher in plaques with moderate or heavy staining of macrophages than in plaques with no or minor macrophage staining, microvessel density 6.1 (4.0-8.3) versus 3.5 (2.2-6.8) /mm^2, respectively (P= 0.012).

Association between angiopoietin expression and microvessel density

The Ang-1 and Ang-2 Western blots both revealed bands at 150 kD (dimer). The negative control blots that were incubated with an isotype antibody did not show a band at 150 kD. The positive controls with Ang-1 and Ang-2 recombinant proteins both showed bands at the same height as the samples (figure 1).

Using a Spearman's rank correlation test, a negative correlation was observed between expression levels of Ang-1 and microvessel density (P=0.001). No significant correlation was observed between expression levels of Ang-2 and microvessel density. Because the local balance between Ang-1 and Ang-2 determines the effect of the Ang-Tie-2 system, we also determined the association between the ratio of Ang-2/Ang-1 and microvessel density. A positive correlation was observed between the ratio of Ang-2/Ang-1 and microvessel density (P=0.002).

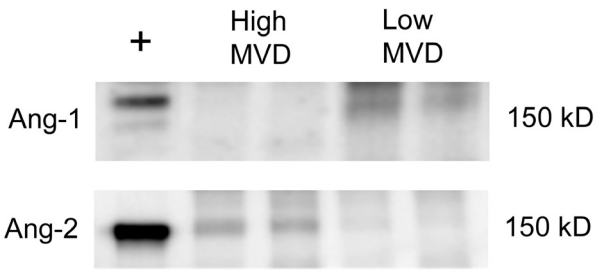

Figure 1.
Western blots of Ang-1 and Ang-2 expression in atherosclerotic plaque. The left lane is a positive control with recombinant protein, indicated with +. The next two lanes are examples of plaques with high microvessel densisty (MVD) showing low Ang-1 expression and high Ang-2 expression. The right two lanes are examples of plaques with low microvessel density showing high Ang-1 and low Ang-2 expression. Bands were observed at 150 kD.

Samples were categorized into quartiles by cutoff points of 25th, 50th, and 75th percentile values of microvessel density. Ang-1 expression was significantly higher in plaques with low microvessel density than in plaques with high microvessel density (P=0.004; figure 2). The ratio between Ang-2 and Ang-1 expression was higher in plaques with high microvessel density than in plaques with low microvessel density (P=0.015; figure 2). Ang-1 expression was higher in plaques with no or minor macrophage staining than in plaques with moderate or heavy macrophage staining (P=0.003). No significant association was observed between macrophage staining and Ang-2 expression.

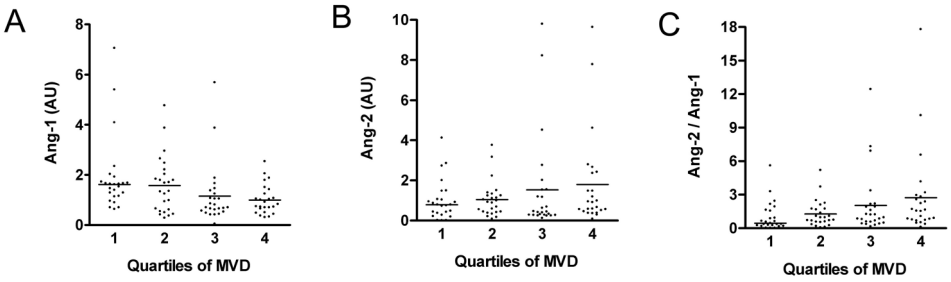

Figure 2
Vertical scatter plots of the association between angiopoietin expression levels (determined by Western blot) and microvessels density. Samples were divided into four equal groups based on quartiles of microvessel density (MVD). A, Negative correlation between Ang-1 and microvessel density (P=0.004). B, Correlation between Ang-2 and microvessel density (not significant). C, Correlation between the ratio of Ang2/Ang1 and microvessel density (P=0.015). AU indicates arbitrary units.

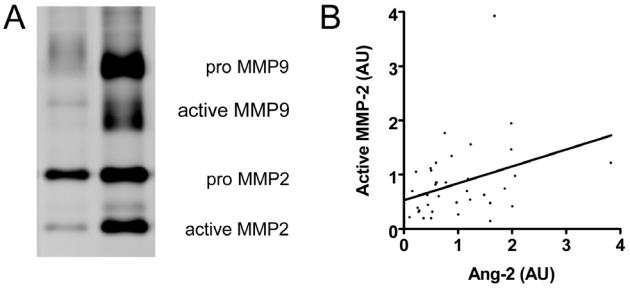

Figure 3
A, Example of zymography gel of atherosclerotic plaque. The left lane shows an example of low levels of active MMP-2 and -9. The right lane shows an example of high levels of active MMP-2 and -9. B, Scatter plot of Ang-2 expression (determined by Western blot) versus active MMP-2 (determined by zymography). AU indicates arbitrary units. A significant correlation was observed between Ang-2 expression and active MMP-2 (P=0.009).

