Vascular progenitor cells in renal and cardiovascular disease

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Thesis University of Utrecht, faculty of Medicine
With a summary in Dutch

Proefschrift Universiteit Utrecht, faculteit Geneeskunde
Met een samenvatting in het Nederlands

ISBN 978-90-393-4838-3

Printed by: Van Setten Kwadraat, Houten, the Netherlands

Cover photography: Krista den Ouden (DAPI nucleoli staining of mouse femoral artery cross-section)

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Vasculaire progenitorcellen in nier- en cardiovasculaire ziekten
(met een samenvatting in het Nederlands)

Proefschrift

ter verkrijging van de graad van doctor aan de Universiteit Utrecht op gezag van de rector magnificus, prof.dr. J.C. Stoof, ingevolge het besluit van het college voor promoties in het openbaar te verdedigen op dinsdag 8 juli 2008 des middags te 4.15 uur

door

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Dit proefschrift werd mede mogelijk gemaakt met financiële steun van de Nederlandse Hartstichting en ZonMw.

Additionele financiële ondersteuning werd gegeven door het St. Antonius Ziekenhuis te Nieuwegein, Amgen, AstraZeneca, Baxter, Boehringer Ingelheim, Bristol-Myers Squibb, Eli Lilly, Genzyme, Kuijkhoven adviesgroep, LEO Pharma, Merck Sharp & Dohme, Novartis, Pfizer, Roche, Sanofi Aventis, Schering-Plough en Shire Pharmaceuticals.

Het onderzoek dat aan dit proefschrift ten grondslag ligt, is mogelijk gemaakt door subsidies van de Nederlandse Hartstichting (2004T022 en 2006R024*)*, ZonMw (2007/12579), NWO (016.036.041), de Nierstichting (PC127 en C.04.2093), de Jacques H. de Jong stichting (2006.01.18), stichting "De Drie Lichten" (22/04) en de Prof. R.J.L. van Ruyven stichting.

Voor Saskia

Contents

1

GENERAL INTRODUCTION

Endothelial homeostasis: pivotal in renal and cardiovascular disease

The endothelium is a continuous single-cell lining of the vascular system that forms a critical barrier between the blood and the perfused tissues. The endothelium has selective permeability and is an active regulator of various processes for which it synthesizes and in part secretes factors such as nitric oxide (NO), platelet-derived growth factor, von Willebrand factor, prostacyclin, endothelin-1, chemokines and adhesion molecules.[1] Maintenance of endothelial integrity plays a central role in the protection against the development of atherosclerotic cardiovascular disease (CVD).[2] CVD risk factors cause loss of endothelial cells or impair endothelial cell physiology, resulting in endothelial dysfunction. Endothelial dysfunction and inflammation are key components in the initiation and propagation of the atherosclerotic process. Dysfunctional endothelium has an impaired capacity to release the vasodilatatory, anti-inflammatory and anti-oxidative NO, which leads to increased vascular permeability and vasoconstriction. Together with induction of a proinflammatory milieu by CVD risk factors, this leads to activation and influx of inflammatory cells into the vascular wall and the initiation of an atherosclerotic lesion.[3,4] In the kidney, the endothelium also has a crucial and highly specialized role in the glomerulus, where it forms a part of the filtration barrier. Glomerular endothelial injury is a major pathogenic factor in various renal diseases and an important determinant of renal disease progression.[5,6]

Regenerating dysfunctional endothelium

Traditionally, it was thought that endothelial repair occurs exclusively through proliferation of local endothelium, stimulated by the release of angiogenic growth factors. In recent years however, it has become clear that bone marrow derived endothelial progenitor cells (EPC) may replace damaged or lost endothelial cells.[7] Using animal hematopoietic chimeras, bone marrow derived cells were shown to incorporate sparsely in 'normal' endothelium, reflecting replacement of endothelial cells undergoing necrosis or apoptosis.[8] We[9] and others[10] have confirmed integration of bone marrow derived endothelial cells in human endothelium. Bone marrow derived cells are a particularly important source of endothelium in neovasculature associated with e.g. hind limb ischemia and myocardial infarction.[11] EPC also incorporate into the damaged glomerulus, differentiate into mature endothelial cells and eventually fully integrate into the resident glomerular endothelium.[12] In addition, apart from incorporation, bone marrow derived cells may have a paracrine effect on resident endothelial cells by secretion of angiogenic growth factors and cytokines such as Vascular Endothelial Growth Factor (VEGF).[13,14] Enhancement of endothelial repair by administration of EPC, enhancing circulating EPC levels or improving EPC function may represent a novel therapeutic target.

Endothelial progenitor cells: origin and characterization

The presence of bone marrow derived EPC in the adult circulation is well established.[7,15] Importantly, multiple types of circulating blood cells may be induced to display endothelial characteristics. In literature, several of these divergent cell types are referred to as EPC. The original description of the 'endothelial progenitor cell' concerned cells that attached to a culture dish *in vitro* after plating unselected peripheral blood mononuclear cells in a specific medium rich in serum and growth factors.[7] Careful characterization of these cultured adherent spindle-shaped endothelial-like cells showed that these cells are mostly monocyte-derived.[16-18] This monocyte-derived EPC, or Type II EPC, can adopt an endothelial phenotype after culture, but lacks many of the cell characteristics that would qualify it as a true 'progenitor' cell (such as a capacity for clonal expansion) and their capacity to form endothelium in vivo has been challenged.[19] However, these cells can be obtained from the blood in relatively high numbers and are potent secretors of angiogenic factors, implying an important

role in promoting angiogenesis and endothelial repair through paracrine stimulation of the resident endothelium.[14,16,20] Indeed, in experimental models *in vivo*, Type II EPC have been shown to incorporate and augment neovascularization after ischemia[21] and secrete angiogenic factors such as VEGF[14]. Another source of circulating cells with the capacity for endothelial differentiation are hematopoietic stem cells (HSC). HSC contain a subfraction of EPC that may be identified using flowcytometry based on the expression of VEGF-R2 (also known as KDR) together with a HSC-marker such as CD34.[22] CD34+KDR+ Type I EPC or 'hemangioblasts' are present in the circulation in low numbers, but represent a defined subset of true progenitor cells as demonstrated by the capacity of CD34+KDR+ cells for clonal expansion into endothelial cell colonies from single cells.[22]

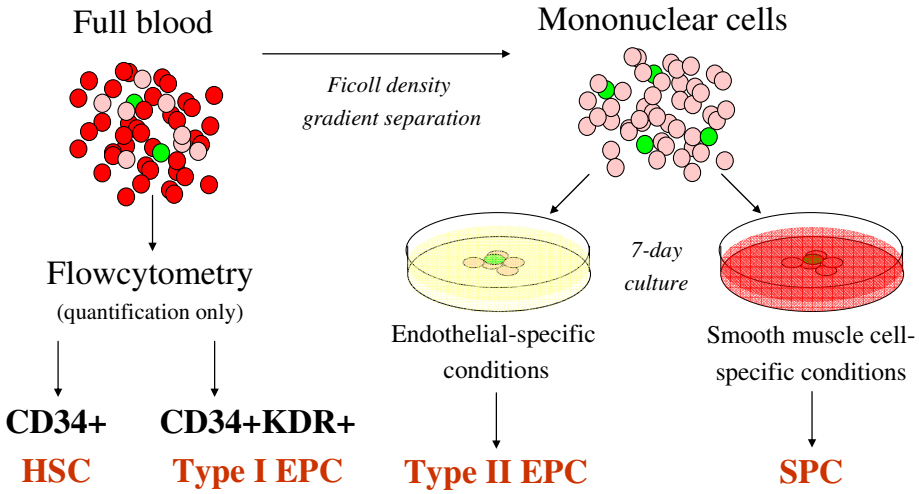

Figure. Methods of identification of circulating vascular progenitor cells in peripheral blood
In full blood, CD34+ hematopoietic stem cells (HSC) and HSC-subfraction CD34+KDR+ Type I endothelial progenitor cells (EPC) can be quantified using flowcytrometry. For identification of monocytic Type II EPC and SPC from blood, mononuclear cells are first isolated from blood using ficoll density gradient separation. Then, mononuclear cells are cultured in media containing specific growth factors for approximately 7 days.

Smooth muscle progenitor cells

Adverse remodeling of the vascular system occurs under various conditions and may involve smooth muscle cells, such as in restenosis due to intimal hyperplasia after endovascular procedures. Previously it was thought that accumulation of smooth muscle cells in the neointima of restenotic lesions was exclusively due to migration and local proliferation of smooth muscle cells from the media or adventitial fibroblasts. However, it was recently shown in bone marrow chimeric animals that smooth muscle cells of bone marrow origin contribute to postangioplasty restenosis.[23] Using similar methods, smooth muscle cells of bone marrow origin were found to contribute to transplant arteriosclerosis and hyperlipidemia-induced atherosclerosis.[24-27] Histological studies of autopsy material of patients who had previously undergone bone marrow transplantation confirmed that smooth muscle cells in human vascular lesions are in part bone marrow derived.[28] Consistently, cells with smooth muscle cell characteristics can be isolated from animal[29,30] and human[31-33] blood by *in vitro* culture. These smooth muscle progenitor cells (SPC) may display characteristics of other mesenchymal-lineage phenotypes such as fibroblasts and have also been referred to as circulating 'fibrocytes' or 'myofibroblast progenitor cells'(MFPC).[33,34] The origin of SPC, their regulation, and how SPC respond to pathophysiological stimuli associated with the development of CVD is not clear. One study found SPC to be mainly derived from CD34+ hematopoietic stem cells[31], but others found circulating endoglin(CD105)+ CD14+ monocytes as the main source of SPC[33]. For targeting intimal hyperplasia, inhibition of SPC incorporation may be desirable, but for atherosclerotic lesions, smooth

muscle cell incorporation and collagen deposition may have beneficial effects as this may increase plaque stability, which is of importance to prevent plaque rupture. In a recent study, SPC injection in atherosclerosis-prone ApoE-/- mice indeed modulated plaque composition towards a more stable plaque phenotype by increasing collagen and actin content and decreasing the incorporation of macrophages. In addition, the total atherosclerotic lesion size was reduced.[35]

Vascular progenitor cell dysfunction in the pro-atherosclerotic milieu

Various studies show that the presence of CVD risk factors may be associated with reduced levels of hemangioblastic Type I and monocytic Type II EPC. In cohort studies of patients with coronary artery disease, decreased levels of circulating CD34+KDR+ Type I EPC were associated with increased age, higher blood pressure, smoking, LDL-cholesterol and the presence of coronary artery disease.[36-38] Importantly, prospective data showed an independent association of lower CD34+KDR+ Type I EPC levels with increased rates of CVD events.[37,38] Monocytic Type II EPC outgrowth in culture has been shown to be reduced in the presence of impaired endothelial function, a high Framingham risk score, Type I and II diabetes, hypertension, renal insufficiency and increased age, in part also in subjects without manifest CVD.[39-42] No prospective cohort studies reporting on the predictive value of monocytic Type II EPC levels for CVD events have been published to date.

In addition to a reduction of circulating cell levels, EPC function may be impaired in the presence of CVD risk factors, reflected by a reduced capacity to proliferate, migrate, adhere to activated endothelial cells, or incorporate in network structures.[40,43,44] This EPC dysfunction was found to occur not only in EPC isolated from peripheral blood, but also in EPC isolated directly from the bone marrow in patients with chronic ischemic heart disease.[45] Less is known about the influence of CVD risk factors on SPC. In Type I diabetic patients, SPC outgrowth was shown to be increased, which was associated with reduced expression of the TGF-β antagonist BMP-6.[34] In one study, circulating levels of SPC-enriched CD105+CD14+ peripheral blood cells were increased in a population of mostly non-diabetic patients with manifest CVD compared to controls, suggesting that SPC levels may be increased in a pro-atherosclerotic milieu.[33]

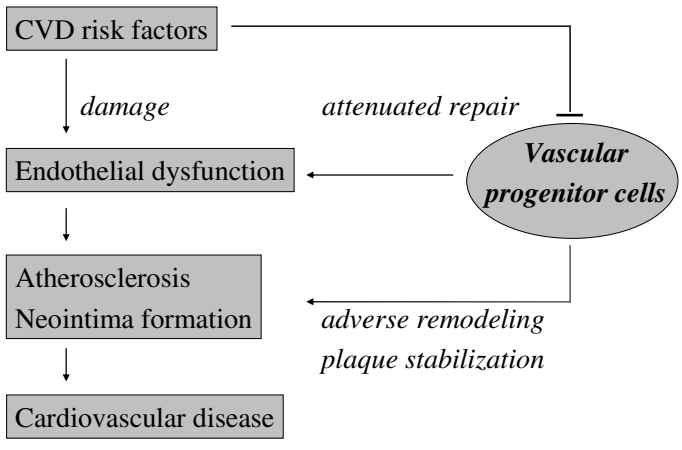

Figure. Proposed general concept of the pathophysiological pathways involved in the development of cardiovascular disease in the presence of cardiovascular risk factors

Taken together, these studies indicate that various cardiovascular risk factors are not only associated with increased endothelial damage, but also with impaired endothelial repair and modulation of vascular remodeling, which further predisposes patients to the development and progression of CVD.

Mobilizing (endothelial) progenitor cells

Regulatory cytokines from peripheral tissues

EPC are detectable in the blood in normal healthy subjects without injury or trauma. Hence, a basal level of EPC is continuously present in the circulation. After vascular injury, EPC levels in the circulation increase. Both peripheral[46] and myocardial ischemia[47] induce an elevation of EPC levels. Interestingly, this is preceded by a plasma peak in VEGF. VEGF is a cytokine known to mobilize EPC.[48] This suggests that the rate of EPC mobilization is in part regulated by cytokines released by ischemic tissue, probably to increase neovascularization and prevent further ischemic damage. Besides VEGF, ischemic tissue has been shown to induce the release of other EPC-mobilizing factors including Stromal-cell Derived Factor (SDF)[49], Granulocyte Colony Stimulating Factor (G-CSF)[50-52] and Stem Cell Factor soluble Receptor (SCF-sR)[53,54].

Regulatory factors for progenitor cell trafficking in the bone marrow

In the bone marrow, progenitor cell mobilization and also proliferation is largely regulated by supporting bone marrow cells, which closely interact with the progenitor cells. These supporting cells consist of a heterogeneous cell population, which together make up the bone marrow stroma and include fibroblasts, endothelial cells and osteoblasts. Progenitor cell proliferation is thought to be predominantly regulated by osteoblasts in the 'osteoblastic niche'. Proliferated progenitor cells then move to the 'vascular niche', where they transmigrate through the sinusoidal endothelial cells lining the sinusoidal vessels, via which they enter the circulation exiting the bone marrow. Regulation of the mobilization involves various cytokines, cell-cell contact molecules and regulatory factors.[55,56]

Active gelatinases Matrix MetalloProtease (MMP)-2 and particularly MMP-9 are released in response to several mobilizing factors, and appear to have an important intermediate role since blocking antibodies against MMP-2 and MMP-9 inhibited the CD34+ transmigration induced by various cytokines.[57] When MMP-9 is released in response to mobilizing factors, membrane-bound kit-ligand is cleaved, releasing soluble kit ligand (sKitL, also known as Stem Cell Factor (SCF)), which triggers progenitor cells to transfer from a quiescent to a proliferative niche.[58,59] Transendothelial migration, induced by SDF-1 signaling through its receptor CXCR4[60], was shown to depend on β_1- and β_2-integrins, PECAM-1[61], and E-Selectin[62] expressed on bone marrow endothelial cells. The exact interplay between these factors is the subject of intense research.

MMP-9 plasma levels have been shown to correlate with the mobilizing response in patients receiving G-CSF.[63] The activation of MMP-9 has been suggested to be nitric oxide-dependent.[64] Several lines of evidence confirm a role for nitric oxide production by endothelial nitric oxide synthase (eNOS) in EPC mobilization. EPC mobilization is impaired in eNOS knockout mice and mice treated with an NO-inhibitor.[64,65] Hyperbaric oxygen, which increases bone marrow nitric oxide levels, increases circulating CD34+ HSC levels.[66] Caveolin, which co-localizes with eNOS in the caveolae and regulates eNOS activity, is required for VEGF/nitric oxide dependent SDF-1 induced EPC mobilization.[67] Based on bone marrow transplantation experiments with eNOS knockout mice and wild-types, it was shown that EPC mobilization in response to a VEGF specifically depends on NO produced in bone marrow stromal cells.[64]

Outline of this thesis

This thesis focuses on the physiology and potential pathophysiological role of circulating progenitor cells in relation to renal and cardiovascular disease, and explores therapeutic modulation of circulating progenitor cells.

Vascular progenitor cell levels in specific conditions associated with increased CVD risk

Circulating progenitor cells have been shown to be affected by the presence of several risk factors for CVD disease. In this thesis we investigated particular disease states known to be associated with increased cardiovascular risk for their effects on vascular progenitor cells. Patients with end-stage renal disease (ESRD) have a strongly increased risk of cardiovascular disease and renal insufficiency is associated with marked endothelial dysfunction and adverse vascular remodeling. Previous studies showed conflicting results on EPC in ESRD patients[44] and SPC had not been investigated. We characterized circulating levels and *in vitro* differentiation of circulating Type I and II EPC and SPC in ESRD patients (chapter 2). In this cross-sectional study, we identified determinants of circulating progenitor cell levels. Furthermore, we evaluated the direct effects of the dialysis-procedure on Type I EPC levels and possible induction of EPC apoptosis as underlying mechanism.

Cardiovascular disease is a prominent long-term complication of the auto-immune disease Systemic Lupus Erythematosus (SLE), for which the induction of endothelial dysfunction is an important pathophysiological component. In chapter 3, we provide an overview of the data supporting these observations and show how autoantibody-formation in SLE may lead to atherosclerotic disease. It was unknown if EPC-levels were affected in SLE-patients. EPC are a subfraction of the hematopoietic stem cells (HSC), which have been shown to be adversely affected in the bone marrow of patients with SLE. We therefore evaluated if peripheral blood HSC and EPC levels were reduced in SLE patients. We measured HSC, Type I EPC and Type II EPC levels in SLE-patients with stable quiescent disease in comparison to those of age-matched healthy controls (chapter 4).

A highly prevalent condition that severely impacts the occurrence of CVD in our society, is the metabolic syndrome. The metabolic syndrome is a clustering of cardiovascular risk factors including abdominal obesity, dyslipidemia, hyperglycaemia, and hypertension.[68] The age-adjusted prevalence is 20-25% in apparently healthy subjects.[69] People with the metabolic syndrome have a 3 to 4 fold increased risk to develop Type II diabetes[70] and a 2 to 3 fold increased risk for future morbidity and mortality of CVD.[70-72] We investigated Type I EPC and HSC levels in obese subjects with the metabolic syndrome compared to normal controls (chapter 10). Importantly, most studies on EPC levels have included patients with manifest CVD. We included apparently healthy subjects without manifest CVD and without treatment, providing insight in EPC levels in an early pathophysiological stage of atherosclerosis.

Potential underlying mechanisms of altered vascular progenitor cell differentiation, mobilization and homing in experimental models

To further understand how vascular progenitor cells are affected by a 'pro-atherosclerotic milieu', we studied progenitor cells in a mouse model for Type I diabetes induced by injection of streptozotocin. We assessed the incorporation of bone-marrow derived cells in neointimal lesions using a mouse chimera previously transplanted with bone marrow from a GFP-congenic strain. As potential mechanism involved, we evaluated the effect of diabetes on SPC differentiation *in vitro* and in particular on the expression of pro-fibrotic TGF-β and TGF-β-anatagonist BMP-6 in diabetic SPC (chapter 5).

As EPC are derived from the bone marrow, we hypothesized that impaired EPC-mobilization might be involved in the reduced EPC levels observed in peripheral blood. For progenitor cell mobilization, the bone marrow vasculature plays a pivotal role and we therefore studied the possible involvement of dysfunctional endothelium in the bone marrow in the streptozotocin-model for diabetes in mice. We measured EPC levels in peripheral blood and bone marrow and investigated the capacity for EPC mobilization from the bone marrow by injection of mobilizing cytokines. We evaluated the structure and regenerative capacity of the bone marrow vascular niche using bone marrow histology and a model of bone marrow recovery from 5-FU-injection. *In vitro*, the influence of diabetes and/or hyperglycaemia on cellular interaction between stromal (endothelial) cells and progenitor cells was investigated with a specific focus on NO (chapter 6).

Effect of diabetes on resident vascular progenitor cells in the vascular wall
EPC have the capacity to stimulate neovascularization. However, there are also angiogenic (progenitor) cells residing in the vessel wall, which are potent contributors to neovascularization.[73] We hypothesized that diabetes would not only attenuate the function of circulating EPC, but also the angiogenic capacity of angiogenic cells in the vessel wall. We therefore studied the phenotype and angiogenic sprout formation from *ex vivo* cultured aorta rings isolated from diabetic and non-diabetic BioBreeding Diabetes-Prone (BBDP) rats. This sprouting-assay provides a tool to specifically study the long-term effect of diabetes on the angiogenic capacity of cells residing in the vessel wall, excluding the direct influence of both circulating factors and circulating cells (chapter 7).

Modulation of vascular progenitor cells
Augmentation of ischemic neovascularization by EPC involves homing of EPC to sites of ischemia. Our group has previously shown that the inflammatory cytokine RANTES is involved in EPC-homing to the injured glomerulus.[74] Therefore, we hypothesized that RANTES might also be critical in ischemia-induced angiogenesis. To test this hypothesis, we employed a rat model for peripheral ischemia by femoral artery ligation.[75] We investigated the microscopic angiogenic response by visualizing capillaries in ischemic muscle and explored the role of RANTES in ischemia-induced angio-genesis using a RANTES receptor antagonist (chapter 8). As RANTES-receptor-antagonism is under investigation as novel anti-atherosclerotic therapy, evaluation of potential adverse effects on ischemic neovascularization is particularly important.

Finally, we assessed if vascular progenitor cells could be therapeutically enhanced. In both mice and humans, PPAR-gamma-agonist rosiglitazone has been shown to augment EPC differentiation.[76,77] Therefore, we tested if the contribution of circulating progenitor cells to renal repair in an experimental animal model of anti-Thy1-glomerulonephritis could be enhanced by rosiglitazone (chapter 9). To enable identification of bone-marrow derived glomerular endothelium, we used chimeric rats previously transplanted with allogenic bone marrow.

Another drug class reported to potentially increase EPC levels is that of the lipid-lowering statins. We therefore evaluated the effect of treatment with intensive lipid-lowering therapy using two regimens with different concentrations of simvastatin but similar lipid-lowering potential by adding the cholesterol-uptake inhibitor ezetimibe to the low-dose simvastatin treatment. For this, we included a cohort of obese subjects with the metabolic syndrome in a randomized cross-over trial including both treatment regimens (chapter 10). In addition, we explored the VEGF, SCF and SCF-sR plasma levels as potential plasma markers for EPC availability.

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2

END-STAGE RENAL DISEASE CAUSES AN IMBALANCE BETWEEN ENDOTHELIAL AND SMOOTH MUSCLE PROGENITOR CELLS

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American Journal of Physiology – Renal Physiology
2007; 292(4): F1132-40

Abstract

Patients with end-stage renal disease (ESRD) on hemodialysis have an increased risk of cardiovascular disease (CVD). Circulating endothelial progenitor cells (EPC) contribute to vascular regeneration and repair, thereby protecting against CVD. However, circulating smooth muscle progenitor cells (SPC) may contribute to adverse vascular remodeling. We hypothesized that an imbalance occurs between EPC and SPC in ESRD patients and sampled progenitor cells from forty-five ESRD patients receiving regular treatment. Our study is the first to show reduced numbers of CD34+KDR+ hematopoietic stem cell (HSC)-derived EPC (Type I EPC). Furthermore, monocyte-derived EPC cultured from mononuclear cells (Type II EPC) were reduced in number and had a reduced capacity to stimulate endothelial cell angiogenesis. In contrast, SPC outgrowth was unaffected. In vitro incubation with uremic serum impaired Type II EPC outgrowth from healthy donor mononuclear cells and did not influence SPC outgrowth. The hemodialysis procedure itself induced HSC apoptosis and caused an acute depletion of circulating EPC. Taken together, the decreased number and impaired function of EPC are compatible with impaired endogenous vascular repair in hemodialysis patients, while the unaffected SPC numbers suggest that the potential of progenitor cells to contribute to adverse remodeling is retained. This EPC-SPC imbalance may contribute to the acceleration of CVD in ESRD patients and could offer novel therapeutic targets.

Introduction

End-stage renal disease (ERSD) is associated with a marked increase in the incidence of atherosclerotic cardiovascular disease (CVD).[1] Endothelial dysfunction is pivotal in this process. Circulating endothelial progenitor cells (EPC) contribute to endothelial regeneration and repair.[2] Reduced EPC numbers may contribute to accelerated atherosclerosis. Several cardiovascular risk factors and the presence of CVD are associated with lower numbers of circulating EPC.[3,4] Furthermore, in patients with coronary artery disease the number of EPC was recently shown to be an independent predictor of cardiovascular events in two prospective cohort studies.[5,6]. In animal models it has been shown that circulating progenitor cells may also differentiate into vascular smooth muscle cells and contribute to atherosclerosis.[7-9] Outgrowth of cells with a vascular smooth muscle/myofibroblast progenitor cell phenotype (SPC) from human blood has been demonstrated.[10,11] SPC numbers were observed to be increased in diabetes mellitus and in coronary artery disease patients.[12,13]

Two types of EPC have been reported. *Type I EPC* are identified using flow cytometry as CD34+ hematopoietic stem cells (HSC) that co-express the endothelial marker KDR. CD34+KDR+ Type I EPC are present in the circulation in low numbers and represent a defined subset of true progenitor cells. *Type II EPC* are identified by *in vitro* culture of peripheral blood mononuclear cells under conditions facilitating outgrowth of angiogenic cells with an endothelial phenotype. Type II EPC can be obtained from the blood in relatively high numbers and are mostly monocyte-derived cells.[14-16] Although the in vivo importance of incorporation of Type II EPC into damaged endothelium has been challenged[17], these cells have been shown to be potent secretors of angiogenic factors, implying an important role in promoting angiogenesis and endothelial repair via paracrine stimulation of the resident endothelium.[14,18,19] SPC can be cultured from peripheral blood mononuclear cells under culture conditions facilitating smooth muscle cell outgrowth.[10,11] The origin of these cells has not been fully elucidated, but similar to the EPC, both CD34+ hematopoietic stem cells[10] and monocytes[13] have been implicated as potential sources of SPC.

We hypothesized that altered vascular progenitor cell differentiation favouring SPC outgrowth and reducing EPC levels and function may contribute to the excess CVD risk in ESRD. We therefore determined the number of Type I EPC and Type II EPC levels and SPC in ESRD patients receiving hemodialysis and maintained on their standard medication. For Type II EPC, we investigated their potential to secrete paracrine angiogenic factors and stimulate endothelial cell angiogenesis. In addition, we investigated the acute effect of a hemodialysis session on Type I EPC.

Methods

Subjects

Forty-five ESRD patients (avg. age 55.9±2.1 yrs; 67% male) on hemodialysis were included from the dialysis units of the University Medical Center Utrecht and Dianet Dialysis Center Utrecht, the Netherlands. Consecutively attending patients were included, with only current infection and malignancy as exclusion criteria. Patients were maintained on their regular medication. Thirty healthy subjects of comparable age and gender (avg. age 50.2±1.1 yrs; 70% male) served as controls. Exclusion criteria for healthy controls were use of medication, hypertension, dyslipidemia, diabetes, renal disease, known cardiovascular disease, current infection, and malignancy. The protocol was approved by the medical ethical committee of the University Medical Center Utrecht.

Type I EPC and HSC flow cytometry

100 µl of EDTA blood was incubated with Fluorescein isothiocyanate (FITC)-conjugated mouse-anti-human CD34 monoclonal antibody (BD Pharmingen, San Diego, USA), Phycoerythrin (PE)-conjugated mouse-anti-human KDR (VEGF-R2) monoclonal antibody (R&D Systems; Minneapolis, USA), Phycoerythrin-Cyanine Dye7 (PECy7)-conjugated mouse-anti-human CD45 monoclonal antibody (BD Pharmingen) and 7-amino-actinomycin D (7AAD; BD Pharmingen) at 4° Celsius. Erythrocytes were lysed in an ammoniumchloride buffer and remaining cells were analyzed by flow cytometry (Beckman Coulter, Fullerton, USA). EPC were identified as CD34+KDR+ cells and quantified relative to the number of granulocytes in the sample, which were identified as CD45+ cells with a forward / sideward scatter pattern typical for granulocytes. Measurements were performed in duplo and the results were averaged for further analysis. Isotype-stained samples served as negative controls.

Annexin V and TUNEL staining of HSC

100 µl of EDTA blood was incubated with FITC-conjugated mouse-anti-human CD34 monoclonal antibody (BD Pharmingen) and Phycoerythrin-Cyanine Dye7 (PECy7)-conjugated mouse-anti-human CD45 monoclonal antibody (BD Pharmingen) at 4° Celsius. Erythrocytes were lysed in an ammoniumchloride buffer and remaining cells were stained with PE-conjugated mouse-anti-human Annexin V (BD Pharmingen) and 7AAD in Annexin V Binding Buffer (BD Pharmingen) at room temperature. Cells were washed and analyzed by flow cytometry (Beckman Coulter). Apoptotic HSC were defined as CD34+AnnexinV+ cells with a low forward scatter and 7-AAD*dim* staining. Isotype-stained samples served as negative controls.

For Tdt-mediated dUTP nick end labelling (TUNEL) of fragmented DNA in CD34+ cells, indicative of late apoptosis, 100 µl of EDTA blood was first incubated with FITC-conjugated mouse-anti-human CD34 monoclonal antibody (BD Pharmingen) for 45 minutes by at 4° Celsius followed by erythrocyte lysis in an ammoniumchloride buffer. Remaining cells were fixed in 2% para-formaldehyde for 30 minutes at room temperature and subsequently permeabilized using 0.1% sodium citrate containing 0.1% Triton-X for 2 minutes on ice and washed in PBS. TUNEL reaction was then performed using the TMR red Roche In situ Cell Death Detection Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Stained cells were washed in PBS and analyzed using flow cytometry.

Type II EPC culture and characterization

Blood samples were collected in EDTA tubes and mononuclear cells (MNC) were isolated using Ficoll density gradient separation (Histopaque 1077; Sigma, St. Louis, USA). MNC were plated on gelatin (Sigma) coated 6-wells plates at a density of 10^7 cells per well in M199 medium (Invitrogen, Breda, the Netherlands) containing 20% fetal calf serum (Invitrogen), 0.05 mg/ml bovine pituitary extract (Invitrogen), 10 units/ml heparin (Leo Pharma, Breda, the Netherlands), and antibiotics (penicillin 100U/ml and streptomycin 100µg/ml; Invitrogen). Medium was changed after 4 days, washing non-adherent cells away. After 7 days, cells were detached for quantification using trypsin-EDTA (Invitrogen) and gentle cell scraping. Type II EPC phenotype was confirmed by the binding of FITC-labeled Ulex Europeus Lectin (Vector, Burlingame, USA) and the uptake of DiI-labeled acetylated LDL (Molecular Probes, Leiden, the Netherlands). Since >90% of attaching cells obtain an endothelial phenotype, the total cell number was automatically counted using a hemocytometer.[2,20] As culture conditions used to generate Type II EPC vary, we confirmed our observations using a second commonly used method for Type II EPC culture, which involves growth on high concentrations

recombinant VEGF-165. For this, we coated 6-well plates with human fibronectin (Becton Dickinson, Alphen aan de Rijn, the Netherlands) and plated 10^7 cells per well in EGM-2 medium (Cambrex, Walkersville, USA), supplemented with 20% fetal calf serum (Invitrogen), 100 ng/ml recombinant VEGF-165 (R&D systems) additional to that supplied in the Singlequots, and antibiotics (penicillin 100U/ml and streptomycin 100µg/ml; Invitrogen). [16]

SPC culture

MNC were plated in 6-wells plates coated with human fibronectin (Sigma) at a density of 5 x 10^6 cells per well and cultured in low-glucose DMEM, supplemented with 20% fetal calf serum, L-glutamine (2mM) and antibiotics (penicillin 100U/ml and streptomycin 100µg/ml; Invitrogen). Medium was changed after 4 days, washing non-adherent cells away. 7 days after the original plating, cells were detached using trypsin-EDTA and a cell scraper. Since the vast majority of cells have a SPC phenotype, identified by the co-expression of alpha-smooth muscle actin and collagen-1, the total adherent cell number was automatically counted using a hemocytometer.[12]

In vitro angiogenesis assay

The capacity of Type II EPC to excrete paracrine angiogenic factors was assessed by testing the effect of Type II EPC conditioned medium on mature endothelial cells. 7-Day Type II EPC were cultured in serum-free endothelial medium (Endothelial Basal Medium-2 supplemented with selected EGM-2 aliquots: hEGF, hydrocortisone, GA-1000, R^3-IGF-1, ascorbic acid and heparin (Cambrex) for 20 hours. Conditioned media were stored at minus 80° Celsius until further processing after removal of non-adherent cells by centrifugation. For further experiments, conditioned media were diluted to correct for the Type II EPC cell number in the original culture. 7.5 x 10^3 early passage Human Umbilical Vein Endothelial Cells (HUVEC) were suspended in conditioned media and placed on matrigel (Chemicon, Ternecula, USA). HUVEC suspended in blanco medium (the serum-free medium with selected EGM-2 aliquots as used to make the conditioned medium) and regular EGM-2 supplemented with 100 ng/ml additional VEGF were used as negative and positive controls. After 20 hours, cells were labeled with Calcein-AM (Molecular Probes), fixed in 4% paraformaldehyde for 30 minutes, and photographed at 50X magnification using an inverted fluorescence microscope. The formation of tubular structures was quantified based on tube length using Scion Image software (Scion Corporation, Frederick, USA) and expressed in arbitrary units.

VEGF Enzyme-Linked ImmunoSorbent Assay (ELISA)

Plasma VEGF levels were measured using commercially available ELISA kits (R&D Systems) according to the manufacturer's instructions. All samples were measured in duplo and averaged for analysis.

Statistical analysis

All data are expressed as mean(SE). Data were analyzed using Graphpad Prism version 4.00 software. Before data set comparison, Gaussian distribution was tested using the normality test and the equality of variances was assessed using the Levene F-Test. Differences between groups were all analyzed using the students' t-test, which was paired where appropriate. For regression analysis, Pearson's correlation coefficients were calculated, except for multinomial values, where model I linear regression was used. A p-value lower than 0.05 was considered statistically significant.

Table 1. Patient characteristics.

ESRD patients (n=45)			
Age (yrs)	55.9 (2.1)	History of CVD	50 %
Gender (male / female)	30 / 15	Hypertension (RR >145/95 or drug use)	79 %
Weight (kg)	74 (5)	Diabetes	28 %
Blood pressure[#] (mm Hg) - diastolic	78 (3)		
- systolic	141 (5)	Medication	
White blood cells (*10^6/ml)	8.3 (0.6)	Antihypertensive drugs [##]	67 %
Mononuclear cells (*10^6/ml)	2.3 (0.2)	Statin	50 %
Granulocytes (*10^6/ml)	6.0 (0.5)	Folic Acid	100 %
Hemoglobin (mmol/l)	7.1 (0.3)	Erythropoietin (darbepoetin-alpha)	80 %
Glucose (mmol/l)	6.4 (0.4)	Avg. dose (µg/week)	49 (6)
Total Cholesterol (mmol/l)	3.40		
LDL (mmol/l)	1.47	Principle causes of ESRD	
HDL (mmol/l)	1.20	Glomerulonephritis	19 %
Triglycerides (mmol/l)	1.63	Hypertension	19 %
		Polycystic Kidney Disease	13 %
Time on dialysis (months)	19.3 (9.3)	Intoxication	13 %
Dialysis session duration (minutes)	195 (5)	Diabetes Mellitus	10 %
Dialysis Kt/V	1.14	Pyelonephritis	10 %
Type of access: Fistel	70 %	Unknown / other	16 %

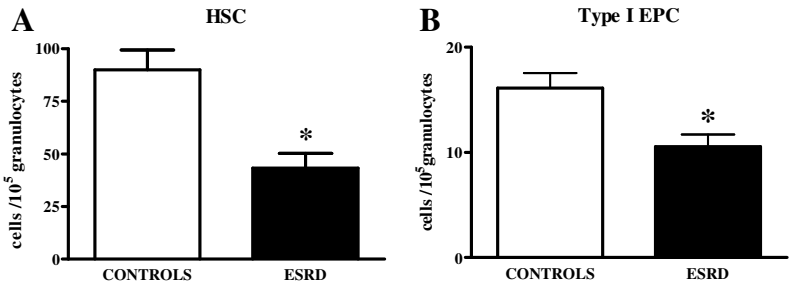
[#] predialysis [##] mainly ACE-inhibitors

Results

Patient characteristics

Patient characteristics are summarized in table 1. Patients had various underlying causes of renal insufficiency. Several other risk factors were present, which were in part the underlying cause of the renal disease, particularly diabetes and hypertension. All patients used medication, including standard drugs for ESRD patients that are not listed such as vitamin supplements and phosphate-binders, but also statins, antihypertensive drugs, and/or erythropoietin.

Figure 1.
Circulating levels of Hematopoietic Stem Cells (HSC) (a) and Type I EPC (b) are reduced in blood samples taken immediately before a hemodialysis session in ESRD patients (n=30) compared to healthy controls (n=15).
**p<0.05*



Type I EPC and total HSC are reduced in ESRD patients

On flow cytometry, CD34+ HSC were readily identifiable and contained a sub fraction of cells staining positive for the endothelial progenitor cell marker KDR. The total number of circulating CD34+ HSC was lower in ESRD patients (43.2 (7.0) vs. 90.0 (9.4) /10^5 granulocytes, 52% reduction; p=0.0003, fig. 1a). The percentage of HSC co-expressing KDR was higher in ESRD patients than in controls (31(3) vs. 20(2)%; p=0.023). The absolute number of Type I EPC in the peripheral blood was significantly lower (10.6 (1.1) vs. 16.1 (1.4) /10^5 granulocytes, 34% reduction; p=0.0046, fig. 1b).

Determinants of Type I EPC numbers

We observed no significant association between the numbers of circulating Type I EPC in our patient population and the presence of concomitant risk factors for cardiovascular disease, such as diabetes (9.8(1.2) vs. 10.7(1.5) Type I EPC /10^5 granulocytes, p=0.72), hypertension (10.0(1.2) vs. 12.5(3.1) Type I EPC /10^5 granulocytes, p=0.382), a history of cardiovascular disease (8.9(1.1) vs. 10.9(1.8) Type I EPC /10^5 granulocytes, p=0.331) and LDL-cholesterol levels (r=-0.135, p=0.63). There was also no association with gender (10.0(1.5) in women vs. 10.7(1.5) cells/10^5 granulocytes in men, p=0.79) or age (r=-0.254, p=0.17). Interestingly, the dose of recombinant erythropoietin (darbepoetin alpha) correlated positively with both the number of HSC (r=0.472; p=0.008, fig. 2a) and Type I EPC (r=0.337; p=0.07 (trend), fig. 2b), as calculated using linear regression modeling. The use of other drugs, including statins, folic acid, or antihypertensives was not related to circulating progenitor cell levels.

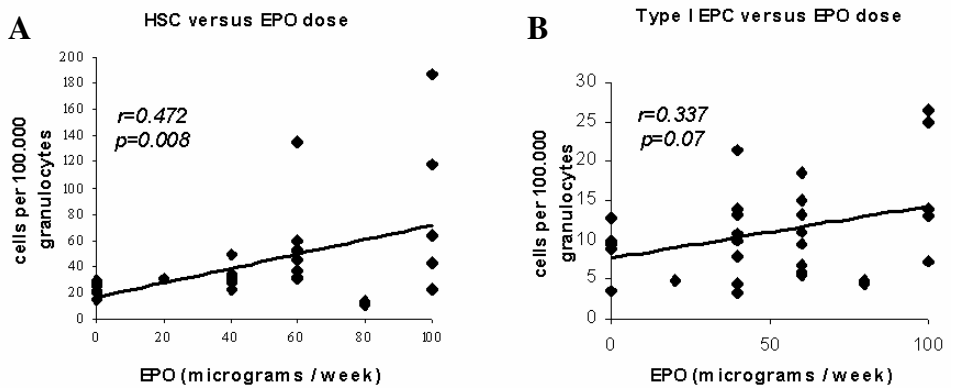


Figure 2.

Circulating levels of HSC (a) and Type I EPC (b; trend) correlated with the weekly dose of exogenously administered recombinant erythropoietin (darbepoetin alpha), as calculated using Type I linear regression model.

Type II EPC outgrowth is impaired in ESRD, while SPC outgrowth is outgrowth is unaffected

After 7 days of culture, Type II EPC expressed KDR, took up DiI-labeled acetylated LDL and bound FITC-labeled lectin, while SPC did not display these endothelial characteristics. Conversely, SPC expressed αSMA, while Type II EPC did not. Type II EPC did express some collagen-1 intracellularly near the plasma membrane, although not as abundant as the SPC, which also secreted it (fig. 3). Quantification of Type II EPC after 7 days of culture revealed that Type II EPC outgrowth from the MNC was reduced by 58 percent in ESRD patients versus controls (3.3 (0.6) vs. 7.9 (1.9) /1000 MNC; p= 0.030, fig. 3). Because Type II EPC culturing conditions are not uniform, we confirmed our observations using a second commonly used protocol[16] in a subgroup of patients. We observed a similar reduction using this method, although with higher efficacy of EPC outgrowth (8.6 (2.7) vs. 15.7 (5.1) Type II EPC / 1000 MNC corresponding with a 45% reduction, n=5; our standard culture method in this subgroup showed a 42% reduction with 5.9 (0.9) vs. 10.1 (3.5) Type II EPC / 1000 MNC). In contrast to the reduction in Type II EPC, SPC outgrowth was not affected in ESRD patients (5.2 (0.7) vs. 5.8 (1.2) SPC / 1000 MNC; p=0.706, fig. 3).

Paracrine angiogenic function of Type II EPC outgrowth is impaired in ESRD

An important mode of action for Type II EPC is the secretion of paracrine acting angiogenic factors. To test the angiogenic capacity of Type II EPC, HUVEC were taken up in Type II EPC conditioned medium and plated on matrigel. Under these conditions, HUVEC formed tubular networks, resembling vascular structures (fig. 4ac). The conditioned medium of Type II EPC from ESRD patients was significantly less effective in stimulating HUVEC angiogenesis than that from healthy controls (+2.2 (2.3) vs. –1.3 (2.6) AU versus basal medium; p=0.034; fig. 4bd).