Angiopoietin expression and matrix metalloproteinases

Ang-2 expression was correlated with both pro- and active MMP-2 (P=0.005 and P=0.009, respectively; figure 3) and with pro MMP-9 (P=0.003). No correlation was observed between Ang-2 and active MMP-9 or between Ang-1 and MMP-2 and -9.

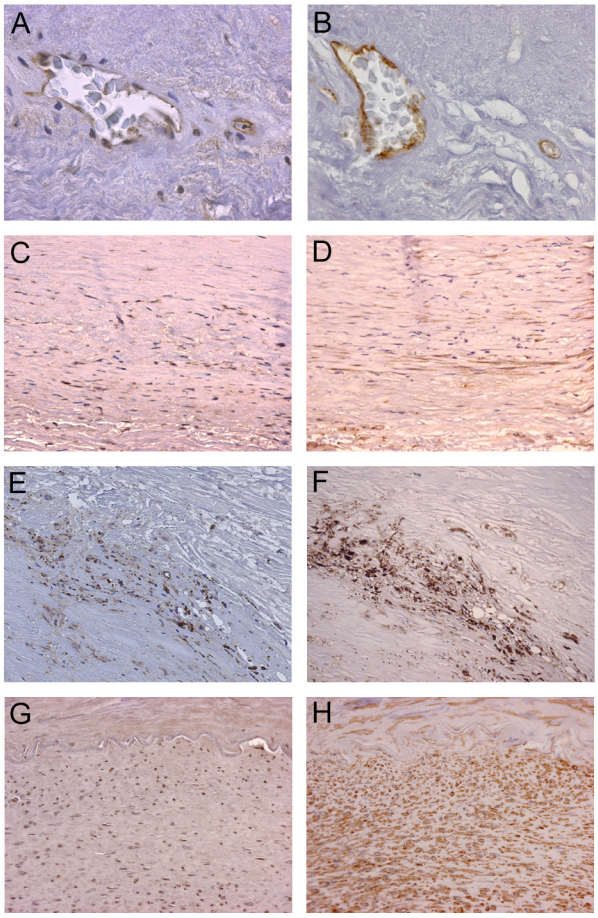

Figure 4
Immunohistochemical detection of Ang-1 and Ang-2 in atherosclerotic plaque. Panel A shows Ang-2 staining in a microvessel in atherosclerotic plaque. Panel B shows a CD34 staining of the adjacent section, identifying the Ang-2 positive cells as endothelial cells. Panel C shows Ang-2 staining in the fibrous cap of an atherosclerotic lesion. Panel D shows an alpha actin staining of the adjacent section, identifying the Ang-2 positive cells as smooth muscle cells. Panel E shows Ang-2 staining at the border of the necrotic core of an atherosclerotic plaque. Panel F shows a CD68 staining of the adjacent section, identifying the Ang-2 positive cells as macrophages. Panel G shows Ang-1 staining at the base of an atherosclerotic plaque near the internal elastic lamina. Panel H shows an alpha actin staining of the adjacent section, identifying the Ang-1 positive cells as smooth muscle cells.

Immunohistochemistry

To study in which cell types the angiopoietins are expressed, we performed an immunohistochemical staining for Ang-1 and 2 in a subset of ten plaques. Immunohistochemical staining of Ang-1 was predominantly observed in smooth muscle cells (figure 4). Ang-2 immunoreactivity was detected in endothelial cells of microvessels, in smooth muscle cells and in macrophage foam cells at the border of the extracellular lipid core (figure 4).

Discussion

Ang-1 and -2 are ligands of the tyrosine kinase receptor Tie-2 that contribute to blood vessel formation during angiogenesis. Ang-1 promotes structural integrity of blood vessels. In contrast, Ang-2 is classically considered as a Tie-2 antagonist, counteracting the stabilizing effects of Ang-1. The local balance between these factors and the level of other angiogenic factors determines whether blood vessels grow, are maintained or become leaky and unstable. We determined the expression levels of Ang-1 and Ang-2 in relation to microvessel density in endarterectomy specimens of patients with significant stenosis of the carotid artery. We found that the balance between Ang-2 and Ang-1 is in favor of Ang-2 in plaques with high microvessel density. To the best of our knowledge this is the first study in which angiopoietin expression levels are determined in association with plaque microvessel density. Our results are in accordance with earlier findings in which high expression levels of Ang-2 were observed in advanced atherosclerotic lesions[15].

We observed an association between microvessel density and an atheromatous inflammatory plaque phenotype. This observation is consistent with earlier findings in which increased microvessel density was associated with plaque hemorrhage, inflammation and rupture[5,16,17]. Microvessels in atherosclerotic lesions are considered to be leaky and instable resulting in intra plaque hemorrhage and plaque progression[17]. The recent observation of diffuse von Willebrand factor staining around plaque microvessels supports the concept of leaky vessels in atherosclerotic plaque[6]. Ang-2 promotes vascular leakage in an *in vivo* situation by destabilization of the endothelium[10,11]. We observed a balance of Ang-1/Ang-2 in favor of Ang-2 in plaques with high microvessel density. These observations suggest a possible role for Ang-2 in the leakiness and rupture of plaque neovessels.