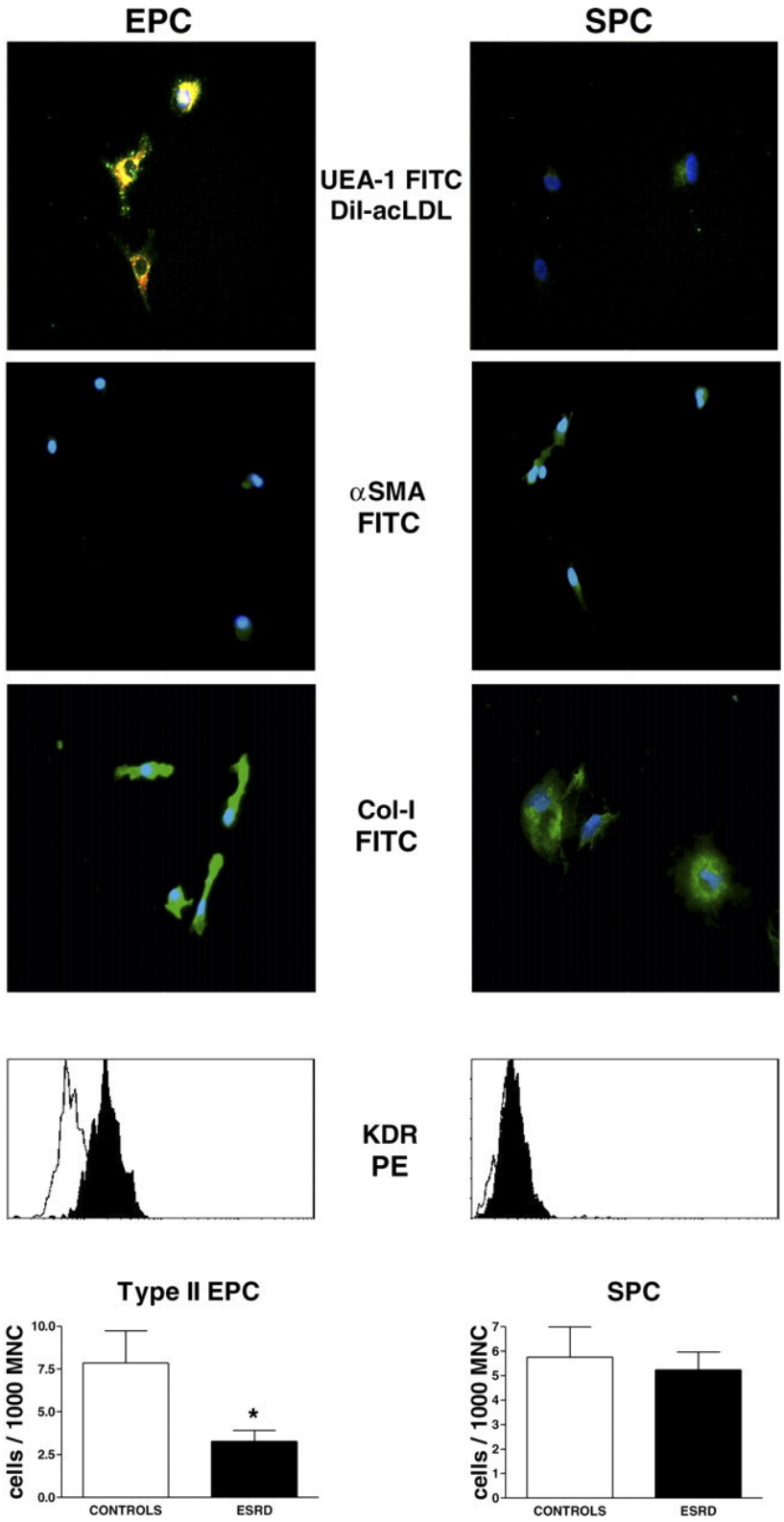


Figure 3. *Type II EPC cultures gave spindle-shaped adherent cells that took up DiI labeled acetylated LDL, bound FITC-conjugated Ulex Europeus Lectin and expressed KDR. SPC cultures gave αSMA and collagen-1 positive cells. SPC did not express endothelial markers. Conversely,Type II EPC did not express αSMA, but did express some collagen-1, although it was not secreted as by the SPC. Stainings of EPC and SPC were performed simultaneously and photographed with identical settings. KDR staining was assessed using flow cytometry with isotype-stained cells as control (filled histogram vs. single line). Numerically, Type II EPC outgrowth from ESRD patients was reduced compared to controls. Numeric SPC outgrowth was not different between ESRD patients and controls. *p<0.05, n=15 patients versus 15 controls*

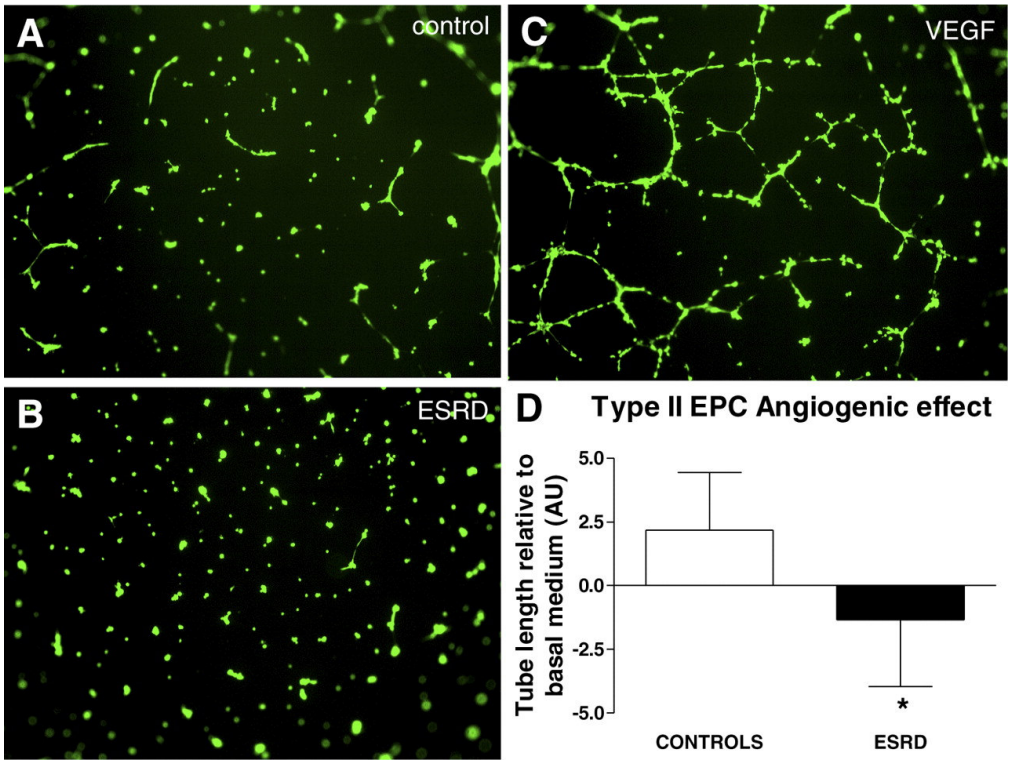
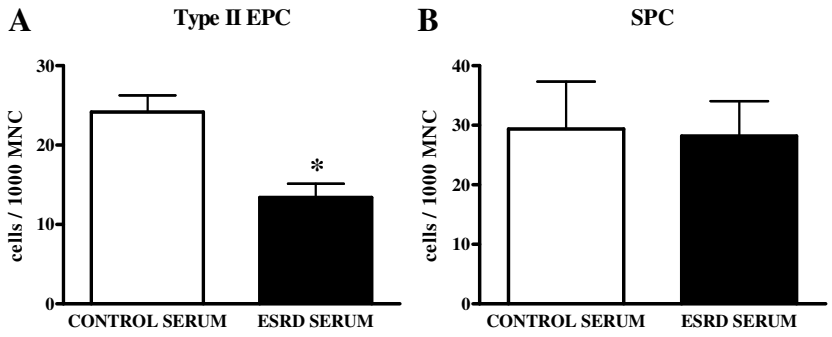


Figure 4. *When taken up in conditioned medium from healthy control Type II EPC, mature endothelial cells (HUVEC) were stimulated to form angiogenic structures when placed on Matrigel (a). Formation of these structures was significantly hampered when HUVEC were incubated with conditioned medium from ESRD patient Type II EPC (b). As a positive control, 100ng/ml recombinant VEGF-165 was used (c). The average length of the formed tubular structures was lower in the assays from ESRD patients than in those from healthy controls and even (non-significantly) lower than those with control basal medium (d). *p<0.05 n=10 patients versus 10 controls*

Uremic serum inhibits Type II EPC, but not SPC, outgrowth

To test if there were direct effects of the uremic environment on Type II EPC outgrowth, we cultured Type II EPC from umbilical cord blood mononuclear cells, substituting fetal calf serum in the medium with serum from either ESRD patients or healthy controls. Uremic serum reduced Type II EPC outgrowth by 46 percent (13.4 (1.8) vs. 24.2 (2.1) /1000 MNC; p=0.004, fig. 5a). SPC outgrowth was unaffected by the uremic serum (28.1 (5.9) vs. 29.3 (7.9) / 1000 MNC; p=0.708, fig. 5b).

Figure 5.
Serum from uremic patients impaired Type II EPC outgrowth (a), without affecting SPC outgrowth (b) from healthy mononuclear cell cultures when compared to serum from healthy controls.
**p<0.05, n=7 patients versus 7 controls in paired analysis of 7 independent experiments*



Hemodialysis causes a depletion of Type I EPC and HSC from the circulation and induces HSC apoptosis

We investigated whether a dialysis session would directly increase EPC numbers. However, Type I EPC levels were *reduced* at the end of the dialysis session as compared to levels before the start of

dialysis (11.1 (1.5) vs. 7.5 (1.2) /10^5 granulocytes; p=0.0054; fig. 6a). To investigate a possible involvement of apoptosis in this process, we measured the fraction of CD34+ HSC that bound Annexin V. Annexin V binds to phosphatidylserine, which is normally located on the inner side of the cell membrane, but translocates to the external side in an early phase during apoptosis, enabling extracellular Annexin V binding. This is distinctive from non-programmed causes of cell death. During dialysis, the fraction of AnnexinV positive HSC increased (27.1 (3.4) vs. 37.1 (4.4), p=0.045, fig. 6b), confirming a role for apoptosis in the observed HSC depletion. HSC apoptosis rates prior to dialysis were comparable to control levels. Additional analysis showed that less than 2% of HSC were TUNEL positive (data not shown), indicating that circulating apoptotic HSC are in an early phase of apoptosis as they in majority do not display signs of late apoptosis, such as TUNEL staining of DNA strand breaks. Binding of Annexin V to Type I EPC could not be studied because the necessary combination of fluorescent label conjugated antibodies is not available.

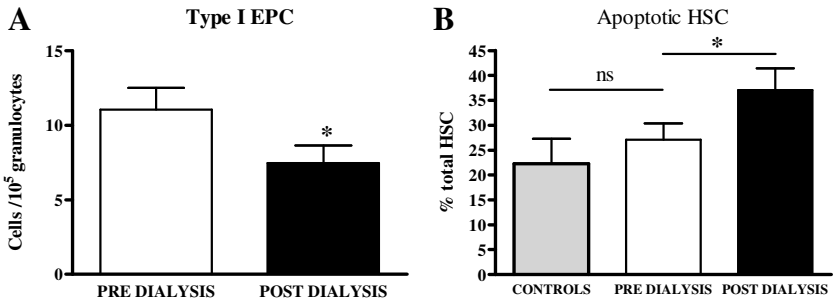


Figure 6.
During dialysis, circulating levels of Type I EPC decreased (a), while inducing progenitor cell apoptosis as evidenced by AnnexinV staining of CD34+ HSC (b).
**p<0.05, n=17 patients before and after dialysis in paired analysis*

VEGF plasma levels are not significantly decreased in ESRD patients
VEGF induces mobilization of EPC and is present in the circulation.[21] We measured plasma VEGF levels, to evaluate if decreased levels of mobilizing stimuli might underlie the reduced EPC levels. However, plasma VEGF was not significantly reduced in ESRD patients (63.4 (16.1) vs. 68.0 (34.2) pg/ml; p=0.891).

Discussion

The present data show that in our population of ESRD patients on regular medication, levels of total HSC, HSC-derived Type I EPC and monocyte derived Type II EPC are markedly reduced as compared to healthy controls, whereas SPC levels are not affected. Furthermore, Type II EPC capacity for paracrine angiogenic stimulation was impaired. Our observations suggest that the uremic environment causes a relative imbalance in vascular progenitor cell differentiation with impaired regenerative potential and enhanced proatherosclerotic tendency as a consequence. Progenitor cell imbalance may have a pathophysiological role in the development of CVD in ESRD patients. In patients with preterminal renal insufficiency EPC and SPC may also influence the progression of renal disease, since EPC have been shown to incorporate in damaged glomerular endothelium[22,23] whilst glomerulosclerosis may be initiated by bone-marrow-derived circulating cells[24]. In our population the marked progenitor cell impairments were observed despite standard medical treatment, indicating that additional therapies aimed at enhancing EPC and/or inhibiting SPC may be of added benefit for ESRD patients.

Our study is the first to show that patients with ESRD have reduced levels of circulating Type I EPC, defined as CD34+ KDR+ cells. Such reductions in Type I EPC are particularly relevant as, at least in patients with coronary artery disease, reduced Type I EPC numbers predicted future cardiovascular events.[5,6] Previous studies have reported decreased levels of the whole CD34+ hematopoietic stem

cell population in ESRD, consistent with our data.[25-27]. However, our observations are in contrast with the single study that has reported on Type I EPC in ESRD by Herbrig et al., who showed no difference despite a (non-significant) decrease in HSC levels.[27] Of note, in our population the reductions in Type I EPC levels were also less pronounced than the reductions in total HSC numbers. Our observations of reduced numeric outgrowth of Type II EPC in our population of ESRD patients on hemodialysis, are in line with several previous reports.[25,26,28] Again contrasting findings were reported by Herbrig et al. who observed an *increased* number of Type II EPC in ESRD patients on hemodialysis.[27] Differences in study methods may play a role. Culture conditions to obtain Type II EPC are not uniform and the methods used by various groups differ on several points. We confirmed the observations using our standard culture conditions [2,20] in a subset of samples using a second commonly used culture method[16]. In a recent review, Herbrig et al. suggested that the apparent discrepancies of their study with those of others might be due to the patient selection, the majority of patients receiving EPO treatment, or the high VEGF levels in their population, which were found to be twofold higher than their controls.[29] Although we did find an association between EPC levels and the EPO dose, overall a majority of our patients received EPO also (80%). The VEGF levels in our study were not higher than in controls and we did not observe a correlation between EPC and VEGF levels, making a determining role for VEGF less likely. Finally, the discrepancy of Herbrig's study with our observations on Type I and Type II EPC and with all previous studies on Type II EPC may be due to their very specific patient selection. They included a highly selected ESRD population without concomitant CVD risk factors and use of various cardiovascular drugs, thereby excluding over 80% of their initial population. Our population consisted of patients on regular treatment with minimal exclusions and thus more representative for the ESRD population as a whole, but heterogeneous in its composition and influencing factors. We found no major statistically significant contributing effects of concomitant individual cardiovascular risk factors or drugs on EPC numbers; however, the statistical power to detect such differences was limited. Cardiovascular risk factors were absent in our control population, where the majority of the ESRD patients in our study had hypertension, diabetes, a history of CVD, dyslipidemia, or a combination of factors. These cardiovascular risk factors such as hypertension can be both cause and consequence of ESRD. An important limitation of our study is therefore that we can all together not dissociate the effect of renal impairment per se with the presence of cardiovascular risk factors. Of note, levels of cardiovascular risk factors may correlate differently with the manifestation of CVD in ESRD patients than in the general population, further complicating any such interpretation.[30]

Similar to what we found in vascular progenitor cell cultures from ERSD patient mononuclear cells, we observed that uremic serum reduced Type II EPC outgrowth from healthy donor cord blood mononuclear cells but did not affect SPC outgrowth. These data indicate that uremic serum contains either impairing toxins or lacks essential stimulants for EPC outgrowth. We cultured mononuclear cells from ESRD patients *ex vivo*, which means that the cells are no longer exposed to the uremic toxins during Type II EPC outgrowth. Despite the non-diseased environment, Type II EPC proved dysfunctional after a 7 days culture period, implying that the impairments caused by the uremic state are to some degree imprinted upon cells.

We observed that apart from altered numeric outgrowth, the paracrine actions of Type II EPC were reduced in ESRD patients as compared to controls. Type II EPC from ESRD patients have previously been shown to exhibit an impaired capacity to migrate, adhere to matrix molecules or mature endothelial cells, and to incorporate into endothelial cell vascular structures.[26-28] However, the particular importance of the paracrine actions of Type II EPC is increasingly recognized, whereas

their role of active participation in the newly formed endothelium is under debate.[14,19] We found that Type II EPC conditioned medium of ESRD patients was less capable of stimulating HUVEC to form vascular structures than that of healthy controls. Hence, in ESRD patients, not only less Type II EPC are available to home to sites of endothelial damage, but they are also less capable of stimulating resident endothelial angiogenesis. The aberrant outgrowth of EPC was not accompanied by changes in SPC outgrowth. We therefore speculate that the regenerative capacity of progenitor cells may be impaired in ESRD, while the capacity of progenitor cells to contribute to fibrosis and adverse remodeling of vascular lesions is unaffected.

Interestingly, a dialysis session did not increase but markedly *reduced* circulating progenitor cells in the circulation. This observation cannot be attributed to changes in circulating blood volume as cell numbers were expressed relative to the number of granulocytes, which moreover did not change significantly during the procedure. Another possibility is EPC sequestration. Leukocyte subpopulations, particularly monocytes but also neutrophils and to some extent lymphocytes, are known to sequestrate during hemodialysis, probably due to complement-activation as a result of contact between the blood and the dialysis membrane. For all leukocytes however, sequestration is a rapidly occurring process that is maximal after approximately 10 minutes, after which circulating leukocyte levels start to rise again and fully recover well before the end of the dialysis session.[31] We cannot exclude the possibility of sequestration of EPC during dialysis, but since we measured decreased progenitor cell levels at the very end of the dialysis session at a time-point when other leukocyte subpopulations are no longer sequestrated, this does not seem likely. Increased apoptosis could also be responsible for the depletion of EPC during hemodialysis. Therefore, we investigated the binding of apoptosis marker Annexin V to circulating HSC, which indeed increased during dialysis, suggesting increased apoptosis to be a causative mechanism. This is in line with other studies showing an induction of apoptosis in the general leukocyte population by hemodialysis.[32]

Repetitive depletion of Type I EPC during dialysis sessions may exhaust the available progenitor cell pools. However, this may only in part explain the decreased progenitor cell levels at baseline. We and others [26] showed that uremia itself has a profound effect on progenitor cells. Furthermore, in patients with severe renal insufficiency but not yet on dialysis, HSC and Type II EPC were decreased compared to controls and increased after the initiation of dialysis.[26] Finally, a recent study demonstrated fully normalized HSC and Type II EPC levels in nocturnal hemodialysis, which is a very intensive dialysis regimen both in frequency and duration resulting in extensive contact of blood with the dialysis membrane, which is highly effective in clearing uremic toxins.[28] Interestingly, previous studies showed unexpected impairment of endothelial function occurring during hemodialysis.[33] This appeared to be related to the type of dialysis filter.[34] It remains to be established if particular hemodialysis methods or materials may ameliorate the induction of progenitor cell apoptosis and depletion during dialysis sessions. All our patients dialyzed with a polysulphone filter, which was previously shown not to impair endothelial function.[34]

We investigated patients receiving standard treatment, including antihypertensives and statins, which are known to increase EPC numbers in other populations.[35,36] However, we did not find differences in progenitor cell levels associated with the use of these drugs in our study. Interestingly, erythropoietin was dose-dependently associated with higher numbers of circulating HSC, and to a lesser extent Type I EPC. Although this observation is likely to be confounded by our cross-sectional study design, it could reflect a beneficial effect of recombinant erythropoietin on EPC levels. Of note, we did not find any correlation with the hemoglobin or erythrocyte levels, suggesting that modulation of EPC

numbers by erythropoietin may be unrelated to effects on erythropoiesis. Erythropietin administration has indeed been shown to upregulate HSC and Type II EPC numbers in patients with advanced renal failure and renal anemia, but also in non-renal conditions, such as congestive heart failure, myocardial infarction and even in healthy controls *in vivo*, as well as *ex vivo* in the absence of the erythroid system.[37-40] These data support a direct stimulating effect on EPC of exogenous erythropoietin.

A well-known endogenous EPC mobilizing factor is VEGF.[21] We measured VEGF plasma levels, which were not different between patients and controls. This is consistent with others who have measured similar or even increased levels.[27,28,41] The reduced EPC levels are therefore not due to a lack of VEGF, although other unidentified mobilizing factors may be deficient.

In conclusion, despite standard medication, levels of circulating HSC and Type I EPC are reduced in the peripheral blood of end-stage renal disease patients on hemodialysis treatment compared to healthy controls. Type II EPC outgrowth from mononuclear cells is impaired under uremic conditions, and these impairments are retained in a non-diseased *ex vivo* environment. Functionally, the secretion of paracrine angiogenic factors by Type II EPC is reduced, hampering their capacity to promote endothelial regeneration. Dialysis induces progenitor cell apoptosis and causes a depletion of EPC from the circulation. In contrast to the impairment in EPC numbers and function, SPC outgrowth was not affected, indicating that the capacity of vascular progenitor cells to contribute to adverse vascular remodeling is retained. These findings may be of importance for understanding the accelerated atherosclerosis in patients with ESRD and offers novel therapeutic targets for the prevention of CVD in these populations. Therapeutically, not only do EPC numbers need to be increased, possibly in combination with inhibition of SPC differentiation, but the restoration of impaired EPC function may be equally important. Erythropoietin may be of particular interest as a candidate drug to enhance EPC-mobilization in the ESRD population, independent of the indications for enhancing erythropoiesis.

Acknowledgements

We want to acknowledge the efforts of patients, nursing staff and nephrologists at the University Medical Center Utrecht and Dianet Dialysis Center Utrecht, as well as our healthy volunteers, for making this study possible. Adele Dijk (UMC Utrecht, the Netherlands) kindly provided the HUVEC used for this study. Dr. Bart Gadella (Utrecht University, the Netherlands) gave excellent technical advice.

Grants

This study was funded by a pilot grant by the Dutch Kidney Foundation. (C.04.2093). PEW is a research fellow of the Dr. E. Dekker program (2004T022) of the Dutch Heart Foundation. MCV is supported by the Netherlands Organization for Scientific Research (NWO VENI-grant 016.036.041).

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3

PREMATURE ATHEROSCLEROTIC CARDIOVASCULAR DISEASE IN SYSTEMIC LUPUS ERYTHEMATOSUS

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Arthritis & Rheumatism 2007; 56(5):1384-96

Abstract

Patients with Systemic Lupus Erythomatosus (SLE) have a markedly increased risk of atherosclerotic cardiovascular disease (CVD) due to both traditional and non-traditional pro-atherosclerotic factors. The effects of autoantibodies take a central role in the disease-specific pathogenesis of the accelerated atherosclerosis in SLE. Autoantibodies can cause (1) dyslipidemia, (2) activation of the immune system, (3) endothelial cell apoptosis, (4) oxidative stress, and possibly (5) a reduction of endothelial progenitor cells. Together with detrimental side effects of medication and consequences of an increased prevalence of traditional risk factors such as hypertension, these effects of autoantibodies result in a chronic low-grade inflammatory state and endothelial dysfunction, eventually leading to atherosclerotic CVD events. Rigorous identification and treatment of traditional cardiovascular risk factors in SLE patients is essential for CVD prevention. This requires better awareness of the importance of adequate treatment of CVD risk factors in SLE of both physicians and patients. Meanwhile, increasing insight in the disease-specific factors may lead to the development of specific interventions in the future.

Abbreviations

β2-GP-I	Beta2-glycoprotein-I	HDL	High-density lipoprotein
ABI	Ankle brachial index	HSP	Heat shock protein
aCL	anti-cardiolipin	IL	Interleukin
ADMA	AsymmetriC dimethylarginine	IMT	Intima media thickness
AMI	Acute myocardial infarction	LAC	Lupus anticoagulant
Anti-ds-DNA	anti-double stranded DNA	LDL	Low-desity lipoprotein
Apo	Apolipiprotein	LPL	Lipoprotein lipase
CAD	Coronary artery disease	MI	Myocardial infarction
CHF	Chronic heart failure	NO	Nitric oxide
CIC	Circulating immune complex	oxLDL	oxidized low-density lipoprotein
CRP	C-reactive protein		
CVA	Cerebrovascular accident	PAF-AH	Platelet-activating factor acetyl hydrolase
CVD	Cardiovascular disease		
EBT	Elecron beam tomography	PMR	Proportional morbidity ratio
EPC	Endothelial progenitor cell	PWA	Pulse wave analysis
FMD	Flow mediated dilation	PWV	Pulse wave velocity
GPRD	General practice research database	SLE	Systemic lupus erythematosus
		VLDL	Very-low-density lipoprotein

Introduction

Systemic lupus erythematosus (SLE) is a complex multi-system disease characterized by chronic inflammation, which may involve virtually every organ. Early in the 20th century SLE was described as a "generally progressive disease terminating fatally"[1], and the first described life expectancy of SLE patients was 3 months to 1 year in 1932.[2] The causes of death were mostly directly associated with periods of active SLE and included renal insufficiency, infection and sepsis. With improving treatment regimens patients live longer, resulting in a current 10-year survival rate of approximately 90%.[3] As survival in SLE patients improved, an increased incidence of cardiovascular disease (CVD) was uncovered with events occurring even in young women. In 1976 Urowitz et al. reported a bimodal pattern of mortality in SLE with early deaths from active disease and late deaths from cardiovascular origin.[4] Several subsequent reports have supported an increased cardiovascular risk among SLE patients. Traditional cardiovascular risk factors such as hypertension and dyslipidemia are more prevalent in SLE patients, and certainly contribute to an increased incidence of CVD.[5] However, cohort studies have shown that the increased cardiovascular risk in SLE patients cannot be fully explained by the higher prevalence of traditional risk factors, and that disease-specific factors play an additional role.[6-8] In this review we will summarize the available data on the incidence of CVD in SLE patients, and provide an overview of atherogenic mechanisms associated with SLE, with a specific focus on how autoantibodies contribute to the premature development of atherosclerosis.

Evidence for increased incidence of atherosclerotic cardiovascular disease in SLE

Three prospective follow-up studies have reported on CVD endpoints in SLE patients in comparison to a reference population without SLE (table 1). The largest study monitored 498 female SLE patients for an average duration of 6.7 years and compared these to age-matched women from the Framingham study. The incidence of myocardial infarction was markedly higher in all age-groups of women with SLE, with a 7-fold higher incidence in women from all age groups combined and a particularly striking more than 50-fold increased risk in those aged 35-44.[9] In a Swedish study myocardial infarction occurred 9 times more frequently in 86 adult SLE patients followed for a period of 6 years than in the general population. A longer disease duration and glucocorticoid treatment were associated with a higher incidence of myocardial infarction.[10] Finally, a smaller prospective study of 47 SLE patients also reported an increased occurrence of CVD endpoints after correction for the expected incidence of CVD based on the prevalence of traditional risk factors.[7]

Two large retrospective studies compared CVD incidence between hospitalized SLE patients and a reference group without SLE based on data obtained from hospital discharge records. As these studies included only hospitalized patients who have more active and more severely manifesting SLE, these studies are not representative for all SLE patients. First, a Swedish study reported a 3-fold higher CVD mortality in 4737 hospitalized SLE patients than in the general population over a period of more than 30 years starting in 1964. Over this period the incidence of CVD deaths in SLE patients remained constant despite a decrease in overall mortality.[11] A second study based on Californian hospital records compared 8742 hospitalized SLE patients with 43710 hospitalized patients without SLE and found SLE patients to be 2 to 4 times more likely to be hospitalized for acute myocardial infarction (AMI), congestive heart failure (CHF) or a cerebrovascular accident (CVA). The increased incidence of CVD occurred particularly in young women aged between 18 and 44 who were estimated to have a more than 8-fold higher chance of MI or CVA and a 11-fold higher chance of CHF than a comparable population without SLE.[12]

Two retrospective studies specifically investigated the incidence of CVD events in SLE cohorts in comparison to what was expected based on the prevalence of traditional risk factors. First, a retrospective study in a cohort of 298 SLE patients showed a 10-fold higher incidence of non-fatal myocardial infarction and a 17.0-fold higher mortality due to coronary heart disease than expected based on their traditional CVD risk factor profile.[6] In line with this, a case-control study comparing 8688 patients with AMI with 33923 controls from the General Practice Research Database (GPRD), holding data from general practitioners in the United Kingdom, found the 41 SLE patients in the database to be 2.67 times more likely to have an AMI than their controls after correction for the presence of traditional risk factors.[8] These studies show that in SLE patients CVD risk estimation based on the Framingham Heart study data underestimates the actual CVD risk, and thereby provide further evidence for the presence of disease-specific mechanisms involved in the increased incidence of CVD in SLE.

Table 1 Studies reporting clinical CVD endpoints for SLE populations versus the general population or healthy controls (in order of appearance in the text)

Study	SLE Patients	Controls	Average Follow-up	Findings
Bessant 2004 [7] *prospective*	n=47	Framingham population	10 yrs, or until death	Higher observed incidences of CVD than predicted. (8.5% vs. 1.4% for CHD. 10.6% vs. 1.0% for stroke)
Fischer 2004 [8] *retrospective case-control*	n=15 SLE with AMI n=26 SLE without AMI	8688 AMI patients 33923 patients without AMI	Not applicable (7.3 yrs search period in GPRD database)	Overall odds ratio of 2.67 for AMI in SLE-patients. Hyperlipidemia and male sex correlated with a higher odds ratio for AMI.
Manzi 1997 [9] *prospective*	n=498 from single medical center	n=2208	6.7 yrs	More CVD events in the SLE population in every age-cohort, with a highest relative risk for MI of 52.4 for the age group of 35-44 yrs old. Older age at diagnosis, disease duration, duration corticosteroid use, hypercholesterolemia, and postmenopausal status correlated with a higher incidence of MI.
Jonsson 1989 [10] *prospective*	n=86 from SLE referral medical center	General population Sweden	4.0 yrs	A 9-fold higher incidence of MI than in the general population. Longer disease duration and the use of corticosteroids correlated with a higher incidence of MI.
Bjornadal 2004[11] *retrospective*	n=4737 from hospital discharge records	general population Sweden	Not applicable (31 yrs search period database)	Overall standardized mortality ratio of 2.97 for CVD; 3.03 for CHD and 2.06 for stroke separately. Highest (16-fold) increase in CHD mortality in age 20-39 yrs. CVD mortality remained constant over time (1964-1995).
Ward 1999 [12] *retrospective*	n=8742 from hospital discharge records California	n=43710 matched hospitalized controls	Not applicable	Higher likelihood for hospitalization for AMI (Proportional morbidity ratio (PMR) 2.27), CHF (PMR 3.8) and CVA (PMR 2.05) of patients aged 18-44 with SLE compared to hospitalized patients without SLE. In older SLE patients there were smaller or no differences with controls. The estimated CVD prevalence more than 8-fold increased for SLE patients aged 18-44. The observations remained significant after adjusting for traditional CVD risk factors.
Esdaile 2001 [6] *retrospective*	n=296 from 2 medical centers	Framingham population	8.6 yrs	Relative risk in SLE patients was 10.1 for non-fatal MI; 17.0 for fatal CHD; 7.5 for overall heart disease; and 7.9 for stroke.

Whether the increased CVD risk in SLE is due to accelerated atherosclerosis or rather a high propensity for thrombotic complications cannot be determined from the epidemiological data above. SLE patients have an increased risk for thrombotic complications, particularly when anti-phospholipid antibodies are present.[13] Of note though, it may clinically be challenging to discern between arterial thrombo-emboli without underlying atherosclerotic pathology and an occluding atherosclerotic lesion, which may also have a thrombotic component. Previously, much attention has been given to the thrombotic propensity in SLE. However, several lines of evidence indicate that atherosclerosis plays an important role in SLE–related CVD, even in young patients. Histopathological studies of post-mortem material from SLE patients showed more extensive and more severe atherosclerotic lesions in vessels from SLE patients than from controls at various sites in

the vasculature.[14-17] In addition, endothelial dysfunction and the presence of subclinical atherosclerosis, assessed using various methods, have been reported in SLE (table 2). Endothelial dysfunction is recognized as an early phenomenon in the development of atherosclerosis, and has been shown to be strongly associated with an increased incidence of future atherosclerotic cardiovascular events.[18] The capacity of the endothelium to release Nitric Oxide (NO) in response to a stimulus is the hallmark of endothelial function. Endothelial function can be assessed in vivo by measuring the dilation of the brachial artery in response to stimuli for endothelial nitric oxide production, such as infusion of acetylcholine or a change in shear stress by temporal inflation of a brachial cuff (Flow Mediated Dilation, FMD).[19] Several studies showed that SLE patients have impaired endothelial function compared to healthy controls assessed as reduced FMD.[20-24] Endothelial function in another cohort of SLE patients was equally impaired as in patients with established cardiovascular disease.[23] The presence of subclinical atherosclerosis can be detected by measuring the Pulse Wave Velocity (PWV) and augmentation index of the pressure waves by performing Pulse Wave Analysis (PWA) using applanation tonometry. Together with the Ankle Brachial Index (ABI),

Table 2. Studies reporting on pre-atherosclerotic or sub-clinical atherosclerotic manifestations in SLE versus matched healthy controls (organized by detection method).

Study	Patients (n)	Controls (n)	Methods	Findings in SLE patients compared to controls
El-Magadmi 2004 [21]	62	38	FMD	Impaired FMD in SLE patients, also after adjustment of classic risk factors.
Lima 2002 [20]	69	35	FMD	Impaired FMD in SLE patients.
Johnson 2004 [22]	5 symptomatic CHD 5 asymptomatic CHD 5 without CHD	5	FMD	SLE patients with symptomatic CHD had a markedly impaired FMD, and SLE patients with no or asymptomatic CHD had a moderately impaired FMD.
Rajagopalan 2004[23]	43	43	FMD	Impaired FMD in SLE patients; equally impaired as CAD patients (also n=43).
Soep 2004 [118]	33	30	FMD	No significant impairment in endothelial dysfunction in SLE patients.
Wright 2006 [24]	32	19	FMD	Impaired FMD in SLE patients. FMD correlated negatively with disease activity. Wave forms analysis suggested alterations in microcirculation of forearm.
Lee 2006 [119]	35	35	Applanation Tonometry	Small Artery Elasticity higher in a population with higher homocysteine, sVCAM-1, oxLDL and CD40L. oxLDL levels, older age at SLE diagnosis and higher disease damage scores correlated inversely with small artery elasticity.
Roman 2005 [28]	101	105	Vascular Ultrasound + Applanation Tonometry	SLE patients more frequently had plaque, but IMT was similar. Arterial stiffness was increased.
Brodszki 2004 [26]	39	55	Vascular Ultrasound	Arterial stiffness was increased in SLE patients, correlating with the mean arterial blood pressure and the use of hormone therapy.
Falaschi 2000 [29]	26	26	Vascular Ultrasound	Higher IMT in juvenile onset SLE patients.
Svenungsson 2001 [30]	26 with CHD 26 without CHD	26	Vascular Ultrasound	More frequent plaque in SLE patients with and without CHD, but only those with CHD had a higher IMT.
Roman 2003 [31]	197	197	Vascular Ultrasound	More frequent plaque, but lower IMT.
Vlachoyiannopoulos 2003 [32]	33	33	Vascular Ultrasound	More frequent plaque, but no difference in IMT.
Cederholm 2004 [72]	26 with CVD 26 no CVD	26	Vascular Ultrasound	Only SLE patients with CVD manifestations had a higher IMT.
Wolak 2004 [33]	51	51	Vascular Ultrasound	More frequent plaque, but no difference in IMT.
Jimenez 2005 [35]	70	40	Vascular Ultrasound	More frequent plaque and at younger age, but no difference in IMT.
Lopez, 2006 [70]	30	27	Vascular Ultrasound	More frequent plaque and higher IMT; IMT correlated with total cholesterol, LDL and triglyceride levels.
Asanuma 2003 [36]	65	69	EBT	More frequent and more severe coronary artery calcification.
Asanuma 2006 [37]	74	85	EBT	More severe coronary artery calcification, correlating with IL-6 levels.

Abbreviations: CAD= coronary artery disease; CHD= coronary heart disease; CVD= cardiovascular disease; EBT= electron beam tomography; FMD= flow mediated dilation; IMT: Intima media thickness

PWV and PWA are indices of vascular stiffness. A high proportion of SLE patients have a decreased ABI[25], echographically measured pulsatile diameter changes showing mechanical alterations of predominantly large vessels[26], and an increase of the PWV and PWA[27,28]. Carotid ultrasonography in SLE patients consistently showed more atherosclerotic plaques in SLE patients than in matched controls but measurements of the intima medial thickness (IMT), also indicative of subclinical atherosclerosis, have been conflicting.[28-35] On Electron Beam computed Tomography (EBT) images, SLE patients proved to have more coronary artery calcification.[36,37]

Few studies focused on the prevalence of CVD in SLE populations with quiescent disease or limited clinical manifestations, e.g. with only dermatological lesions. Three studies reporting impaired endothelial function in SLE patients included cohorts with low disease activity.[21,22,24] However, the majority of studies included heterogeneous SLE patients groups. The larger cohort studies provide only limited clinical information but will have included patients with renal disease, vasculitis, recurrent infections, and those using high-dose immunosuppressive drugs. The smaller studies do not have the power for subgroup analysis. It is therefore unclear whether the results and conclusions from the studies summarized above can be extrapolated to all SLE. CVD risk in mild SLE cases may be relatively low, overestimating their risk when based on that of the overall SLE population as generally reported.

Mechanisms underlying atherosclerosis in SLE

Current concept of the development of atherosclerosis

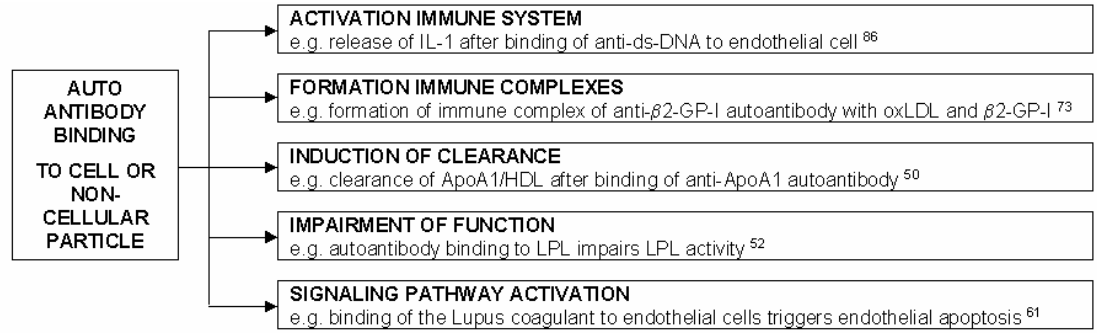
The development of atherosclerosis is a highly complex process (for comprehensive reviews see e.g. [38] and [39]). Here we will briefly summarize some of the key elements. Endothelial dysfunction and inflammation play pivotal roles in the initiation and propagation of the atherosclerotic process. Loss of the functional integrity of the endothelium may be structural due to loss of endothelial cells, or functional when the capacity of the endothelium to respond to physiological and pathological stimuli is impaired despite adequate covering by endothelial cells. In both conditions the endothelium will be dysfunctional with an impaired capacity to release the vasodilatatory, anti-inflammatory and anti-oxidative NO. This increases vascular permeability and induces vasoconstriction. In addition, various pathological stimuli activate the immune system and initiate the release of proinflammatory cytokines, creating a proinflammatory milieu. Together with an impaired anti-inflammatory defense of dysfunctional endothelium, this leads to activation and influx of inflammatory cells into the vascular wall. Atherosclerotic lesions are characterized by the presence of large numbers of inflammatory cells, including monocytes/macrophages, mast cells, dendritic cells, and T-cells. Infiltrated macrophages take up oxLDL, which is the oxidized form of LDL and one of the most important atherogenic peroxidation products. OxLDL uptake by macrophages results in foam cell formation, constituting the characteristic fatty streak observed in early atherosclerotic lesions. Further plaque development is highly influenced by intraplaque inflammatory processes, affecting thrombocyte aggregation, smooth muscle cell and fibroblast proliferation, and matrix production. During lesion progression the remodeling of the plaque and the extent of inflammatory infiltrate determine whether a plaque will be stable or unstable. Rupture of an unstable plaque eventually precedes a clinical syndrome as plaque rupture initiates thrombus formation and occlusion of the diseased blood vessel.

The role of autoantibody formation

The production of a large variety of autoantibodies is a prominent pathogenic feature of SLE and contributes to the accelerated atherosclerosis. Premature atherosclerotic lesions have been shown to contain IgG-containing immune complex deposits.[40] Autoantibody production and binding to proteins or cells can induce several responses (figure 1):

- activation of the immune system, including the release of inflammatory mediators and (further) activation of inflammatory cells;
- formation of atherogenic circulating immune complexes (CIC);
- accelerated clearance of the autoantibody-bound protein or cell by inflammatory cells, which may cause loss of atheroprotective components;
- impairment of the function of the auto-antibody-bound protein or cell;
- modulation of intracellular signaling of an autoantibody-bound cell, including the induction of apoptosis.

Figure 1. Mechanisms of atherogenic autoantibody interference



In SLE, this contributes to a number of pro-atherogenic pathophysiological phenomena, including (1) dyslipidemia, (2) activation of the immune system, (3) endothelial cell apoptosis, (4) oxidative stress, and possibly (5) a reduction of endothelial progenitor cells. Together with detrimental side effects of medication, consequences of an increased prevalence of traditional risk factors such as hypertension, and dysregulation of the immunesystem, the effects of autoantibody-binding result in a chronic low-grade inflammatory state and endothelial dysfunction, eventually leading to atherosclerotic CVD events in SLE (figure 2).

'Traditional' risk factors and effects of immunosuppressive medication

Several 'traditional' risk factors such as hypertension and dyslipidemia, are more prevalent in SLE patients (table 3). This is related to both disease-specific factors and effects of the (immunosuppressive) medication. Treatment with corticosteroids has been implicated as a risk factor for atherosclerosis, because it may induce hyperlipidemia, hyperglycemia, hypertension and obesity, in addition to being an independent risk factor for cardiovascular disease suggesting it may have direct atherogenic properties.[41] Several studies in SLE patients have shown that prolonged prednisone use is associated with atherosclerotic changes[5,42,43], although others found contradicting results.[44] Roman et al. found that more aggressive immunosuppressive therapy, including higher doses of prednisone and the use of cyclophosphamide or hydroxychloroquine, was associated with the absence of plaque.[31] A non-significant association was found between use of azathioprine and presence of plaque in this study, which is in contrast with a prospective study by Doria et al in which a higher IMT was observed in patients using azathioprine (significant in univariate analysis only).[43] For

hydroxychloroquine use, a protective effect against CAD has previously been described[41], and has been suggested to be related to a cholesterol level lowering effect[41,45]; although this is not universally supported.[46] Observed associations between the use of (immunosuppressive) medication and vascular changes are confounded because disease severity directly relates to both indication and effect of drug use.