An abnormal balance between Ang-1 and -2 with downregulation of Ang-1 and upregulation of Ang-2 has also been observed in other blood vessel diseases with an instable vessel wall such as brain arteriovenous malformations (which often present as cerebral hemorrhage), infantile hemangioma and menorrhagic endometrium[18,19,20].

In addition, a shift of the ratio between Ang-1 and Ang-2 in favor of Ang-2 has been described in human tumors and is considered to play an important role in tumor microvessel development[21]. In renal cell carcinoma, in which large hemorrhages due to unstable tumor microvessels are frequently observed, high expression levels of Ang-2 have also been described[22].

We observed an association between Ang-2 expression and MMP-2 activity. This observation is in concordance with previous studies in the oncology field. Ang-2 induced MMP-2 activity has been described in cells of human brain tumors[23]. Considering the observed association between Ang-2 and MMP-2 activity, MMP-2 might play a role in Ang-2 induced destabilizing effects on microvessels.

We detected Ang-1 immunohistochemical staining in smooth muscle cells, which is consistent with previous observations in which Ang-1 expression was also predominantly observed in pericytes and smooth muscle cells[24,25]. We found Ang-2 immunoreactivity in endothelial cells of microvessels. Previous studies have also described expression of Ang-2 in endothelial cells[15,26]. In addition, we observed Ang-2 staining in smooth muscle cells, which is consistent with earlier reports[24,25, 7]. At the border of the extracellular lipid core we detected staining of Ang-2 in macrophage derived foam cells. Ang-2 expression in macrophages has earlier been described in human macrophages of the synovial capsule of rheumatoid arthritic patients, which is also a disease with chronic inflammation as is atherosclerosis[28]. In addition, Ang-2 transcription and excretion has also been demonstrated in murine macrophages[29].

Recent observations in immunology suggest that next to its role in angiogenesis, the angiopoietin Tie-2 signaling system is also important in inflammatory responses[30]. Angiopoietin-2 thereby primes the endothelium to respond to proinflammatory cytokines such as TNF-alpha which may induce the expression of adhesion molecules and subsequent extravasation of inflammatory cells[31]. In other diseases with chronic inflammation, such as rheumatoid arthritis, high expression levels of Ang-2 have also been observed[32]. Further studies are needed to explore the exact role of the angiopoietins in plaque inflammation.

VEGF is an important regulator of angiogenesis that is also involved in plaque neovessel formation[33]. The present study was limited by the fact that we did not study VEGF expression. The influence of VEGF on plaque angiogenesis might be more important than the influence of Ang-1 and Ang-2. Future studies are needed to further explore the exact contribution of the different angiogenic factors on plaque angiogenesis. Another limitation of the study is that we did not measure angiopoietin levels in the plasma of the patients. Plasma levels of angiogenic factors might also exert effect on atherosclerotic plaque.

In conclusion, Ang-1 and Ang-2 are expressed in human atherosclerotic plaque. In

plaques with high microvessel density, the balance between Ang-1 and Ang-2 is in favor of Ang-2. Ang-2 expression in atherosclerotic plaque is associated with MMP-2 activity. These results suggest a role for Ang-2 and Ang-2 induced MMP-2 activity in the development of (unstable) plaque microvessels.

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Part IV | Chapter 9

9

General discussion

Patients suffering from the cardiovascular diseases myocardial infarction (MI), atherosclerosis and Hereditary Hemorrhagic Telangiectasia (HHT) all suffer from diseased blood vessels and/or dysfunctional myocardium. Cardiovascular repair in these diseases is not only mediated by local cells, but current studies suggest that also peripheral blood derived cells, cytokines and growth factors are able to positively influence repair of malfunctioning tissue. In this thesis several aspects of cardiovascular repair have been explored.

Peripheral blood derived cells are involved in cardiovascular repair

Ischemic heart disease, including acute MI, is world wide the leading cause of death[1]. Currently, the use of primary percutaneous intervention and a variety of medicines for MI has led to a higher survival rate. However, many patients surviving MI have considerably damaged hearts, resulting in decreased life expectation due to the development of heart failure[2]. Over the last years, cellular therapy post MI has extensively been studied to reduce infarct size and restore dysfunctional cardiac tissue[3]. Various progenitor cell subsets are mobilized from the bone marrow into the peripheral circulation after MI[4-6] (Chapter 4), and X-Y mismatch transplantation studies suggest incorporation of endogenous blood derived cells into the heart and vasculature[7;8]. Moreover, increased levels of cytokines and growth factors are found in the peripheral blood[9;10] (Chapter 4), these may reduce myocardial apoptosis, enhance cardiomyocyte survival, stimulate angiogenesis, local cellular repair and progenitor cell mobilization and homing.