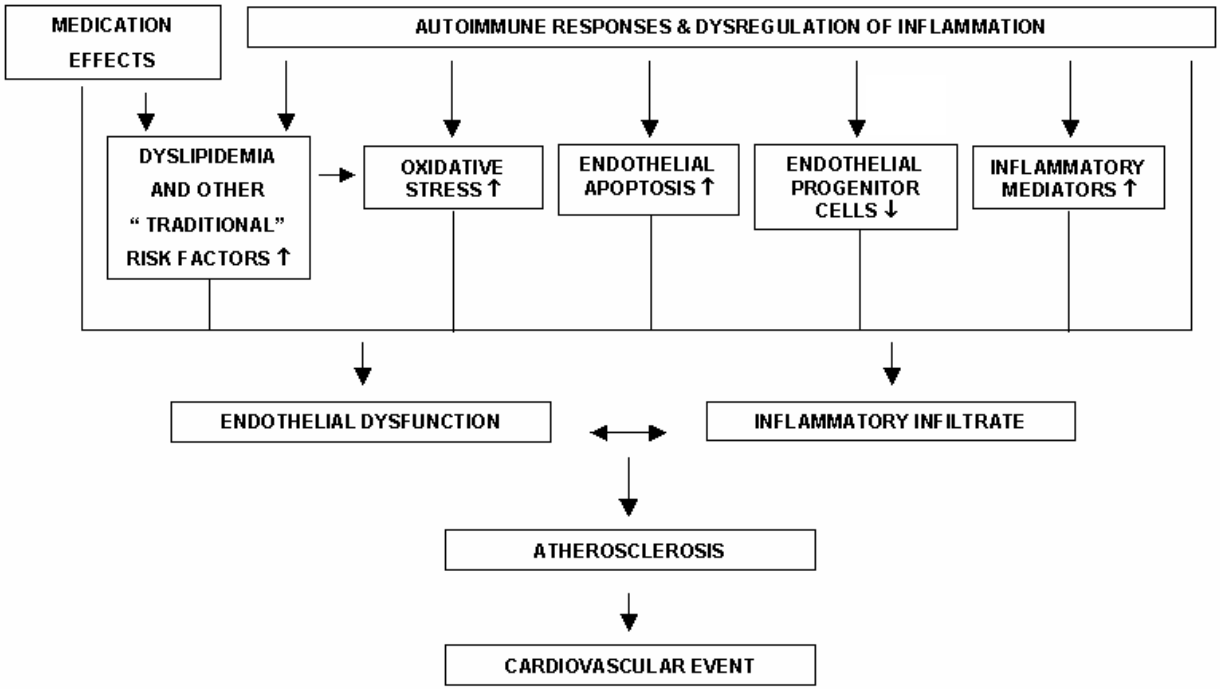
Table 3. Traditional CVD risk factors known to be more prevalent in SLE patients than in controls

Traditional Risk factors	Non-traditional risk factors
Hypertension [5,6,21,35,42,43,120,121]	Use of corticosteroids [5,42,43]
Hypercholesterolemia [5,6,9,35,42,43,120,121]	Pro-inflammatory HDL [53]
Hypertriglyceridemia [21,30,36,120]	Endothelial apoptosis [23,57,58]
Hyperhomocysteinemia [30,36,120]	Anti-HSP autoantibodies [58,74,101]
Low HDL [55,122]	Increased ox-LDL levels [67]
High BMI [27,42,123]	High levels circulating immune complexes [73]
Insulin resistance [52]	High ADMA levels [77]
Metabolic Syndrome [52]	Elevated levels inflammatory cytokines [37,80,83-85,87,88]
Diabetes Mellitus [120]	(IL-1, IL-6, IL-12, IL-18, MCP-1, IFN-γ, TNF-α,
Earlier menopause [9,42,120]	TNF-α- associated receptors, ICAM-1, VCAM-1, E-selectin)
Renal impairment [120]	Dendritic cell CD86-overexpression [92]
Sedentary lifestyle [120]	T-cell CD40L upregulation [93]
Elevated CRP [13, 30, 42, 123]	Decreased endothelial progenitor cell levels [108]
Smoking [13, 121]	

Traditional risk factors may be influenced by SLE-specific factors. Atherogenic actions of anti-phospholipid antibodies may relate to effects on lipid levels. Antibodies against phospholipids and other lipid transport components adversely affect the lipid profile and induce lipid peroxidation. 30-50% of SLE patients produce anti-phospholipid antibodies, including anti-cardiolipin (aCL), lupus anticoagulant (LAC) and anti-beta2-glycoprotein-I (β2-GP-I).[47,48] Other components of lipid particles against which autoantibodies are formed in SLE are the HDL-component apolipoprotein (Apo)-A1 and lipid-associated enzyme lipoprotein lipase (LPL).[13,49-52] Lipid-poor Apo A-I is the major component of the atheroprotective HDL and is involved in the uptake of cholesterol from the vessel wall, including from foam cells. Autoantibody binding to HDL / ApoA-I leads to enhanced clearance of HDL and reduced HDL levels[50], which impairs anti-inflammatory protection, increases lipid peroxidation, and enhances foam cell formation causing stress to the endothelium. Furthermore, the ability of HDL isolated from SLE patients to prevent LDL oxidation was shown to be less than for HDL isolated from controls. In 44% of SLE patients (versus 4% of controls) the HDL acted even *pro*inflammatory. Higher levels of proinflammatory HDL were observed in SLE patients with CAD, supporting a pro-atherogenic role of this dysfunctional type of HDL in SLE patients.[53] LPL hydrolyses circulating triglycerides and LPL impairment results in hypertriglyceridemia. In SLE, binding of autoantibodies to the LPL enzyme impair the enzyme activity [52,54] and anti-LPL antibody titres correlate with triglyceride levels, disease activity, and with markers of inflammation.[49,51] A second function of LPL is the degradation of VLDL with subsequent conversion into LDL. The high VLDL and low LDL levels observed in SLE may therefore be explained by the impaired LPL activity.[54] The result is that SLE patients have relatively low LDL levels, together with low HDL, high VLDL, and high triglyceride levels.[55] Elevated triglycerides, elevated VLDL, and low HDL are traditional cardiovascular risk factors, but it remains to be established whether the correlations with

individual lipid components and CVD observed in cohorts such as in the Framingham heart study may be extrapolated to the SLE population as several lipid spectrum components are influenced by the disease. A prospective study showed an increased rate of CVD events in SLE patients when total cholesterol levels were high (above 5.2 mmol/l), but did not compare the observed CVD risk increase with that calculated using the Framingham Risk Score.[56] Particularly the HDL levels may be difficult to interpret because HDL may be dysfunctional.

Figure 2. Overview of potential mechanisms underlying the pathophysiology of accelerated CVD in SLE



Endothelial cell apoptosis

The majority of SLE patients produce a heterogeneous array of antibodies that bind to the endothelium.[57,58] Several in vitro studies with cultured endothelial cells have identified antibodies derived from SLE sera that induce endothelial apoptosis, although other studies found no effect on apoptosis at all.[59,60]. A specific target is Heat Shock Protein (HSP)-60, which is present in the endothelial membrane, and causes endothelial apoptosis upon binding of anti-HSP60 antibodies.[58] Furthermore, LAC[61,62] and antibodies against double-stranded DNA (anti-ds-DNA)[63] can bind to endothelium and induce endothelial apoptosis. In vivo, apoptotic or damaged cells may circulate in the blood for a limited time after detaching from the vessel wall. In SLE patients, the number of apoptotic circulating endothelial cells is increased[23], indicating that the SLE-associated induction of endothelial apoptosis observed *in vitro* also occurs *in vivo*. Endothelial apoptosis contributes to the loss of endothelial integrity and thereby to the initiation of atherosclerosis.[64] Indeed, the number of circulating apoptotic endothelial cells correlates with endothelial (dys)function in SLE patients.[23] Apoptotic endothelium is also pro-thrombotic.[65] In SLE and particularly in those patients producing antiphospholipid antibodies, the prothrombotic consequences of endothelial cell apoptosis are further aggravated because circulating levels of Annexin V, thought to "shield" apoptotic cells from coagulation proteins and platelets, are reduced[66] whereas levels of plasma tissue factor, a major pro-coagulant, are increased and correlated with levels of apoptotic endothelial cells[23]. Increased endothelial apoptosis may represent an important mechanism for development of both atherosclerosis and thrombosis in SLE.

Increased oxidative stress and impaired antioxidant defense

Oxidative stress is high in SLE, particularly because of enhanced lipid peroxidation. Plasma levels of oxLDL are elevated and correlate with the presence of CVD.[67] In addition, oxLDL and minimally modified LDL (LDL with early oxidative changes) have been shown to be immunogenic.[68] Autoantibodies against OxLDL are elevated in SLE patients[67,69,70] and facilitate OxLDL uptake by macrophages.[71] Epitopes for this immune response are Phosphorylcholine and Lysophosphatidylcholine.[67] Platelet-Activating Factor-Acetyl Hydrolase (PAF-AH), which hydrolyses the atherogenic LDL-related phospholipid Platelet-Activating Factor, may play a role in the formation of oxLDL. PAF-AH activity was increased in SLE patients, particularly in those patients with CVD, and was associated with LDL and oxLDL levels.[72] β2-GP-I is an apolipoprotein (ApoH) that inhibits the uptake of oxLDL and is thereby atheroprotective. However, β2-GP-I may also serve as an autoantigen, in which case circulating immune complexes may be formed of β2-GP-I, anti-β2-GP-I autoantibodies, and oxLDL.[73] Such circulating immune complexes were indeed detected in SLE patients.[74] When a circulating immune complex is formed, the inhibitory effect of β2-GP-I on oxLDL uptake is impaired by the anti-β2-GP-I autoantibody. In addition, macrophage Fcγ-receptors may bind the anti-β2-GP-I autoantibody, enhancing the uptake of the immune complex and thereby of oxLDL, and facilitating foam cell production.[75]

Several anti-oxidant defenses are impaired in SLE. HDL has antioxidant properties by preventing the oxidation of LDL and inhibiting oxLDL uptake by monocytes. As described above, HDL levels are decreased in SLE due to anti-HDL antibodies. In addition, anti-HDL antibodies may impair the activity of paraoxonase, an antioxidant enzyme associated with HDL.[76] A powerful antioxidant produced by the endothelium itself is nitric oxide. Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of the nitric oxide producing enzyme nitric oxide synthase, and is produced from methylated arginine. High levels of ADMA are associated with endothelial dysfunction and a high risk of acute coronary events.[77] Anti-ds-DNA autoantibodies, a hallmark feature of SLE, can induce the methylation of arginine in vitro and form ADMA[78], further contributing to endothelial dysfunction. Indeed, in a recent cross-sectional study of SLE patients, high ADMA levels correlated with anti-ds-DNA antibody titres and were associated with the occurrence of cardiovascular events.[79]

Dysregulation of inflammation

In SLE, the major pro-atherosclerotic pathogenic derangements are a proinflammatory state and endothelial dysfunction. The proinflammatory state is characterized by an activated immune system with elevated levels of pro-inflammatory cytokines and dysregulation of inflammatory cell responses.

In SLE, levels of inflammatory cytokines are elevated both in active and inactive periods of the disease[80], indicating that there is a chronic low-grade inflammation that increases in periods of disease exacerbation. Several inflammatory intermediates are well known pro-atherogenic compounds. CRP is widely used as a marker of inflammation, but is also pro-inflammatory and pro-atherogenic itself.[81] In SLE, CRP production is increased[82], although limited in comparison with other autoimmune diseases[83]. CRP levels are associated with the presence of atherosclerosis in SLE patients.[42,84]

IL-1, IL-6, IL-12, IL-18, MCP-1, IFN-γ, and TNF-α, all potent pro-inflammatory cytokines and involved in atherogenesis, are increased in SLE.[37,80,83-85] For IL-1α and IL-1β, it has been shown that anti-ds-DNA antibodies directly stimulate their release.[86] The atheroprotective IL-10 and IL-1 receptor antagonist, the endogenous inhibitor of IL-1, are also elevated in SLE[80], but to which extent

this counterbalances the proatherosclerotic cytokine actions is not known. The elevated levels of these pro-inflammatory cytokines mobilize and activate inflammatory cells. Patients with SLE have also been shown to have higher plasma levels of adhesion molecules VCAM-1[87], ICAM-1, and E-Selectin[88]. These adhesion molecules facilitate the recruitment of inflammatory cells from the blood to the vessel wall and subendothelial space. Elevated plasma levels of various adhesion molecules are associated with the presence of clinically manifest CVD.[89-91]

Within the atherosclerotic plaque, antigen-presenting macrophages and dendritic cells activate T-cells, predominantly inducing a Th1 response.[39] Several factors influence this process in SLE patients. First, the expression of co-stimulatory molecules, which regulate T-cell activation upon interaction with antigen-presenting cells, is modulated. Antigen-presenting dendritic cells from SLE patients have been shown to spontaneously over express CD86, which is an important costimulatory molecule.[92] On the other hand, T-cells from SLE patients show a higher basal level of expression of another important costimulatory molecule, CD40L. In addition, CD40L expression remains up regulated for a longer period of time after T-cell activation.[93] CD40-CD40L interaction may also facilitate atherogenesis via T-cell independent mechanisms, and both CD40 and its ligand are highly present in atherosclerotic plaques.[94,95]

Second, stimuli for T-cell activation are present in higher levels in SLE patients. Ox-LDL is an important antigen for T-cells in the atherosclerotic plaque[96] and oxLDL levels are increased in SLE[67]. T-cells are also activated by Heat Shock Proteins (HSP), a family of proteins that are upregulated during stress stimuli and have protective properties in the response to tissue injury.[97] HSP-60 has been found to be present in the atherosclerotic plaque.[97] HSP may activate T-cells and subsequently stimulate B-cells to produce anti-HSP autoantibodies. Anti-HSP autoantibodies have been shown to be associated with CVD in several studies.[98-100] SLE patients produce autoantibodies against various HSP, including HSP-60[58] and HSP-65[74,101].

Finally, inhibition of T-cell activation may be impaired in SLE. T-cell activation is inhibited by the HDL-component ApoA1, and ApoA1 levels are reduced in SLE patients.[76] Also Th2 cytokines, such as IL-10, inhibit Th1 activation. However, in SLE IL-10 appears not to result in protection against atherosclerosis, as an IL-10 polymorphism resulting in decreased IL-10 levels was associated with a lower CVD incidence in SLE patients.[102] An explanation for this may be the immune-stimulatory effects of IL-10, including enhanced production of anti-dsDNA antibodies by IL-10 induced B-cell activation.[103,104]

Defective Endothelial Regeneration by Endothelial Progenitor Cells

Endothelial cell loss by endothelial apoptosis and other causes of endothelial damage may be compensated by endothelial repair. Although it was previously thought that lost or damaged endothelium was exclusively replaced by migrating and proliferating neighboring endothelium, in recent years it has become clear that progenitor cells originating from the bone marrow contribute to this process.[105-107] These Endothelial Progenitor Cells (EPC) comprise a subset of the hematopoietic stem cell population, display early endothelial characteristics, and are capable of homing to sites of endothelial injury via the blood and developing into fully functional endothelial cells.[105-107] We have shown that SLE patients have decreased numbers of circulating EPC, reflecting an impaired capacity for endothelium regeneration, which may contribute to accelerated atherosclerosis.[108] Increased Annexin V binding to the circulating progenitor cells suggested increased apoptosis as the underlying mechanisms of EPC deficiency.[108] In line with this, SLE serum was found to induce hematopoietic

stem cell apoptosis.[109,110] This has been reported to be due to binding of IgG to the hematopoietic stem cells, implicating a direct autoantibody-mediated mechanism[109], although this was not confirmed by another study in which a non-IgG serum component proved to be the critical factor.[110] In our study, we included patients with SLE that were in clinical remission indicating that EPC levels are chronically low, even when the SLE is in a quiescent phase. Since patients with systemic sclerosis and active rheumatoid arthritis also have decreased EPC numbers, this impairment may occur in a broader range of autoimmune diseases.[111-113]

Conclusion and clinical implications

In summary, there is strong evidence that SLE patients have a markedly increased risk of atherosclerotic cardiovascular disease by both traditional and non-traditional pro-atherosclerotic factors. However, many questions remain unanswered. From available studies it is not clear whether the increased CVD risk in SLE extends to SLE patients with limited disease manifestation. Furthermore, although several lines of evidence support that autoantibody formation plays a role in the pathogenesis of the accelerated atherosclerosis, the mechanisms through which this induces a chronic low-grade inflammatory state and endothelial dysfunction are complex and have not been fully elucidated.

The high incidence of CVD has not decreased with advances in the treatment of SLE, which have been characterized by improving effectiveness of immunosuppression whilst minimizing the side-effects.[11] This would suggest that suppressing the general disease activity may be insufficient to normalize CVD risk in SLE patients, although at least one large cross-sectional suggests an association of more intensive immunosuppressive therapy and absence of atherosclerotic plaque[31]. So far no specific interventions, e.g. targeting the production or actions of atherogenic autoantibodies, have been developed. Treatment of traditional risk factors therefore remains to be the main focus for CVD risk reduction in SLE. Currently, traditional cardiovascular risk management in SLE is not optimal.[114] SLE patients should be regarded as a high-CVD-risk population, for example similar to patients with diabetes, in whom prompt identification and stringent treatment of CVD risk factors is recommended.[115,116] Recently, guidelines for CVD risk management in SLE patients were proposed, targeting mainly the traditional CVD risk factors and centering around lifestyle advice and the use of traditional drugs such as statins, ACE-inhibitors and aspirin.[115] However, thus far clinical trials investigating to what extent these proposed interventions may reduce CVD risk in SLE patients are lacking. A recent effort to initiate such a trial failed, because of the 662 patients originally selected only 41 were enrolled, of which 22 dropped out within 4 months after entering the trial. One of the main reasons for failure of the trial was a lack of enthusiasm for participation in the trial among both patients and clinicians.[117] This suggests that the awareness of the importance of CVD in SLE should be increased in both physicians and patients. Physicians need to focus rigorously on the identification and treatment of traditional cardiovascular risk factor in SLE patients. In future, selective interventions targeting SLE-specific atherosclerosis pathophysiology will hopefully provide further benefit.

Acknowledgements

This work was financially supported by foundation “De Drie Lichten” in the Netherlands (grant no. 22/04). PEW is a research fellow of the Dr. E. Dekker program (2004T022) of the Dutch Heart Foundation. MCV is supported by the Netherlands Organization for Scientific Research (NWO VENI-grant 016.036.041).

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4

HAEMATOPOIETIC AND ENDOTHELIAL PROGENITOR CELLS ARE DEFICIENT IN QUIESCENT SYSTEMIC LUPUS ERYTHEMATOSUS

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Annals of the Rheumatic Diseases 2007; 66(7):865-70

Abstract

Objectives

SLE is associated with a high prevalence of cardiovascular disease (CVD). Circulating Endothelial Progenitor Cells (EPC) contribute to vascular regeneration and repair, thereby protecting against atherosclerotic disease. EPC are derived from CD34+ Haematopoietic Stem Cells (HSC), which have an increased propensity for apoptosis in the bone marrow of SLE patients. We aimed to determine whether circulating HSC and EPC are reduced in SLE, contributing to an increased cardiovascular risk.

Methods

Progenitor cells were sampled from 15 female SLE patients in prolonged clinical remission from their disease and 15 matching healthy controls. HSC and CD34+KDR+ EPC were quantified by flow cytometry. Annexin V staining was used to identify apoptotic cells.

Results

SLE patients had reduced levels of circulating CD34+ HSC and CD34+KDR+ EPC, associated with increased HSC apoptosis. Compared to controls, the fraction of HSC that could be identified as EPC was higher in SLE patients, consistent with a primary defect of HSC. EPC outgrowth from mononuclear cells, which depends mainly on CD34-negative cells, was unaffected.

Conclusions

SLE patients have lower levels of circulating HSC and EPC, even during clinical remission. Our data suggest that increased HSC apoptosis is the underlying cause for this depletion. These observations indicate that progenitor cell mediated endogenous vascular repair is impaired in SLE, which may contribute to the accelerated development of atherosclerosis.

Introduction

Systemic Lupus Erythematosus (SLE) is a complex autoimmune disorder with a strong predisposition for atherosclerotic cardiovascular disease (CVD).[1,2] As much as a 50-fold increase in the incidence of myocardial infarction has been reported in young female SLE patients.[3] A higher prevalence of traditional risk factors such as hypertension and dyslipidemia cannot fully account for the increased incidence of CVD.[4] Disease-specific factors must therefore also play a role.

Dysfunction of the endothelium is pivotal in the initiation and progression of atherosclerotic CVD. SLE is associated with impaired endothelial function, in part related to enhanced endothelial cell apoptosis.[5-7] Bone marrow-derived Endothelial Progenitor Cells (EPC) in the circulation contribute to maintenance and repair of the endothelium and enhance angiogenesis.[8,9] EPC levels correlate positively with endothelial function[10] and low EPC levels were shown to independently predict cardiovascular events in patients with coronary artery disease, supporting a pathophysiological role of EPC deficiency in CVD.[11,12] EPC constitute a bone-marrow derived subpopulation of the CD34+ haematopoietic stem cells (HSC), identified by the co-expression of the VEGF-receptor Kinase Domain Receptor (KDR) as CD34+KDR+ EPC.[9] In bone marrow aspirates from patients with active SLE increased apoptosis of HSC has been observed, which may explain the defective haematopoiesis.[13] Whether progenitor cells are also impaired during disease remissions, whether the EPC subpopulation is affected, and whether this translates to reduced HSC and EPC levels in the circulation has not been reported.

We hypothesized that even during prolonged clinical remission HSC and consequently circulating EPC are reduced in SLE patients, impairing endothelial repair and thereby contributing to an increased propensity for the development of atherosclerotic disease.

Methods

Study population

Fifteen consecutive female SLE patients attending the lupus clinic of the University Medical Centre Utrecht were included in this study. Inclusion criteria were meeting at least four classical American College of Rheumatology criteria for SLE[14], having clinically inactive disease for at least one year, and taking a maximal daily dose of 10 mg prednisone. Exclusion criteria were current pregnancy, the use of statins, and impaired renal function (Cockcroft-Gault estimated creatinine clearance of <80 ml/min). Fifteen healthy women matched for age and smoking behaviour served as controls. At the time of blood sampling, R.H.W.M.D scored clinical disease activity on a scale of 0 to 10 (0- no activity; 10- severe disease activity) and determined the Systemic Lupus Erythematosus Activity Index (SLEDAI).[15] All subjects gave written consent before entering the study, which was approved by the Medical Ethics Committee of the University Medical Centre Utrecht.

Assessment of arterial stiffness

As surrogate marker of subclinical atherosclerosis, arterial stiffness was assessed by measuring the Ankle-Brachial Index (ABI), Pulse Wave Velocity (PWV), and the pulse wave Augmentation Index (AI). For determination of the PWV and AI applanation tonometry was performed using the SphygmoCor2000 device according to the manufacturers instructions.

Flow cytometry for circulating EPC

EDTA blood was incubated with anti-CD34-FITC (BD Pharmingen, San Diego, USA), anti-KDR-PE (R&D Systems; Minneapolis, USA), and anti-CD45- PE-Cy7 (BD Pharmingen) antibodies and 7-amino-actinomycin-D (7-AAD; BD Pharmingen). Erythrocytes were lysed in an ammonium chloride buffer. CD34+KDR+ Type I EPC were quantified in duplicate from around 200.000 events per sample (average of 192957 events; range 125426 to 316852) using a flow cytometer (Beckman Coulter, Fullerton, USA). For HSC and EPC identification, first the CD34+ HSC were gated based on FITC signal and appropriate sideward scatter (SS) in the lymphocyte/monocyte range. Next, CD34+ cells evaluated for the expression of KDR based on the presence of a concomitant PE signal. In addition, the number of CD45+$FS_{high}SS_{high}$ granulocytes in the sample was determined on a forward-sideward scatter (FS-SS) plot of cells positive for panleucocyte marker CD45, which were gated on a histogram of the PE-Cy7 signals. EPC numbers per ml blood were subsequently estimated based on full blood granulocyte count made using a haematocytometer. Typical examples are depicted in figure 1A. Isotype-stained samples served as negative controls.

Measurement of apoptotic HSC

EDTA blood was incubated with anti-CD34-FITC and anti-CD45-PE-Cy7 antibodies. After erythrocyte lysis, cells were stained with anti-AnnexinV-PE (BD Pharmingen) and 7-AAD in Annexin V Binding Buffer (BD Pharmingen) and analysed by flow cytometry. Apoptotic HSC were defined as CD34+AnnexinV+FS_{low} 7-AAD_{dim} cells. For these analyses, also around 200.000 events were analysed per sample. A reduced FS debris threshold was used, as apoptotic cells have a decreased FS. First, CD34+ HSC were identified and gated, followed by gating for CD34+ cells which bound AnnexinV. These cells were plotted on a scatterplot for their FS and 7AAD signal. CD34+AnnexinV+ HSC with a low FS and intermediate 7AAD staining were considered to be apoptotic HSC. The intermediate 7AAD staining is named “dim”; indicating that cells take up small amounts of the dye, which is characteristic for apoptosis. Fully viable cells with completely intact cell membranes keep all 7AAD out of the cell and are 7AAD-negative. Dead cells take up large amounts of 7AAD, resulting in a so-called ‘bright’ signal. Typical examples are depicted in figure 1B. Isotype-stained samples served as negative controls.

EPC culture from mononuclear cells and functional characterization

EPC-mediated repair depends not only on CD34+KDR+ circulating cells, but also on a second type of EPC, which can be cultured from peripheral blood mononuclear cells. These EPC are mainly derived from CD34-negative cells, particularly CD14+ monocytes, and are thought to be predominantly of importance for the secretion of paracrine angiogenic factors.[16-18] To obtain these EPC, mononuclear cells were isolated from EDTA blood using Ficoll density gradient separation (Histopaque 1077; Sigma, St. Louis, USA) and plated on fibronectin-coated plates in EGM-2 medium (Cambrex, Walkersville, USA), supplemented with 20% fetal calf serum (FCS; Invitrogen), 100 ng/ml VEGF-165 (R&D systems), and antibiotics. For characterization of the secretion of angiogenic factors, EPC cultured for 7 days were placed in serum-free Endothelial Basal Medium-2 supplemented with selected aliquots (hEGF, hydrocortisone, GA-1000, R^3-IGF-1, ascorbic acid and heparin) for 20 hours, yielding EPC-conditioned medium. Human Umbilical Vein Endothelial Cells (HUVEC; kindly provided by Adele Dijk, Utrecht, the Netherlands) were placed on matrigel (Chemicon, Ternecula, USA) in the EPC-conditioned conditioned media, which had been diluted to correct for EPC numbers in the original culture. The tubular structures formed by the HUVEC after 20 hours were labelled with Calcein-AM (Molecular Probes) and tube length was measured using Scion Image software (Scion Corporation, Frederick, USA).[19]

Figure 1. Gating strategies for flow cytometry

CD34+ HSC, CD34+KDR+ EPC, and CD45+ granulocytes were identified and quantified in the sample. Based on the granulocyte count in the full blood made using a hematocytometer, the number of HSC and EPC per ml of blood was estimated. Typical examples of a control and SLE patient are shown (A).

HSC with decreased membrane integrity were identified based on intermediate 7AAD staining. To evaluate if these cells were apoptotic, the binding of AnnexinV to HSC was evaluated. CD34+ cells were considered apoptotic when they were AnnexinV-positive and had a low FS and 7AAD-dim staining. Typical examples of a control and SLE patient are shown (B).

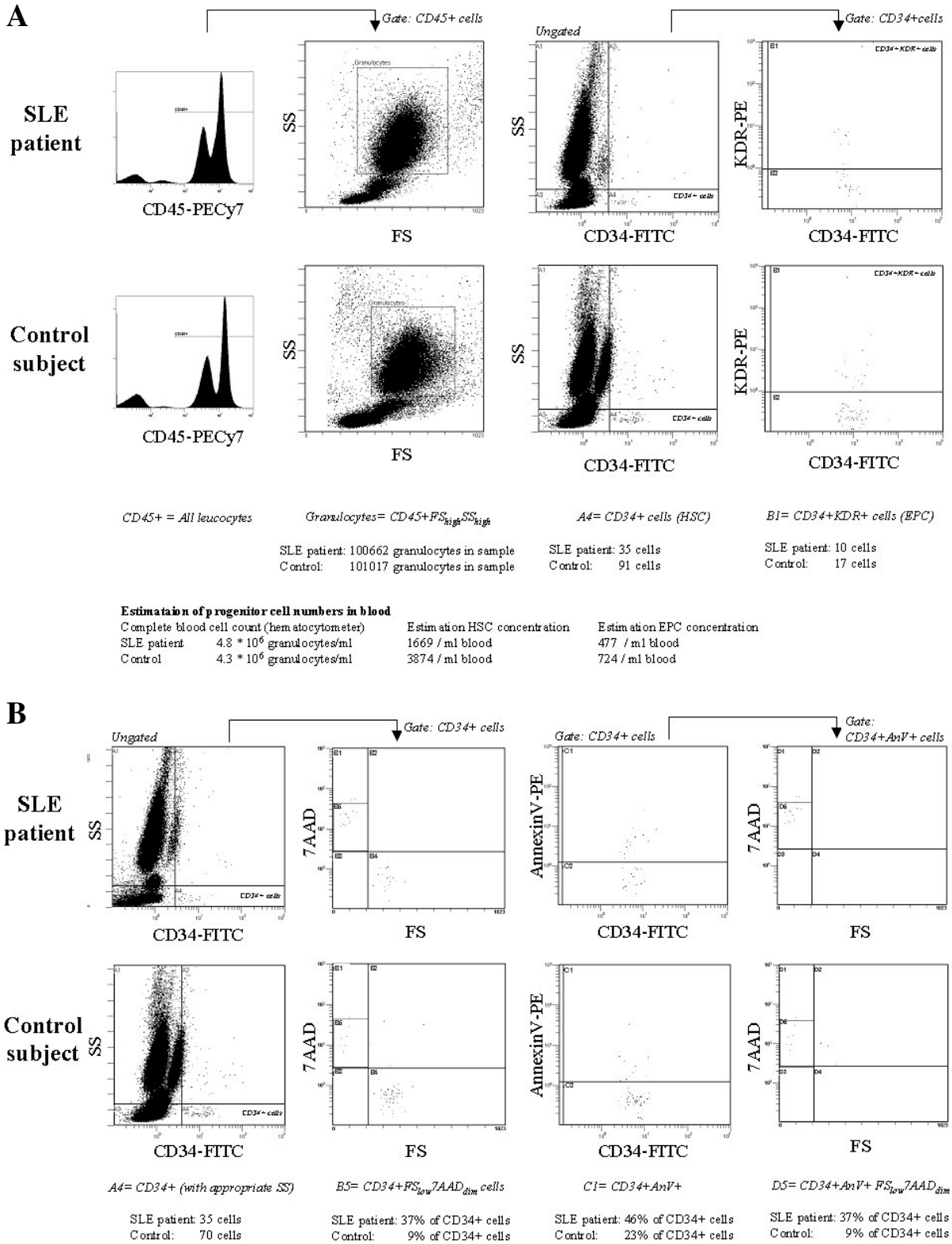

Table 1. Characteristics of SLE patients and healthy controls

	Patients (n=15)	Controls (n=15)	p-value
Age (yrs)	36.6 ± 2.9	36.8 ± 2.7	0.95
Smoking	4/15	4/15	1.00
RR - Systolic (mm Hg)	128 ± 5	128 ± 3	0.99
RR - Diastolic (mm Hg)	76 ± 3	77 ± 2	0.82
Ankle-Brachial Index	0.92 ± 0.03	0.92 ± 0.01	0.86
Resting heart rate (bpm)	75 ± 3	75 ±3	0.88
Weight (kg)	77.3 ± 5.2	72.3 ± 3.0	0.41
BMI	27.4 ± 2.3	24.8 ± 0.9	0.30
Waist – Hip ratio	0.82 ± 0.02	0.77 ± 0.01	0.03 *
Premenopausal	11/15	13/15	0.37
History of occlusive CVD	2/15	0/15	0.15
Hypertension †	6/15	3/15	0.24
White blood cell count (*10^6/ml)	6.2 ± 0.4	7.4 ± 0.5	0.09
Haemoglobin level (mmol/l)	8.5 ± 0.2	9.2 ± 0.4	0.11
Platelets (*10^6/ml)	225 ± 23	267 ± 13	0.11
Cholesterol total (mmol/l)	4.52 ± 0.19	5.16 ± 0.27	0.04 *
LDL cholesterol (mmol/l)	2.88 ± 0.18	3.15 ± 0.19	0.23
HDL cholesterol (mmol/l)	1.19 ± 0.09	1.52 ± 0.07	0.01 *
VLDL cholesterol (mmol/l)	0.45 ± 0.05	0.49 ± 0.04	0.43
Triglycerides (mmol/l)	1.59 ± 0.17	1.68 ± 0.12	0.64
ApoA1 (mg/dl)	112 ± 5	138 ± 5	<0.01 *
ApoB (mg/dl)	73 ± 4	80 ± 4	0.25
Aortic PWV (m/sec)	6.3 ± 0.5	6.1 ± 0.3	0.72
Brachial PWV (m/sec)	6.9 ± 0.2	7.2 ± 0.4	0.54
Carotid AI (%)	24.0 ± 3.5	26.7 ± 3.0	0.56
Radial AI (%)	13.9 ± 3.1	14.7 ± 4.2	0.88
Time from diagnosis (months)	138 (18 – 329)		
Clinical activity (scale 0-10)	0 (0 – 2)		
SLEDAI	2 (0 – 4)		
Antiphospholipid antibodies detectable	3/15		
dsDNA (IU/ml)	46 ± 21		
C3 (g/L)	1.00 ± 0.05		
C4 (g/L)	0.14 ± 0.01		
Medication			
Prednisone	8/15	0/15	
Azathioprine	5/15	0/15	
Hydroxychloroquine	8/15	0/15	
Antihypertensive drugs	2/15	0/15	
Anticoagulants	3/15	0/15	
Bisphosphanates	3/15	0/15	
Supplementary calcium	7/15	0/15	
Oral contraceptives	2/15	4/15	
Framingham risk score ‡	-5 (-13 – 11)	-4 (-14 – 9)	0.97
10 yrs risk of CHD (%)‡	1 (1 – 13)	1 (1 – 9)	0.80

** p<0.05*
† Hypertension was defined as having a systolic blood pressure of ≥140 mmHg, a diastolic blood pressure of ≥ 90 mm Hg, or use of antihypertensive drugs.
‡ calculated using the Coronary Disease Risk Prediction Score Sheet for Women; see http://www.nhlbi.nih.gov/about/framingham/riskwom.pdf

VEGF Enzyme-Linked ImmunoSorbent Assay (ELISA)
VEGF levels were measured in blood plasma and in cell culture supernatant in duplicate using a commercially available ELISA kit (R&D Systems) according to the manufacturer's instructions.

Statistical analysis
Data are expressed as mean±SEM or median(range) and analysed using SPSS version 11.5 and Graphpad Prism version 4.00 software. Students' t-test was used to compare continuous variables and the Mann-Whitney test for categorical variables. For regression analysis, Pearson's correlation coefficients were calculated except for multinomial values, where model I linear regression was used. A p-value lower than 0.05 was considered statistically significant.

Results

Subject characteristics
Subject characteristics are presented in table 1. All patients had been clinically in remission for at least one year and disease activity was rated as low by the treating physician on a 0 to 10 scale (median 0 (0 – 2)) and SLEDAI (median 2 (0 – 4)). Patients used immunosuppressive drugs in low dosages. 8 patients used prednisone (5.7(5-10) mg/day; in 5 patients combined with azathioprine). 8 patients used hydroxychloroquine (in 3 combined with prednisone). Six patients had previously suffered from nephritis, but did not have an impaired renal function at the time of the study. Three patients were positive for antiphospholipid antibodies, of whom two had a history of thrombo-embolic events. Blood pressure, BMI, and LDL-cholesterol were not significantly different between patients and controls. A slightly higher waist to hip ratio and lower HDL-cholesterol with associated lower Apo-A1 lipoprotein levels were observed in SLE patients. A history of cardiovascular disease and the presence of hypertension was more prevalent in the SLE group, although not statistically significant. The Framingham risk score and associated 10-years risk for coronary heart disease (CHD) were not higher in the SLE patients than in controls.

Arterial stiffness is not increased in the SLE patients
The ABI in the SLE patients was similar to that in controls (0.92±0.03 vs. 0.92±0.01; p=0.86). The aortic and brachial PWV were not different between groups (6.3±0.5 vs. 6.1±0.3 m/s; p=0.72 and 6.9±0.2 vs. 7.2±0.4 m/s; p=0.54 respectively). Analysis of the pulse wave forms did not reveal a significant difference in the AI (24.0±3.5 vs. 26.7±3.0%; p=0.56 and 13.9±3.1 vs. 14.7±4.2%; p=0.88 for carotid and radial wave forms respectively). Taken together, arterial stiffness in our SLE population was not evidently increased in comparison to the controls.

HSC and circulating EPC are reduced in the SLE patients
The total number of circulating CD34+ HSC was lower in SLE patients than in healthy controls (1339±151 vs. 3813±517 /ml blood, 65% reduction; p<0.0001, fig. 2A). There was also an absolute reduction of circulating EPC numbers in SLE patients compared to the controls (488±66 vs. 907±159 /ml blood, 46% reduction; p=0.021, fig. 2B), although the fraction of CD34+ HSC that co-expressed the EPC-marker KDR was higher in SLE than in controls (37±3 vs. 25±4%; p=0.015).

HSC apoptosis is increased in SLE patients
SLE patients showed more 7-AAD positive HSC than controls (15±2 vs. 8±1%; p=0.014), suggestive of apoptosis. This was in contrast to the CD34 negative cells, the vast majority of the leukocytes, in which 7-AAD uptake was low and not statistically different between the groups (0.18±0.02 vs.

0.18±0.02%; p=0.88). Annexin V staining confirmed a higher fraction of apoptotic CD34+ cells in SLE patients than in healthy controls (39.5±6.2 vs. 22.3±5.0%; p=0.032, fig. 2C).

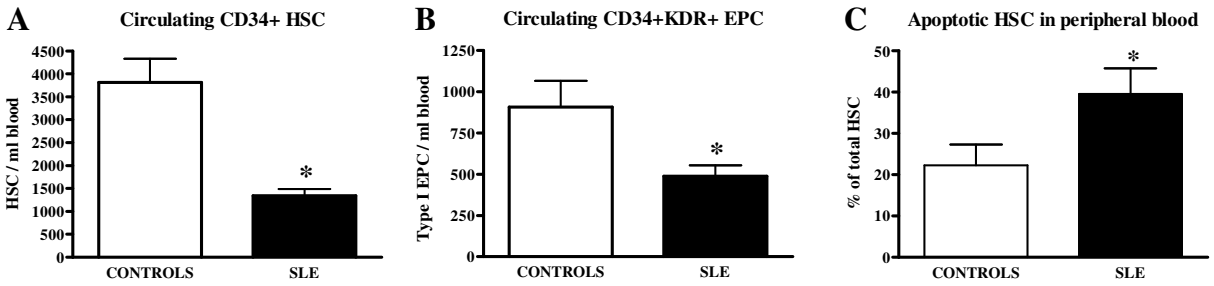

Figure 2. Circulating HSC and EPC levels
In SLE patients, circulating numbers of CD34+ HSC(A) and CD34+KDR+ Type I EPC (B) were lower than in healthy controls. The fraction of circulating CD34+HSC that was identified as being apoptotic based on AnnexinV-staining and typical FS-low/ 7-AAD-dim staining pattern, was increased in SLE patients (C).

Mononuclear-cell derived cultured EPC are numerically and functionally unaffected
EPC outgrowth from isolated blood mononuclear cells cultures was numerically equal in SLE patients compared to controls (1.54±0.3 vs. 1.52±0.4 per 100 MNC originally plated; p=0.97, fig. 3AB). Stimulation of mature endothelial cell angiogenesis by EPC conditioned medium was comparable for EPC from SLE patients and controls in (139±14 vs. 134±12 AU tube length; p=0.81, fig. 3CD). VEGF levels, an angiogenic factor secreted by cultured EPC in high amounts[20], were not different in the conditioned medium, which is consistent with an intact release of paracrine proangiogenic factors (177±38 vs. 166±34 ng secreted per well in 20 hours; p=0.83).

Determinants of EPC numbers
Higher ABI (r=0.532; p=0.042) and lower carotid (r=-0.525; p=0.045) or radial (r=-0.580; p=0.023) AI were associated with higher EPC levels. Cholesterol levels were inversely related to EPC numbers (r=-0.587; p=0.021). Disease duration and activity, either scored on a clinical scale or by SLEDAI, did not show a significant correlation with circulating progenitor cell levels, although it must be noted that the limited range in the data for clinical activity and duration made correlation analysis for these parameters insensitive. There was no correlation of EPC numbers with dsDNA antibody titres (r=0.317, p=0.25) or the levels of complement factors C3 (r=0.111, p=0.71) and C4 (r=-0.008, p=0.98). The presence of anti-phospolipid antibodies was also not associated with increased or decreased EPC levels (451±154 vs.. 497±76 cells/ml, p=0.79). Plasma VEGF was not significantly different between SLE patients and controls (78±10 vs. 81±17 pg/ml; p= 0.90).

The use of prednisone, regardless of the concomitant use of azathioprine, was not associated with circulating HSC numbers (1415±233 in patients using prednisone vs. 1252±198 /ml in those who did not p=0.61) or EPC levels (529±110 vs. 442 ± 93 /ml; p=0.56). Even though patient numbers were low, hydroxychloroquine use tended to be dose dependently associated with higher EPC numbers (r=0.458; p=0.086 in linear regression model with 377±74 vs. 509±136 vs. 631±138 EPC per ml for 0 (n=7), 200 (n=3), and 400 (n=4) mg daily dose respectively). Furthermore, when comparing patients using any dose of hydroxychloroquine (n=8) with those without (n=7), hydroxychloroquine use tended to be associated with lower arterial stiffness parameters (ABI 0.97±0.04 vs. 0.87±0.08; p<0.05; aortic PWV 5.8±0.4 vs. 6.8±0.8m/s; p=0.31, carotid AI 19.2±5.9 vs. 29.6±1.8%; p=0.14, and radial Augmentation Index 8.4±3.6 vs. 20.2±4.4%; p=0.058) and lower cholesterol levels (4.17±0.25 vs. 4.93±0.23; p<0.05).

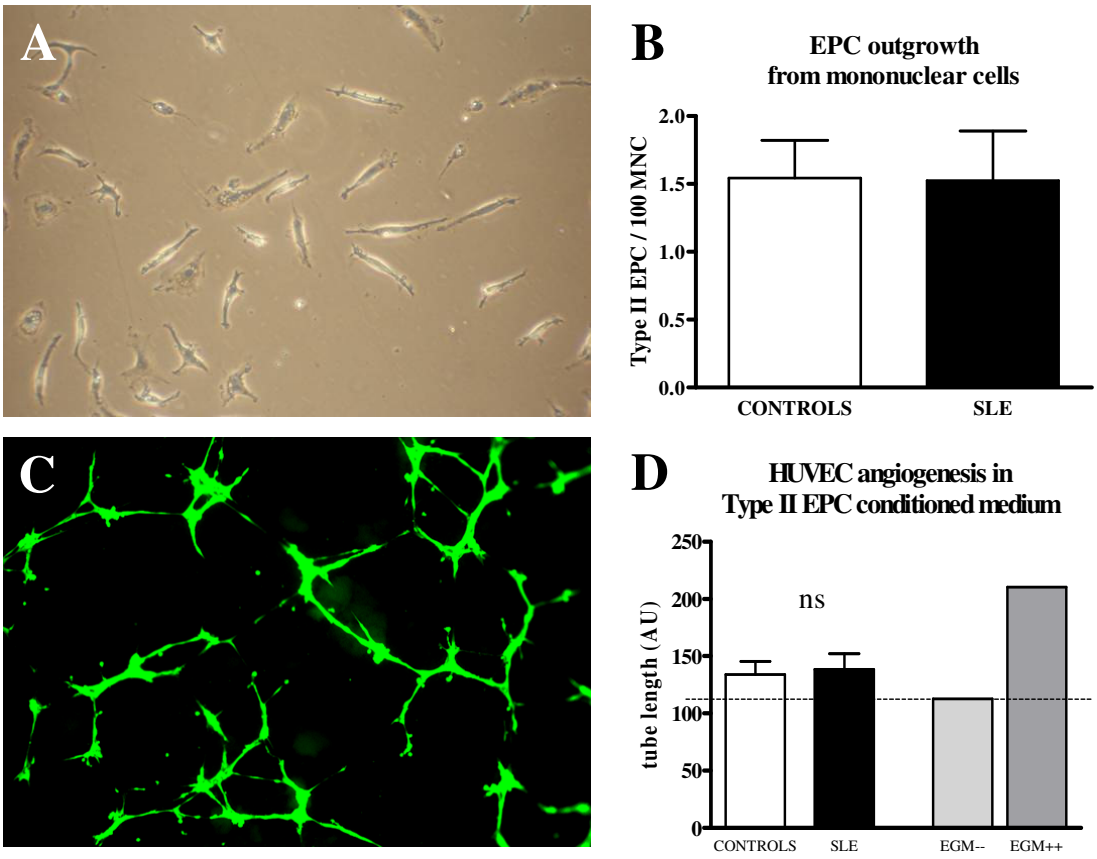

Figure 3. EPC outgrowth from mononuclear cells in vitro and their capacity for paracrine angiogenic stimulation

EPC cultured from peripheral blood mononuclear cells in a 7-day period gave a numerically identical outgrowth in SLE patients as the healthy controls (A: phase-contrast at 100X magnification; B: quantification). Functionally, stimulation of HUVEC angiogenesis by EPC conditioned medium was not statistically different (C: Calcein-AM-labelled HUVEC vascular networks on Matrigel, visualized by fluorescence microscopy at 50X magnification; D: quantification based on HUVEC tube length, with a reference line for the effect of non-conditioned blanco EGM-- medium and a positive control of EGM++ medium supplemented with VEGF and bFGF) .

Discussion

The present study is the first to show that SLE patients in clinical remission have decreased levels of circulating CD34+ HSC compared to a population of matched healthy controls. Moreover, SLE patients have reduced numbers of CD34+KDR+ EPC, a specialized HSC subpopulation serving as progenitor cells for vascular endothelium. Our data suggest an increase in the propensity of HSC cells to undergo apoptosis as the underlying mechanism of the lowered HSC and EPC levels. EPC have been proposed to play a key role in endothelial repair, thereby protecting against atherosclerosis. Hence, decreased levels may contribute to the increased risk of SLE patients for developing CVD.

Apoptosis rates of circulating cells such as lymphocytes[21] and neutrophils[22], and also of mature endothelial cells [7,23] have been reported to be increased in SLE patients. One study reported increased HSC apoptosis and decreased CD34+ HSC levels in the bone marrow of patients with active SLE.[13] In the present study, 7-AAD uptake was higher in circulating CD34+ HSC from SLE patients. Increased Annexin V binding, a phenomenon specific for apoptosis, confirmed higher levels of apoptotic circulating CD34+ HSC in SLE patients. Interestingly, we did not observe increased apoptosis in CD34-negative leucocytes in our population with inactive disease. This is consistent with a previous study, in which increased lymphocyte apoptosis was observed in patients with active

disease but not with inactive SLE[24], although others found no correlation with disease activity[25]. Our finding of increased levels of circulating apoptotic HSC could be the result of either increased induction of apoptosis, impaired clearance of apoptotic HSC[26], or a combination of both. The observation of reduced levels of HSC and EPC suggests increased induction of apoptosis, which is consistent with previous observations of induction of apoptosis of CD34+ HSC by serum from SLE patients *in vitro*.[27,28] Increased HSC apoptosis has also been observed at the bone marrow level in SLE patients.[13] We show that this is reflected in peripheral blood and affects the CD34+KDR+ EPC subpopulation. Our observations that, although absolute levels of CD34+KDR+ EPC were lower, the proportion of CD34+ HSC expressing KDR was higher in SLE patients compared to controls, suggest that CD34+ HSC apoptosis is the primary defect with consequent decrease in CD34+KDR+ EPC. Consistently, EPC outgrowth from unselected mononuclear cells, which is known to depend mainly on CD34-negative cells[17], was unaffected. Reduced HSC levels in SLE indicate that there is a limited capacity for haematopoietic renewal, which may contribute to the cytopenias observed in these patients. Reduced EPC levels suggest an impairment of endothelial regeneration, while in SLE the demand for this regeneration is higher due to excessive endothelial cell loss.

The SLE population investigated by us consisted of patients in clinical remission at the time of the study. Therefore, our findings suggest that the negative effects of the presence of SLE on EPC-mediated endothelial repair are chronic and not confined to periods of active disease in which many inflammatory intermediates and drug effects are present. The suppressive effect on progenitor cells may in part be related to the higher prevalence of several traditional risk factors in our population. We indeed found clearly reduced levels of atheroprotective HDL and its major component ApoA1 in our population, as well as a higher waist-hip ratio. The limited sample size precluded stratification for the various CVD risk factors and we could therefore not assess if reduced EPC levels are an independent risk factor for CVD in this population. However, the calculated Framingham risk scores were low and not statistically different in SLE patients and controls. Moreover, no significant differences in arterial stiffness assessed by ABI, PWV, and AI were observed. Therefore, SLE specific factors, which are present even during inactive disease, appear to be responsible for reducing circulating endothelial and haematopoietic progenitor cell numbers, possibly in part through effects on 'traditional' CVD risk factors.

Serum from leukopenic SLE patients was reported to induce HSC apoptosis and limit HSC colony forming capacity of HSC isolated from peripheral blood or bone marrow of healthy donors.[27,28] This may be related to autoantibody formation. One study reported binding of IgG autoantibodies to HSC[27]; however, others found a non-IgG serum component to be the critical factor.[28] Autoantibodies from SLE patients have been demonstrated to induce apoptosis in cultured endothelial cells[7,23], but whether autoantibodies are also responsible for inducing apoptosis in EPC has not been investigated. In our study, neither circulating progenitor cell levels nor progenitor cell apoptosis rates correlated with dsDNA or antiphospholipid autoantibody titres. Increased CD34+ HSC cell apoptosis as underlying mechanism for EPC deficiency may not only occur in SLE, but also in other autoimmune diseases. In patients with rheumatoid arthritis the rate of HSC apoptosis in bone marrow aspirates was also enhanced.[29] In other pro-atherosclerotic conditions with reduced HSC and EPC levels, i.e. diabetes and renal failure, progenitor cell apoptosis was not significantly increased.[19,30]

EPC-mediated repair depends not only on CD34+KDR+ circulating EPC, but also on a second type of EPC, which can be cultured from peripheral blood mononuclear cells. These cultured EPC have also been reported to be reduced in the presence of traditional CVD risk factors[10]. We found outgrowth of

these EPC to be unaffected both numerically and functionally in our study. This may be consistent with observations that these EPC are mainly derived from CD34-negative cells, particularly CD14+ monocytes[16-18], whereas our observations in SLE suggest a defect in the CD34+ HSC in a quiescent disease state.

Our study was not set up to evaluate the effect of drug treatment and the cross-sectional design and limited sample size issue a need for caution in the interpretation of observed associations with the use of particular drugs. We observed higher levels of EPC in patients using hydroxychloroquine. The use of hydroxychloroquine was associated with significantly lower total cholesterol and less arterial stiffness. This is consistent with previous reports on reduced cholesterol levels in SLE patients using hydroxychloroquine.[31,32]

In conclusion, circulating CD34+ HSC and CD34+KDR+ EPC are markedly reduced in SLE patients even when the disease is in clinical remission and despite the patients receiving standard treatment. HSC apoptosis is increased, which may be the underlying cause of the observed decreased progenitor cell levels. The observed enhanced HSC apoptosis and EPC deficiency may identify a novel pathophysiological component underlying the increased risk of CVD observed in SLE patients. Prevention of EPC apoptosis and increasing EPC levels may offer a new therapeutic approach to prevent premature atherosclerosis and CVD in SLE patients independent of the disease activity.

Acknowledgements

The study was financially supported by foundation “De Drie Lichten” in the Netherlands (grant no. 22/04). PEW is a research fellow of the Dr. E. Dekker program (2004T022) of the Dutch Heart Foundation. MCV is supported by the Netherlands Organization for Scientific Research (NWO VENI-grant 016.036.041). We thank Coby Slee, Judith Wiersma and Petra de Bree for their practical assistance, Geesje Dallinga-Thie (Erasmus MC, Rotterdam, The Netherlands) for measuring the lipid profiles, Peter Boer and Wim Busschers for advice on statistical analysis, Gerry Ligtenberg for useful discussions on the study setup, and the patients and healthy volunteers for their cooperation.

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5

IMBALANCED TGF-β/BMP-6 EXPRESSION AND INCREASED OUTGROWTH OF CIRCULATING SMOOTH MUSCLE PROGENITOR CELLS IN A TYPE I DIABETES MOUSE MODEL

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in revision

Abstract

Background
Diabetic patients experience exaggerated intimal hyperplasia after endovascular procedures. Recently it has been shown that circulating smooth muscle progenitor cells (SPC) contribute to intimal hyperplasia. We hypothesized that SPC differentiation would be increased in diabetes and focused on imbalanced TGF-β/BMP-6 signaling as potential underlying mechanism.

Methods
We isolated SPC from C57Bl/6 mice with streptozotocin-induced diabetes and controls. SPC differentiation was evaluated by immunofluorescent staining for αSMA and collagen Type I. SPC mRNA expression of TGF-β and BMP-6 was quantified using real-time PCR. Intima formation was assessed in cuffed femoral arteries. Homing of bone-marrow derived cells to cuffed arterial segments was evaluated in animals transplanted with bone marrow from GFP-transgenic mice.

Results
We observed that SPC differentiation was accelerated and numeric outgrowth increased in diabetic animals (24.6±8.8 vs. 8.3±1.9 per HPF after 10 days, $p<0.05$). Quantitative real-time PCR showed increased expression of the profibrotic growth factor TGF-β and decreased expression of the antifibrotic growth factor BMP-6 in diabetic SPC. Intima formation in cuffed arterial segments was increased in diabetic mice (intima/media ratio 0.68±0.15 vs. 0.29±0.06, $p<0.05$). In GFP-chimeric mice, bone-marrow derived cells were observed in the neointima (4.4±3.3 cells per section) and particularly in the adventitia (43.6±9.3 cells per section).

Conclusions
In conclusion, in a diabetic mouse model, SPC outgrowth is increased and SPC TGF-β/BMP-6 expression is imbalanced. Altered TGF-β/BMP-6 expression may facilitate SPC outgrowth. This may contribute to exaggerated intimal hyperplasia in diabetes as bone-marrow derived cells home to sites of neointima formation.

Introduction

Diabetes mellitus greatly increases the risk of cardiovascular disease (CVD) and adversely affects the outcome after endovascular procedures. Diabetic patients experience higher rates of restenosis due to intimal hyperplasia.[1,2] Previously it was thought that accumulation of smooth muscle cells in the neointima of restenotic lesions was exclusively due to migration and local proliferation of medial smooth muscle cells or adventitial fibroblasts. However, it was recently shown in bone marrow chimeric animals that smooth muscle cells of bone marrow origin contribute to postangioplasty restenosis.[3] Consistently, cells with smooth muscle cell characteristics can be isolated from animal[4,5] and human[6-8] blood. These smooth muscle progenitor cells (SPC) may display characteristics of other mesenchymal-lineage phenotypes such as fibroblasts and have also been referred to as circulating 'fibrocytes' or 'myofibroblast progenitor cells'.[8,9] As these cells appear to lack the expression of several specialized smooth muscle proteins such as h-caldesmon and desmin after incorporation, their potential to adopt a phenotype comparable to a mature smooth muscle cell must be considered limited.[10] We have shown that in Type I diabetic patients, the outgrowth of cells with a smooth muscle/myofibroblast phenotype from cultured mononuclear cells was increased and that BMP-6 expression in these cells was down regulated.[9] Our present study aims to bring further evidence for enhanced SPC differentiation in diabetic conditions in the better-controlled experimental setting of an inducible diabetic mouse model. In addition, we evaluated the effect of diabetes on TGF-β-expression in cultured SPC, as TGF-β is known to counteract BMP-6 signaling and enhance intimal hyperplasia. We hypothesized that inducing Type I diabetes in mice enhances SPC differentiation and numeric outgrowth with decreased BMP-6 expression and increased TGF-β expression in diabetic SPC.

Materials and Methods

Animals and induction of diabetes

Diabetes was induced in male, eleven week old C57BL/6 mice (Harlan, Horst, the Netherlands) by a single intraperitoneal injection with 200 mg/kg streptozotocin (STZ; Serva, Heidelberg, Germany; n=11 vs. 9 controls). Insulin-releasing pellets (Linbit, Linshin, Scarborough, Canada) were placed subcutaneously, providing a low insulin dose that is below normal physiological levels and is still associated with marked (more than twice upper limit of normal) hyperglycemicia, but prevents severe catabolism and spontaneous deaths. Blood glucose levels were measured using a portable glucose meter (Medisense Precision Xtra; Abbott Laboratories, Bedford, USA). HbA1c was determined in EDTA anti-coagulated blood by HPLC method. All experiments were approved by the local ethics committee on animal experiments.

To enable tracking of bone-marrow derived cells *in vivo*, in a separate set of experiments, we transplanted bone marrow from GFP-mice ($5x10^6$ cells i.v. / animal) to lethally irradiated animals (700cGy whole-body γ-irradiation delivered by linear accelerator). Peripheral blood chimerism was evaluated using flowcytometry on peripheral blood leukocytes after lysing erythrocytes with an ammonium chloride lysis buffer. Chimeric animals were required to have at least 90% GFP-positive leukocytes to be included in the experiments.

Cuff model of intima hyperplasia

Five weeks after onset of diabetes, a non-constrictive polyethylene cuff (0,4 mm inner diameter, 0,8 mm outer diameter, length 2 mm, Portex, Kent, UK) was loosely placed around both femoral arteries. 21 days after cuff placement, cuffed arterial segments were harvested after perfusion with 0.9% saline containing 0.1 mg/ml nitro-glycerine at 120 mm Hg for 5 minutes, fixed in formaldehyde and

embedded in paraffin. Serial 5 μm cross-sections were obtained at 200 μm intervals over the length of the cuffed femoral artery segment for histological analysis. 4 equally spaced cross sections of each arterial segment were stained with Elastin Von Gieson staining and the intimal and medial cross-sectional areas were measured using computerized morphometric analysis (Soft Imaging Systems, Münster, Germany). Neointimal smooth muscle cells were identified using biotinylated mouse-anti-human-αSMA antibody (clone 1A4, Sigma) and streptavidin-peroxidase/ TRITC-Tyramide Signal Amplification (TSA) system (PerkinElmer, Boston, USA) and counted.

Cryostat sections from neointimal lesions in cuffed arterial segments (n=18 cuffed segments from 9 animals) and control non-cuffed arterial segments from GFP-chimeric animals were stained with TRITC-conjugated mouse-anti-human-αSMA antibody and DAPI. GFP-positive cells in neointima and adventitia were identified using direct fluorescencence microscopy and the average number of incorporating cells per section was quantified. For detailed evaluation of fluorescence patterns, selected sections were scanned using a confocal fluorescence microscope. To exclude possible misinterpretation by autofluorescence or fluorescence channel bleed-through artefacts, GFP-epifluorescence was confirmed by measuring emission wave length spectrum.

Smooth muscle progenitor cell culture

SPC were obtained by culturing spleen mononuclear cells on fibronectin-coated dishes in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% heat-inactivated fetal calf serum to facilitate smooth muscle cell differentiation. After 4 and 10 days in culture, SPC were identified by immunofluorescent staining for αSMA using biotinylated mouse-anti-human αSMA antibody (Sigma) and streptavidin-peroxidase/ TSA system, and staining for collagen type 1 using a goat-anti-human collagen type 1 polyclonal antibody (Southern Biotechnology Associates, Birmingham, USA) and peroxidase labeled rabbit anti-goat immunoglobulin with the TSA system. DAPI was used for visualization of cell nuclei. Double-positive cells for both αSMA and collagen type 1 were counted as SPC and quantified per 200-fold magnification high power field (HPF). Isotype-stained sections served as controls.

To verify that spleen mononuclear cell giving rise to SPC in culture were indeed bone-marrow derived cells and not spleen stroma, we performed a control experiment in which we transplanted bone marrow from GFP-mice ($5x10^6$ cells i.v. / animal) to lethally irradiated animals (700cGy whole-body γ-irradiation delivered by linear accelerator; n=4). After peripheral blood chimerism was established (>90% GFP+ on flow cytometry), two of four animals received STZ to induce diabetes. Similar to our main study protocol, after 8 weeks diabetes, spleen mononuclear cells were isolated, analyzed for GFP-expression on flow cytometry, and placed in SPC-culture. After 7 days in culture, SPC were detached using trypsin/EDTA and analyzed for GFP-expression on flow cytometry.

TGF-β and BMP6 mRNA expression by quantitative real-time PCR

Total RNA was extracted from SPC with RNeasy columns (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed to cDNA using oligo-dT, random hexamers, and Superscript reverse transcriptase (Invitrogen, Carlsbad, CA, USA). Taqman quantitative real-time PCR reactions were performed in duplicate on an ABI Prism 7700 Sequence Detection System using pre-designed primer sets for TGF-β1, BMP-6, and housekeeping gene β-actin (Taqman assays-on-demand, Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). mRNA expression was quantified using the comparative Ct method.

Statistical analysis

All data are presented as mean ± SEM. The Mann-Whitney test was used to compare means between groups. A value of $p<0.05$ was considered statistically significant.

Results

Course of diabetes

Glucose levels exceeded twice the upper limit of normal within 2 days after STZ injection. Blood glucose levels in diabetic mice remained at least above twice the upper limit of normal during the course of the experiment (average 24.5±1.6 mmol/l). Blood glucose levels in control mice ranged from 6.5 to 9.5 mmol/l (average 8.5±0.5 mmol/l; $p<0.001$ vs. diabetic mice). At termination after a total of 8 weeks hyperglycemia, HbA1c levels were 8.3±0.4% in STZ diabetic mice compared to 4.9±0.5% in control mice ($p<0.0001$).

Intimal hyperplasia is exaggerated after cuff-induced vascular injury in diabetic mice

Some concern was raised whether STZ-induced diabetes is an adequate model to study diabetes-associated vascular complications as intimal hyperplasia was not increased in STZ-induced diabetic rats in two studies.[11,12] We therefore checked to see if the STZ-induced diabetic mice in our study displayed an exaggerated neointima formation as we expected. Indeed, twenty-one days after vascular injury the intima-media ratio was higher in diabetic animals than in controls (0.68±0.15 versus 0.29±0.06; $p<0.05$, fig. 1), corresponding with an increased number of intimal αSMA-positive cells (31±4 vs. 17 ± 2 cells per cross-section; $p<0.005$, fig. 1).

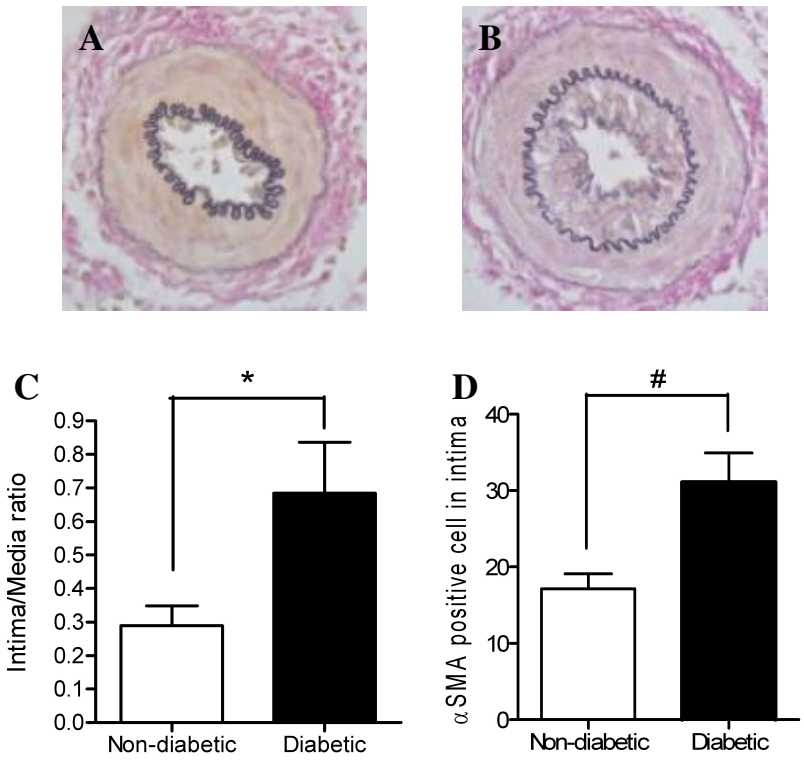


Figure 1. Intima formation in cuffed femoral artery segments

*Representative pictures of Elastin Von Gieson stained cuffed femoral artery sections from non-diabetic (A) and diabetic animals (B). 21 Days after vascular injury intima/media ratio is higher in diabetic animals (C) and intimal lesions of diabetic animals contain more aSMA positive cells as compared to control animals (D). *p<0.05, # p<0.005*

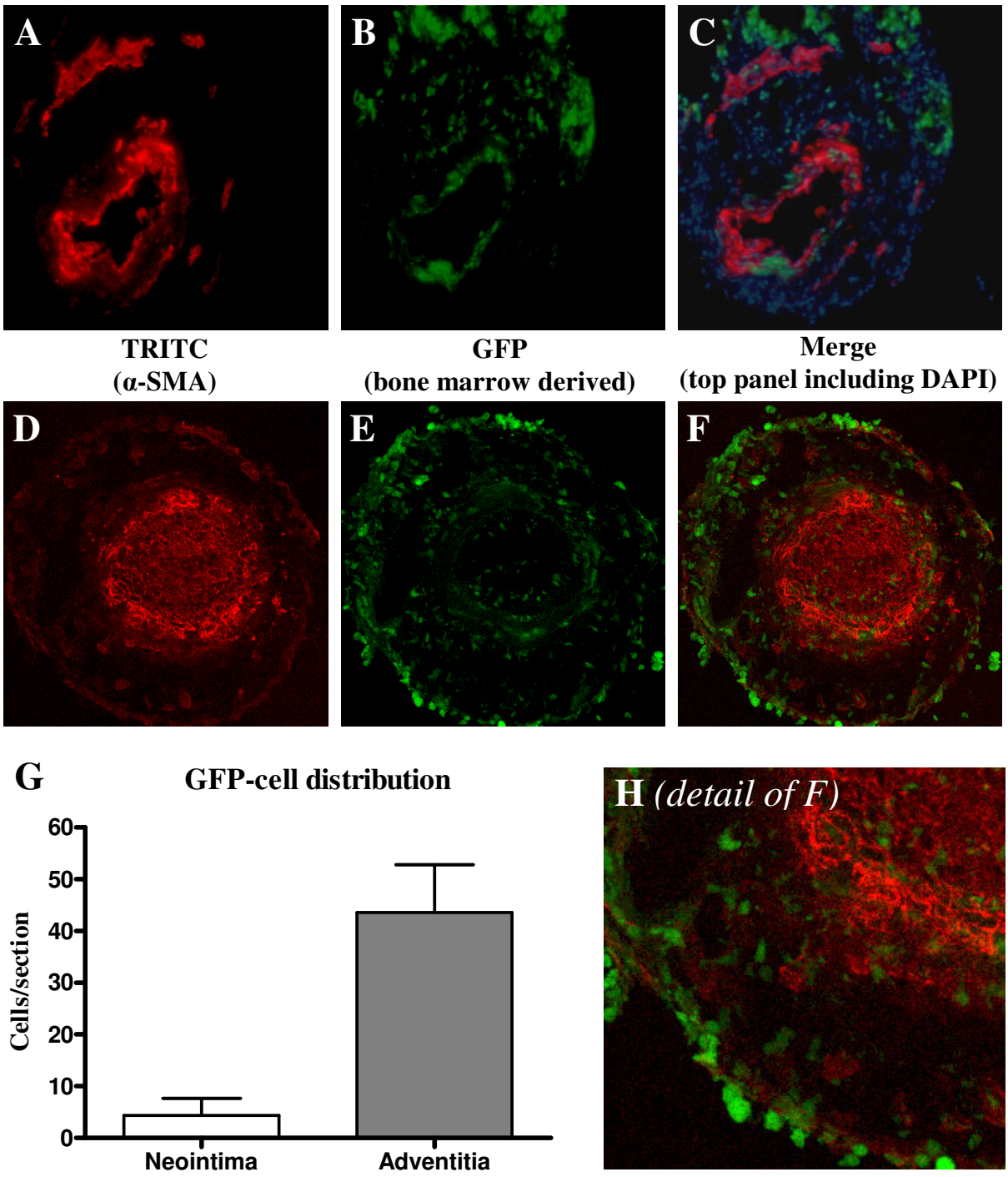


Figure 2. GFP-positive bone-marrow derived cells incorporate into the vessel wall at sites of neointimal formation.

Sections from cuffed femoral artery segments from GFP-chimeric animals show incorporation of bone-marrow derived cells. TRITC-labeled α-SMA-positive and bone-marrow derived GFP-positive cells are shown in separate fluorescence channels (A/D and B/E respectively) and in overlay images (C/F, including DAPI for C). Panels A/B/C show representative pictures using a regular fluorescence microscope, which was used to quantify GFP-positive cell incorporation (G). Substantial numbers of GFP-positive cells incorporated into the cuffed femoral artery segment, particularly in the adventitia (G). Panels D/E/F show a striking example of a section of a cuffed arterial segment that is completely occluded by neointima, which has been visualized using a confocal fluorescence microscope. A detail of the merged picture F is shown in H, illustrating that most of the bone-marrow derived GFP-positive cells are located in the adventitia, although various GFP-positive cells can be found dispersed throughout the neointima.

Bone-marrow derived cells home to sites of neointima formation

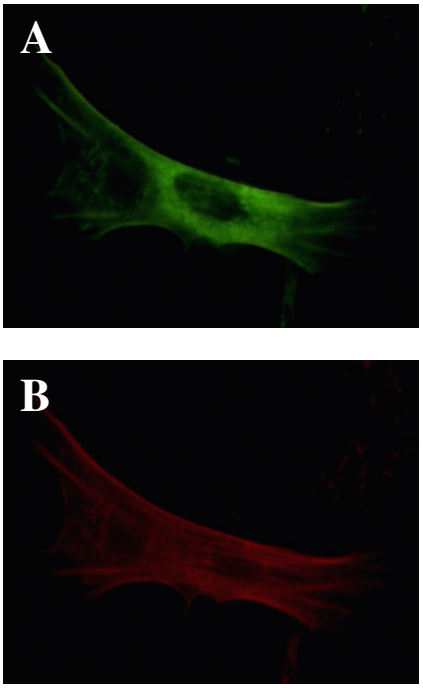
In cuffed arterial segments of GFP-chimeric animals, GFP-positive bone-marrow derived cells were observed in the neointima (4.4±3.3 cells per section, fig. 2) and particularly in the adventitia (43.6±9.3 cells per section, fig. 2). These cells were in majority α-SMA negative. In non-cuffed control arterial segments bone-marrow derived cells were observed only sporadically in the adventitia.

SPC differentiation is accelerated under diabetic conditions

Under SPC culture conditions, a proportion of spleen mononuclear cells became adherent to the culture dish, adopted an elongated morphology and started expressing α-SMA and collagen type 1 (fig. 3AB). After 4 days of culture, 20±6% of adherent cells from control animals were double-positive for both α-SMA and collagen type 1 and were thus identifiable as SPC, while more than 50±4% of adherent cells had differentiated into SPC in cultures from diabetic animals ($p<0.001$). After 10 days of culture, nearly 100% of adherent cells was positive for SPC markers in cultures from both diabetic and control mice.

SPC number is increased in diabetic mice

The absolute number of SPC cultured from diabetic animals after 4 days was higher than from controls (7.5±1.4 vs. 2.3±0.4 per HPF; $p<0.01$, fig. 3C). After 10 days, total cell number in both groups had increased further than the original number of adherent cells on day 4, indicating that SPC proliferate in culture. The total number of SPC in cultures from diabetic mice remained substantially higher compared to cultures from controls (24.6±8.8 vs. 8.3±2.0 per HPF; $p<0.05$, fig. 3C)



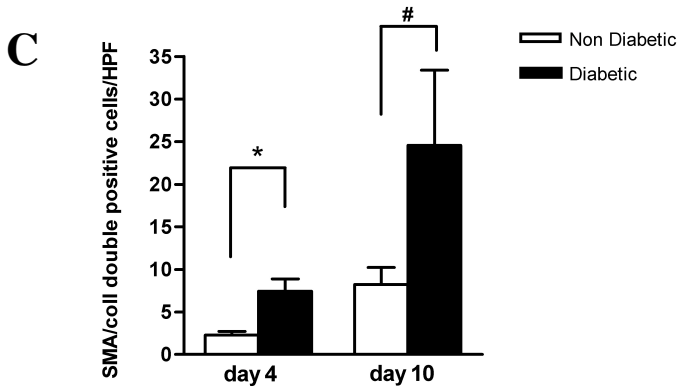


Figure 3. SPC characterization and quantification of outgrowth in vitro from mononuclear cells.
Cultured mouse SPC expressed collagen type I (detected by FITC-labeled antibody; A) and α-smooth muscle actin (detected by TRITC-labeled antibody; B) demonstrated by immunofluorescent staining. The number of SPC obtained after 4 and 10 days culture (C) is higher in diabetic animals (black bars) than in non-diabetic animals (white bars).
** p<0.01, # p<0.05*

SPC cultured from spleen mononuclear cells are bone marrow derived

A control experiment in GFP bone marrow transplanted mice showed that indeed the vast majority of mononuclear cells isolated from the spleen are bone marrow derived (range: 91-94% GFP-positive, n=4). Also after culturing the spleen mononuclear cells to SPC, most proved bone marrow derived in both control and diabetic animals (range 79-91%), indicating that contaminating spleen stroma is at most a minor factor in SPC cultures from spleen.

mRNA expression of BMP6 is decreased and of TGF-β increased in diabetic SPC
Quantitative RT-PCR of SPC cultured for 10 days showed a 2.7-fold down regulation of BMP-6 in SPC derived from diabetic mice in comparison with control SPC ($p<0.05$, fig. 4A). TGF-β expression in diabetic SPC was 4.2-fold up regulated compared to control SPC ($p<0.001$, fig. 4B).

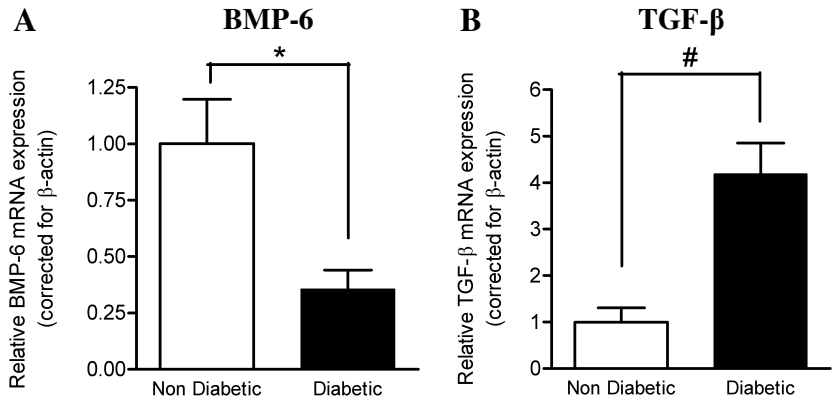


Figure 4. BMP-6 and TGF-β mRNA expression in SPC
*Quantitative PCR showed decreased BMP-6 (A) and increased TGF-β (B) mRNA expression in SPC from diabetic animals compared to controls. mRNA levels are expressed relative to the mean expression in the non-diabetic control group in SPC cultured for 10 days. * p<0.05, # p<0.001*

Discussion
In the present study, we show increased SPC levels in diabetic mice, associated with an increased expression of TGF-β and decreased expression of BMP-6 in the diabetic SPC. This imbalanced TGF-β/BMP-6 expression may underlie enhanced SPC differentiation and expansion from diabetic mononuclear cells *ex vivo* and may be of consequence for expanding intimal lesions to which SPC home. In GFP-chimeric animals we show that substantial numbers of bone-marrow derived cells incorporate into sites of neointimal formation, particularly the adventia, where these cells may represent a relevant source of TGF-β production.

Using bone marrow chimeric animals, we show that part of the neointimal cells are of bone-marrow origin, consistent with recent reports by others[3]. It was shown that smooth muscle cells of bone marrow origin also contribute to transplant arteriosclerosis and hyperlipidemia-induced atherosclerosis.[13-16] Histological studies of autopsy material of patients after bone marrow transplantation confirmed that smooth muscle cells in human vascular lesions are in part bone marrow derived.[17] The origin of SPC, their regulation, and how SPC respond to pathophysiological stimuli associated with the development of CVD has not been fully elucidated. One study found SPC to be mainly derived from CD34+ hematopoietic stem cells, but others found circulating endoglin(CD105)+ CD14+ monocytes as a main source of SPC[8]. Interestingly, circulating levels of these CD105+CD14+ cells were increased in a population of mostly non-diabetic patients with manifest CVD compared to controls, suggesting that SPC levels may be increased in a pro-atherosclerotic milieu.[8] We have previously reported increased SPC differentiation in Type I diabetic patients, in which we cannot exclude an influence of a presence of subclinical atherosclerotic disease.[9] Our current observation of enhanced SPC levels in mice with inducible diabetes provides strong additional evidence for the involvement of diabetes in enhancement of SPC differentiation.

TGF-β enhances the outgrowth of SPC-like collagen-secreting pericytes from mononuclear cell cultures.[18] BMP-6 may act as an inhibitor of TGF-β, as BMP-6 was shown to attenuate TGF-β-induced upregulation of a variety of SMC differentiation markers, including smooth muscle actin, in mature smooth muscle cells.[19] Diabetes is associated with higher TGF-β levels in the circulation.[20,21] Therefore, we assessed the expression TGF-β and BMP-6 in SPC from diabetic animals. We observed increased TGF-β and decreased BMP-6 expression in diabetic SPC, which could explain the accelerated maturation and increased outgrowth of diabetic SPC in culture. Furthermore, since circulating SPC home to sites of injury and constitute a significant proportion of neointimal cells[3,15] and in our observations also a major source of adventitial cells, enhanced TGF-β and reduced BMP-6 production may exert paracrine effects on resident smooth muscle cells. Over-expression of TGF-β induces formation of a cellular and matrix-rich intima even in uninjured arteries[22], while inhibition of TGF-β signalling attenuates intimal hyperplasia and remodeling after vascular injury[23,24]. We indeed observed aggravated intima-formation in the diabetic mice in our study.

In this study we used spleen mononuclear cells because of the low numbers of circulating cell numbers in mice. This is different from our previous human study in which SPC were isolated from peripheral blood.[9] Spleen mononuclear cells are a reservoir for circulating mononuclear cells and permit higher cell numbers to be obtained from individual animals. Using GFP-bone marrow transplanted animals, we confirmed that cultured splenic SPC are indeed from hematological lineage.

Our study has several limitations. Identification of SPC required *in vitro* culture for several days as a-SMA and/or collagen-I expression could not be detected by immunocytochemistry on freshly isolated cells. Therefore, we cannot determine if the numeric differences observed by us in SPC cultures of diabetic animals after 4 days was the result of an increased level of circulating SPC or if it is attributable to enhanced proliferation or survival *in vitro*. Also, we were unable to reliably quantify TGF-β and BMP-6 protein levels in the cultured SPC and thus did not exclude if posttranscriptional modulation may have attenuated the effects of the presence of diabetes on TGF-β and BMP-6 gene expression.

The present data are consistent with our previous observations that SPC numbers are increased in Type I diabetic patients and lack BMP-6.[9] Increased SPC numbers have also been observed in (non-diabetic) patients with manifest coronary artery disease[8]; however, whether SPC from these patients have altered TGF-β/BMP-6 expression has not been studied. Further studies are required to evaluate if inhibition of SPC outgrowth, e.g. by modulating the TGF-β/BMP-6 expression, may reduce intimal hyperplasia in diabetes.

Acknowledgements

The reported work was funded by the Netherlands Organization for Scientific Research (NWO VENI-grant 016.036.041 to MCV) and the Dutch Heart Foundation (Dr. E. Dekker Research Fellowship 2004T022 to PEW). Jaap A. Joles and Paula Martens are acknowledged for excellent advice and technical assistance.

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6

DYSFUNCTION OF THE BONE MARROW VASCULAR NICHE IMPAIRS ENDOTHELIAL PROGENITOR CELL MOBILIZATION IN DIABETES

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Abstract

Introduction
Circulating Endothelial Progenitor Cells (EPC) are reduced in diabetes. This may be a consequence of impaired mobilization of EPC from the bone marrow. The 'vascular niche' in the bone marrow plays a critical role in EPC mobilization. We hypothesized that under diabetic conditions, mobilization of EPC from the bone marrow to the circulation is impaired due to dysfunction of the bone marrow vascular niche.

Methods
EPC were quantified using flowcytometry as Sca-1^+ Flk-1^+ cells in peripheral blood from streptozotocin-induced diabetic C57Bl6 mice and buffer-injected littermates under steady-state conditions and after injection of EPC-mobilizing cytokines (250μg G-CSF + 50μg SCF per kg BW /day during 5 days). Bone marrow stromal cells were cultured to confluence and eNOS mRNA and protein levels were measured using real time PCR and ELISA. The capacity for progenitor cell support in the bone marrow environment was studied in co-cultures of human CD34+ hematopoietic stem cells (HSC) and primary mouse stroma and human endothelial cells (HUVEC) by evaluation of CFU-generating capacity over time. In vivo, mice were subjected to 5-FU challenge as model for bone marrow vascular niche function.

Results
EPC levels were lower at baseline in diabetic mice (7.7±0.9 vs. 13.3±1.9 x10^3 per ml blood; $p<0.05$) and the mobilization in response to injection of mobilizing cytokines was attenuated. CD34+ HSC support by diabetic stroma was reduced compared to control stroma (45±10 vs. 75±7 CFU/well; $p=0.023$). In the presence of hyperglycemia, CD34+ HSC supported by endothelial cells gave less CFU over time. In vivo, diabetic mice had a histologically aberrant bone marrow vasculature and failed to recover from 5-FU injection, while controls successfully regenerated the bone marrow. Diabetic stromal cell mRNA for the NO-producing enzyme eNOS was 3.94 times downregulated ($p<0.01$) and eNOS protein levels reduced (1.9±0.4 vs. 4.1±0.4 AU; $p<0.01$). Inhibition of NO-production using L-NAME impaired endothelial support of CD34+ HSC.

Conclusion
EPC mobilization is impaired under experimental diabetic conditions, which is associated with attenuated bone marrow stromal cell eNOS levels and impaired function of the bone marrow vascular niche in vitro and in vivo. Reduced levels of circulating EPC in diabetes may undermine endothelial repair and contribute to the development of cardiovascular disease.

Introduction

Premature cardiovascular disease (CVD) is a major complication in diabetic patients.[1] Diabetes is associated with endothelial cell dysfunction and impaired neovascularization after ischemia.[2-4] The intact endothelium and maintenance of endothelial integrity play a central role in protecting against the development of atherosclerotic vascular disease.[5] Dysfunctional endothelium is characterized by a decreased bio availability of nitric oxide (NO) and increased generation of reactive oxygen species (ROS).[5-7] Endothelial progenitor cells (EPC) circulate in peripheral blood and contribute to restoring damaged or lost endothelium and ischemic neovascularization.[8] EPC are a specialized subset of hematopoietic stem cells (HSC), capable of endothelial differentiation[9] and secretion of angiogenic growth factors and cytokines.[10,11] EPC originate from the bone marrow, from which they are mobilized in response to mobilizing cytokines, such as VEGF[12], G-CSF[13,14], SCF-sR[15], and SDF[16]. Supporting bone marrow stromal cells largely regulate progenitor cell proliferation and mobilization. The stroma consists of a heterogeneous cell population that includes fibroblasts, endothelial cells and osteoblasts. Progenitor cell proliferation and mobilization is thought to be predominantly regulated by osteoblasts in the 'osteoblastic niche' and sinusoidal endothelium in the 'vascular niche'.[17-19] Based on bone marrow transplantation experiments between eNOS knockout mice and wild-type controls, it was shown that EPC mobilization in response to VEGF specifically depends on NO produced in bone marrow stromal cells; and not the progenitor cells themselves.[20]

Previous studies showed reduced EPC levels in Type I and Type 2 diabetic patients[21,22]. Various other CVD risk factors are also associated with reduced EPC levels.[23-25] Reduced levels of circulating EPC in peripheral blood may undermine the regenerative potential of the endothelium and thus contribute to accelerated CVD. Indeed, in prospective cohort studies, lower levels of EPC were associated with poor event-free survival.[26,27] Interestingly, impaired NO availability, as measured by flow-mediated brachial artery reactivity, correlates with reduced EPC numbers in the peripheral circulation of patients at risk for cardiovascular disease.[24] This may suggest that endothelial dysfunction and reduced EPC levels share a common pathogenic mechanism. Although circulating levels of EPC may also be reduced by increased turnover or extravasation, recent experimental evidence in diabetic murine models support a role for impaired mobilization in diabetes.[28,29] eNOS in diabetic bone marrow displays an aberrant enzyme function[30] and increasing NO-availability by hyperoxia restored EPC levels in diabetic animals[29]. Taken together, this supports that impaired bone marrow NO-production is involved in impaired EPC mobilization in diabetes. As NO-production is a hallmark feature of endothelial function, it may be speculated that it is also vital for proper function of the 'vascular niche'. We therefore hypothesized that in diabetic hyperglycemic conditions, EPC levels are reduced due to an impaired EPC-mobilization from the bone marrow due to dysfunction of the bone marrow vascular niche.