In a mouse MI model, injection of hepatocyte growth factor (HGF) or vascular endothelial growth factor (VEGF) intramyocardially post MI, decreases infarct size and improves cardiac function. This is likely caused by increasing the tolerance of cardiomyocytes to ischemia and reducing apoptosis[11]. Increasing HGF and VEGF levels by injecting mesenchymal stem cells over expressing HGF / VEGF or systemic infusion of HGF 1 day post MI also ameliorates cardiac function[11;12]. Furthermore, clinical studies infusing peripheral blood or bone marrow progenitor cells in the infarct related artery after MI report a moderate improvement of the left ventricular function[13]. Taken together these results suggest that cells, cytokines and growth factors present in peripheral blood may reduce infarct size and positively influence cardiac remodeling, either by differentiation or by paracrine mechanisms – the delivery of angiogenic and pro-survival factors.

CD26 has a crucial role in mononuclear cell homing

As discussed in Chapter 2, the SDF-1α / CXCR4 axis plays an important role in cell mobilization and homing in cardiovascular disease. In the ischemic myocardium high levels of SDF-1α are produced, resulting in the recruitment of CXCR4 expressing cells from the circulation to the ischemic heart[14;15]. Similarly, in the bone marrow high levels

of SDF-1α are produced, resulting in the retention of CXCR4 expressing cells[16]. CXCR4+ cells are mobilized from the bone marrow when stromal SDF-1α drops. Cell mobilization from the bone marrow is also mediated by CD26[17]. CD26 is an endopeptidase known to negatively regulate the SDF-1α / CXCR4 axis by cleaving the amino-terminal peptide from SDF-1α and by co-internalization of CXCR4[18;19].

In this thesis, we show directly, using a mouse model for HHT-1, and indirectly, using the REPERATOR patient population, that CD26 has a pivotal role in peripheral blood derived cell homing towards the infarcted myocardium. In Chapter 5 we report that in patients with acute MI a high cellular CD26 expression decreases the migratory capacity of MNCs towards SDF-1α and thereby negatively influences cardiac function post MI. I*n vitro* inhibition of CD26 by sitagliptin results in increased migration capacities. Furthermore, in Chapter 6 and 7, we show that HHT-1-MNCs have decreased homing capacities towards SDF-1α. In an *in vivo* mouse model for MI, homing of infused MNCs could be normalized by pre-treating cells with a CD26 inhibitor. This shows that the reduced homing capacity of HHT-MNCs is indeed due to high cellular levels of CD26.

Recently Zaruba and coworkers showed in mice that systemic CD26 inhibition in combination with G-CSF treatment, increases cell homing to a myocardial infarct area, reduces cardiac remodeling and improves cardiac function[20]. Together with our observations, this suggests that systemic inhibition of CD26 after MI may enhance cell homing and result in improved cardiac function. However, since CD26 is also essential for progenitor cell mobilization from the bone marrow, accurate timing of CD26 inhibition post MI might be of great importance[17;21].

Cytokines and growth factors

E*ndoglin in* HHT-1 *and myocardial infarction*

Endoglin (CD105, Eng) is an accessory TGFβ type III receptor and is primarily expressed on proliferating endothelial cells, but at low levels on mononuclear cells (MNCs) and monocytes. In chapter 6, we show that MNCs from HHT-1 patients – which are Eng haploinsufficient – exhibit reduced recruitment and subsequent vessel formation after MI when compared to MNCs from healthy controls. In patients with acute MI, monocyte expression of Eng is relatively high directly after MI and drops from one week onwards (Chapter 4). These results may suggest that expression of membrane bound Eng is important to support cardiovascular repair. Besides membrane bound Eng, a soluble form of Eng (sEng) exists in blood plasma and tissues[22-24]. We show in Chapter 4 that sEng – similarly to membrane bound Eng – is high directly after MI and drops from one week onwards. Intriguingly, a previous study has shown that a greater decrease in sEng correlates with poor survival rate post MI[25]. Furthermore, high levels of sEng lead to endothelial dysfunction[22] and induction of arteriovenous malformations in VEGF

induced angiogenesis[24]. Also TGFβ signaling may be impaired due to the elevated sEng levels as sEng seems to function as a scavenger for TGFβ. Therefore, contrary to membrane bound Eng, low sEng levels seem to be favorable after acute MI.

Angiopoietin expression in atherosclerotic plaques and MI

The angiogenic factor angiopoietin (Ang)-1 is a Tie-2 receptor agonist, and stabilizes vessels by increasing the interaction between endothelial cells[26] by stimulation of sphingosine kinase 1 activity[27] and pericyte recruitment[28]. Ang-2 looses endothelial cell interaction by antagonizing Ang-1 – Tie-2 signaling[29]. We reported that atherosclerotic plaques with high microvessel density have relatively high levels of Ang-2 compared to the expression of Ang-1 (Chapter 8). Furthermore, high Ang-2 expression is positively correlated with MMP-2 activity, important for plaque destabilization. Interestingly, a higher Ang-2 to Ang-1 ratio is found in patients 1 week post MI, suggesting a more angiogenic environment and may promote vessel sprouting (Chapter 4). These observations suggest beneficial effects of increased Ang-2 levels after MI by favoring angiogenesis that may positively affect cardiac function. However, increased Ang-2 levels may have detrimental effects in atherosclerotic plaques leading to relatively leak microvessels within the plaque that may result in plaque destabilization.