Methods

Animal model of diabetes

Diabetes was induced in C57Bl/6 mice by intraperitoneal injection of 200 mg/kg streptozotocin (STZ, Serva, Germany). Buffer injected mice served as controls. Blood glucose levels were measured with a portable glucose meter (Medisense, Abbot). Diabetic mice received suboptimal insulin treatment by subcutaneous implantation of an insulin releasing pellet (Linbit, Linshin, Canada) to prevent lethal diabetes. Diabetic animals were required to have a non-fasting blood glucose level of >15 mmol/l to be included in the study. All protocols were approved by an animal ethical committee of either the

University Medical Center Utrecht, the Netherlands, or the Weill Cornell Medical Center, New York, USA.

Quantification of EPC in blood and bone marrow
EPC in peripheral blood and bone marrow were quantified by flow cytometry. EDTA anticoagulated blood or bone marrow cell suspension flushed from mice femurs was stained with α-Sca-1-FITC (BD Pharmingen) and α-Flk-1-PE (BD Pharmingen). Erythrocytes were lysed in an ammonium chloride buffer and remaining cells analyzed on a FC-500 flow cytometer (Beckman Coulter). For peripheral blood samples, EPC numbers per ml of blood were estimated based on their relative proportion to the leucocytes in the flow cytometry sample and the total number of leucocytes in a complete blood cell count made on a hematocytometer.

From bone marrow cell suspensions, an additional EPC quantification was performed by culturing bone marrow cells at a density of $2x10^6$ cells/ well on fibronectin-coated cover slips in 24-wells plates in EGM-medium supplemented with 20% FCS, 100ng/ml VEGF (RnD Systems) and penicillin/streptomycin (Gibco). After 4 days, non-adherent cells were washed away and adherent cells were incubated with DiI-labeled acetylated-LDL (Invitrogen), fixed in paraformaldehyde, stained with FITC-labeled BS1-lectin (Bioconnect), DAPI, and then mounted on slides using Vectashield (Vector Labs, Burlingame, CA). EPC were identified as ac-LDL / lectin double-positive cells using a fluorescence microscope and quantified using the average cell count in 3 random high-power fields.

Assessment of EPC mobilization
EPC mobilization was assessed after subcutaneous injections of 250 µg/kg granulocyte-colony stimulating factor (G-CSF, Neupogen, Amgen) and 50 µg/kg stem cell factor (SCF, Amgen) in 0,9% NaCl for five consecutive days (experimental days 0 to 4). EPC in peripheral blood were quantified at baseline and at experimental days 2, 4, 7, and 10.

Isolation of human CD34+ hematopoietic stem cells
Human CD34+ hematopoietic stem cells (HSC) were isolated from umbilical cord blood by magnetic activated cell sorting (MACS) using the commercially available CD34+ isolation kit (Miltenyi Biotech, Auburn, CA) according to the manufacturer's instructions. In brief, mononuclear cells were isolated using Ficoll-density gradient separation (Amersham Biosciences, Piscataway, NJ) and incubated with microbead-conjugated anti-CD34-antibodies and FcR-blocking solution. Cells were passed over a selection column (LS column, Miltenyi Biotech) placed in a magnetic field. After removal of the column from the magnetic field, positive cells were eluded and the procedure was repeated using a second column. Purity of selected CD34+ cells was evaluated on flow cytometry using FITC-conjugated anti-CD34-antibody (BD Pharmingen). Mean purity was 91% (range 71-96%) in the isolations performed for the experiments in this study.

Ex vivo model for bone marrow stroma – progenitor cell interaction
Primary mouse bone marrow stroma cells (BMSC) were obtained by isolating the plastic-adherent fraction from crude bone marrow cell suspensions. Bone marrow cells were flushed from mouse femurs using RPMI medium and cultured in DMEM (Gibco) containing 20% FCS and penicillin/streptomycin (Gibco) at a density of $1x10^7$ cells per T25 culture flask. Medium was changed after one week and subsequently every 2-3 days until cells reached confluence. Mouse BMSC were passed into a 12-wells plate and co-cultured with $1x10^5$ human isolated CD34+ HSC

from cord blood in X-VIVO-20 medium (Biowhittaker) containing 2% FCS. After 10 days, non-adherent and adherent cells detached using trypsin-EDTA were pooled and a fraction was plated in methylcellulose medium containing hematopoietic growth factors (Methocult complete, StemCell Technologies, Vancouver, BC). The number of colony forming units (CFU) was quantified after 14 days culture.

In vitro model for the bone marrow vascular niche
An endothelial cell line generated from human umbilical vein endothelial cells (HUVEC) was grown to confluence in 12-wells plates. $1x10^5$ CD34+ HSC were plated on top of the confluent HUVEC in IMDM medium (Gibco) containing 0 or 30mM added D-Glucose (Sigma Aldrich, St. Louis, USA). A small volume of fresh medium was added every 2-3 days and excessive medium was carefully removed with minimal aspiration of non-adherent cells every two weeks. After 2, 4 and 6 weeks, the number of non-adherent cells was counted and then pooled with the adherent co-cultured cells, which were detached using trypsin-EDTA (Gibco). A fraction of these cells was plated in methylcellulose medium containing hematopoietic growth factors (Methocult complete, StemCell Technologies) and evaluated for generation of CFU after 14 days culture.

Assessment of direct effects of hyperglycemia on CD34+ HSC survival and migratory function
Isolated CD34+ HSC were incubated overnight in IMDM medium containing 10% FCS and 0 or 30mM added D-Glucose, or 30mM D-Mannitol (Sigma Aldrich) as osmotic control. Cell number was assessed using a Bürker-Türk counting chamber, counting only viable cells based on Trypan-Blue exclusion. Equal cell numbers were taken up in IMDM medium containing 1% FSC and 0 or 30mM added D-Glucose, or 30mM D-Mannitol and placed in transwell insert (5µm pore size, Corning Costar, Cambridge, MA) in a 24-well migration system with 100 ng/ml SDF-1 (RnD Systems) or vehicle added to the bottom wells. After 4 hours, migrated cells were counted using an automated cell counter.

Bone marrow histology
Excised femurs were fixed overnight in 4% paraformaldehyde on a shaker at room temperature, decalcified using a commercially available decalcifying solution (Richard-Allen Scientific, Kalamazoo, MI) for 75 minutes, dehydrated and then embedded in paraffin. 5 µm sections were cut longitudinally in the center region of the femur. Hematoxylin and Eosin staining was performed to assess general histology. To identify sinusoidal endothelium, bone marrow sections were stained for VEGF-R3. In brief, deparaffinized sections were pretreated with DAKO target antigen retrieval solution (DAKO Cytomation, Carpenteria, CA), blocked with donkey serum and avidin/biotin blocking solution (DAKO Cytomation) and incubated with rat-anti-mouse VEGF-R3 antibody (BD Pharmingen) overnight. Staining was visualized using anti-rat IgG-biotin, HRP-conjugated streptavidin and 3,3-diaminobenzidine (DAB). Sections were briefly counterstained with hematoxylin and cover slipped using cytoseal (Richard-Allen Scientific).

In vivo model for vascular niche function: 5- fluorouracil challenge
Control and diabetic mice were intravenously injected with 250 mg per kg body weight 5-fluorouracil (5-FU; American Pharmaceutical Partners, Schaumburg, IL). White blood cell (WBC) and platelet counts were monitored in peripheral blood samples using a hematocytometer at baseline and after 4,7,10,14 and 21 days.

Statistical analysis

Data are expressed as mean±SEM and were analyzed using SPSS version 11.0 software. After testing for normal distribution of data and equality of variances, differences between groups were analyzed using a two-tailed students' t-test for two-group comparison or ANOVA with Newman-Keuls post-hoc test to compare between three or more groups. A p-value of <0.05 was considered statistically significant.

Results

Decreased EPC levels in peripheral blood under steady state conditions are associated with impaired mobilization after G-CSF/SCF injection in diabetic mice

During steady-state conditions (baseline in fig. 1) peripheral blood Sca-1+Flk-1+ EPC levels were lower in diabetic mice than in controls (7.7±0.9 vs. 13.3±1.9 $x10^3$ per ml blood; p<0.05). After injection of G-CSF and SCF, a robust EPC mobilization was observed in control mice (p<0.001 for time interaction in ANOVA analysis). EPC levels reached levels of 30.5±3.2 $x10^3$ EPC per ml blood after maximal stimulation at day 4, corresponding to a 129% increase with a 17.2±3.0 $x10^3$ EPC per ml blood absolute difference (fig. 1). EPC levels remained high during injections from day 0 to 4 and returned to baseline after injections were stopped. In contrast, EPC levels only marginally increased in diabetic mice after G-CSF/SCF injections and a statistically significant difference with baseline levels was not achieved (p=0.68 for time interaction in ANOVA analysis). EPC levels reached levels of 12.0±1.3 $x10^3$ EPC per ml blood after maximal stimulation at day 4, corresponding to a 56% increase with a 4.3±0.8 $x10^3$ EPC per ml blood absolute difference (fig. 1).

Total Sca-1+ cells and c-kit+ progenitor cell populations in peripheral blood also displayed lower baseline levels and a blunted response to G-CSF/SCF in diabetic animals, but for these populations, the impairment in mobilization was not as severe in diabetic mice (not shown).

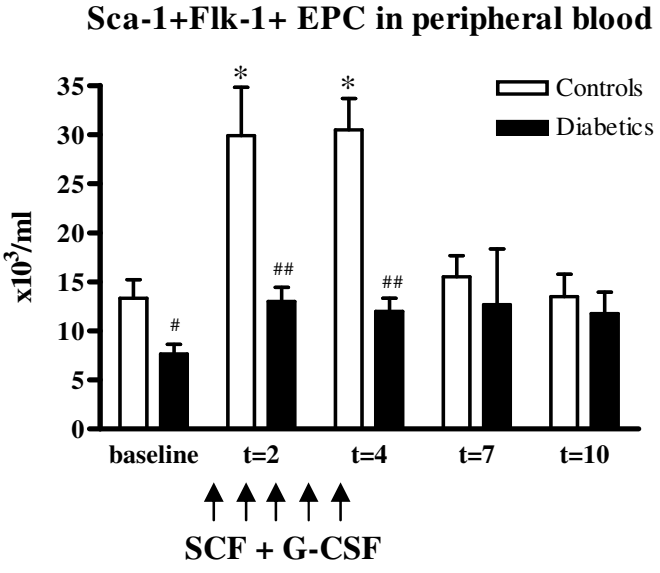

Figure 1. EPC mobilization in response to cytokine injection

Diabetic animals have reduced levels of Sca1+Flk-1+ EPC in peripheral blood under steady-state (baseline) conditions compared to controls.

In controls, a significant mobilization of EPC was observed after injection with mobilizing cytokines G-CSF and SCF (from day 0 to 4) and EPC levels returned to baseline after cessation of cytokine injection.

In contrast, diabetic animals showed a diminished mobilization response after cytokine injection (p<0.001 for interaction of time and diabetes in 2-way ANOVA).

 # p<0.05 compared to controls

p<0.01 compared to control

** p<0.01 compared to baseline*

Bone marrow EPC levels are unaffected in diabetic mice

The number of bone marrow EPC isolated per femur was not significantly different between diabetic and control mice (13.2±3.7 vs. 10.5±3.4 $x10^3$; p=ns). Consistently, quantification of EPC by *ex vivo* culture of bone marrow cells also revealed no significant differences in cell number (24.1±2.2 vs. 24.4±5.3 per high power field; p=ns).

Diabetic bone marrow stroma is impaired in supporting hematopoietic progenitor cells and contains reduced eNOS levels

Plastic-adherent bone marrow stromal cells, a heterogeneous cell population including fibroblasts, endothelial cells, osteoblasts and potentially various other cell types, from diabetic and control primary cultures of mouse bone marrow cell suspensions were grown to confluence to serve as feeder layers for co-cultured human CD34+ HSC. Stromal cell morphology and growth pattern was similar between cultures from diabetic and control animals (fig. 2). However, the number of CFU cells derived from 10-day co-cultures was significantly lower for diabetic stroma versus control stroma (45±10 vs. 75±7 CFU/well; p=0.023, fig. 2). The distribution over the various types of CFU colony outgrowth was not affected (not shown). Of note, cells were cultured using identical medium with a standard d-glucose concentration. Diabetic stroma lysates had reduced levels of NO-producing enzyme eNOS as determined by ELISA (1.9±0.4 vs. 4.1±0.4 AU, p=0.0055). Quantitative real-time PCR for eNOS mRNA showed a 3.9 fold reduced expression in diabetic stroma (p<0.01), suggesting that the decreased stromal cell eNOS levels were due to inhibited eNOS transcription.

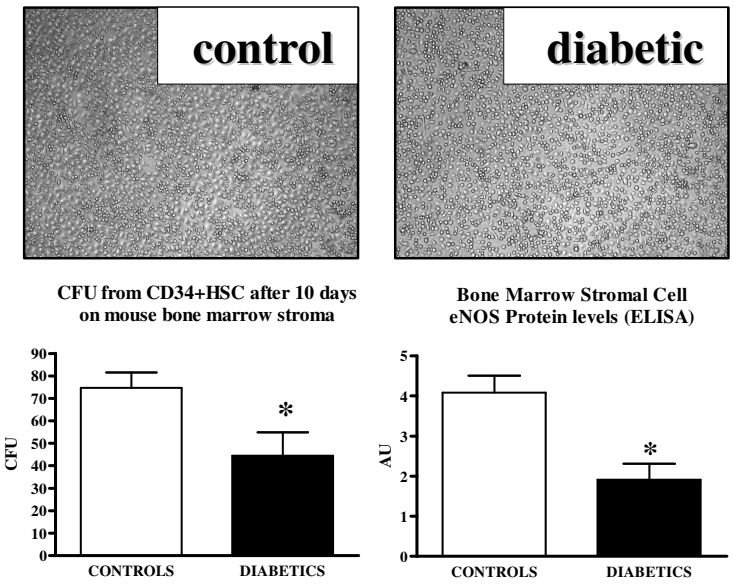

Figure 2. Progenitor cell support and eNOS protein levels of diabetic versus control bone marrow stromal cells
*Plastic-adherent bone marrow stromal cells were grown to confluence from isolated bone marrow cell suspensions. Stromal layers from control and diabetic mice had a comparable morphology and growth pattern. Stromal layers were then used as feeder layer for human CD34+ HSC. The number of hematopoietic colonies (CFU) derived from 10-day co-cultures was significantly lower for diabetic stroma than for control stroma. Also, diabetic stroma lysates had reduced levels of NO-producing enzyme eNOS as determined by ELISA (expressed in Arbitrary Unit, AU). * p<0.05*

Hyperglycemia impairs progenitor cell support by endothelial cells in a co-culture model for the 'vascular niche'

To study if the impairment for supporting progenitor cells by stromal cells in diabetic conditions might be reflected in endothelial cells serving as a model for the 'vascular niche', we co-cultured human CD34+ HSC with HUVEC in regular versus hyperglycemic conditions. Indeed, CD34+ HSC cultured on endothelium in the presence of hyperglycemia generated less non-adherent cells and less CFU-progenitor cells (fig. 3). The distribution over the various types of CFU colony outgrowth was not affected by hyperglycemia (not shown).

Dysfunctional endothelium by NO-synthesis inhibition is impaired in supporting progenitor cell expansion

A hallmark feature of dysfunctional endothelium is an impaired capacity to generate bioavailable nitric oxide (NO)[5]. Diabetes/hyperglycemia has been previously shown to impair endothelial NO-release in vivo[31] and in vitro[32]. We therefore investigated to capacity of dysfunctional endothelium to support progenitor cells by co-culturing endothelial cells with HSC in the presence of NO-synthesis inhibitor L-NAME. Interestingly, NO-inhibited dysfunctional endothelium was impaired in supporting progenitor cell expansion (fig. 4).

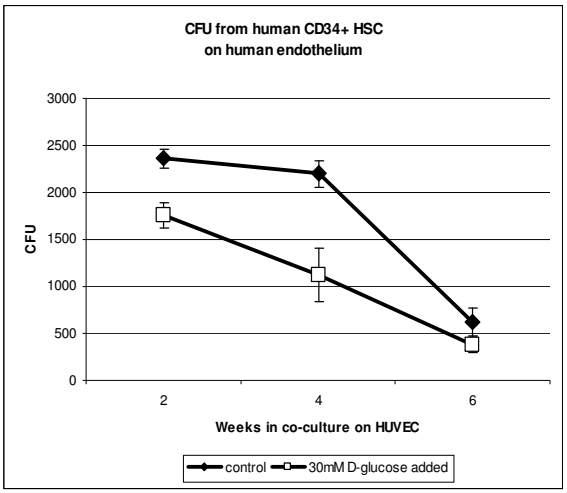

Figure 3. Progenitor cell support in the in vitro model of bone marrow vascular niche under hyperglycemic conditions
Human CD34+ HSC were co-cultured with HUVEC for six weeks and evaluated for the production of CFU at several time points. Fewer CFU were obtained from CD34+ HSC co-cultured on endothelium in the presence of hyperglycemia than in control conditions.

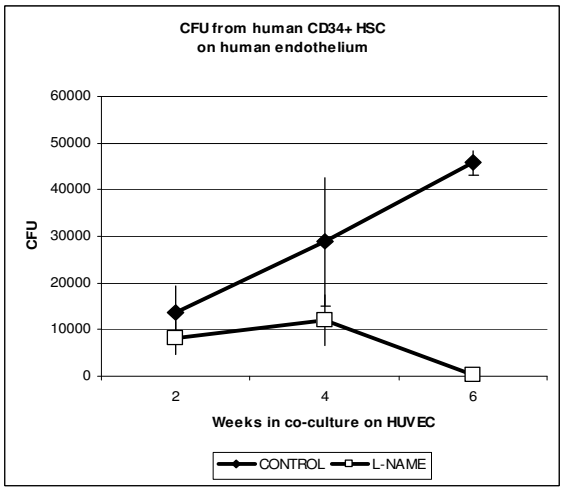

Figure 4. Progenitor cell support in the in vitro model for the bone marrow vascular niche with inhibition of Nitric Oxide production
Human CD34+ HSC were again co-cultured with HUVEC for six weeks and evaluated for the production of CFU at several time points. Fewer CFU were obtained from CD34+ HSC co-cultured on endothelium in the presence of NO-synthesis inhibitor L-NAME.

Diabetic animals have histologically disorganized vasculature

On HE staining, diabetic bone marrow showed an apparently normal cellularity, consistent with an unaffected progenitor and stroma content, but sinusoidal vessels appeared distended and tortuous (fig. 5). A specific staining for sinusoidal vessels based on the expression of VEGF-R3 confirmed the aberrant disorganized structure of sinusoidal bone marrow vessels (fig. 5).

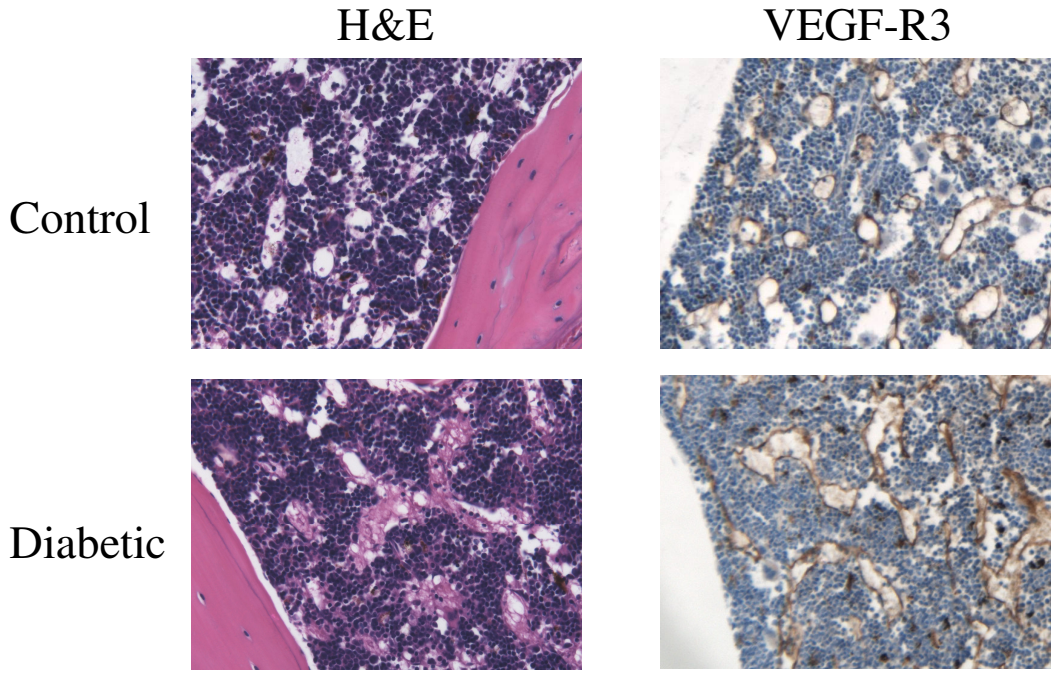

Figure 5. The diabetic bone marrow displays histological signs of aberrant vasculature
On HE staining, diabetic bone marrow showed an apparently normal cellularity, but sinusoidal vessels appeared distended and tortuous. VEGF-R3 staining for bone marrow sinusoidal vessels confirmed the aberrant disorganized structure.

Diabetic bone marrow fails to recover after 5-FU challenge

Diabetic and control animals were injected with 5-FU. 5-FU injection causes destruction of proliferating bone marrow progenitor cells and leads to a depression in peripheral blood WBC and platelet numbers. Recovery depends on the presence and function of quiescent multipotent stem cells, but also on bone marrow angiogenesis to recreate a functional vascular niche.[17] After 5-FU injection we observed a reduction in both WBC and platelets which was maximal at 7 days after injection (fig. 6). As expected, control animals fully recovered with restoration of WBC levels and platelets, displaying a typical thrombocytosis after recovery. In contrast, diabetic animals died between day 10 and 14 with severe peripheral blood leukopenia and thrombopenia (fig. 6).

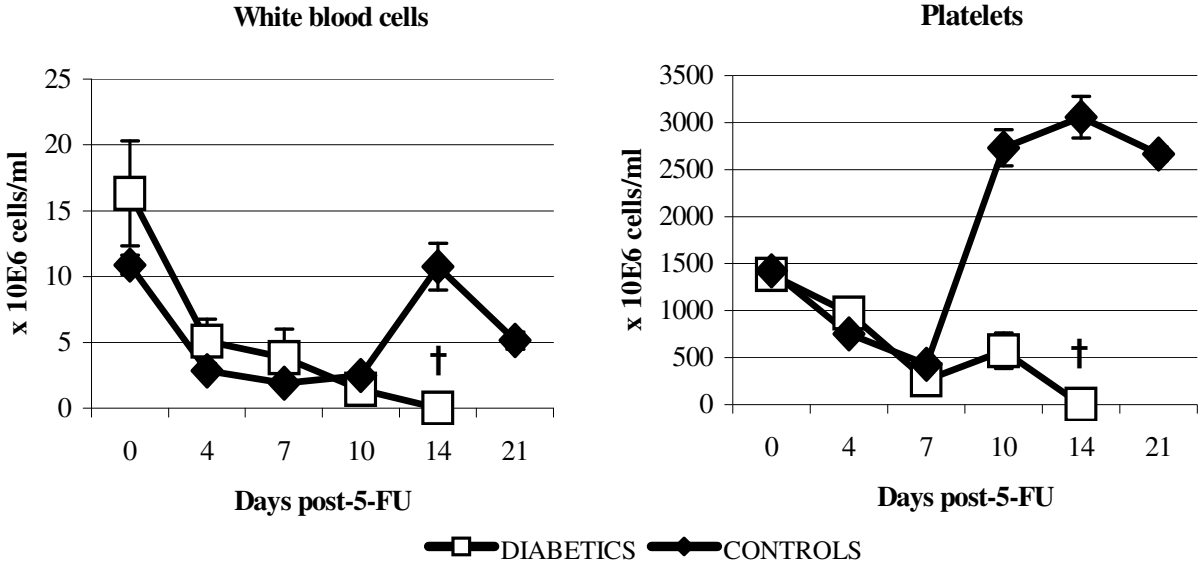

Figure 6. Diabetic mice do not recover after a 5-FU challenge

After 5-FU injection, a reduction in both WBC and platelets was observed in control and diabetic animals, which was maximal at 7 days after injection. Control animals fully recovered with restoration of WBC levels and platelets, displaying a typical thrombocytosis after recovery. In contrast, diabetic animals died between day 10 and 14 with severe peripheral blood leukopenia and thrombopenia.

Discussion

Chronically reduced EPC levels that are unresponsive to induction of mobilization may hamper maintenance and repair of the vascular endothelium and impair neovascularization in response to ischemia in diabetes. In this study we show that impaired mobilization of endothelial and hematopoietic progenitor cells in diabetes is associated with dysfunction of the bone marrow stromal environment. Our *in vitro* and *in vivo* data demonstrate that diabetes affects the endothelial component of the bone marrow stroma, and indicate that a defect in the bone marrow 'vascular niche' from which progenitor cells are mobilized leads to the impaired mobilization of endothelial and hematopoietic progenitor cells observed in diabetes. eNOS levels in diabetic stroma were reduced and NO-inhibition impaired endothelial support of progenitor cells, suggesting a potential role for attenuated endothelial NO-release in diabetic stroma in dysfunctional progenitor cell mobilization.

Dysfunctional mobilization may underlie chronically lower peripheral blood EPC levels at steady-state, as well as during induction of mobilization by the release of mobilizing cytokines. In our experiments we used exogenous administration of the cytokines, G-CSF and SCF as mobilizing stimuli. These cytokines may be endogenously released e.g. in the case of an ischemic event. In patients with acute myocardial infarction, G-CSF was shown to be upregulated and correlated with

circulating CD34+ progenitor cell levels.[33] We simultaneously injected SCF as SCF was reported to have a synergistic effect progenitor cell mobilization with G-CSF.[34] In control mice, we indeed observed a more than two-fold increase in circulating EPC. However, the presence of diabetes impaired this mobilization response. In the diabetic mice no significant rise in circulating EPC occurred. Our flow cytometric and *ex-vivo* culture analysis of bone marrow cell suspensions, found bone marrow-derived EPC levels not to be reduced in diabetes, indicating that the attenuated mobilization response is not due to reduced pools of progenitor cells present in the bone marrow. In line with this, histology of the bone marrow showed unaffected cellularity.

The sinusoidal vessels form the 'vascular niche' and are an anatomical and functional barrier between the hematopoietic compartment and the peripheral circulation. Bone marrow endothelial cells of the sinusoidal vessels regulate progenitor cell differentiation and mobilization.[17] To evaluate the effects of diabetes on the interaction between supporting bone marrow stroma cells and progenitor cells, we employed two *in vitro* models. First, we assessed primary diabetic stroma. We cultured plastic-adherent stromal bone marrow fractions from mice and co-cultured these with human CD34+ HSC. In this co-culture model, HSC were supported to survive and give rise to CFU after an *ex vivo* culture period of 10 days. We observed that fewer CFU were obtained from co-cultures using (non-diabetic) HSC and bone marrow stroma from diabetic mice than in those using bone marrow stroma from control mice, indicating that diabetes induces an impairment in the bone marrow stroma to support progenitor cells. Interestingly, these observations were made in non-hyperglycemic culture conditions, suggesting that diabetic stromal cell impairment is at least to some extent imprinted upon the cells. In a second *in vitro* model, we co-cultured isolated human endothelial cells with human CD34+ HSC and found that in hyperglycemic conditions, fewer CFU were obtained from equal numbers of plated HSC. This indicates that progenitor cell support by endothelium is impaired by the presence of hyperglycemia. This supports that diabetes is associated with a pathophysiological impairment of the interaction between progenitor cells and endothelial cells, which is critical for progenitor cell mobilization from the bone marrow vascular niche.

In vivo, we assessed the bone marrow vasculature in diabetes by regular histology. We observed disorganization of sinusoidal vessels in histological sections of the bone marrow of diabetic mice. To further assess the *in vivo* function of the bone marrow vasculature, we injected animals with 5-FU. This is causes destruction of cycling hematopoietic cells as well as bone marrow vasculature. Recovery from 5-FU injection depends on both hematopoietic stem cell repopulation and bone marrow angiogenesis for restoration of the vascular niche.[17] Diabetic mice proved to be dramatically impaired in recovering from 5-FU challenge and died during the experiment from bone marrow failure while controls recovered. This failure of diabetic animals to recover from the 5-FU challenge is consistent with dysfunction of the 'vascular niche'. A limitation to this experiment is that we cannot exclude that the hyperglycemic condition had an effect on the long-term repopulating stem cells in the bone marrow. Overnight exposure to hyperglycemia did not have any marked effects on CD34+ HSC *in vitro*.

Further studies are required to identify the pathways involved in this disrupted cellular interaction. In the regulation of mobilization, various cytokines, cell-cell contact proteins and other regulatory factors participate. A critical pathway involves MMP-9 –mediated cleavage of membrane-bound kit-ligand, releasing soluble kit ligand or SCF, which triggers progenitor cells to transfer from a quiescent to a proliferative niche.[35,36] The activation of MMP-9 has been shown to be nitric oxide-dependent.[20] In this study, we found reduced levels of the NO-synthesizing enzyme eNOS in diabetic bone marrow

stroma. Also, we observed that inhibition of eNOS reduced the capacity of endothelial cells to support HSC to give rise to CFU. Several lines of evidence confirm a role for nitric oxide production by endothelial Nitric Oxide Synthase (eNOS) in EPC mobilization. eNOS knockout mice and mice treated with an NO-inhibitor have an impaired EPC mobilization.[20,37] Hyperbaric oxygen, which increases bone marrow nitric oxide levels, increases circulating HSC levels.[29] Caveolin, which co localizes with eNOS in the caveolae regulating eNOS activity, is required for VEGF/nitric oxide dependent SDF-1 induced EPC mobilization.[38] Consistently, increasing NO-availability by hyperoxia restored EPC mobilization in diabetic animals.[29]

In conclusion, we propose a novel concept of 'vascular niche dysfunction' underlying impaired endothelial progenitor cell mobilization and availability in the circulation. Dysfunction of the endothelium composing the vascular niche carries a high homology with the endothelial dysfunction in the arterial systems of diabetic patients. Potentially, therapeutic interventions known benefit arterial endothelial function may also benefit endothelial function in the vascular niche. Such effects may be mediated through enhancement of reduced endothelial NO production as this is pivotal in arterial endothelial function and, as we show in this study, also for endothelial support of progenitor cells. Several common pharmacological interventions that increase NO-production also favorably affect progenitor cells levels and progenitor cell mobilization, such as treatment with statins[39-41], estrogen[42,43] or PPAR-gamma-agonists[44-46], HDL-infusion[47] and physical training[48]. We propose that these actions may be mediated via stimulation of NO-production in bone marrow endothelium. Indeed, for statins it was confirmed that the induced increased in circulating EPC was associated with increased bone marrow eNOS levels.[49] Vascular niche dysfunction with reduced EPC levels may not only occur in diabetes, but also in other pro-atherosclerotic conditions associated with impaired NO-availability.[21,23-25]

Acknowledgements

The technical advice from Dr. Geurt Stokman and Dr. Jaklien Leemans from the Amsterdam Medical Center, the Netherlands, is gratefully acknowledged.

Funding sources

The reported work was supported by the Dutch Heart Foundation (Dr. E. Dekker Grant 2004T022 and travel grant 2006R024), ZonMW (AGIKO grant ZonMw (2007/12579), the Jacques H. de Jong Foundation (grant 2006.01.18) and the Prof. R.L.J. van Ruyven Foundation.

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7

ANGIOGENIC SPROUTING FROM THE AORTIC VASCULAR WALL IS IMPAIRED IN THE BB RAT MODEL OF AUTOIMMUNE DIABETES

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Microvascular Research 2008;75(3):420-425

Introduction

Diabetes mellitus is associated with impaired neovascularization leading to reduced revascularization of ischemic tissue, impaired wound healing, embryonic vasculopathy, and organ transplant rejection in diabetic patients.[1,2] On the other hand, pathologically enhanced neovascularisation is also observed in diabetes, contributing to diabetic retinopathy, diabetic nephropathy, and possibly atherosclerotic plaque destabilization.[1,2] Neovascularization is a complex process, involving growth factors, cytokines, and both resident endothelial cells as well as circulating cells. Circulating factors in the diabetic milieu directly influence neovascularization. Hyperglycemia decreases endothelial cell proliferation *in vitro*[3] and myocardial interstitial fluid from dogs with experimental diabetes impairs angiogenic tube formation by cultured endothelial cells.[4] The diabetic milieu also influences circulating cells. Monocytes from diabetic patients respond poorly to angiogenic chemotactic factor VEGF-A[5] and circulating angiogenic endothelial progenitor cells were previously shown to be numerically reduced and functionally impaired in diabetes.[6]

It is currently unclear what the effect of diabetes is on angiogenic cells residing in the vessel wall, which are potent contributors to neovascularization.[7] We hypothesized that diabetes would attenuate their angiogenic capacity and therefore assessed angiogenic sprout formation from ex vivo cultured aorta rings isolated from diabetic and non-diabetic biobreeding diabetes-prone (BBDP) rats. We furthermore determined the phenotype of the outgrowing sprout cells. This sprouting-assay provides a tool to specifically study the long-term effect of diabetes on the angiogenic capacity of cells residing in the vessel wall, thereby excluding the direct influence of both circulating factors and circulating cells.

Material and Methods

Biobreeding diabetes-prone BBDP rats

Both male and female BBDP/Wor rats were used. BBDP rats spontaneously develop autoimmune diabetes due to the absence of regulatory T cells and are used to model human Type 1 diabetes.[8] Age-matched neonatally thymectomized BBDP rats and pre-diabetic BBDP rats served as non-diabetic controls. All animal use was in accordance with the guidelines of the "Principles of laboratory animal care" and the Animal Ethics Committee of the University Medical Center Groningen. In this study, two experimental groups were included. Group 1 (long-term group) had a long diabetic course (~45 wks diabetes) and consisted of 15 diabetic BBDP rats and 8 age-matched neonatally thymectomized non-diabetic BBDP rats. Group 2 (short-term group) had a relatively short diabetes duration and consisted of 8 diabetic BBDP rats (~6 wks diabetes; ~17 wks of age) and 7 pre-diabetic BBDP rats (8 wks of age). Diabetic rats were suboptimally treated with insulin by subcutaneous implantation of slow-release Linplant insulin-pellets (LinShin, Scarborough, Canada).

Aortic ring sprouting assay

Aortas were harvested from the diabetic and non-diabetic BBDP rats under sterile conditions and flushed with saline to remove residual blood. Then ~1mm (group 1) and 0.65mm (group 2) thick aortic rings were transversally cut manually or using an automatic tissue chopper (McIlwain Tissue Chopper, The Mickle Laboratory Engineering Co. LDT., Gomshall, Surrey, England), respectively. Aortic rings were centrally positioned in the wells of 96-well tissue culture plates containing cold liquefied BD Matrigel™ Basement Membrane Matrix (BD Biosciences, Alphen aan den Rijn, The Netherlands), and overlaid with EC medium (RPMI1640 medium containing 2 mM L-glutamine, 50 µg/ml endothelial cell growth factor (ECGF), 5 units/ml heparin, 100 U/ml penicillin, 100 µg/ml

streptomycin and 20% fetal calf serum). Cultures were performed in duplicate (group 1) or triplicate (group 2). Plates were then placed at 37°C 5% CO_2, allowing the matrix solution to solidify. Sprout length in the long-term diabetic group (group 1) was measured after 10 days of culture. In the short-term diabetic group (group 2), sprout length was measured after 4, 7, 10 and 14 days of culture in order to evaluate kinetics of sprout formation, with having medium replenished at the moment of measurements. Sprout length was quantified (expressed in arbitrary units) as mean maximal sprout length from the perimeter of the aortic ring to the most distal tip of the angiogenic sprout in four quadrants of each aortic ring under an inverted microscope at 40-fold magnification using a microscopic grid.

Sprout cell isolation

To evaluate the absolute number of sprout cells, sprouts growing in Matrigel from the short-term diabetics and pre-diabetics (group 2) were isolated and counted. Medium and aorta rings were removed from the Matrigel cultures after which remaining sprouts and Matrigel were incubated with dispase (BD, Alphen aan den Rijn, The Netherlands, 2.5 caseinolytic units/well) at 37°C, 5% CO_2 for 2 hours to ensure complete dissociation. Cultures were performed in triplicate and after enzymatic digestion the total solution obtained from triplicate cultures was diluted in 10 ml PBS. Absolute numbers and viability of isolated sprout cells were determined using a haemocytometer and Trypan Blue exclusion. Viability was consistently >98% (not shown). Cell counting was performed after 4, 7, 10 and 14 days of culture in Matrigel. Cells isolated after 10 days of culture in Matrigel were either replated in fresh EC medium for continued cell culture (described below) or resuspended in PBS + 2.5% BSA for cytospots. Cytospots were prepared by spinning a total of $5x10^4$ isolated cells onto glass-slides (5 min., 550 rpm) using a Shandon Cytospin 4 (Thermo Fisher Scientific).

Evaluation of sprout cell proliferative capacity

To determine the proliferative status of sprout cells, cytospots ($5x10^4$ cells/spot) of cells isolated after 10 days of culture in Matrigel were immunostained for Ki67, a marker which is expressed during all active phases of cell replication but not in resting cells. Cytospots were acetone-fixed for 12 min and dried for 1 hr at room temperature (RT). Blockade of endogenous peroxidase (0.03% H_2O_2 in PBS for 10 min) was followed by incubation (1 hr, RT) with α-Ki67 monoclonal antibody (DakoCytomation Denmark A/S, Glostrup, Denmark). Subsequently, cytospots were incubated (30 min, RT) with horseradish peroxidase-conjugated rabbit-α-mouse antibody (DakoCytomation Denmark A/S, Glostrup, Denmark) followed by visualisation with diaminobenzidine (DAB, Sigma-Aldrich, Zwijndrecht, The Netherlands). Cells were counterstained with hematoxylin and coverslipped in Depex mounting medium. All $Ki67^+$ cells per cytospot ($5x10^4$ cells) were counted.

To further evaluate the proliferative capacity of isolated sprout cells after replating, $2x10^4$ cells/well were seeded in EC medium in 6-well tissue culture plates that were precoated (1 hr) with 1 ml/well Matrigel (1 mg/ml). Cells were cultured for 96 hrs (4 days) after which they were trypsinized and counted using a haemocytometer and Trypan Blue exclusion. In some experiments cells were cultured in Matrigel-coated 8-wells chamberslides (Lab-Tek™, Nunc, VWR International, Amsterdam, The Netherlands) for phenotypic analysis.

Phenotypic analysis of sprout cells

To demonstrate an EC-phenotype of isolated sprout cells, cytospots were stained using the following antibodies: α-CD31 (clone TLD-3A12, mIgG1, BD Pharmingen), α-Flk-1 (clone A-3, mIgG1, Santa Cruz Biotechnology), mAb HIS43[9] (own produced hybridoma tissue culture supernatant, mIgG1) and α-alpha-SMA (vascular smooth muscle cells clone 1A4, mIgG2a, DakoCytomation A/S). After

aceton fixation (12 min, -20°C) cytospots were incubated with primary monoclonal antibodies (1 hr, RT). Cytospots were then incubated (30 min, RT) with TRITC-conjugated goat-α-mouse IgG1 and FITC-conjugated goat-α-mouse IgG2a antibodies (both from Southern Biotechnology Associates, Birmingham, Alabama, USA) diluted in PBS + 3% normal rat serum. After nuclear staining with DAPI cytospots were embedded in Citifluor. Cytospots were analyzed on a Confocal Laserscanning Microscope (TCS SP2, Leica, Microsystems Nederland B.V., Rijswijk, The Netherlands).

Statistical analysis

Data are expressed as mean±SEM and were analyzed using GraphPad Prism 4.0 software (GraphPad Software Inc., San Diego, USA) using a two-tailed Students' t-test. A p-value of <0.05 was considered statistically significant.

Results

Diabetes onset and glycaemic control

Non-thymectomized BBDP rats developed diabetes with a median onset of diabetes at the age 84 days (group 1; long-term) and 75 days (group 2; short-term), respectively. Age-matched control thymectomized BBDP rats did not become diabetic. In group 1 mean follow-up time was 324 and 386 days for the diabetic and thymectomized non-diabetic BBDP rats, respectively. Mean blood glucose level after long-term diabetes was 15.1±0.3 mmol/L versus 5.3±0.1 mmol/L in thymectomized non-diabetic controls (fig. 1A, $p<0.0001$). In group 2 mean follow-up time was 120 and 56 days for the short-term diabetic and pre-diabetic BBDP rats, respectively. Mean blood glucose level after diabetes onset was 20.8±1.0 mmol/L versus 7.4±0.3 mmol/L in pre-diabetic controls.