Statins in acute myocardial infarction

To date, Statins, 3-hydroxy-3-methylglutaryl co-enzyme A reductase inhibitors, are used for primary and secondary prevention to reduce cardiovascular morbidity and mortality[30]. Experimental animal studies showed that early statin treatment after MI inhibits reperfusion damage and dramatically decreases the infarct size[31;32]. Interestingly, statin treatment was also able to enhance progenitor cell mobilization[33-35]. However, the role of statins in reducing reperfusion damage in a clinical setting is unclear[36-39]. Here, we observed no effect on reperfusion damage in our clinical pilot trial investigating early statin treatment during primary PCI, as assessed by detailed MRI measurements of the left ventricular function (Chapter 3). However, early statin treatment after MI did enhance $CD133^+$ progenitor cell mobilization (Chapter 4). No association between progenitor cell mobilization and cardiac function was observed, which could be explained by 1) $CD133^+$ progenitor cells are unable to reach the damaged myocardium, 2) $CD133^+$ progenitor cell numbers – despite their mobilization – are too low or 3) $CD133^+$ progenitor cells simply do not affect cardiac function.

Future perspectives

Cellular therapy aiming at restoration of dysfunctional heart and/or vasculature has been widely studied the past years, but the optimal therapy for clinical patients is still

under debate. In this thesis we investigated various aspects of cardiovascular repair. Protocols currently being explored for cardiac regeneration mainly use cell isolation from the patient and subsequent local infusion in the diseased heart with or without *in vitro* manipulation of these cells. However, this has many disadvantages as it is laborious, time consuming, expensive and *ex vivo* cell processing may result in DNA changes, incorporation of animal derived proteins or bacterial contamination. Many of these disadvantages can be circumvented when agents can be used which preserve the damaged tissue (either to stimulate local cell survival and angiogenesis or to decrease apoptosis and necrosis), stimulate progenitor cell mobilization from the bone marrow or stimulate progenitor cell homing to an infarct area. Inhibition of CD26 is an intriguing approach, since this will increase progenitor cell homing to an ischemic area. CD26 inhibition was originally developed for the treatment of type II diabetes since it results in an enlarged availability of glucagon like peptide-1 (GLP-1) and glucose dependent insulinotropic peptide (GIP) which results in increased insulin release and decreased glucagon release[40]. Recently two CD26 inhibitors have been approved for the treatment of type II diabetic patients: sitagliptin (Januvia, Merck Sharp & Dohme) and vildagliptin (Galvus, Novartis). Therefore, clinical application of CD26 inhibition, making use of its off-label effects, might result in increased cardiac function post MI remains to be investigated (Figure 1). Currently patients are recruited for a clinical study investigating the effect of combined treatment of G-CSF and CD26 inhibition after MI (ClinicalTrials.gov, trial number NCT00650143). Furthermore, HHT-1 – "no option" – patients suffering from very severe nose-bleeds are also treated with sitagliptin to assess the effect of CD26 inhibition on the frequency and severity of their epistaxis.

Systemic inhibition of CD26 may however also lead to adverse effects since patients taking the CD26 inhibitor sitagliptin are reported to have an increased risk for infections[40]. Experimental animal studies have previously suggested that a proinflammatory state after MI deteriorates cardiac function[41;42]. Similarly, in a rat model for MI systemic infusion of irradiated apoptotic blood derived MNCs is shown to further reduce the infarct area compared to infusion of non-irradiated MNCs. These results suggest that the heart benefits from the delivery of pro-angiogenic factors while negative effects due to local inflammation may be circumvented[43]. Furthermore, a recent study, using CXCR4 heterozygous mice, shows a decreased inflammatory response post MI and a decreased infarct area, however, this is not accompanied by an improvement in cardiac function[44]. By inhibiting CD26 shortly after MI, local inflammation may be enhanced due to increased homing capacities of CXCR4+ monocytes and lymphocytes to the MI area, and deteriorate cardiac function. Accurate timing of CD26 inhibition after MI therefore seems highly important as it theoretically may also deteriorate cardiac function. Not only

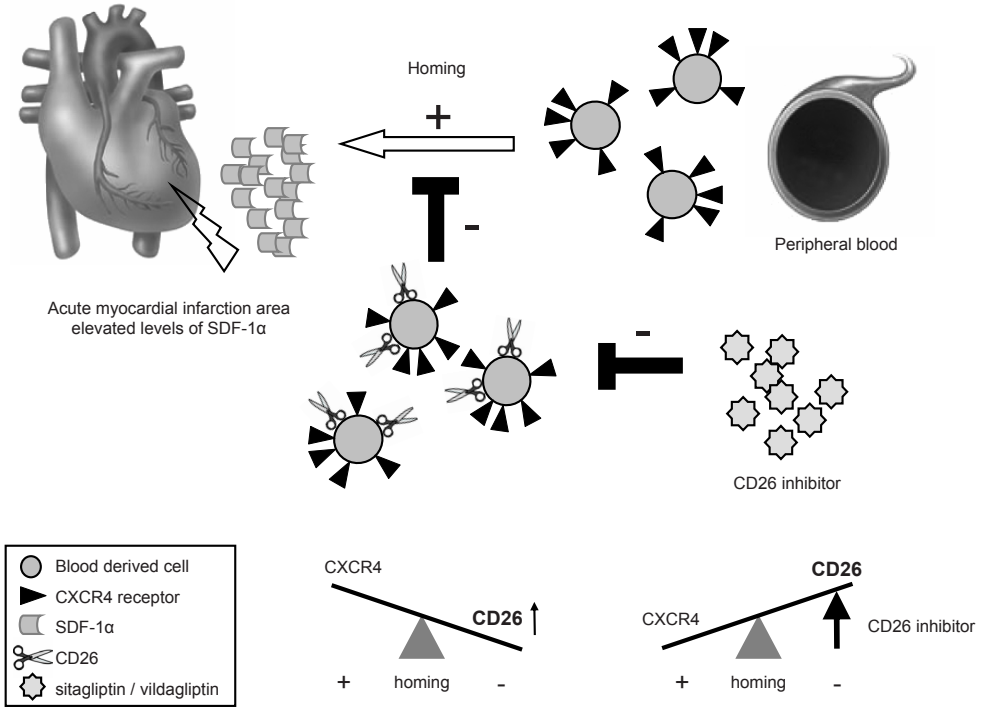

Figure 1
The SDF-1α/CXCR4 axis after acute MI is negatively regulated by CD26. Inhibition of CD26 results in increased homing of peripheral blood derived cells to an infarct area.

patients suffering from MI, but also HHT-1 patients may benefit from CD26 inhibition as it may restore their dysfunctional peripheral blood cell mediated vascular repair. In conclusion, CD26 inhibitors are of great interest enhancing cell mediated homing to an infarct area and improve blood cell mediated vascular repair. Further clinical trials will be needed to assess the effects of CD26 inhibitors in cardiovascular disease.

In conclusion, the concept of systemic infusion of selected agents which reduce infarction size and reduce deleterious cardiac remodeling by stimulation of local cells and systemically available progenitor cells represents a promising approach for the treatment of cardiovascular diseases. In the future standard medical care in patients presenting with acute MI might not only consist of primary PCI, anti-coagulants, beta-blockers and ACE-inhibitors, but also treatment with various cytokines / growth factors which reduce local apoptosis, increase survival as well as agents which enhance progenitor cell mobilization and homing. However, further research is needed to determine and select the optimal therapy.

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Summary

Patients suffering from myocardial infarction (MI), atherosclerosis and Hereditary Hemorrhagic Telangiectasia type 1 (HHT-1) all have diseased and dysfunctional blood vessels. Cardiovascular repair in these diseases occurs not only locally, but also peripheral blood (progenitor) cells and cytokines/growth factors positively contribute to the repair of malfunctioning tissue. In this thesis several aspects of cardiovascular repair have been explored. First, we show that in MI patients relatively large infarctions or unfavorable hemodynamic conditions result in increased progenitor cell mobilization and pro-angiogenic cytokine/growth factor levels in the circulation. Secondly we show that, in MI and HHT-1 patients, homing of (progenitor) cells from the peripheral blood to an infarct area is stimulated by low levels or inhibition of the endopeptidase CD26. Furthermore, in MI patients, low CD26 levels are associated with an improved cardiac function and inhibition of CD26 on (progenitor) cells improved their homing. CD26 inhibition may therefore improve cardiac outcome.

In a clinical pilot trial, we furthermore investigated whether pre-medication with atorvastatin in ST-elevated-MI reduces reperfusion damage. We did not find any effects of pre-treatment with atorvastatin on cardiac function, microvascular perfusion or MI size. Finally, we show that in atherosclerotic plaques with a relatively large number of microvessels, high levels of Angiopoietin-2 are found. These high Angiopoietin-2 levels may result in leaky microvessels and subsequent plaque destabilization.

Taken together, the results presented in this thesis show that MI results in mobilization of (progenitor) cells and production of high levels of pro-angiogenic cytokines/growth factors, which may improve cardiovascular repair. Furthermore, our results suggest that CD26 inhibition is a promising tool to increase homing of systemically available cells which may have beneficial effects on cardiovascular repair and function, but a clinical trial is needed to demonstrate this.