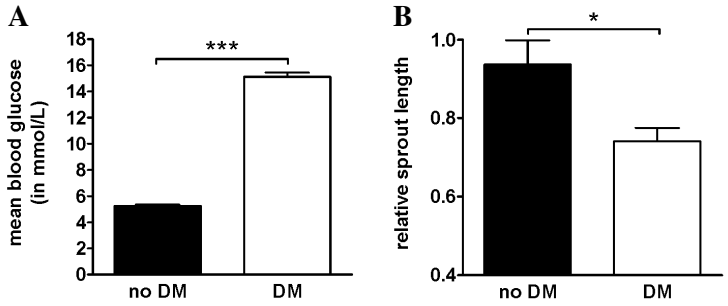

Figure 1. Long-term diabetes (~45 wks) impairs aortic sprouting.
*(A) Mean blood glucose levels in group 1: long-term diabetics (DM, n=15) and age-matched thymectomized non-diabetic controls (no DM, n=8). (***p<0.0001) (B) Quantification of the relative sprout length from aortic rings revealed significantly reduced (*p<0.05) sprout formation in vitro after exposure to hyperglycemic conditions in diabetic BBDP rats in vivo compared to age-matched thymectomized non-diabetic control BBDP rats.*

Angiogenic sprouting from aorta rings is impaired in diabetic animals

In both diabetic and non-diabetic BBDP rats we observed sprouts starting to emerge from the vascular wall and growing outward after 4 days of culture in Matrigel. All rings generated sprouts of comparable morphology although sprout length and sprout density was reduced in the diabetic rats. In group 1 (long-term group) maximal sprout length in aortic rings measured at day 10 was significantly lower in diabetic rats than those from thymectomized non-diabetic controls (0.94±0.06 vs. 0.77±0.03, $p<0.05$, fig. 1B). In order to determine the kinetics of sprout development, sprout length was determined in the short-term diabetics and pre-diabetic BBDP rats at 4, 7, 10 and 14 days after culture in Matrigel. Figure 2A shows representative photomicrographs depicting reduced sprout formation in rings from diabetic rats (right panel) compared with rings from pre-diabetic rats (left panel).

Quantitative analysis revealed reduced sprout length in diabetic rats at 4, 7 and 10 days after culture in Matrigel reaching the level of statistical significance at day 4 ($p<0.01$) and day 10 ($p<0.05$) compared with pre-diabetic rats (fig. 2B). After 14 days of culture in Matrigel sprouts had reached the border of the well, and thereby maximal sprout length, in rings from both diabetic and pre-diabetic rats.

Our microscopic analysis of developing sprouts suggested reduced sprout density in rings obtained from diabetic rats. Reduced sprout length and sprout density is anticipated to result in the presence of decreased numbers of sprout cells in diabetic rats compared with pre-diabetic rats. To test this assumption we determined the absolute number of cells that could be isolated from sprouts that had grown out of aortic rings from short-term diabetic and pre-diabetic BBDP rats. Cells were isolated by enzymatic digestion using dispase followed by counting using a haemacytometer. As shown in figure 2C absolute numbers of cells isolated from sprouts from diabetic rats were significantly reduced (at days 4, 7 and 14) compared with the numbers of cells isolated from sprouts from pre-diabetic rats.

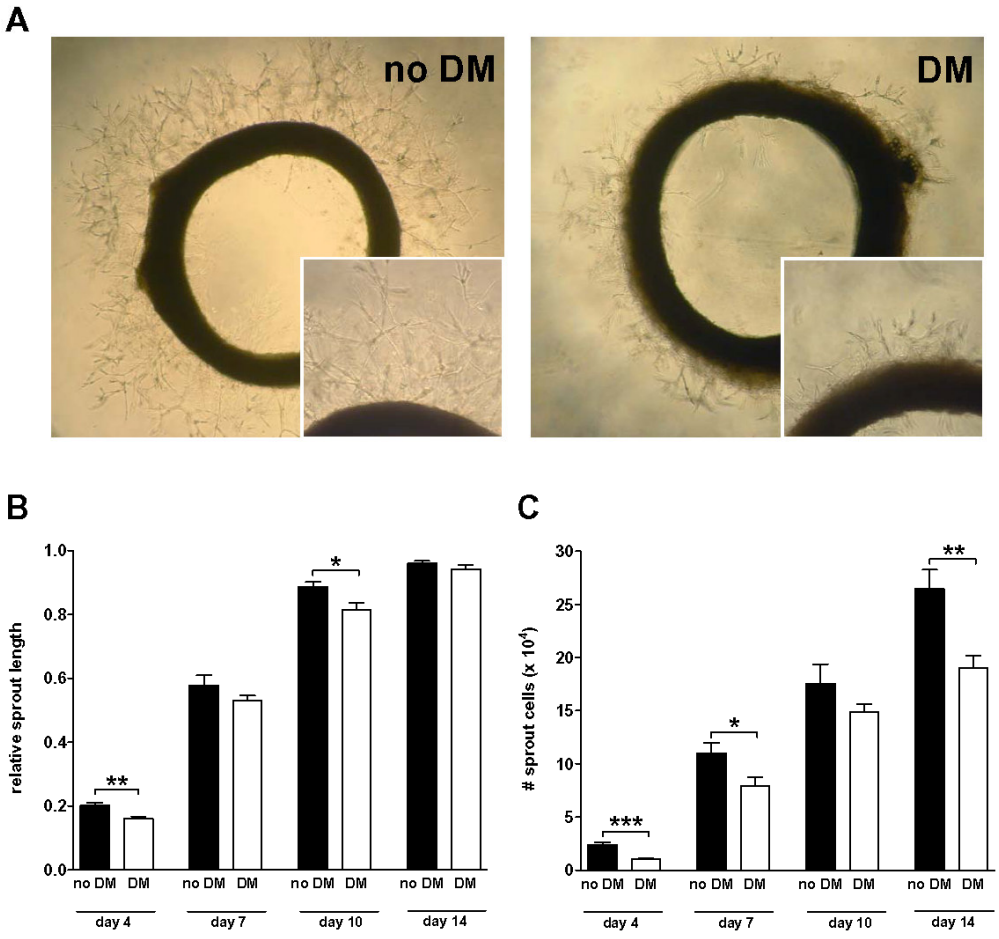

Figure 2. Kinetics of in vitro sprout-formation in aortic rings from short-term (~6 wks) diabetic (DM) and pre-diabetic (no DM) BBDP rats.
(A) Representative photomicrographs of aorta rings from pre-diabetic (no DM; left panel) and diabetic (DM; right panel) BBDP rats after 4 days of culture in Matrigel. (B) Quantification of sprout length from aortic rings revealed reduced sprout formation in vitro after exposure to hyperglycemic conditions in diabetic BBDP rats in vivo (compared to pre-diabetic BBDP rats) after 4, 7, and 10 days of culture in Matrigel. ($p<0.05$, ** $p<0.01$) (C) Quantification of the absolute numbers of sprout cells revealed significantly reduced numbers in diabetic rats (DM) compared with pre-diabetic (no DM) control rats after 4, 7 and 14 days of culture. (* $p<0.05$,** $p<0.01$, *** $p<0.0001$)*

Aortic sprout cells from diabetic rats have a lower proliferative status

A possible explanation for reduced sprout length and density in diabetic rats is a reduced proliferative capacity of sprout cells. To test this possibility we determined the proliferative status of sprout cells by performing an immunostaining for the proliferation marker Ki67 on cytospots of cells isolated from sprouts that were cultured for 10 days in Matrigel. Figure 3A shows the nuclear staining for Ki67 in dividing cells. Compared with pre-diabetic controls, diabetic rats had significantly less Ki67-positive cells per $5x10^4$ spotted cells ($p<0.01$, fig. 3B) suggesting a lower proliferation status of sprout cells derived from diabetic rats.

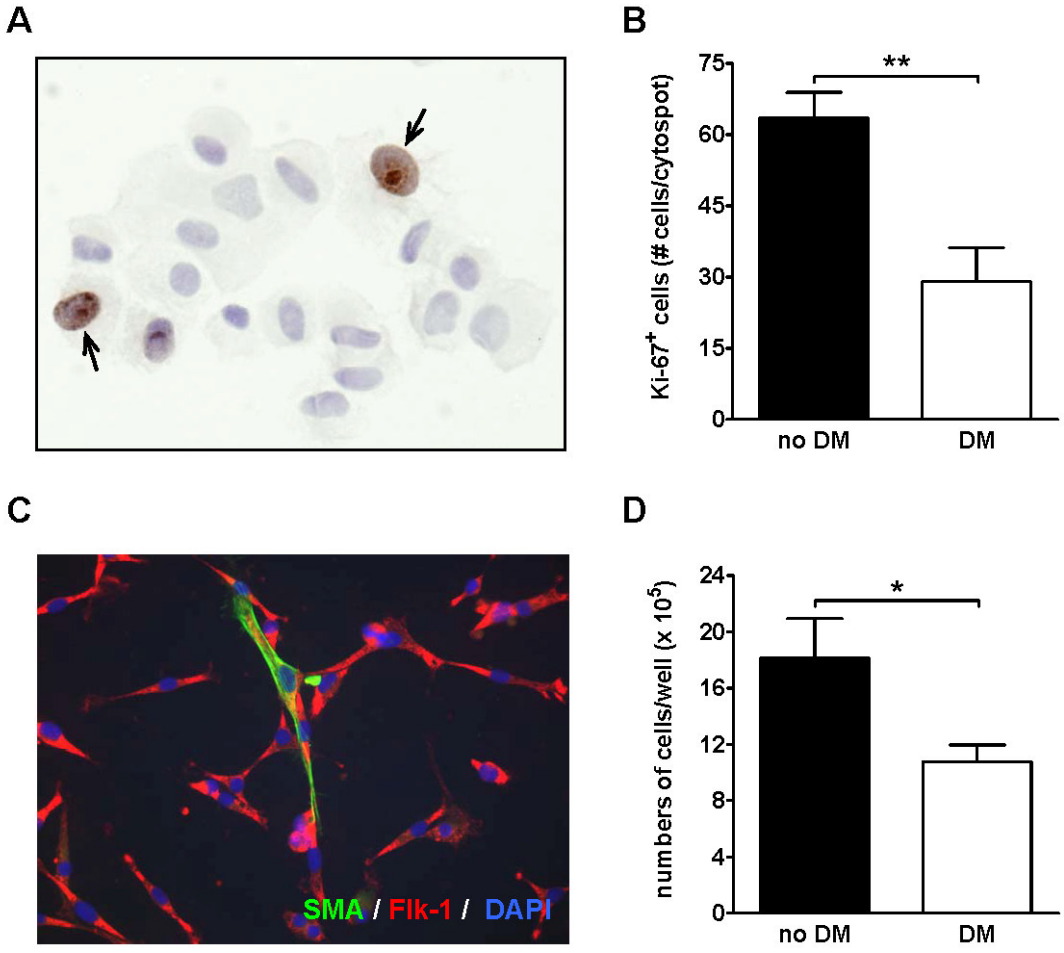

Figure 3. Sprout cells from short-term diabetic BBDP rats have a decreased proliferation status
*(A) Representative photomicrograph of cytospots of sprout cells isolated after 10 days of culture in Matrigel and stained with the proliferation marker Ki67. Arrows indicate proliferating $Ki67^+$ cells. (B) Quantification of $Ki67^+$ cells on cytospots ($5x10^4$ cells/spot) shows significantly reduced numbers of positive cells in diabetic rats (DM, n=7) compared with pre-diabetic rats (no DM, n=7). (** $p<0.01$) (C) Virtually all in vitro expanded isolated sprout cells express Flk-1 (VEGFR2) (red) whereas occasionally smooth muscle α-actin (α-SMA)-positive myofibroblasts were detected (green) (magnification x400). (D) In vitro culture of isolated sprout cells for 4 days in EC medium shows significantly reduced expansion of cells derived from short-term diabetic rats (DM, n=8) compared with pre-diabetic control rats (no DM, n=7).*

Aortic sprout cells from diabetic rats maintain their low proliferation status in vitro

To study whether sprout cells derived from diabetic rats maintain their reduced proliferative capacity under normoglycemic conditions *in vitro*, a fixed number of $2x10^4$ cells was seeded and cultured in EC medium in 6-well plates. After 4 days of culture seeded cells had differentiated into elongated cells that expressed Flk-1 (VEGFR2) suggesting an endothelial phenotype. Occasionally SMA^+

myofibroblasts were detected in these cultures (fig. 3C). After 4 days of culture, cells were trypsinized and counted. As shown in figure 3D, *in vitro* culture of sprout cells obtained from diabetic rats resulted in significantly ($p<0.05$) reduced cell numbers compared with pre-diabetic controls. These data indicate that sprout cells isolated from aortic rings obtained from diabetic rats have reduced proliferative capacity which persists under normoglycemic conditions *in vitro*.

Aortic sprout cells express endothelial markers

To demonstrate that the sprout cells are predominantly endothelial cells, additional immunofluorescent stainings were performed on cytospots of sprout cells isolated after 10 days of culture on Matrigel using antibodies against the EC-markers CD31, Flk-1 (VEGFR2) and the antigen recognized by mAb HIS43. As shown in figure 4, most sprout cells stained positive for CD31 and Flk-1 and to a somewhat lesser extent HIS43. Occasionally a SMA^+ myofibroblast was detected. Similar results were obtained on isolated sprout cells that were cultured for another 4 days (not shown). Together, these data indicate that the majority of the sprout cells are endothelial cells.

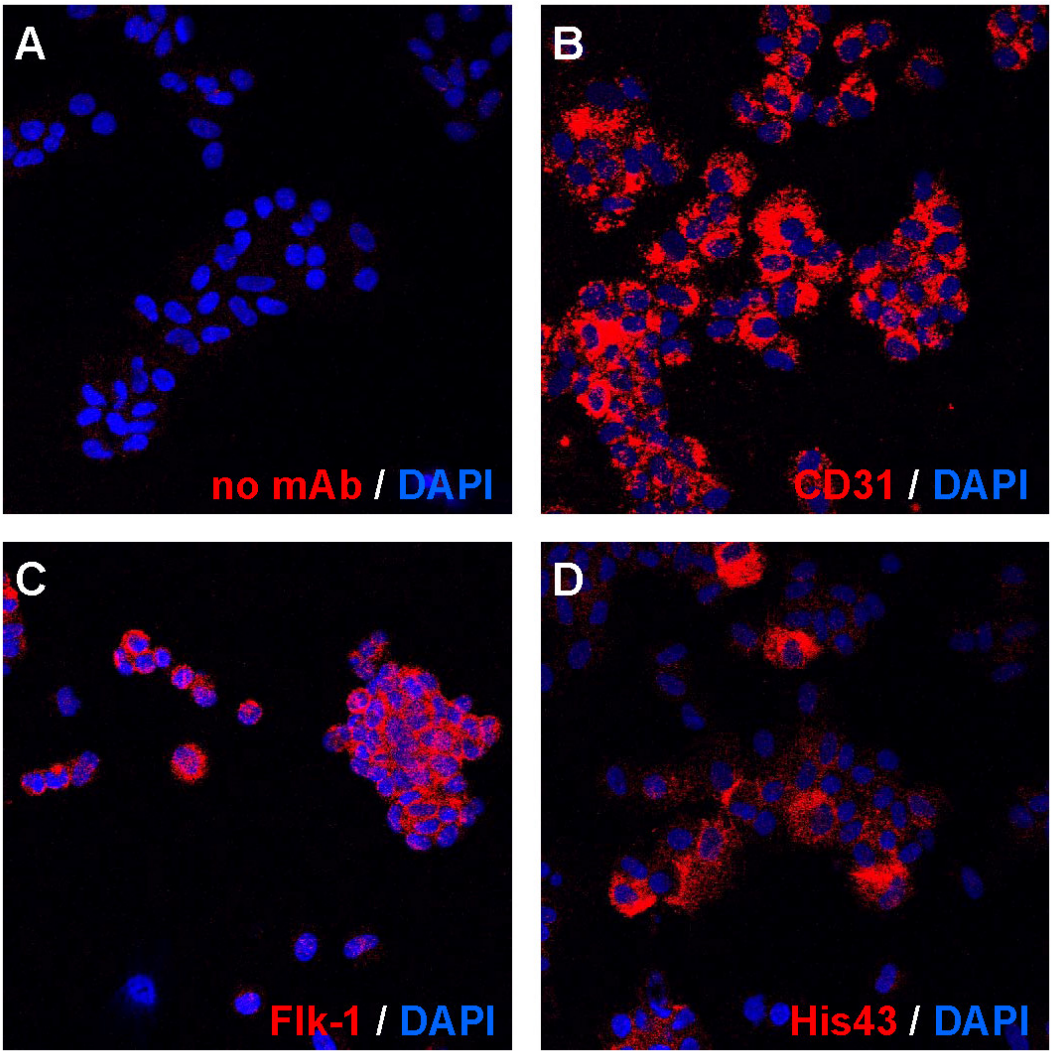

Figure 4. Immunofluorescent staining of sprout cell cytospots (cells isolated after 10 days of culture in Matrigel) indicates an endothelial cell phenotype.
(A) negative control (no primary antibody added), (B) CD31 (PECAM) expression, (C) Flk-1 (VEGFR2) expression, (D) protein expression recognized by HIS43. (magnification x630).

Discussion

Understanding how diabetes affects neovascularization is important for the development of pro- or anti-angiogenic therapeutic strategies. Our data from isolated vessels show that the effects of diabetes on neovascularisation include impaired angiogenic sprouting from cells residing in the vascular wall, independent of effects on circulating cells or circulating angiogenic/anti-angiogenic factors. As the vascular rings and isolated sprout cells were cultured *ex vivo* under normoglycemic conditions, the angiogenic impairment of diabetic sprouting cells is at least to some extent imprinted upon the cells. The model of aortic ring sprouting provides a method to specifically evaluate the effect of potential pro- or anti-angiogenic interventions at the level of the resident cells in the vascular wall. To what extent the impairment observed in this study *ex vivo* is physiologically relevant *in viv*o remains to be established.

Acknowledgements

The reported work was supported by the Netherlands Organization for Scientific Research (NWO VENI-grant 016.036.041 to MCV, VENI-grant 916.46.104 to JLH), the Dutch Heart Foundation (Dr. E. Dekker Grant 2004T022 to PEW) and the Ubbo Emmius Foundation (to GO).

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8

RANTES IS REQUIRED FOR ISCHEMIA-INDUCED ANGIOGENESIS, WHICH MAY HAMPER RANTES-TARGETED ANTI-ATHEROSCLEROTIC THERAPY

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Thrombosis & Haemostasis 2008;99(4):794-795
(letter to the editor)

The cytokine RANTES (Regulated upon Activation, Normal T cell Expressed and Secreted) is involved in initiation of atherosclerotic plaque formation[1] and inhibition of RANTES-receptor activation with Met-RANTES, an amino-terminal-modified methionylated form of RANTES acting as a competitive receptor inhibitor, has been shown to effectively inhibit the development of both atherosclerosis[2] and neointima formation[3]. RANTES secretion from fatty tissue is increased in obese insulin-resistant mice and obese human patients with the metabolic syndrome, which may contribute to an accelerated development of atherosclerosis.[4] Based on these data, it was suggested that inhibiting RANTES-receptors (CCR1 and CCR5) might offer therapeutic potential by targeting the development of atherosclerosis associated with the metabolic syndrome.[4,5] However, atherosclerosis, neointima-formation and angiogenesis share common initiating events involving homing of circulating cells to activated endothelium.[6] Various platelet-derived chemokines are thought to play a bimodal role in affecting both angiogenesis and atherosclerosis.[1,7] For the atherosclerotic patient, it may be of importance to preserve the capacity for angiogenesis during anti-atherosclerotic therapy as neovascularization may be required to restore blood flow to ischemic tissues in case of vessel occlusion. Indeed, studies suggest a role for RANTES in modulating arterial remodeling by affecting the influx of inflammatory cells.[8] We aimed to study whether inhibition of RANTES-receptor activation impairs ischemia-induced angiogenesis.

We injected rats intraperitoneally daily with 200µg Met-RANTES (kind gift from Peter J.Nelson, University of Munich, and Amanda Proudfoot, Serono, Geneva.; n=6) or saline control (n=4) starting prior to subjecting the animals to unilateral hind limb ischemia by femoral artery ligation. The contra-lateral hind limb was sham-operated and served as internal control. Rats were sacrificed after 14 days of ischemia and the gastrocnemic muscles were harvested and snap frozen in isopenthane. Capillaries were visualized by staining 5µm thick sections for endogenous alkaline phosphatase activity with eosin counterstaining. The capillary density was quantified by computer-assisted analysis and expressed as an ischemic to non-ischemic muscle ratio by dividing the average number of capillaries per square millimetre of muscle tissue in sections from the ischemic limb by that in the control limb. ED-1 positive cells were visualized using mouse-anti-rat ED-1 antibody (kindly provided by Ed Dub, Amsterdam, the Netherlands), rabbit-anti-mouse-peroxidase (Dako Cytomation, Carpenteria, CA), and 3,3-diaminobenzidine (DAB; Sigma, Zwijndrecht, the Netherlands). ED-1 positive cells were quantified per muscle fibre based on evaluation of at least three high-power fields by a blinded observer. All data are presented as mean±SEM. A student's t-test was used for statistical analysis and a p-value of <0.05 was considered statistically significant.

We found that saline treated animals had a 75% increase in capillary density in the ischemic hind limb based on comparison to the sham-operated limb (ischemic to non-ischemic muscle ratio=1.75±0.14, fig. 1). In contrast, Met-RANTES treated animals did not show an increase in capillary density following ischemia (ratio= 0.94±0.18, fig. 1). The capillary density ratio was significantly lower in the Met-RANTES treated group compared to saline treated controls (p=0.008, fig. 1). ED-1 positive cell influx was not significantly reduced in Met-RANTES treated animals versus vehicle-treated controls (0.64±0.13 vs. 0.74±0.19 ED-1 positive cells per muscle fibre, p=ns).

From these data we conclude that the presence of functional RANTES is required for angiogenesis following peripheral ischemia. This is in line with previously observed reduced corneal neovascularization in mice deficient in RANTES-receptor CCR5.[9] The exact mechanism through which RANTES regulated ischemia-induced angiogenesis remains unclear. The influx of ED-1 positive moncytes/macrophages was not affected by Met-RANTES. Interestingly, we have found that

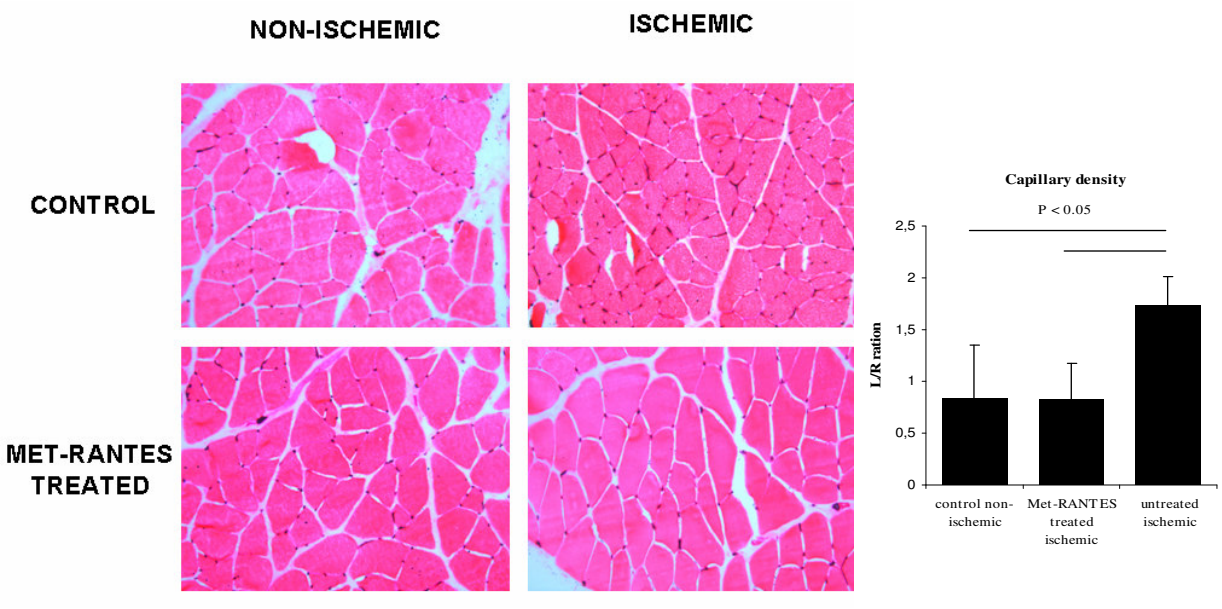

Figure 1. Representative images of muscle sections and quantification of capillary density

Met-RANTES inhibited the influx of angiogenic endothelial progenitor cells (EPC) in experimental glomerulonephritis.[10] Further studies in bone-marrow transplanted animals are required to evaluate if RANTES is also involved in EPC-homing to ischemic muscle. Also, our study focused on peripheral ischemia. Whether neovascularization following cardiac ischemia is similarly affected by inhibition of RANTES receptor activation was not investigated.

The implications for the potential effects of RANTES inhibition in the atherosclerotic patients are speculative. Inhibition of inflammatory cell influx in atherosclerotic plaques is desirable. Angiogenesis is important for neovascularization of ischemic tissues, but interestingly, angiogenesis also occurs in the atherosclerotic plaque, where it is suggested to contribute to plaque progression and destabilization.[11] Therefore, inhibition of RANTES receptor activation may serve as potential therapeutic strategy for reducing the progression of atherosclerosis by inhibition of inflammation or inhibition of plaque angiogenesis, but may have adverse effects by impairing neovascularization of ischemic tissue.

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9

AMELIORATION OF ANTI-THY1-GLOMERULONEPHRITIS BY PPAR-γ AGONISM WITHOUT INCREASE OF ENDOTHELIAL PROGENITOR CELL HOMING

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American Journal of Physiology - Renal Physiology 2008;294(2):F379-384

Abstract

Impaired glomerular endothelial integrity is pivotal in various renal diseases and depends on both the degree of glomerular endothelial injury and the effectiveness of glomerular endothelial repair. Glomerular endothelial repair is in part mediated by bone marrow derived endothelial progenitor cells (EPC). PPAR-γ agonists have therapeutic actions independent of their insulin sensitizing effects, including enhancement of EPC function and differentiation. We evaluated the effect of PPAR-γ-agonist rosiglitazone (4 mg/kg/day) on the course of anti-Thy1-glomerulonephritis in rats. Rosiglitazone limited the development of proteinuria and prevented plasma urea elevation (8.1±0.4 vs. 12.5±1.1 mmol/l, p=0.002). Histologically, inflammatory cell influx was not affected, but rosiglitazone-treated rats did show fewer microaneurysmatic glomeruli on day 7 (26±3 vs. 41±5%, p=0.01) and reduced activation of matrix production with reduced renal cortical TGF-ß, PAI-1 and Fibronectin-1 mRNA expression. However, bone marrow derived endothelial cell glomerular incorporation was not enhanced (3.1±0.4 vs. 3.6±0.3 cells per glomerular cross section; p=0.31). Rosiglitazone treatment in non-nephritic rats did not influence proteinuria, urea or renal histology. In conclusion, treatment with PPAR-γ-agonist rosiglitazone ameliorates the course of experimental glomerulonephritis in a non-diabetic model, but not through enhancing incorporation of bone marrow derived endothelial cells in the glomerulus.

Introduction

Glomerular endothelial injury is a pivotal pathogenic factor in various renal diseases. The glomerular endothelium can recover from injury by replacing lost or damaged endothelial cells. Progression of renal disease may not only depend on the degree of glomerular endothelial injury, but also on the effectiveness of endothelial repair. Traditionally, it was thought that glomerular endothelial repair occurs exclusively through proliferation of local endothelium, stimulated by the release of angiogenic growth factors. However, we[1] and others[2] have observed that damaged glomerular endothelium in human renal disease may also be regenerated from circulating bone marrow derived endothelial progenitor cells (EPC). EPC incorporate into the damaged glomerulus, differentiate into mature endothelial cells and eventually fully integrate into the resident endothelium.[3] In addition, EPC secrete angiogenic factors such as VEGF, which may stimulate endothelial repair by resident endothelial cells.[4] Enhancement of glomerular endothelial repair either by administration of EPC, enhancing the EPC levels or improving EPC function using specific stimuli may represent a novel therapeutic target.

The anti-Thy1-glomerulonephritis model involves both renal injury and repair. It is induced by a single injection of an antibody directed against Thy1, which is expressed on glomerular mesangial cells. This results in a complement-mediated mesangiolysis with secondary endothelial damage.[5,6] Interestingly, the model is self-limiting and fully reversible. Endothelial repair and restoration of glomerular capillary structures is a prominent component of recovery in the model[6] and involves angiogenesis of glomerular capillaries[7], stimulated by the release of angiogenic factors VEGF and bFGF[6,8,9]. Consistently, the limited upregulation of angiogenic factors in uninephrectomized rats injected with anti-Thy-1.1. antibody is associated with a progressive course of the glomerulonephritis.[10] We have shown that glomerular endothelial repair after anti-Thy1-glomerulonephritis is in part dependent on bone marrow derived EPC.[11]

Recently, it was observed that PPAR-γ-agonist rosiglitazone, a thiazolidinedione, can promote EPC differentiation and accelerate EPC-mediated reendothelialization of a denuded segment of the femoral artery in mice.[12] Furthermore, the number and function of EPC cultured from human blood was enhanced by rosiglitazone *in vitro*.[12,13] We therefore hypothesized that rosiglitazone treatment would ameliorate the course of anti-Thy-1-glomerulonephritis due to enhanced contribution of bone marrow derived EPC. Here we demonstrate that rosiglitazone indeed improves the course of anti-Thy-1-glomerulonephritis. However, this favorable effect on renal function could not be explained by enhanced EPC-mediated replacement of damaged glomerular endothelium.

Methods

Animals

Male 11-week-old Brown Norway/RijHsd and Wag/RijHsd rats weighing 280-300 g (Harlan, Horst, the Netherlands) housed in a 12/12 hour light dark cycle and receiving food and acidified water *ad libitum* were used for all experiments. The animal ethics committee of our institution approved all protocols.

Experimental Design

For the first study, 16 Brown Norway (BN) rats were treated with PPAR-γ agonist rosiglitazone (GlaxoSmithKline) starting 3 days prior to anti-Thy-1-injection (day –3) and compared to 12 BN controls. Rosiglitazone was mixed through powdered chow, and food intake was measured. The

average dose received during the study was 4 mg/kg bodyweight/day. Anti-rat Thy-1.1 monoclonal antibody (ER4, 1 mg/kg bodyweight) was injected intravenously on day 0 in all animals except for four rosiglitazone-treated rats, who served as non-nephritic controls. Rats were placed in metabolic cages with free access to food and water at several time points to collect 24-hour urine samples. Systolic blood pressure was measured in conscious animals by tail cuff sphygmomanometry (IITC, San Diego, CA, USA). The animals were sacrificed at day 7 and day 28, and kidneys were excised after perfusion at 120 mmHg with ice-cold 0.9% saline. Kidney specimens were transversely cut in 1mm slices and snap-frozen in liquid nitrogen or fixed in 4% buffered formaldehyde and embedded in paraffin.

For the second study BN rats underwent an allogenic bone marrow transplantation with WagRij bone marrow ($WagRij_{BM}$→Brown Norway) as previously described[11], allowing identification of bone marrow derived cells in the rat chimeras based on the expression of WagRij-specific MHC-I haplotype. A minimal chimerism of 90% was required to enter the study protocol. Of 16 $WagRij_{BM}$→Brown Norway bone marrow transplanted rats 8 were treated with rosiglitazone starting at day –3. All rats received anti-Thy-1 on day 0 and were terminated at day 7.

Measurements of proteinuria, plasma urea, and urinary NO_x excretion
Urinary protein concentration was determined by Bio-Rad Protein assay (Bio-Rad laboratories GmbH, München, Germany) in 24-hour urine samples, plasma urea was measured enzymatically (Elitech, Sees, France), and urinary total NO_x using a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Tallinn, Estonia).

Renal histology
Renal morphology was evaluated using Periodic Acid Schiff (PAS) stained 5 μm paraffin sections based on at least 50 glomeruli per kidney section. The percentage of glomeruli containing microaneurysms was counted in sections from rats terminated at day 7.

Renal cortex mRNA isolation and real-time PCR
The renal cortex was separated from the medullary region in 1mm frozen kidney slices and total renal cortex mRNA was isolated using the Qiagen RNA isolation kit (Qiagen, Venlo, the Netherlands) according to manufacturer's instructions. For real-time (RT) PCR, 3 μg of total RNA was reverse-transcribed using random hexamers and the TaqMan Reverse Transcription Reagents (Applied Biosystems; Nieuwerkerk a/d IJssel, the Netherlands). RT-PCRs for rat TGF-β, PAI-1, Fibronectin-1, nephrin and housekeeping gene TBP were performed using mRNA-specific Taqman Primer/Probe assay mixes and universal PCR mastermix (Applied Biosystems). PCR products were amplified during 45 cycles (95°C for 15 sec followed by 60°C for 1 min for each cycle) and analyzed on a RT- PCR cycler (ABI Prism 7900; Applied Biosystems). mRNA expression was quantified using the comparative Ct method with the first control sample as calibrator sample. All samples were assayed in duplicate and averaged for analysis.

Immunohistochemistry
Inflammatory cell influx into the glomerulus was assessed by ED-1 immunohistochemical staining on 5 μm paraffin sections using mouse-anti-rat ED-1 antibody (kindly provided by Ed Dub, Amsterdam, the Netherlands), rabbit-anti-mouse-peroxidase (Dako Cytomation, Carpenteria, CA), and swine-anti-rabbit-peroxidase (Dako Cytomation). ED-1 staining was visualized using 3,3-diaminobenzidine (DAB; Sigma, Zwijndrecht, the Netherlands). ED-1 positive cells were counted in at least 20

glomeruli per kidney. Fibronectin staining was performed on pepsin-pretreated paraffin sections using HRP-conjugated rabbit-anti-human fibronectin antibody known to cross-react with rat fibronectin (Dako Cytomation) and visualized with Nova Red peroxidase substrate (Vector Laboratories, Burlingame, CA). Endothelial cells were identified on frozen sections using anti-RECA-1 (Rat Endothelial Cell Antigen-1; Serotec, Hilversum, the Netherlands) and Nova Red peroxidase substrate (Vector Laboratories).

To identify donor bone marrow (WagRij) derived endothelial cells in bone marrow transplanted animals, 6 μm cryostat sections were incubated with WagRij-specific anti-MHC-I U9F4 (kindly provided by J. Rozing, Groningen, the Netherlands) and anti-RECA-1 (Serotec) antibodies, which were biotinylated and visualized using the DAKO- Animal Research Kit (DAKO, Carpenteria, CA) and tyramide-FITC and tyramide-TRITC respectively (TSA systems, PerkinElmer Life Sciences, Boston, MA), after blocking sections using the avidin and biotin blocking kit (Vector laboratories). Nuclei were stained with DAPI and sections were mounted with Vectashield (Vector Laboratories). The total number of glomerular U9F4 positive and U9F4/RECA double-positive cells were counted in at least 10 glomeruli per kidney section.

To assess endothelial cell proliferation, animals were injected with bromodeoxyuridine (BrdU) 16 hours prior to termination. 6 μm cryostat sections were stained for RECA-1 as described above and then incubated with swine-anti-BrdU-HRP (Abcam, Cambridge, UK), tyramide-FITC, and DAPI. The number of RECA-1/BrdU double-positive cells was counted in at least 10 glomeruli per kidney section. An additional staining for RECA-1 and Ki-67 was performed using biotinylated mouse-anti-rat-Ki-67 (clone MIB-5, DAKO) and tyramide-FITC to confirm the frequency of proliferating endothelial cells identified based on BrdU incorporation.

Two blinded observers performed quantification of stains. Immunofluorescent double-stainings were quantified at 630-fold magnification using a Leica DMR microscope (Leica GmbH, Wetzlar, Germany). Confocal laser-scanning microscopy confirmed the specificity of the fluorescent staining and was used to photograph the sections. Non-fluorescent staining was quantified at 400-fold magnification using a light microscope (Olympus BX40, Zoeterwoude, the Netherlands). For all stains, isotype-stained sections served as controls.

Statistical analysis

All values are expressed as mean ± SEM. Data were analyzed using SPSS version 11.0 software. Differences between groups were analyzed using the two-tailed students' t-test, one-way or two-way ANOVA where appropriate. A p-value of <0.05 was considered significant.

Table 1. Proteinuria in nephritic animals (mg/24hr)

*Proteinuria levels (in mg/24 hrs ± SEM) before and at several time points after aThy1 injection in rats with and without rosiglitazone treatment. * p<0.05 compared to baseline # p<0.05 between groups; n=10 controls vs. 12 rosiglitazone-treated animals at baseline and on day 3 and 7; n=6 vs. 6 on day 14, 21 and 28.*

	Baseline	Day 3	Day 7	Day 14	Day 21	Day 28
Controls	14 ± 1	36 ± 6 *	77 ± 14 *	53 ± 10 *	18 ± 1 *	24 ± 5
Rosiglitazone	15 ± 1	34 ± 3 *	51 ± 7 *	15 ± 2 #	14 ± 1 #	11 ± 1 *

** p<0.05 vs. baseline; # p<0.05 vs. control at the same time-point*

Results

Rosiglitazone was well tolerated

No adverse effects were observed with rosiglitazone treatment, notably no edema or hyperphagia. Food intake and weight gain was not affected by rosiglitazone treatment. All nephritic rats experienced a transient decrease in bodyweight after induction of anti-Thy-1-glomerulonephritis, but rosiglitazone intake remained stable. Systolic blood pressure tended to be slightly reduced with rosiglitazone treatment (111±3 vs. 118±2 mmHg, p=0.08).

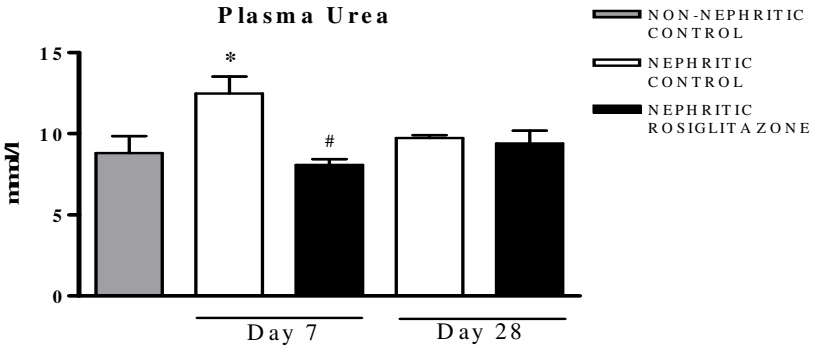

Figure 1. Plasma urea levels
Plasma urea levels in rats with and without rosiglitazone treatment at day 7 and 28 after aThy1 injection, and in non-nephritic rats with rosiglitazone treatment.
** p<0.05 vs. non-nephritic control at day 7*
 # p<0.05 vs. nephritic control at day 7

Rosiglitazone ameliorates renal function impairment in anti-Thy-1-glomerulonephritis

Induction of glomerulonephritis caused an increase of proteinuria in all nephritic rats peaking at day 7 (table 1). Rosiglitazone treatment ameliorated the increase in proteinuria and lead to an earlier recovery to baseline levels with proteinuria returning to normal values at day 14 vs. day 21 in controls. Rosiglitazone did not affect proteinuria in non-nephritic rats (data not shown). Rosiglitazone prevented the significant increase in plasma urea observed in nephritic control rats on day 7 after anti-Thy-1-injection (12.5±1.1 vs. 8.1±0.4 mmol/L on day 7, p=0.002, fig. 1). On day 28 plasma urea levels of all nephritic controls had normalized to baseline levels and were not significantly different between nephritic rats with or without rosiglitazone treatment and non-nephritic rats (9.4±0.8; 9.7±0.2 and 8.8±1.0 mmol/L respectively, p=0.67, fig. 1).

Rosiglitazone reduces microaneurysm formation and activation of extracellular matrix production

Inflammatory cell influx is an early key event in the development of anti-Thy1-glomerulonephritis. Rosiglitazone treatment did not reduce the number of ED-1-positive cells in the glomeruli at day 7 (6.2±0.5 vs. 7.3±0.5, p=0.15). Also, glomerular endothelial cell surface by semi-quantitative assessment of RECA-staining was not significantly influenced by rosiglitazone treatment (1.62±0.30 vs. 1.38±0.22 on an semi-quantitative score between 0 and 4; p=0.52). However, rosiglitazone treated animals did have a significantly lower percentage of micro-aneurysmatic glomeruli at day 7 after anti-Thy-1-injection than controls (26±3 vs. 40±5%, p=0.01).

Rosiglitazone-treated animals also had reduced activation of matrix production as renal cortex mRNA expression of TGF-β was reduced (1.5-fold; p=0.006, fig. 2A) together with reduced expression of TGF-β target genes PAI-1 and fibronectin-1 (2.4 and 1.8 fold down regulation; p<0.001 and p=0.02 respectively, fig. 2BC). Immunohistochemical staining for fibronectin confirmed that rosiglitazone reduced extracellular matrix production at the protein level (2.01±0.35 vs. 1.31±0.11 on a semi-quantitative score between 0 and 4 for extent and intensity of glomerular fibronectin staining; p=0.04, fig. 2DE). The number of BrdU/RECA double-positive cells was not different between rosiglitazone treated and control animals and relatively low (0.065±0.046 vs. 0.058±0.038 /HPF; p=0.90). Double-staining for Ki-67 and RECA confirmed the low incidence of proliferating endothelial cells in both

groups regardless of treatment. Urinary excretion of nitrate/nitrite (NOx), metabolites of NO, tended to be higher in rosiglitazone treated animals than in controls (12.0±0.5 vs. 9.9±1.0 µmols/24hrs, p=0.07).