Samenvatting in het Nederlands

Patiënten met een hartinfarct, slagaderverkalking of met de ziekte van Rendu-Osler-Weber (Hereditary Hemorrhagic Telangiectasia type 1) hebben zieke, niet goed functionerende bloedvaten en/of beschadigd hartspierweefsel. Reparatie van dit zieke weefsel wordt niet alleen verzorgd door locale bloedvat- en/of hartspiercellen, maar ook (voorloper)cellen uit het bloed en signaaleiwitten zoals groeifactoren kunnen het weefselherstel positief beïnvloeden. In dit proefschrift zijn verschillende aspecten van reparatie van zieke bloedvaten en hartspierweefsel onderzocht.

Wereldwijd is het krijgen van een hartinfarct een van de belangrijkste oorzaken van ziekte en sterfte. Een hartinfarct is meestal het gevolg van slagaderverkalking (atherosclerose). Door erosie, destabilisatie en vervolgens scheuren van de verkalking (plaque) kan een bloedstolsel in de kransslagader ontstaan wat een totale afsluiting van het bloedvat veroorzaakt. Hierdoor ontstaat een tekort aan zuurstof en voedingsstoffen waardoor een gedeelte van het hart afsterft. Dit kan leiden tot een verminderde hartfunctie wat uiteindelijk hartfalen tot gevolg heeft.
Op dit moment zijn er diverse behandelingen van een hartinfarct mogelijk. Allereerst is het van groot belang om binnen enkele uren na het ontstaan van de bloedvatafsluiting het bloedvat open te maken, waardoor het tekort aan zuurstof en voedingsstoffen wordt opgeheven. Dit gebeurt met een dotterbehandeling. Verder zijn er vele medicijnen beschikbaar (o.a. cholesterolverlagers, beta-blokkers, aspirine en ACE-remmers) die de hartfunctie en doorbloeding van het hart positief beïnvloeden en/of de levensverwachting na een hartinfarct vergroten. Als de hartfunctie ernstig verminderd is en behandeling met medicijnen tekortschiet, kan het nodig zijn een harttransplantatie te verrichten. Omdat dit een ingrijpende behandeling is, er een tekort aan donoren bestaat en er kans op afstoting van het getransplanteerde hart is, zijn de laatste jaren alternatieve behandelingsmethoden intensief onderzocht. Stamceltherapie is zo'n methode. Na een hartinfarct verplaatsen stam- of voorlopercellen zich vanuit het beenmerg naar het bloed om vervolgens deels naar het hartinfarct te bewegen. Deze reactie van het lichaam suggereert dat deze cellen de grootte van het hartinfarct misschien kunnen verkleinen en/of de hartfunctie positief beïnvloeden. Patiëntenstudies waarbij voorlopercellen uit het bloed of beenmerg als mononucleaire cel fractie in het hartinfarct gebied werden ingespoten toonden een lichte verbetering van de hartfunctie. Op dit moment is het nog niet duidelijk hoe dit precies werkt. Voorlopercellen vormen mogelijk nieuwe hartspiercellen, maar kunnen ook een gunstig effect hebben op het zieke hartspierweefsel door stimulatie van bloedvatvorming (angiogenese), remming van de ontstekingsreactie of door stimulatie van voorlopercellen die deel uitmaken van het hart.

In **hoofdstuk 2** wordt een literatuuroverzicht gegeven van de beweging van (voorloper) cellen tussen beenmerg, bloed en hart en hun rol in reparatie van het hart en/of bloedvaten.
Vervolgens worden in **deel II** van dit proefschrift studies bij patiënten met een hartinfarct beschreven. In **hoofdstuk 3** worden de resultaten van de REPERATOR studie beschreven. In deze studie zijn wij nagegaan of het geven van een cholesterolverlager voor de dotterbehandling bij een hartinfarct de hartfunctie positief beïnvloedt. Eerdere experimentele dierstudies toonden dat heel vroege behandeling met een cholesterolverlager na een hartinfarct de schade vermindert die ontstaat bij de overgang zuurstofarme naar zuurstofrijke omgeving. Onze resultaten tonen geen gunstig of ongunstig effect van vroege behandeling van patiënten met cholesterolverlagers na een hartinfarct.
Vervolgens worden in **hoofdstuk 4** de effecten van een hartinfarct op het aantal voorlopercellen en concentraties signaaleiwitten in het bloed onderzocht. Onze resultaten laten zien dat bij relatief grote hartinfarcten of verminderde hartfunctie, er meer voorlopercellen en hogere concentraties groeifactoren in het bloed aanwezig zijn. Eén week na het hartinfarct blijkt de vroege behandeling met een cholesterolverlager het aantal $CD133^+$ voorlopercellen in het bloed verder te verhogen. Het aantal voorlopercellen in het bloed of de concentratie groeifactoren heeft echter geen relatie met het herstel van de hartfunctie. Het lichaam lijkt dus te reageren op grote hartinfarcten of een verminderde hartfunctie met meer voorlopercellen en meer groeifactoren, maar deze cellen en eiwitten beïnvloeden de hartfunctie niet. Mogelijk omdat de voorlopercellen hun weg naar het infarct niet kunnen vinden.
In **hoofdstuk 5** is daarom de beweging van cellen uit het bloed naar het beschadigde hartspierweefsel verder onderzocht (homing). Na een hartinfarct vonden wij een verhoogd niveau van het enzym CD26 op de celmembraan van mononucleaire cellen, waardoor deze cellen minder goed in staat zijn om naar SDF-1α te bewegen. Het verhoogde niveau van het enzym CD26 op de celmembraan is gerelateerd aan een slechter herstel van de hartfunctie na een hartinfarct. Remming van CD26 herstelt het bewegen van deze cellen naar SDF-1α. Bij patiënten met een hartinfarct zou daarom behandeling met een CD26 remmer kunnen resulteren in een betere hartfunctie en kleiner hartinfarct.