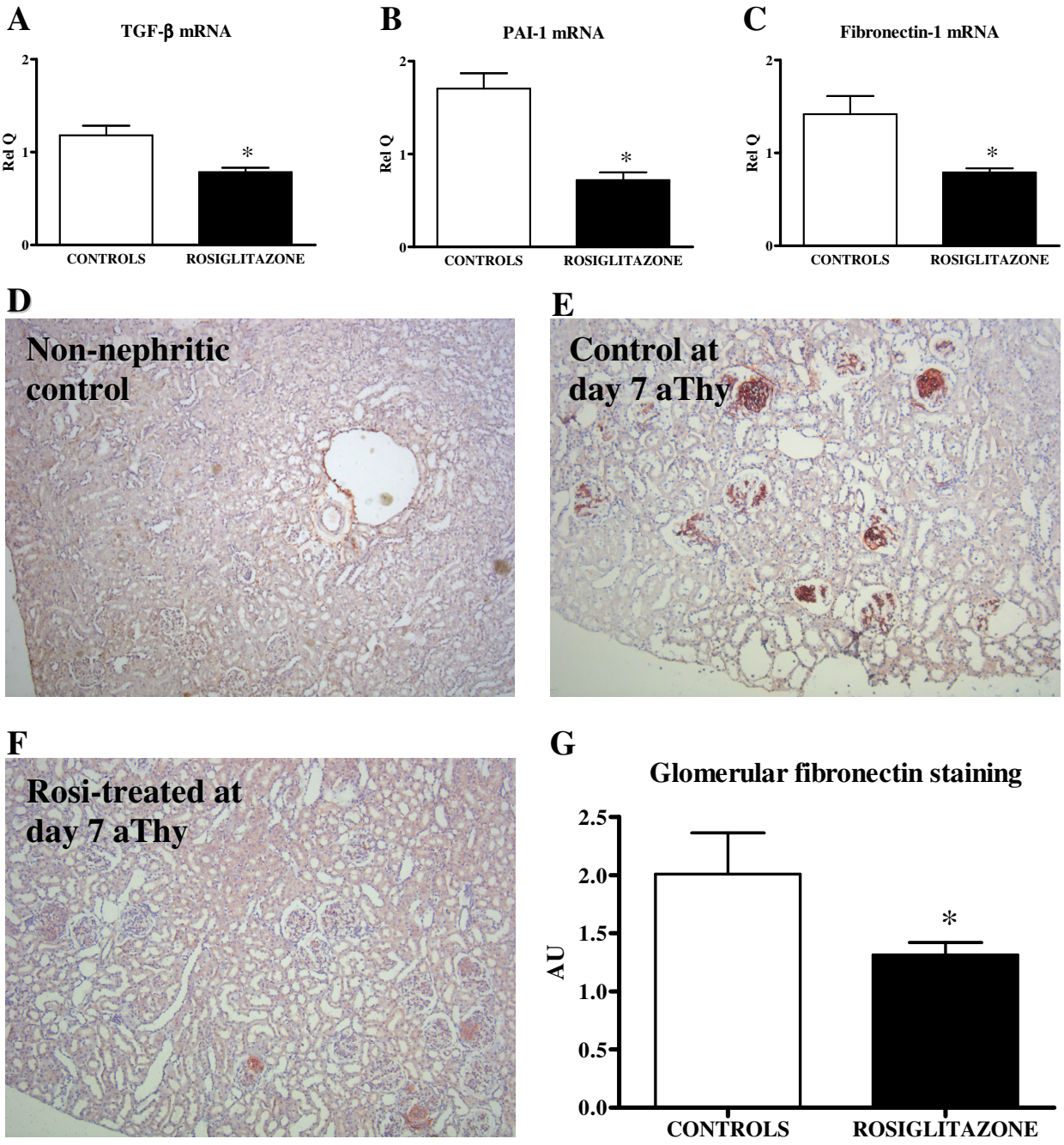

Figure 2. Activation of extracellular matrix production on day 7 after a-Thy1 glomerulonephritis
*Renal cortical mRNA levels of TGF-β (A), PAI-1 (B) and fibronectin-1 (C) were lower in rosiglitazone-treated animals. Staining for fibronectin confirmed reduced activation of extracellular matrix production. Typical examples of glomerular fibronectin staining in a non-nephritic rat (D) and in control (E) and rosiglitazone-treated (F) rats at day 7 after anti-Thy1-glomerulonephritis are shown. Semi-quantitative assessment of extent and intensity of staining performed by a blinded observer showed a significant reduction with rosiglitazone of glomerular fibronectin staining at day 7 after induction of a-Thy1 glomerulonephritis(G); n=8 kidneys from 4 controls vs. 12 kidneys from 6 rosiglitazone-treated nephritic animals. * p<0.05 vs. controls*

Renal nephrin expression in anti-Thy1-glomerulonephritis is not attenuated by rosiglitazone

As treatment with PPAR-γ-agonist pioglitazone was previously shown to attenuate the development of proteinuria through downregulation of renal nephrin in passive Heymann nephritis[14], we measured renal cortical nephrin mRNA levels, but did not find a statistically significant effect of rosiglitazone treatment (1.44±0.32 vs. 1.17±1.18 Rel Q; p=0.45).

Rosiglitazone does not affect the glomerular incorporation of bone marrow derived endothelial cells after anti-Thy1-glomerulonephritis

To evaluate a possible modulating effect of rosiglitazone on the incorporation and differentiation of bone marrow derived progenitor cells, we performed immunofluorescent stainings on kidney sections from Brown Norway rats with anti-Thy1-glomerulonephritis that had previously undergone bone marrow transplantation from WagRij rats. Rats were sacrificed 7 days after injection with anti-Thy-1, at which time point we had previously seen the highest number of incorporated bone marrow derived endothelial cells.[11] Double-staining with WagRij-specific anti-MHC-I U9F4 antibody and endothelial specific RECA antibody confirmed the presence of bone marrow derived endothelial cells in the glomeruli (fig. 3), but the number of these cells per glomerulus was not influenced by rosiglitazone treatment (3.1±0.4 vs. 3.6±0.3 U9F4/RECA double-positive cells per glomerular cross section; p=0.31, fig. 3). The total number of bone marrow derived cells was also equal (6.0±0.5 vs. 6.8±0.5 U9F4-positive cells per glomerular cross section; p=0.27, fig. 3).

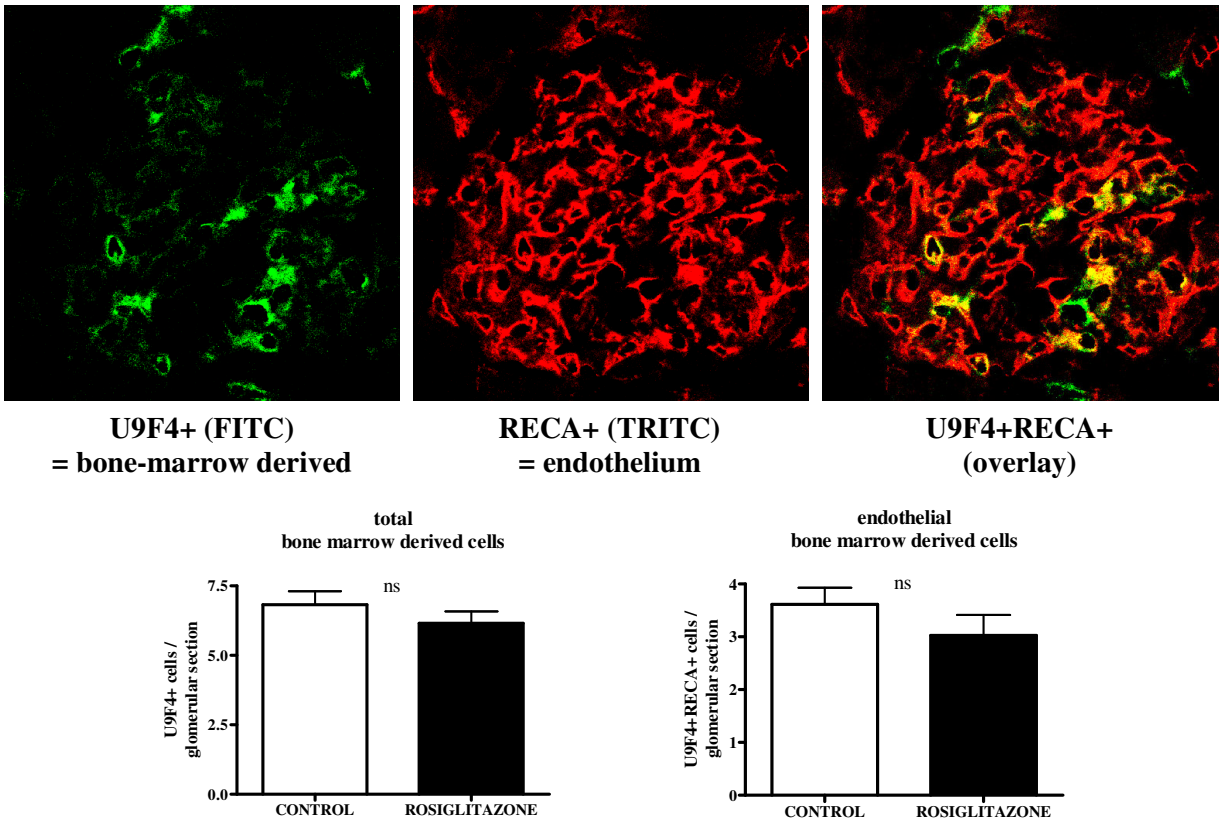

Figure 3. Identification and quantification of bone marrow derived endothelial cells in the glomeruli

Immunofluorescent images showing U9F4-positive bone marrow derived cells (FITC-channel), RECA-positive endothelial cells (TRITC channel) and a partial overlap in the overlay, identifying U9F4/RECA double-positive bone marrow derived endothelial cells. Quantifications of the total number of U9F4-positive bone marrow derived cells and U9F4/RECA double-positive bone marrow derived endothelial cells averaged per glomerular cross section at day 7 after aThy1-injection do not show an effect of rosiglitazone treatment (± SEM, ns=non significant).

Discussion

Our data show that treatment with the PPAR-γ-agonist rosiglitazone ameliorates the impairment of renal function following induction of anti-Thy-1-glomerulonephritis. Rosiglitazone partly prevented the development of proteinuria, led to an earlier normalization of the proteinuria, fully prevented plasma urea elevation, attenuated microaneurysm-formation, and limited activation of matrix production after induction of the glomerulonephritis. However, rosiglitazone did not increase the participation of bone marrow derived progenitor cells in glomerular endothelial repair.

PPAR-γ-agonists are widely used in the treatment of Type II diabetes mellitus, but may have therapeutic potential based on drug actions beyond the metabolic effects. Several experimental studies in rats showed favorable effects of PPAR-γ-agonism on non-diabetic renal pathology[15], including anti-GBM antibody-induced crescentic glomerulonephritis[16], passive Heymann nephritis[14], the development of glomerulosclerosis after 5/6 nephrectomy[17] and renal ischemia-reperfusion induced damage[18]. The course of anti-Thy-1-glomerulonephritis is determined by the degree of inflammatory cell invasion and inflammatory damage[19], as well as by the degree of endothelial repair[7]. Although PPAR-γ-agonism may have anti-inflammatory effects[20], we did not observe a significant effect of rosiglitazone on the glomerular influx of ED-1-positive monocytes/macrophages in our study. In other animal glomerulonephritis models, the consequences of PPAR-γ-agonist treatment on inflammatory cell glomerular influx have been variable as both enhancement (in anti-thymocyte induced glomerulonephritis[21]) and inhibition (in crescentic[16] and passive Heymann[14] glomerulonephritis) have been reported. The lack of effect of rosiglitazone on the influx of ED-1-positive monocytes/macrophages or endothelial cell loss in our model suggests that in our study the inflammatory stimulus from anti-Thy1 binding to the mesangium and subsequent loss of endothelial cells was not influenced by rosiglitazone treatment. However, we cannot exclude some effect of rosiglitazone on the induction of glomerular/endothelial damage, as damage and repair of mesangial and endothelial cells occur simultaneously in this model. Furthermore, even in the absence of structural differences, functional effects on inflammatory or glomerular cells may underlie the observed attenuating effects.

As endothelial repair also involves glomerular endothelial cell proliferation[8] we assessed the effect of rosiglitazone on endothelial cell proliferation in our study, but we did not observe an enhancement of glomerular endothelial proliferation and could not detect a difference in glomerular endothelial cell content. This is in line with *in vitro* studies performed by others, in which rosiglitazone inhibited rather than stimulated endothelial cell proliferation.[22] Endothelial integrity does not only rely on the number of cells, but also on their function. A hallmark feature of endothelial function is the capacity of the endothelium to produce nitric oxide.[23] EPC are also capable of producing NO.[24] Several studies have shown that enhanced NO availability improves the clinical course of anti-Thy-1-glomerulonephritis[25,26], presumably via stimulation of eNOS. Consistently, enhancement of nitric oxide stimulated cGMP production by soluble guanylate cyclase slows progression of anti-Thy1-glomerulonephritis.[27] Rosiglitazone has been shown to enhance NO-production by increasing eNOS transcription in isolated endothelial cells and eNOS phosphorylation in mice hearts[28] and isolated endothelial cells[29]. In human Type 2 diabetic patients, rosiglitazone treatment was shown to increase intrarenal NO levels, which was associated with improvement of renal hemodynamics and reduction of proteinuria.[30] This suggests a renoprotective effect of rosiglitazone mediated by improvement of renal endothelial function. A similar beneficial effect of rosiglitazone on endothelial function may have contributed to ameliorating the course of anti-Thy-1-glomerulonephritis in our study, as rosiglitazone treatment was associated with increased urinary levels of NO metabolites (NOx),

although this did not reach statistical significance. Increased glomerular NO levels by rosiglitazone may have contributed to vasodilation of the afferent arterioles[31], thereby limiting the development of microaneurysmata.

We previously demonstrated that the regenerated glomerular endothelium during recovery from anti-Thy-1-glomerulonephritis originates in part from bone marrow derived EPC.[11] Although we observed no significant differences in endothelial cell content between the groups, this may have been caused by a high variability in RECA-positive cell quantification, probably due to the irregular RECA staining pattern in the injured glomerulus. Thus this does not exclude a potential effect on endothelial cell proliferation or incorporation of bone marrow derived endothelial cells, particularly since the number of bone marrow derived cells in untreated nephritic rats is modest (3 cells per glomerular section in this study, which is in accordance with our previous observations[11]). Rosiglitazone is known to stimulate EPC function and differentiation in mice[12] and humans[12,13], which appears to be a drug class effect as PPAR-γ-agonist pioglitazone has similar effects in mice[32] and humans[33]. We therefore specifically investigated the effect of rosiglitazone on the contribution of EPC to restoration of glomerular endothelium in our model. Using a rat allogenic bone marrow transplantation model we show that treatment with rosiglitazone does not enhance the number of incorporated bone marrow derived glomerular endothelial cells in the recovering glomerulus. We cannot exclude that rosiglitazone may not have enhanced the incorporation/proliferation of bone marrow derived endothelial cells because circulating endothelial progenitor cell levels do not increase in rats. However, as an increase in circulating EPC with PPAR--γ-agonist treatment occurs both in mice[12,32]and humans[12,13,33], we think this is not likely.

In conclusion, we have shown that PPAR-γ-agonism with rosiglitazone ameliorates the course of anti-Thy-1-glomerulonephritis, which may be consistent with enhanced regeneration of glomerular endothelium. However, this could not be attributed to stimulation of the incorporation of bone marrow derived EPC to glomerular recovery. Our study does bring further evidence that thiazolidinediones may have beneficial effects on non-diabetic renal disease, including attenuation of the course of acute glomerulonephritis.

Acknowledgements
The technical assistance of Paula Martens, Ria de Winter and Nel Willekes is gratefully acknowledged.

Grants
The reported work was supported by a grant by the Dutch Kidney Foundation. (PC127). PEW is a research fellow of the Dr. E. Dekker program (2004T022) of the Dutch Heart Foundation. The Netherlands Organization for Scientific Research supports MCV (NWO VENI-grant 016.036.041).

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10

LIPID-LOWERING THERAPY RESTORES REDUCED ENDOTHELIAL PROGENITOR CELL LEVELS IN OBESE MEN WITH THE METABOLIC SYNDROME WITHOUT MANIFEST CARDIOVASCULAR DISEASE

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in revision

Abstract

Aims

EPC contribute to endothelial regeneration and thereby protect against cardiovascular disease (CVD). Patients with manifest CVD have reduced EPC levels, but it is not clear if this also occurs in subjects at high CVD risk without manifest atherosclerotic disease. Therefore, we aimed to first, measure circulating levels of Endothelial Progenitor Cells (EPC) in subjects without manifest CVD but at high cardiovascular risk due to obesity and presence of the metabolic syndrome. Second, we evaluated the effect on EPC levels of two lipid-lowering treatments.

Methods and Results

Circulating CD34+KDR+ EPC levels were reduced by nearly 40% in obese men with the metabolic syndrome compared to non-obese healthy controls (331±193 vs. 543±164 EPC/ml, p=0.006). In a randomized double-blind cross-over study comparing intensive lipid-lowering treatment using 80mg simvastatin mono-treatment with combination treatment of 10mg simvastatin and 10mg ezetimibe, we found a similar treatment effect on EPC levels. Secondary analyses of these data suggested that both treatment regimens had increased circulating EPC to control levels (626±428 after combination treatment, $p<0.01$; 524±372 EPC/ml after monotherapy, $p<0.05$). Serum levels of EPC-mobilizing factor SCF-sR correlated with reduced EPC levels and normalized concurrently with treatment.

Conclusion

EPC levels are reduced in apparently healthy men with abdominal obesity and the metabolic syndrome, even in the absence of manifest CVD. This is important as EPC contribute to endothelial regeneration and thereby protect against CVD. SCF-sR may be a candidate serum marker of circulating EPC levels. Treatment with low-dose statin with ezetimibe combination therapy or high-dose statin monotherapy has similar effects on the reduced EPC levels.

Introduction

The intact endothelium and maintenance of endothelial integrity play a central role in protecting against the development of atherosclerotic cardiovascular disease (CVD).[1] Cardiovascular risk factors cause loss of endothelial cells or impair endothelial cell physiology. In recent years, it has become clear that bone marrow derived endothelial progenitor cells (EPC) may replace damaged or lost endothelial cells.[2]. However, the presence of CVD is associated with lower numbers of circulating EPC.[3,4] Interestingly, the reduced EPC numbers correlated with the impairment of endothelial function.[5] Reduced EPC levels quantified as CD34+KDR+ cells on flowcytometry were also found to be an independent predictor of cardiovascular events in patients with coronary artery disease in two prospective cohort studies.[6,7] These observations are consistent with a pathogenic role of decreased endothelial protection due to reduced levels of circulating EPC in atherosclerotic disease progression. However, these findings are mostly based on studies in patients with manifest CVD. Whether the presence of CVD risk factors affects circulating CD34+KDR+ EPC levels in subjects in the general population without manifest CVD and thus in an earlier pathophysiological state, is not as clear. We hypothesized that reduced EPC levels are present in apparently healthy subjects without manifest atherosclerotic disease but at high CVD risk. We therefore studied EPC levels in obese men recruited from the general population with metabolic abnormalities consistent with the metabolic syndrome, which is a highly prevalent condition, and in age-matched healthy volunteers. Furthermore, as restoring reduced EPC levels may improve endothelial protective capacity and attenuate the development of CVD, we evaluated the effect of lipid-lowering therapy on circulating EPC levels in these men. Statins have previously been shown to increase EPC-levels in mice[8-10] and patients with manifest CVD[11,12]. It is not clear if the increase in EPC levels observed with statin treatment is fully mediated by the LDL-lowering effect. In support of a pivotal role of LDL lowering are the inverse correlation between EPC numbers and LDL plasma levels in hypercholesterolemic patients[13] and the observation that non-pharmacological cholesterol reduction by changing dietary habits increases EPC levels[14]. However, a previous study in chronic heart failure patients showed that lipid-lowering therapy with 10 mg simvastatin but not 10 mg ezetimibe monotherapy enhanced EPC levels[12], which may point towards a pleiotropic action of statins. Although ezetimibe monotherapy is not commonly used clinically, combination therapy of ezetimibe and a statin is an effective LDL-lowering strategy and becoming regular practice. However, there may be uncovered disadvantages of this combination therapy because of the decreased statin dose. Less effective stimulation of circulating EPC levels may be such a disadvantage. To date this has not been studied. We performed a double blind randomized cross-over trial to compare the effects on EPC levels of high-dose HMG-CoA-reductase inhibitor simvastatin (80 mg) monotherapy with combination therapy of low dose simvastatin (10 mg) and the cholesterol-absorption inhibitor ezetimibe (10mg).

Methods

Subjects

The Institutional Review Board of the University Medical Center Utrecht approved the study protocol and the study was conducted in accordance with the Declaration of Helsinki. All participants in the study gave their written informed consent. Study subjects were recruited from the general population by posted advertisements and advertisement in the local newspaper asking obese men with a waist circumference of >102 cm to come to our clinic for further screening. 32 subjects were screened for inclusion and exclusion criteria. We included 20 male subjects between 18 and 70 years of age with a body mass index (BMI) of > 25 but < 35 kg/m^2 who also met the ATP III criteria for the metabolic syndrome, i.e. three or more of the following criteria: waist circumference > 102 cm; blood pressure

≥ 130 mmHg systolic or ≥ 85 mmHg diastolic; serum triglycerides ≥ 1.70 mmol/l (150mg/dl); low high-density lipoprotein (HDL) cholesterol < 1.04 mmol/l (40 mg/dl); fasting glucose ≥ 6.1 mmol/l (110 mg/dl). Glucose level ≥7.8 mmol/l after a standardized oral glucose tolerance test was also regarded as fulfilling the glucose criterion. Ten age-matched healthy male controls were recruited by posted advertisements, who were allowed to meet no more than two of the ATP-III criteria for the metabolic syndrome.

Exclusion criteria were smoking, thyroid disease (TSH >5 mU/l with clinical symptoms of hypothyroidism), hepatic disease (ASAT or ALAT > 2 times upper limit of normal), renal disease (serum creatinine >1.7 times the upper limit of normal), a history of CVD (coronary heart disease, cerebrovascular disease or peripheral arterial disease), use of drugs for the primary prevention of cardiovascular disease including lipid lowering and antihypertensive medication, blood pressure ≥ 180/110 mmHg, BMI > 35 kg/m^2, HbA1c > 6.5% or triglycerides > 8.0 mmol/L. All participants in this study were assessed on an individual basis by trained research physicians or research nurses during a screening visit to our hospital using a standard score sheet and identical calibrated machines to perform anthropometric measurements. Routine physical examination was performed prior to entering the study protocol. Flowcytometric analysis of EPC was performed on an individual basis immediately after blood collection. Blood plasma was isolated using a standard protocol and stored at -80 degrees celcius until further analysis, which was performed in single batches including samples from both controls and study subjects before and after treatment.

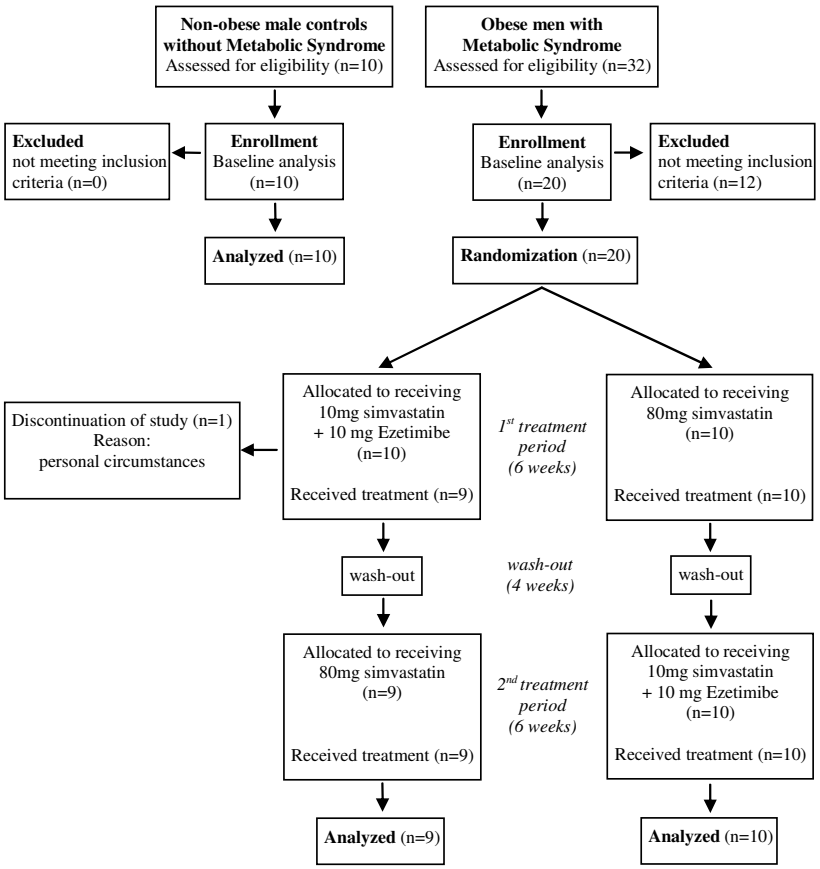
Subjects meeting inclusion and exclusion criteria were enrolled in the study without further selection based on the screening order until the target sample size was achieved. The pre-specified target sample size of 10 controls and 20 obese subjects with the metabolic syndrome was based on previous studies at our laboratory involving EPC-quantification in populations at increased CVD risk.[15,16] Obese subjects with the metabolic syndrome were entered in a randomized, cross-over, double blind trial. Randomization was performed at the pharmacy department with the use of sub-blocks with 4 subjects per sub-block. A number was assigned to each participant, which was used as a code for the data collection and analysis period of the study. After a baseline visit, subjects were randomized to a treatment order of receiving either low dose (10mg) simvastatin combined with 10mg cholesterol-absorption inhibitor ezetimibe or high-dose (80mg) simvastatin monotherapy first. Subjects were asked not to make changes in regular physical activity during the study or to try to lose weight. The effect of treatment was assessed after 6 weeks drug-use. Following a 4 week washout period, subjects received the alternative treatment for again 6 weeks. Randomization was blinded to the study subjects, participating research physicians and nurses and other members of the study group. The pills for the two treatments were specially prepared for our study by the pharmacy department at our institution and carried no label. There were no visual differences between the pills containing the simvastatin monotherapy and those containing the simvastatin/ezetimibe combination therapy. Data collection an analysis was performed using coded samples. Laboratory analyses were performed in batches including all samples from all subjects at all time points where possible. The code for treatment order was broken only after all of the data collection and analysis had been completed. Study enrolment and completion of patients and controls, as well as schematic overview of the study design is shown in a patient flow diagram in figure 1.

Lipid profile, hsCRP, VEGF, SCF, and SCF-sR measurements

Fasting blood was sampled for all measurements. Total cholesterol, HDL cholesterol, and LDL-cholesterol were analyzed using commercially available assays (Wako, Osaka, Japan) with a Cobas

Mira auto analyzer (Roche, Basal, Switzerland). VLDL-cholesterol was calculated (VLDL-cholesterol = total cholesterol minus LDL-cholesterol and minus HDL-cholesterol). Plasma triglycerides were measured using a commercially available assay (Unimate, Roche), as were apolipoprotein A and B levels (Wako). hsCRP, VEGF, SCF, and SCF-sR levels were measured by commercially available ELISA according to the manufacturer's instructions (R&D systems, Minneapolis, USA).

Figure 1. Patient flow diagram



Anthropometric measurements

Weight and height were measured and BMI was calculated as weight to height squared. Waist circumference was measured halfway between the lower rib and the iliac crest. Body fat percentage was estimated by using Omron body fat monitor BF306 (Omron, Matsusaka, Japan). Blood pressure was measured using a semiautomatic oscillometric device (Omega 1400, Invivo research laboratories Inc., Oklahoma, USA).

Flow cytometry for circulating EPC and HSC

EDTA blood was incubated with anti-CD34-FITC (BD Pharmingen, San Diego, USA), anti-KDR-PE (R&D Systems), and anti-CD45- PE-Cy7 (BD Pharmingen) antibodies. Erythrocytes were lysed in an ammonium chloride buffer. CD34+ Hematopoietic Stem Cells (HSC) and CD34+KDR+ EPC were quantified in duplicate relative to the number of CD45+$FS_{high}SS_{high}$ granulocytes in the sample using a flow cytometer (Beckman Coulter, Fullerton, USA). HSC and EPC numbers per ml blood were subsequently estimated based on a full blood granulocyte count made using a hematocytometer. Isotype-stained samples served as negative controls.

Statistical analysis

Data are expressed as mean±SD and were analyzed using SPSS version 12.0 or Graphpad Prism version 4.0 software. Normal distribution of data and equality of variances were tested where needed. The population of interest for this study were obese men with metabolic syndrome in the general population. During data collection and analysis, all samples were coded and investigators were blinded to treatment order designation in the randomized controlled trial. Primary data analyses were a) a comparison between EPC levels in obese men with the metabolic syndrome and healthy controls using a students' t-test and b) a comparison between the treatment effect of Simvastatin Monotherapy and Simvastatin/Ezetimibe Combination Therapy using a Student's t-test using data from the randomized controlled cross-over trial included in this study on a per protocol basis. Secondary analyses were a) a comparison between baseline and post-treatment EPC levels for the obese men with the metabolic syndrome using a repeated-measures ANOVA and b) a comparison between post-treatment EPC levels for the obese men with the metabolic syndrome and control levels using regular ANOVA. The Newman-Keuls post-hoc test was used to compare between data sets after ANOVA. Additional, hypotheses-generating, analyses have included similar comparisons between control and obese men with the metabolic syndrome at baseline and after the two treatments, for measurements other than EPC-levels. Here, Bonferroni correction for multiple testing was not included. Regression analysis was performed using linear univariate models. A p-value of <0.05 in two-sided analysis was considered statistically significant.

Table 1. Baseline subject characteristics and effect of treatment on general parameters

	Controls	MetS Baseline	MetS after 6 weeks 10mg Simvastatin + 10mg Ezetimibe	MetS after 6 weeks 80mg simvastatin
Metabolic syndrome components				
Triglycerides (mmol/l)	1.70 ± 0.51	1.67 ± 0.56	1.22 ± 0.39#	1.15 ± 0.49 #
HDL-cholesterol (mmol/l)	1.27 ± 0.28	1.14 ± 0.26	1.12 ± 0.26	1.14 ± 0.31
Glucose (mmol/l)	5.2 ± 0.7	6.2 ± 0.7 *	6.1 ± 0.8	6.1 ± 0.6
Waist circumference (cm)	96 ± 6	111 ± 7 *	110 ± 7	111 ± 6
Systolic blood pressure (mmHg)	124 ± 8	138 ± 13 *	131 ± 8	135 ± 13
Diastolic pressure (mmHg)	87 ± 8	89 ± 6	87 ± 5	86 ± 6
Total number of MetS components according to ATP III criteria	0 (0-2)	3 (3-5)	-	-
Other parameters				
Age (years)	46 ± 6	52 ± 8	-	-
Height (m)	1.85 ± 0.06	1.83 ± 0.06	-	-
Weight (kg)	86.9 ± 8.7	100.3 ± 11.5*	100.3 ± 11.3	100.9 ± 11.2
Body mass index (kg/m^2)	25.5 ± 2.3	30.2 ± 2.5*	30.1 ± 2.6	30.2 ± 2.5
Body fat (%)	26 ± 4	31 ± 3*	30 ± 3#	31 ± 3‡
Hemoglobin (mmol/l)	9.7 ± 0.6	9.1 ± 1.0	9.2 ± 0.7	9.4 ± 0.7
Platelets (exp^9/l)	246 ± 71	221 ± 47	214 ± 35	193 ± 39 #
Leucocytes (exp^9/l)	5.5 ± 0.7	5.6 ± 1.1	5.6 ± 1.2	5.5 ± 1.1
Total cholesterol (mmol/l)	5.38 ± 1.09	5.58 ± 0.92	3.75 ± 0.85 #	3.71 ± 0.86 #
LDL-cholesterol (mmol/l)	3.53 ± 0.94	3.73 ± 0.67	2.08 ± 0.45 #	2.10 ± 0.53 #
VLDL-cholesterol (mmol/l)	0.58 ± 0.27	0.71 ± 0.25	0.56 ± 0.37 #	0.46 ± 0.25 #
Apolipoproteïn A (g/l)	123 ± 15	114 ± 15	109 ± 18	112 ± 17
Apolipoproteïn B (g/l)	85 ± 20	98 ± 16	71 ± 18 #	70 ± 17 #
hs-CRP (mg/l)	0.8 ± 0.7	2.9 ± 1.8 *	3.7 ± 3.1	3.9 ± 3.9
10-years risk for coronary heart disease based on FRS charts **	6.4 ± 3.1	11.1 ± 5.6 *	-	-

*Values represent mean ± SD or median (range); * p<0.05 compared to controls, # p<0.05 compared to baseline, ‡ p<0.05 compared to alternative treatment group; ** based on Framingham Risk Score (FRS) sheet: http://www.nhlbi.nih.gov/about/framingham/riskmen.pdf; MetS=Metabolic Syndrome*

Results

Subject characteristics

Besides abdominal obesity, which was the primary screening criterion, the majority of subjects had hypertension and evidence of insulin resistance with high fasting blood glucose levels and/or impaired oral glucose tolerance test. Overall, HDL levels were non-significantly lower compared to control subjects and triglyceride levels were not different from controls. hsCRP levels were higher in the obese men with the metabolic syndrome. The estimated 10-years risk for coronary heart disease based on the Framingham risk score was nearly twofold on average in the obese men with metabolic syndrome compared to controls (11.1±5.6 vs. 6.4±3.1 %, p=0.023). None of the subjects used any medication, except two subjects who used ranitidine and terazosin respectively, which were considered unlikely to interfere with the study. Further details on study subject characteristics are included in table 1. Body weight remained constant during the trial, indicating that subjects adhered to the request not to change diet or exercise patterns.

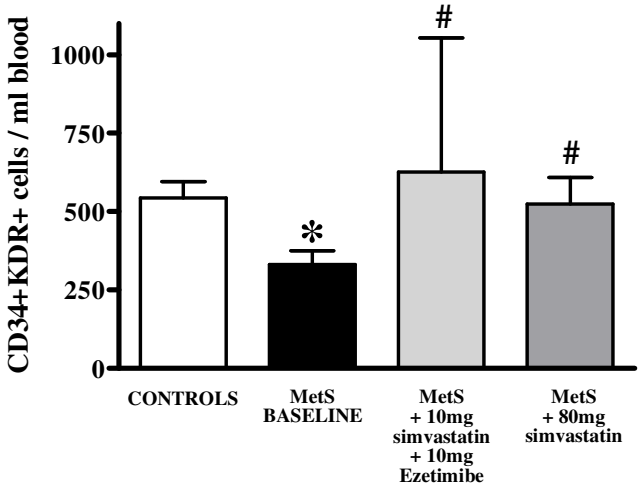


Figure 2. EPC levels are reduced in subjects with the metabolic syndrome. Both lipid-lowering treatments increased EPC levels.
*Circulating levels of CD34+KDR+ EPC quantified per ml blood were reduced in obese men with the metabolic syndrome (MetS) when compared to matched controls. Combination treatment of 10 mg simvastatin with 10 mg ezetimibe lowered LDL-cholesterol similarly as monotherapy with 80 mg simvastatin. There were no significant differences between the two treatment arms. Mean±SD cells/ml. *p<0.05 compared to controls;#p<0.05 compared to baseline*

EPC levels are reduced in obese men with the metabolic syndrome

Circulating EPC levels were 39% lower in obese men with the metabolic syndrome than in controls (331±193 vs. 543±164 /ml blood, p=0.006, fig. 2). The number of CD34+ hematopoietic stem cells was 18% lower, but this was not statistically significant (2509±1117 vs. 3065±1167 /ml blood, p=0.220).

Univariate analysis of obese men with the metabolic syndrome at baseline and controls combined did not show significant correlations between EPC levels and any of the individual components of the metabolic syndrome, although trends towards inverse relationships were observed for EPC levels and waist circumference as well as systolic blood pressure (table 2). The number of components of the metabolic syndrome according to the ATP III criteria present in the individuals was significantly associated with lower circulating EPC levels (standardized regression coefficient beta= -0.517, p=0.004). Significant correlations were observed for reduced EPC levels and the BMI (beta= -0.387, p=0.038) and the body fat percentage (beta= -0.389, p=0.037). Other parameters, such as age (beta= -0.222, p=0.246) and hsCRP levels (beta= -0.138, p=0.475) did not show a significant correlation.

Intensive lipid-lowering therapy with both treatment regimens increases EPC levels

Both intensive lipid-lowering treatment regimens effectively reduced plasma LDL-cholesterol and triglycerides levels, while HDL cholesterol levels remained unchanged (table 1). The combination of

Table 2. Regression analysis for EPC levels in healthy controls and obese men with the metabolic syndrome at baseline combined

	Standardized beta-coefficient	p-value	Non-standardized beta-coefficient (95% CI)
Metabolic syndrome components			
Triglycerides (mmol/l)	-0.164	0.396	-64 (-215 to 88)
HDL-cholesterol (mmol/l)	0.155	0.422	120 (-181 to 421)
Glucose (mmol/l)	-0.298	0.117	-74 (-168 to 20)
Waist circumference (cm)	-0.301	0.113	-7 (-15 to 2)
Systolic blood pressure (mmHg)	-0.361	0.055	-6 (-12 to 0)
Diastolic pressure (mmHg)	-0.060	0.757	-2 (-14 to 10)
Total number of MetS components according to ATP III criteria	-0.517	0.004 *	-72 (-118 to -25)
Other parameters (sign. only)			
Body mass index (kg/m^2)	-0.386	0.038 *	-24 (-47 to -1)
Body fat (%)	-0.389	0.037 *	-20 (-39 to -1)

10 mg ezetimibe and low dose (10mg) simvastatin therapy resulted in equal LDL-reduction as compared to high dose (80mg) simvastatin monotherapy. Surprisingly and not in line with previous studies[17], hsCRP-levels increased after treatment. As this was also not statistically significant, this is most likely attributable to chance.

In our primary analysis of the effect of the two treatment regimens, we found that there was no statistically significant difference in treatment effect (calculated as mean difference in achieved EPC levels, which was -103 EPC/ml blood with a 95% confidence interval of -363 to 157). In a secondary analysis of the data, including also the individual baseline EPC levels, we found that EPC levels increased during both treatment regimens. During 10mg simvastatin/ 10mg ezetimibe combination therapy, EPC levels increased to 626±428 /ml blood (89% increase, $p<0.05$ compared to baseline, fig. 2). Simvastatin monotherapy (80 mg) resulted in 524±372 EPC /ml blood (58% increase, $p<0.05$ compared to baseline, fig. 2). The difference between the achieved EPC levels was no longer reduced compared to controls.

The individual increase in the number in circulating EPC did not correlate with the individual reduction in LDL ($r=0.268$, $p=0.267$ for simvastatin/ezetimibe combination therapy and $r=0.295$, $p=0.221$ for simvastatin monotherapy), although it must be noted that this analysis was insensitive for detecting a correlation at this level as the heterogeneity in cholesterol-lowering was small between subjects.

Table 3. Regression analysis of progenitor cell mobilizing cytokines with progenitor cell levels

	CD34+ HSC			CD34+KDR+ EPC		
	Standardized beta-coefficient	p-value	Non-standardized beta-coefficient (95% CI)	Standardized beta-coefficient	p-value	Non-standardized beta-coefficient (95% CI)
VEGF	0.411	0.027*	9.1 (1.1 to 17.2)	0.433	0.019*	1.7 (0.3 to 3.2)
SCF	0.007	0.970	0.1 (-2.4 to 2.5)	0.275	0.149	0.3 (-0.2 to 0.7)
SCF-sR	0.431	0.019*	46.6 (8.1 to 85.1)	0.493	0.007*	9.7 (2.9 to 16.4)

Serum VEGF and SCF-sR correlate with progenitor cell levels at baseline and are modulated by lipid-lowering treatment

Serum levels of endogenous EPC-mobilizing cytokine VEGF correlated positively with EPC levels (table 3) in the overall dataset of control subjects and subjects with the metabolic syndrome at baseline. VEGF levels in men with the metabolic syndrome were lower than in controls, although not statistically significant (55.1±51.8 vs. 66.6±53.9, p=0.58, fig. 3). Serum levels of progenitor-cell mobilizing SCF were significantly reduced in the metabolic syndrome (1013±151 vs. 1162±210 pg/ml; p=0.04, fig. 3), but did not correlate with circulating progenitor cell levels (table 3). However; serum levels of the soluble form of SCF receptor c-kit, soluble SCF-receptor (SCF-sR), were both significantly reduced in subjects with the metabolic syndrome compared to controls (53±8 vs. 63±9; p=0.003, fig. 3) and correlated with HSC and EPC levels (table 3).

After lipid lowering treatment with both 10mg simvastatin/ 10mg ezetimibe combination therapy and 80 mg simvastatin monotherapy, VEGF levels were substantially reduced to 38.7±35.8 pg/ml (p<0.01, fig. 3) and 36.3±32.9 pg/ml (p<0.01, fig. 3) respectively. Thus EPC levels increased while VEGF levels decreased despite a positive correlation between VEGF and EPC levels at baseline. Serum SCF-sR however, increased to control levels in conjunction with the observed normalization of circulating EPC-levels after treatment with both simvastatin monotherapy (to 68±21 ng/ml; p<0.05, fig. 3) and 10mg simvastatin/ 10mg ezetimibe combination therapy (to 68±19 ng/ml; p<0.05, fig. 3).

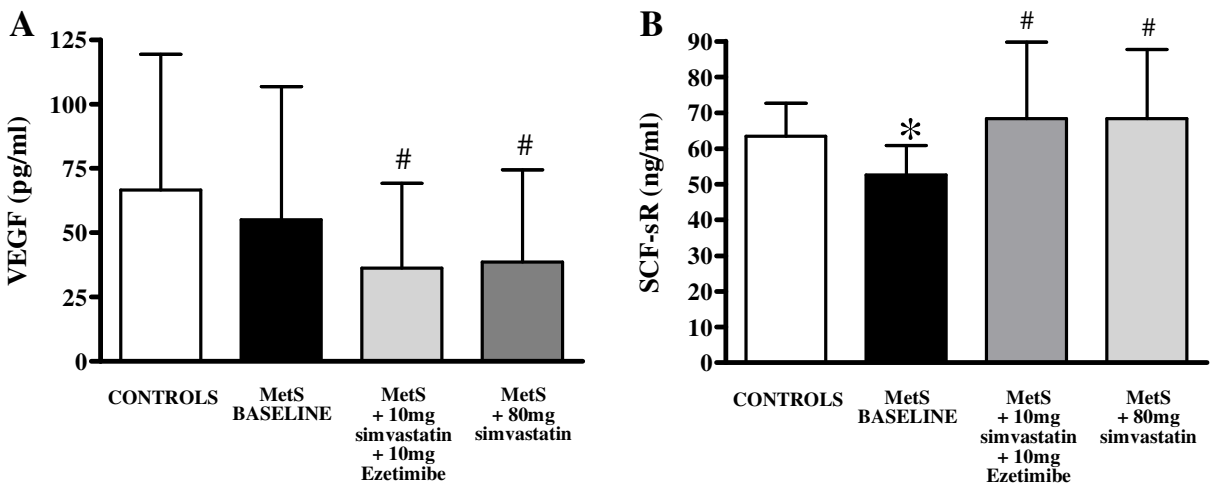


Figure 3. Serum levels of progenitor cell mobilizing cytokines VEGF and SCF-sR before and after treatment
*At baseline, serum VEGF levels were lower in subjects with the metabolic syndrome, although this was not statistically significant (A). After both lipid-lowering treatment regimens, VEGF levels further decreased (A). Serum SCF-sR levels were significantly reduced in subjects with the metabolic syndrome at baseline compared to controls and increased to control levels after both treatment regimens (B). Mean±SD pg or ng/ml * p<0.05 versus controls. # p<0.05 versus baseline*

Discussion

Here we report that obese men with the metabolic syndrome but without diabetes or manifest CVD have low levels of circulating EPC; approximately 40% lower than in age-matched controls without the metabolic syndrome. Obesity-associated parameters BMI and body fat percentage were significant clinical predictors of low EPC levels in univariate analysis. The number of ATP III criteria for the metabolic syndrome correlated inversely with the level of circulating EPC. In addition, our findings that serum levels of the soluble form of SCF receptor c-kit, soluble SCF-receptor (SCF-sR) correlated with HSC and EPC levels, were significantly reduced in subjects with the metabolic syndrome, and

increased to control levels after lipid lowering treatment in conjunction with changes in circulating EPC-levels, suggest the potential use of SCF-sR as a serum marker of circulating progenitor cell levels.