In **deel III** beschrijven we vervolgens studies rond hart- en bloedvat reparatie bij patiënten met de erfelijke ziekte van Rendu-Osler-Weber (Hereditary Hemorrhagic Telangiectasia type-1 of HHT-1). Deze ziekte wordt veroorzaakt door mutaties in het gen dat codeert voor Endoglin. Endoglin is een eiwit dat van groot belang is voor de groei van bloedvaten. In **hoofdstuk 6** hebben wij de effecten van een hartinfarct

onderzocht in een muismodel voor HHT-1, de endoglin heterozygote muis. We laten zien dat bij een tekort aan Endoglin de hartfunctie na een hartinfarct meer achteruitgaat dan in normale (wildtype) muizen. Vervolgens hebben wij mononucleaire cellen van gezonde mensen of van HHT-1 patiënten in het bloed van deze muizen gespoten na een hartinfarct. De mononucleaire cellen van gezonde mensen bleken in staat de hartfunctie na een hartinfarct te herstellen, terwijl cellen van HHT-1 patiënten geen effect hadden. In **hoofdstuk 7** zijn we vervolgens nagegaan wat hiervan de oorzaak was. Mononucleaire cellen van HHT-1 patiënten bleken een verhoogd niveau van CD26 op hun celmembraan te hebben. Behandeling van deze HHT-1 mononucleaire cellen met een CD26 remmer resulteerde vervolgens in normalisatie van de beweging naar een hartinfarct gebied (homing). Op dit moment is het mogelijk CD26 te remmen met een medicijn dat eigenlijk op de markt is voor mensen met suikerziekte. Hierdoor zou het op korte termijn mogelijk zijn het effect van deze remmer op hartfunctie-herstel bij patiënten te onderzoeken.

Daarnaast hebben wij in **hoofdstuk 8** het ontstaan van bloedvaten op plaatsten van slagaderverkalking onderzocht. Wanneer er in zo'n verkalking relatief veel bloedvaatjes groeien is er een relatief grote hoeveelheid eiwit wat kenmerkend is voor jonge, niet stevige bloedvaten – Angiopoietine-2. Mogelijk draagt dit bij aan het scheuren van deze slagader verkalking, wat bijvoorbeeld een hartinfarct kan veroorzaken.
Tot slot worden in **hoofdstuk 9** de resultaten van de voorgaande hoofdstukken bediscussieerd.

De onderzoeksresultaten beschreven in dit proefschrift zorgen voor nieuwe inzichten in de reactie van het lichaam op een hartinfarct, de ziekte van Rendu-Osler-Weber en slagaderverkalking. Deze resultaten geven een basis voor nieuwe therapieën voor deze invaliderende ziekten, waaronder de remming van het eiwit CD26. Remming van dit eiwit in experimentele studies zorgt voor een beter herstel van de hartspier na een hartinfarct. Het is nu van belang dat therapeutische remming van dit eiwit onderzocht gaat worden in patiënten studies. Deze nieuwe inzichten dragen mogelijk bij aan het slagen van celtherapie voor patiënten met een hartinfarct.

Dankwoord

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Curriculum Vitae

Simone Post was born on September 11th, 1978 in Haarlem. In 1997, after graduating from secondary school (Stedelijk Gymnasium, Haarlem), she started her study Medicine at the University of Utrecht. During her medical study she performed her scientific internship at the department of Orthopedics, University Medical Center Utrecht. During this internship she investigated the role of systemically delivered mesenchymal stem cells in fracture healing. She obtained her medical degree in February 2004. Subsequently she worked as an AGNIO Cardiology in the Jeroen Bosch Medical Center, Den Bosch. In January 2005 she started as a PhD candidate at the Department of Cardiology, University Medical Center Utrecht, supervised by prof. Pieter Doevendans, prof. Gerard Pasterkamp, dr. Marie-José Goumans and dr. Benno Rensing. The results of her research are presented in this thesis. In December 2006 she started her Cardiology training at the department of Internal Medicine, St. Antonius Hospital Nieuwegein. Since January 2009 she works at the Department of Cardiology in the St. Antonius Hospital Nieuwegein and Lucas Hospital in Apeldoorn.