To the best of our knowledge, our study is the first to report an effect of the presence of common CVD risk factors on levels of circulating CD34+KDR+ EPC determined by flow cytometry in obese subjects at high CVD risk due to the presence of the metabolic syndrome recruited from the general population without manifest cardiovascular disease. Metabolic syndrome is a highly prevalent condition that severely impacts the occurrence of CVD in our society. The metabolic syndrome is a clustering of cardiovascular risk factors including abdominal obesity, dyslipidemia, hyperglycaemia, and hypertension.[18] The age-adjusted prevalence is 20-25% in seemingly healthy subjects.[19] People with the metabolic syndrome have a 3 to 4 fold increased risk to develop Type II diabetes[20] and a 2 to 3 fold increased risk for future morbidity and mortality of CVD.[20-22] Therefore, the subjects included in our study have a substantially increased risk of developing CVD and may have pre-atherosclerotic vascular changes or subclinical atherosclerosis[23]. Reduced EPC levels preceding the development of manifest cardiovascular disease is consistent with an early pathophysiological role, which might relate to a decreased capacity to replace damaged or lost endothelial cells. Consistently, in a population of middle-aged subjects from the general population, reduced EPC levels were an independent predictor of an increased intima-media thickness of the carotid artery, which is a strong indicator of subclinical atherosclerotic disease.[24]

We identified circulating EPC using flow cytometry for CD34+ hematopoietic stem cells (HSC) that co-express the endothelial marker KDR. CD34+KDR+ EPC are present in the circulation in low numbers and represent a defined subset of true progenitor cells. Importantly, prospective data showed an independent association of lower CD34+KDR+ EPC levels with increased rates of CVD events.[6,7] In cohort studies of patients with coronary artery disease, decreased levels of circulating CD34+KDR+ EPC were associated with increased age, higher (systolic) blood pressure, smoking, LDL-cholesterol and the presence of coronary artery disease per se.[4,6,7] However, these study populations consisted of patients with manifest cardiovascular disease. Other studies reported on numbers of EPC identified by *in vitro* culture of peripheral blood mononuclear cells under conditions facilitating outgrowth of angiogenic cells with an endothelial phenotype. Associations have been reported between decreased EPC outgrowth in culture and impaired endothelial function, Framingham risk score, Type I and II diabetes, hypertension, renal insufficiency and increased age, in part also in subjects without manifest CVD.[5,15,25,26] However, these cultured EPC constitute a different cell population and are mostly monocyte-derived cells.[27,28] In apparently healthy subjects with occasional presence of cardiovascular risk factors, numeric outgrowth of cultured EPC does not correlate with the number of circulating CD34+KDR+ cells.[29] Our current study shows that subjects from the general population at increased CVD risk but without manifest CVD do indeed have reduced CD34+KDR EPC levels.

Our data from our double blind randomized cross-over trial show that in previously untreated obese men with the metabolic syndrome, the effect on EPC levels of simvastatin/ezetimibe combination treatment is not inferior to a high dose statin treatment achieving an equal reduction in LDL-cholesterol. Secondary analysis of the treatment effects compared to baseline showed that intensive lipid-lowering therapy can normalize decreased CD34+KDR+ EPC levels, even when average LDL-levels were not significantly above control values. Statins have previously been shown to increase EPC-levels in mice[8-10], in patients with coronary artery disease[11], and in patients with chronic heart

failure[12]. In *in vivo* experimental injury models, statin therapy increased EPC levels while restoring age-related impaired neovascularization in mice[30] and augmented EPC-mediated reendothelialization of denuded arterial segments in rats.[31] *In vitro*, statins exerted direct effects on EPC, enhanced EPC differentiation[8,32], enhanced EPC proliferation[9,33], inhibited oxidative-stress induced EPC-apoptosis[34], reduced the rate of senescence[33,35], and enhanced their migratory[9] and angiogenic function[36]. These *in vitro* observations are made in the absence of cholesterol products in their milieu, suggesting that these are LDL-lowering independent effects of statins. Notably, a previous study showed that lipid-lowering therapy with 10 mg simvastatin but not 10 mg ezetimibe enhanced EPC levels in chronic heart failure patients[12], also consistent with a pleiotropic cholesterol-independent effect of statins on EPC levels. This might possibly also imply that low-dose statin/ ezetimibe combination treatment may be less effective in restoring reduced EPC levels than high-dose statin monotherapy. However, in our population, high-dose statin and low-dose statin in combination with ezetimibe resulting in similar LDL-reduction, were equally effective in enhancing EPC levels. This suggests a more important role for LDL-reduction in contrast to potential pleiotropic effects of statin therapy for the observed EPC-increase in our patients. In line with this, non-pharmacological cholesterol reduction by changing dietary habits also increased EPC levels.[14] In addition, in hypercholesterolemic patients, EPC function was impaired and EPC numbers were reduced and inversely correlated with LDL plasma levels.[13] However, an alternative explanation could be that the maximal effect of simvastatin for increasing EPC levels in our study had already been achieved at a dose of 10 mg.

The mechanism through which lipid-lowering therapy restored reduced EPC levels in our study population remains speculative. *In vitro*, oxidized LDL directly inhibits EPC differentiation and accelerates the rate of senescence through reducing telomerase activity.[37-39] Statin therapy increased the activity of the NO-producing enzyme eNOS in rat bone marrow[40] and experimental evidence shows that bone marrow NO-production is pivotal in EPC mobilization from the bone marrow.[10,41] eNOS activation is also thought to mediate VEGF-induced EPC mobilization[42]. We observed a baseline correlation between serum VEGF levels and circulating EPC numbers, but while EPC levels increased, serum levels of VEGF decreased after lipid-lowering treatment, consistent with previous observations[43-45]. This supports an effect of statins (or lipid-lowering therapy in general) on an intermediate in the signaling pathway of VEGF, such as eNOS. Lower VEGF levels without reducing EPC levels may be beneficial for (sub)clinical atherosclerosis, since plasma VEGF stimulates plaque neovascularization[46], which in turn causes plaque progression[46] and destabilization[47]. On the other hand, VEGF is also capable to provide a vascular cytoprotective activity through the release of NO and PGl2 which can prevent the recruitment, adhesion and transmigration of proinflammatory cells.[48] Furthermore, VEGF is also capable to promote endothelial cell migration and proliferation.[48] Thus, based on the knowledge available at this time, it is difficult to predict the expected clinical consequence of a reduction of VEGF serum levels.

Interestingly, serum levels of SCF-sR were significantly reduced in subjects with the metabolic syndrome compared to controls and increased to control levels after lipid lowering therapy, concurrently with the restoration of EPC levels. SCF-sR is produced by various tissues including hematopoietic cells and vascular endothelium, and induces mobilization of hematopoietic progenitor cells from the bone marrow to the circulation.[49] How lipid-lowering therapy affects SCF-sR production and if this represents a mechanistic link remains to be established. However, our data indicate that SCF-sR might serve as a surrogate indicator of circulating progenitor levels that adequately modulates during EPC-mobilizing therapy.

Whether SCF-sR and/or CD34+KDR+ EPC levels are useful for cardiovascular risk assessment in clinical practice cannot be concluded from our study. EPC levels are increasingly recognized as novel intermediate cardiovascular endpoint that independently correlate with cardiovascular outcome. Various studies in recent years have underscored the pathophysiological role of EPC and their potential as novel therapeutic target. In contrast to some other intermediate endpoints, changes in EPC levels can be assessed on a short-term, which allows monitoring for effect of treatment. Although it is feasible to quantify EPC from a small volume of peripheral blood in routine clinical practice, this is a costly procedure. A plasma measurement of e.g. SCF-sR may therefore represent a superior candidate for use in clinical practice. Further studies are required to assess if including quantification of EPC or related factors provide sufficient additional information over the current risk indicators to be effectively implemented in clinical practice.

Our study has several limitations. We did not investigate if the elevation of EPC levels observed with lipid-lowering therapy after 6 weeks in our population extends beyond that time period. This may be important to evaluate in future trials as a recent non-randomized observational cohort study of patients with coronary artery disease long-term statin therapy was associated with *decreased* rather than *increased* EPC levels.[35] Also, we did not investigate if cessation of lipid-lowering treatment was associated with a decline in EPC levels and with this did not verify if EPC levels had returned to baseline levels at the end of the washout period. Another limitation of our study is that we have assessed circulating CD34+KDR+ EPC only and not ex vivo cultured monocytic EPC. In contrast to the monocytic EPC, CD34+KDR+ EPC can not be obtained in sufficient quantities to allow assessment of functional characteristics.

In conclusion, EPC levels are significantly lower in obese men with the metabolic syndrome, even without manifest vascular disease. This suggests that reductions in EPC levels are a relatively early and potentially etiological pathophysiological phenomenon in the development of atherosclerosis, potentially contributing to an increased risk for future cardiovascular events in these patients. Intensive lipid-lowering therapy using either high-dose simvastatin or a combination of low-dose simvastatin with ezetimibe is equally effective in modulating EPC levels and secondary analysis of our data suggests that such treatment may even fully normalize reduced EPC levels. If the EPC mobilizing effect of lipid-lowering (statin) therapy proves structural on the long-term, this may contribute to reducing CVD risk through restoring the endothelial regenerative capacity in subjects with metabolic syndrome.

Funding

The reported work was supported by the Netherlands Organization for Scientific Research (NWO VENI-grant 016.036.041 to MCV), the Dutch Heart Foundation (Dr. E. Dekker Grant 2004T022 to PEW), and the Jacques H. de Jong Foundation (grant 2006.01.18).

Acknowledgements

Geesje Dallinga-Thie (Erasmus MC, Rotterdam, The Netherlands) measured the lipid profiles for this study.

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11

SUMMARY AND DISCUSSION

Circulating vascular progenitor cells can restore dysfunctional endothelium and contribute to physiological but also pathophysiological vascular and renal remodeling in response to injury. In this thesis we show that the presence of cardiovascular risk factors (renal insufficiency, SLE, diabetes and metabolic syndrome), resulting in a 'pro-atherosclerotic milieu', affects the number, function and differentiation of circulating progenitor cells. Potential underlying causes are impairment of EPC mobilization, increased EPC apoptosis and modulation of TGF-β/BMP signaling. For diabetes, we established that this affects not only circulating angiogenic progenitor cells, but also impairs angiogenic cells residing in the vessel wall. Modulation of deregulated progenitor cell physiology represents a novel therapeutic target.

Specific observations in various conditions associated with increased CVD

Progenitor cells in renal disease

In chapter 2, we show that in patients with end-stage renal disease, EPC differentiation is impaired and the capacity of EPC to stimulate angiogenesis is reduced. We also found that dialysis sessions induce EPC apoptosis, resulting in a temporary aggravated reduction of circulating EPC. In addition, we show that SPC differentiation towards a myofibroblast/smooth muscle cell lineage was unaffected, indicating that the capacity for adverse remodeling was retained. Bone-marrow derived cells incorporate into the injured glomerulus after anti-Thy1 glomerulonephritis, replacing damaged endothelium ([1] and chapter 9). Progression of renal disease does not only depend on the degree of glomerular endothelial injury, but also on the effectiveness of endothelial repair.[2,3] Therefore, reduced progenitor cell differentiation towards the endothelial lineage may hamper renal recovery from injury. In addition, bone-marrow derived cells incorporating into the kidney may also have adverse effects when these cells differentiate into myofibroblasts. Glomerulosclerosis may even be initiated by bone-marrow-derived circulating cells.[4] As SPC-differentiation is not affected in uremic patients, adverse sclerotic remodeling through SPC remains intact.

Progenitor cells in systemic lupus erythematosus (SLE)

SLE patients suffer from markedly increased rates of cardiovascular disease. We have identified various ways through which auto-antibody formation may contribute to accelerated atherosclerosis in SLE (chapter 3). We report that women with quiescent SLE have reduced EPC levels (chapter 4). This may be one of the pathophysiological mechanisms contributing to the development of CVD in SLE-patients. The reduced EPC levels were associated with reduced HSC levels, of which the deficiency was even more pronounced. Reduced HSC levels in SLE indicate that there is a limited capacity for hematopoietic renewal, which may contribute to the cytopenias observed in these patients. Interestingly, the defect in EPC in SLE was limited to HSC-derived Type I EPC, where Type II EPC were unaffected. This is different from other pro-atherosclerotic conditions, where Type I and Type II EPC are generally similarly reduced. One explanation may be that the primary defect in SLE is in the CD34+ HSC, resulting in a concomitant reduction in HSC-derived Type I EPC. We observed increased fractions of circulating apoptotic HSC. Others have previously shown that SLE serum induces hematopoietic stem cell apoptosis.[5,6] and that HSC-apoptosis was increased in bone marrow cell suspensions obtained from SLE-patients.[7]

Progenitor cells in diabetes

Micro- and macrovascular complications are the major cause of morbidity and mortality in diabetes. In chapters 5 and 6, we show that in an inducible model of Type I diabetes, EPC levels are reduced, while SPC outgrowth is increased. In chapter 5, we report that the increased SPC-outgrowth is

associated with increased TGF-β and decreased BMP-6 expression. This is in line with previous studies in Type I diabetic patients by our group.[8,9] Interestingly, circulating levels of a SPC-enriched population of CD105+CD14+ cells were increased in a cohort of mostly non-diabetic patients with manifest CVD compared to controls, suggesting that SPC levels are increased in a pro-atherosclerotic milieu that not necessarily includes diabetes.[10] Circulating SPC home to sites of injury including the neointima.[11,12] We found SPC to be a major source of adventitial cells. Enhanced TGF-β and reduced BMP-6 production by (adventitial) SPC may exert paracrine effects on resident smooth muscle cells. Indeed, in diabetic mice we observed aggravated neointima formation.

We observed that diabetes not only negatively affects circulating angiogenic cells, but also angiogenic cells residing in the vessel wall (chapter 7). By culturing aortic rings from diabetic rats ex vivo, we excluded a contribution from circulating cells. Angiogenic sprouting from diabetic vessels was reduced, which was associated with a decreased proliferative potential of vascular wall angiogenic cells. This impairment was observed in ex-vivo non-diabetic conditions, indicating that the influence of the diabetic milieu is to some extent imprinted on the angiogenic cells.

Progenitor cells in the metabolic syndrome

We have shown that obese men with the metabolic syndrome but without diabetes or manifest CVD have lower levels of circulating EPC than age-matched controls without the metabolic syndrome (chapter 10). As this occurs prior to any symptoms of CVD, reduced EPC-levels may be an early pathophysiological phenomenon in these subjects. Interestingly, we found that serum levels of the soluble form of SCF receptor c-kit, soluble SCF-receptor (SCF-sR) correlated with HSC and EPC levels, were significantly reduced in subjects with the metabolic syndrome, and increased to control levels after lipid lowering treatment in conjunction with changes in circulating EPC-levels. This suggests the potential use of SCF-sR as a serum marker of circulating progenitor cell levels.

Decreased levels of circulating endothelial progenitor cells in the presence of cardiovascular risk factors: common pathways with specific twists

We and others have demonstrated reduced peripheral blood EPC levels in the presence of CVD risk factors however, the exact mechanisms underlying this reduction are not clear. It is important to appreciate that EPC levels in peripheral blood are only one element of a complex and dynamic process. Reduced EPC levels in the circulation may be the result of 1) decreased production of EPC in the bone marrow; 2) Decreased mobilization of EPC from the bone marrow; 3) Decreased EPC proliferation or differentiation after mobilization; 4) Increased loss of EPC form the circulation by EPC apoptosis; or 5) by enhanced recruitment to the vessel wall or any sequestrating tissue bed. As the pathophysiology of the variety of CVD risk factors associated with reduced EPC levels is diverse, the underlying causes for reduced EPC levels in peripheral blood may also be heterogeneous.

EPC production in the bone marrow in the presence of cardiovascular risk factors

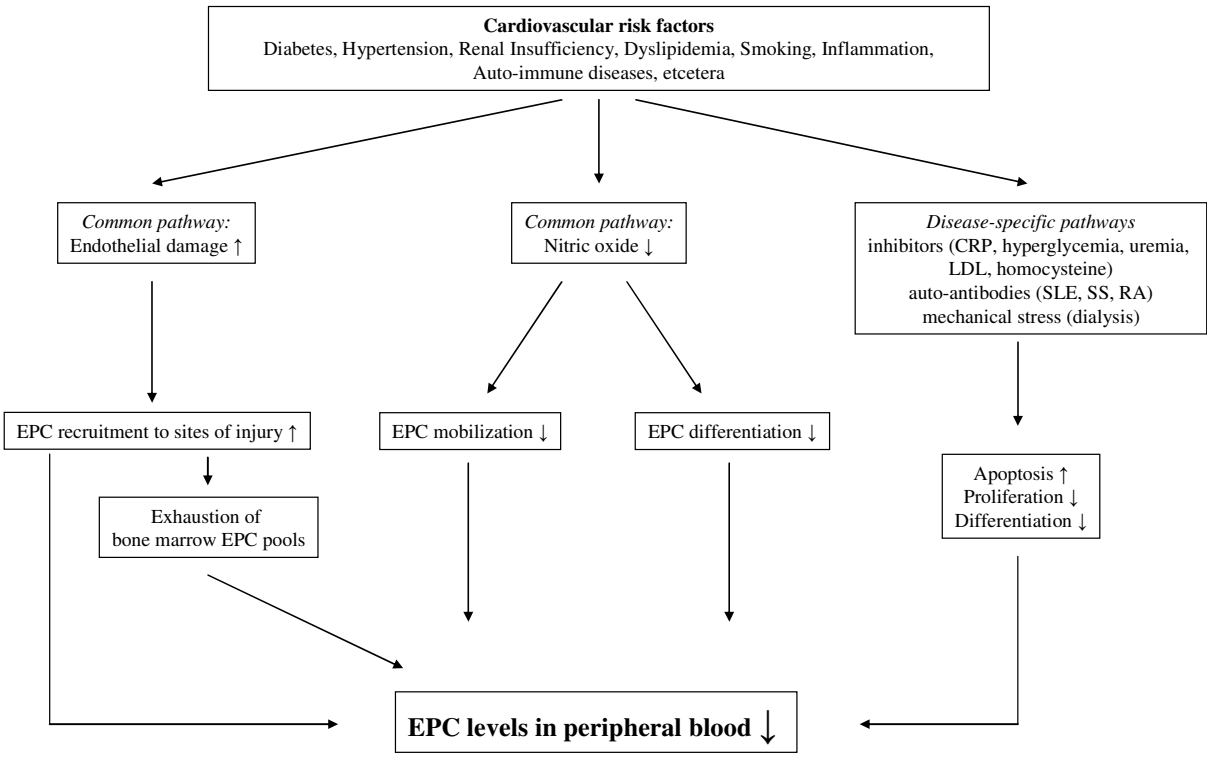
Little is known about the effects of CVD risk factors on EPC production in the bone marrow. In chapter 6 we have shown that in experimental diabetes mellitus, EPC levels in the bone marrow were not affected, while peripheral blood EPC levels were reduced. One human study investigated CD34+ HSC progenitor cell levels in bone marrow of patients with dilated cardiomyopathy in comparison to a group of healthy controls with normal cardiac structure and function.[13] In this study, bone marrow EPC levels were comparable to control levels in patients with non-ischemic cardiomyopathy, but reduced in patients with ischemia as underlying cause of the cardiomyopathy. It may be speculated that in the latter group of patients, EPC mobilization has been repetitively stimulated by the release of

mobilizing cytokines from the ischemic heart. Taken together, these data suggest that EPC production in the bone marrow is not directly affected by the presence of CVD risk factors, but that after prolonged exposure to the 'pro-atherosclerotic mileu' in which repetitive EPC-mobilization is induced, particularly in response to intensive mobilization stimuli such as ischemia, EPC production may get 'exhausted'.

EPC mobilization in the presence of cardiovascular risk factors

In chapter 6, we propose that 'vascular niche dysfunction' may underlie impaired endothelial progenitor cell mobilization and availability in the circulation. We show that impaired mobilization of endothelial and hematopoietic progenitor cells in diabetes is associated with dysfunction of the bone marrow stromal environment. Our *in vitro* and *in vivo* data demonstrate that diabetes affects the endothelial component of the bone marrow stroma, and indicate that a defect in the bone marrow 'vascular niche' from which progenitor cells are mobilized leads to the impaired mobilization of endothelial and hematopoietic progenitor cells observed in diabetes. eNOS levels in diabetic stroma were reduced and NO-inhibition impaired endothelial support of progenitor cells, suggesting a potential role for attenuated endothelial NO-release in diabetic stroma in dysfunctional progenitor cell mobilization. Dysfunction of the endothelium composing the vascular niche carries a high homology with the endothelial dysfunction in the arterial systems of diabetic patients, where NO-availability is also reduced.

Figure. Pathophysiological mechanisms involved in reduced peripheral blood EPC levels



EPC proliferation or differentiation in the presence of cardiovascular risk factors

Various factors have been identified that are present in specific pro-atherosclerotic conditions, which have direct effects on EPC proliferation and/or differentiation. In chapter 2 we show that uremic serum contains factors that inhibit EPC outgrowth *in vitro*. The inflammatory milieu may be of influence through CRP, which induces EPC apoptosis and inhibits EPC outgrowth *in vitro*.[14] Hyperglycemia or hyperinsulinemia in diabetic patients may directly inhibit EPC differentiation and

proliferation.[15,16] *In vitro*, oxidized LDL directly inhibits EPC differentiation and accelerates the rate of senescence through reducing telomerase activity.[17-19] Homocysteine reduced EPC outgrowth *in vitro* through accelerating the onset of EPCs senescence and thus inhibiting proliferation.[20,21]

Loss of EPC from the circulation

Circulating EPC may be reduced by loss of EPC due to increased apoptosis in the presence of cardiovascular risk factors. In chapter 2 we show that dialysis in ESRD patients induces a decrease in the number of circulating progenitor cells, which is associated with increased EPC apoptosis. However; during steady-state conditions, no difference in EPC apoptosis was observed. In chapter 4, we show that in SLE patients with reduced EPC levels, EPC apoptosis is increased even in quiescent disease conditions. Our findings are in line with the observations of increased HSC apoptosis and decreased CD34+ HSC levels in the bone marrow of patients with active SLE.[22] Increased CD34+ HSC cell apoptosis as underlying mechanism for EPC deficiency may occur in a broader range of autoimmune diseases associated with reduced EPC levels, since the rate of CD34+ HSC apoptosis was also found to be higher in bone marrow aspirates from patients with rheumatoid arthritis[23] in whom EPC levels are reduced, particularly during periods of active disease[24,25]. In contrast, progenitor cell apoptosis was found not to be significantly increased in diabetic[26] and uremic[27] patients.

Enhanced recruitment of EPC to sites of vascular regeneration

Another cause for loss of EPC from the circulation may be that recruitment of EPC to the vessel wall, developing neovasculature, or any sequestrating tissue bed is increased. Indeed, it was shown that intravenously injected ^{111}In-oxine-labeled bone marrow cells home to atherosclerotic lesions in the aortas of apolipoprotein E-deficient mice based on high-resolution whole-body helical single photon emission computed tomography (SPECT) detection. However, most cells sequestrated in the spleen and liver or relocated to the bone-marrow.[28]

Reduced peripheral blood EPC-levels levels: common pathways with specific twists

There are multiple mechanisms through which EPC levels in peripheral blood may be reduced in the presence of cardiovascular risk factors. It is remarkable that a wide array of cardiovascular risk factors so consistently share the phenomenon of reduced EPC levels in peripheral blood. Impaired NO-availability is a pathophysiological phenomenon that is also a common feature of various CVD risk factors. Impaired NO availability, as measured by flow-mediated brachial artery reactivity, correlated with reduced EPC numbers in the peripheral circulation of patients at risk for cardiovascular disease.[29] Accumulating evidence, from this thesis and from other studies[30,31], suggests that impaired NO-availability impairs EPC mobilization and differentiation. Impaired NO-availability may be a common pathophysiological mechanism involved in reduced EPC levels in the presence of CVD risk factors. On top of this, risk-factor specific mechanisms such as increased apoptosis or inhibition of proliferation and differentiation through direct effects on EPC play a role.

Modulation of progenitor cell differentiation in the presence of cardiovascular risk factors

In addition to effects on EPC levels, SPC levels may be affected by CVD risk factors. As shown in chapter 5 and consistent with previous observations from our group[9], diabetes is associated with enhanced SPC differentiation, in association with increased TGF-β and decreased BMP-6 expression in diabetic SPC. In ESRD, SPC outgrowth was not affected while EPC differentiation was impaired, resulting in a relative EPC/SPC imbalance. Others have shown that circulating levels of SPC-enriched CD105+CD14+ peripheral blood cells were increased in a population of mostly non-diabetic patients

with manifest CVD compared to controls.[10] Interestingly, SPC-mediated mobilization and contribution to intimal hyperplasia following carotid artery ligation was increased in eNOS knockout mice.[32] This suggests that NO availability may be pivotal in regulating EPC/SPC balance. Consistently, in this thesis we found that ESRD and diabetes, both conditions associated with reduced NO availability, favoured SPC differentiation while EPC levels were reduced. Taken together, enhancement of SPC-mediated remodeling is observed in a pro-atherosclerotic milieu.

Current challenges

Defining EPC

EPC-literature is confusing as nomenclature is used interchangeably for cells with different origin, characteristics and physiological function. One might argue that the nomenclature chosen for the cells described in the hallmark paper by Asahara et al. in Science 1997 was incorrect. Asahara coined the term "endothelial progenitor cells" for cells that have been referred to in this thesis as Type II (monocytic) EPC. As these lack crucial requirements for a 'progenitor' and represent more of a 'plastic monocyte', naming these cells endothelial *progenitor* cells has understandably provoked much debate. However, with more than one thousand papers including the name "endothelial progenitor cell" in the decade following the Asahara paper (pubmed search 1997 to 2007 for "endothelial progenitor cell(s)" renders 1254 results) and mostly referring to the monocytic EPC, efforts to alter the nomenclature (into e.g. 'cultured/circulating angiogenic cells (CACs)'[33]) have had little success. The findings in chapter 4 in SLE patients may serve as an example emphasizing that Type I and Type II EPC need be regarded as a distinct cell types that may be differentially modulated. Although continued use of the term "EPC" for divergent cell types seems inevitable, authors should at least be clear about their definition of an 'EPC' for any particular manuscript.

EPC as risk indicator for CVD

EPC levels are increasingly recognized as novel intermediate cardiovascular endpoint. Type I CD34+KDR+ EPC independently correlate with cardiovascular outcome.[34,35] EPC levels are negatively affected by a wide array of CVD risk factors and can increase in response to treatment. In contrast to some other intermediate endpoints, changes in EPC levels can be assessed on a short-term, which allows monitoring for effect of treatment. EPC can be quantified from a small volume of peripheral blood in routine clinical practice, although this is a costly procedure. A plasma protein measurement may therefore represent a superior candidate for use in clinical practice. In chapter 10, we show that serum levels of SCF-sR were significantly reduced in subjects with the metabolic syndrome compared to controls and increased to control levels after lipid lowering therapy, concurrently with the restoration of EPC levels. Further investigations are required to evaluate if SCF-sR might serve as a surrogate indicator of circulating progenitor levels. With a wide array of alternative (novel) CVD risk markers available, quantification of EPC or associated markers will need to bring substantial additional value to be worth implementing in clinical practice.

Therapeutic modulation of EPC levels

In chapter 10, we show that intensive lipid-lowering therapy using either simvastatin or simvastatin/ezetimibe combination therapy normalized reduced EPC levels in a population of subjects with obesity and metabolic syndrome, who are at markedly increased CVD risk. Whether this will lead to actual reduction in CVD mortality remains to be established. In an experimental model for assessing bone-marrow mediated endothelial recovery after glomerulonephritis, we investigated the effect of PPAR-gamma-agonist treatment as this was previously shown to increase EPC levels, at

least in part by stimulating EPC differentiation[36-39]. However, although treatment with PPAR-gamma-agonist rosiglitazone did ameliorate the course of anti-Thy1 glomerulonephritis, we did not observe an increase in EPC-incorporation. In ESRD patients, we observed a correlation between the erythropoietin dose and EPC levels. In SLE-patients we observed a trend towards such a correlation for hydroxychloroquine. As the latter observations were made in cross-sectional studies, these require further evaluation in randomized controlled trials to exclude confounding. For erythropoietin, there is evidence from animal and human studies that it indeed enhances EPC levels.

The mechanisms through which these interventions work remain to be further elucidated. To enhance EPC-mediated repair, potential targets for intervention are 1) increasing mobilization of EPC from the bone marrow; 2) Increasing EPC differentiation and proliferation after mobilization, while protecting EPC against apoptosis; and 3) enhancing the recruitment to the vessel wall by augmenting EPC homing.

For statins, it was shown in an animal model that an induced increase in circulating EPC was associated with increased bone marrow eNOS levels.[40] Enhancing bone marrow nitric oxide levels may facilitate EPC mobilization. Interestingly, various other interventions with a protective effect on CVD are known to enhance nitric oxide and have now also been found to increase EPC-levels, such as estrogen[41,42], PPAR-gamma-agonist treatment[36-38,43], HDL-infusion[44], angiotensin II receptor antagonism[45], and physical training[46]. Several factors such as G-CSF and GM-CSF that are, known to mobilize progenitor cells, have been shown to also mobilize EPC and successfully augmented EPC-mediated neovascularization of ischemic tissue in experimental animals models.[47,48] Clinical trials have investigated the effect of G-CSF administration to enhance EPC-mediated repair in humans after myocardial infarction, but these results have so far been disappointing.[49] Novel pharmacological agents are under investigation, such as CXCR4-antagonist AMD3100[50] and eNOS-promotor enhancer AVE9488[51], which mobilize EPC to the peripheral blood. AMD3100 acts through inhibition of SDF-1-mediated regulatory restriction of progenitor cell transendothelial migration in the bone marrow, which leads to rapid mobilization of progenitor cells upon acute administration of AMD3100, although these effects may not be sustained with chronic adminstration.[52] AVE9488 acts through increasing bone marrow eNOS levels[51], which leads to increased progenitor cell mobilization through mechanisms described earlier. Whether these will result in clinical benefit remains to be established.

Enhancement of EPC proliferation, differentiation and survival can also be achieved by drugs currently available. For example, simvastatin rapidly activates the protein kinase Akt in EPC, thereby enhancing proliferation and cell survival.[53] PPAR-gamma-agonist rosiglitazone protects against CRP-induced EPC-apoptosis and inhibition of proliferation.[54], while stimulating EPC differentiation[36,54]. Erythropoietin enhances EPC proliferation and differentiation.[55,56] Angiotensin II receptor antagonist valsartan protects against angiotensin II induced EPC senescence and inhibition of proliferation.[57]

We evaluated if treatment with PPAR-gamma agonist rosiglitazone could stimulate EPC-homing to the injured glomerulus, but we did not observe a detectable increase in the number of incorporated EPC after recovery from anti-Thy1 glomerulonephritis (chapter 9). EPC-homing involves CC- en CXC-chemokines with associated receptors and therapeutic modulation of these signaling pathways has been identified as key target to direct EPC to sites of injury.[58] However, these families of chemokines are also involved in homing of inflammatory cells, including to developing atherosclerotic or neointimal lesions.[59] One of these chemokines is RANTES. RANTES-inhibition using RANTES receptor antagonist Met-RANTES has been shown to attenuate the development of

atherosclerosis and neointimal lesion.[60,61] However, we have previously observed that Met-RANTES treatment inhibits EPC-homing to the injured glomerulus.[62] Here, we show that treatment with Met-RANTES inhibits ischemia-induced angiogenesis (chapter 8). RANTES-inhibition as anti-atherosclerotic therapy may be hampered by adverse effects on EPC-mediated repair. Vice-versa, future strategies aimed at enhancing EPC-homing will need to act specifically on EPC to avoid activation of the influx of inflammatory cells.

Therapeutic modulation of SPC levels

Interestingly, various drugs that stimulate EPC mobilization or differentiation, appear to have opposite effects on SPC. PPAR-γ-agonist rosiglitazone stimulates EPC function and differentiation while inhibiting SPC differentiation.[36] Similarly, HMG-CoA-reductase inhibitor pravastatin increased the number of EPC while decreasing the number of SPC in peripheral blood mononuclear cell cultures.[63] Treatment with a novel angiotensin II receptor blocker reduced SPC outgrowth *in vitro* and decreased the number of SPC in neointimal hyperplasia after mechanical arterial injury in mice.[64] As NO availability may be pivotal in regulating EPC/SPC balance[32], pharmacologically increasing NO-availability may be expected to inhibit SPC-levels. Although inhibition of SPC differentiation may attenuate adverse vascular remodelling in neointima formation, there may be adverse effects on plaque stability that deserve careful consideration.[65]

Therapeutic transplantation of EPC for ischemic neovascularization

To explore the effect of promoting EPC-mediated neovascularization, bone marrow or peripheral blood mononuclear cell suspensions have been injected into ischemic tissue, in some cases after *ex vivo* EPC-differentiation and expansion. EPC-transplantation has extensively been tested in experimental animals.[66-70] Several pioneering human studies involving autologous bone marrow cell transplantation have demonstrated clinical benefit in patients with occlusive cardiovascular disease, particularly for myocardial infarction.[71,72] However, the absolute therapeutic effect size observed in these clinical trials for these complicated, invasive, and costly procedures is limited and some trials did not show any effect at all.[73]

There appears to be a discrepancy between the substantial results in animal models and limited success in human clinical medicine. A likely explanation for this is that the EPC from patients with manifest CVD are dysfunctional and that transplantation of these autologous dysfunctional EPC results in suboptimal stimulation of neovascularization. Indeed, infusion of bone marrow cells from patients with ischemic cardiomyopathy in an animal model of peripheral ischemic injury, showed that these cells have a markedly diminished capacity to enhance neovascularization compared to cells from healthy controls.[74] Although it could relate to lower EPC concentration in the unselected bone marrow cell suspensions used, it could also be a functional defect. This is supported by the observation that SDF-1 induced migration of the cells *in vitro* was reduced.[74]

For optimal autologous cell therapy strategies, dysfunctional EPC may need to be 'pre-treated' in order to restore their neovascularization potential. In experimental settings, this option is currently being explored. Hypoxic preconditioning augmented the capacity of human EPC to enhance experimental neovascularization *in vivo*.[75] Dysfunctional EPC, which have reduced SDF-1-responsiveness, may be primed with high ex-vivo concentrations of SDF-1, which increases their potential to home to activated endothelium.[76] Modulation of NO by ex-vivo pre-treatment of bone marrow mononuclear cells with endothelial NO synthase enhancer AVE9488 was shown to enhance their functional activity.[77] Indeed, the various drugs identified as having a positive effect on EPC-

functionality may be applied in high doses to cell suspensions ex vivo prior to transplantation. One clinical trial added atorvastatin to cell suspensions in culture before intracoronary transplantation of EPC in patients with myocardial infarction.[78] Unfortunately, this trial did not investigate if the addition of atorvastatin affected the outcome of EPC transplantation.

In addition to optimizing the functionality of transplanted cells, many other questions remain: should cells be harvested from bone marrow or from (mobilized) peripheral blood? Is ex-vivo differentiation to EPC required prior to transplantation? What is the best time-point for transplantation after an acute ischemic event? What is the best route for administration (intracoronary infusion or intramyocardial injection)? Which patients will benefit most from transplantation?

In conclusion

Progenitor cells from the bone marrow circulate in the blood and contribute to physiological and pathophysiological vascular and renal remodeling. CVD risk factors influence the mobilization, differentiation and function of these progenitor cells. This occurs through various mechanisms, which include common pathways relevant for various CVD risk factors, and risk-factor specific mechanisms. Vascular progenitor cell levels represent a novel therapeutic target in cardiovascular and renal disease.

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12

NEDERLANDSTALIGE SAMENVATTING

In dit proefschrift wordt de rol van in het bloed circulerende voorlopercellen besproken bij hart- en vaatziekten (HVZ) en nierziekten. Bij specifieke ziekten die samengaan met een versnelde atherosclerose (vaatwandverkalking) wordt beschreven hoe het circulerende aantal, de functionele eigenschappen en de uitrijping tot endotheelcel of gladde-spiercel beïnvloed wordt. Door analyse van signaalmoleculen en experimenten in diermodellen is onderzocht welke factoren een rol spelen bij het veranderde gedrag van de voorlopercellen. Vervolgens is geëvalueerd of dit medicamenteus te sturen is.

De rol van endotheel, gladde spiercellen en hun circulerende voorlopercellen

Alle bloedvaten zijn bekleed met een enkel cellaagje zogeheten endotheel. Dit endotheel is van groot belang voor het goed functioneren van de vaten, want het vormt de kritische barrière tussen het bloed en het achterliggend weefsel. Het endotheel reageert op signaalstofjes of geactiveerde cellen in het bloed alsook vanuit de weefsels en geeft zelf ook voortdurend signaalstofjes af in reactie daarop. Op die manier wordt gereguleerd hoe de vaten zich moeten aanpassen aan de behoefte van de weefsels ter plekke en elders in het lichaam. Een belangrijke signaalstof van het endotheel is stikstofoxide (Nitric Oxide, NO). Stikstofoxide afgegeven door het endotheel zorgt ervoor dat gladde spiercellen om het vat zich ontspannen, waardoor het vat meer open komt te staan. Ook voorkomt het dat er ontstekingscellen de vaatwand in kunnen dringen en beschermt het de vaatwand tegen schadelijke oxidanten door deze weg te vangen. De aanmaak van voldoende stikstofoxide is een belangrijk kenmerk van gezond endotheel met een goede endotheelfunctie. Risicofactoren voor atherosclerose zoals een hoge bloeddruk, roken of suikerziekte, hebben gemeen dat zij het endotheel beschadigen en ontsteking bevorderen. Beschadigd endotheel heeft een verminderd vermogen om stikstofoxide af te geven. Hierdoor wordt de vaatwand gevoeliger voor het binnendringen van ontstekingscellen en beschadiging door oxidanten. Zo kunnen ontstekingscellen zich ophopen en ontstaat uiteindelijk een atherosclerotische plaque. Ook in de nier is endotheel van groot belang. In de nier wordt al het bloed gefilterd door talloze kleine vaatkluwentjes, de zogeheten glomeruli. Deze glomeruli bestaan voor een groot deel uit endotheel en net zoals in de vaatwand is gezond endotheel mede bepalend voor een goed functioneren van de nier. Beschadigd endotheel leidt tot problemen met de nierdoorbloeding en beperkt de kwaliteit van de filterfunctie van de nier, waardoor bijvoorbeeld eiwitten de urine in lekken.

Tot ongeveer 10 jaar terug werd gedacht dat beschadigd endotheel uitsluitend vervangen werd door omliggend endotheel middels celdeling. Het blijkt echter dat het beenmerg een andere belangrijke bron van endotheelvervanging vormt. Vanuit het beenmerg komen er voortdurend endotheelvoorlopercellen (een soort stamcellen) in het bloed die zich een weg vinden naar plekken van beschadigd endotheel en daar uitrijpen tot endotheelcel. Dit gebeurt zowel in de vaatwand als in de glomeruli van de nier.

Naast voorlopercellen voor endotheel bleken er ook beenmergafkomstige voorlopercellen voor gladde-spiercellen in het bloed te circuleren. Gladde-spiercellen vormen een spierlaag in de vaatwand. In sommige omstandigheden kunnen gladde spiercellen echter problemen veroorzaken voor de vaatdoorgankelijkheid. Met name na vaatwandbeschadiging zoals bij dotterprocedures of het plaatsen van stents na een hartinfarct, kunnen vaten dichtgroeien met gladde-spiercellen. Dit gebeurt deels door celdeling van lokale gladde-spiercellen en deels door beenmergafkomstige gladde-spiercelvoorlopercellen. Gladde-spiercelvoorlopercellen worden ook gevonden in atherosclerotische plaques. Mogelijk is hun rol daar een gunstige, omdat de aanwezigheid van gladde-spiercellen in plaques leidt tot stevige, stabiele plaques die niet scheuren, hetgeen de meeste problemen veroorzaakt.

De invloed van risicofactoren voor HVZ op circulerende voorlopercellen
In hoofdstuk 2 wordt beschreven dat bij patiënten met een ernstig gestoorde nierfunctie die daardoor afhankelijk zijn van dialyse, verminderde aantallen circulerende endotheelvoorlopercellen in het bloed aanwezig zijn. Ook blijken deze cellen minder goed in staat tot het stimuleren van vaatvorming door endotheel. Tijdens het dialyseren wordt gezien dat het aantal endotheelvoorlopercellen nog verder daalt in het bloed, hetgeen samengaat met een verhoogd aantal endotheelvoorlopercellen dat tot apoptose (geprogrammeerde celdood) overgaat. De uitrijping van gladde-spiercelvoorlopercellen is daarentegen geheel ongestoord. Dit is van belang, omdat bij dialysepatiënten een zeer sterk verhoogd voorkomen van atherosclerose gezien wordt, waarbij de stoornissen in de endotheelvoorlopercellen een nieuw ontdekte bijdragende oorzaak vormen. Ook het dichtgroeien van vaten door gladde-spiercellen is een probleem bij dialysepatiënten, in het bijzonder van de dialyseshunt. Beïnvloeding van de uitrijping van circulerende voorlopercellen kan een nieuwe therapeutisch aangrijpingspunt vormen. In hoofdstuk 2 wordt beschreven hoe opvalt dat de gebruikte dosis eytropoietine samengaat met hogere endotheelvoorlopercellen. Verder onderzoek is nodig om te onderzoeken of dit wellicht gebruikt kan worden voor het verhogen van het aantal endotheelvoorlopercellen in dialysepatiënten.

In hoofdstuk 3 en 4 wordt ingegaan op factoren die bijdragen aan de versnelde ontwikkeling van atherosclerose bij patiënten met de autoimmuunziekte Systemische Lupus Erythematosus (SLE). Hoofdstuk 3 geeft een overzicht van de studies die gedaan zijn naar het voorkomen van HVZ bij SLE en de op dit moment bekende oorzakelijke factoren. In het bijzonder is duidelijk gemaakt via welke mechanismen de vorming van een antistof gericht tegen lichaamseigen materiaal (kenmerkend voor een autoimmuunziekte) kan leiden tot versnelde atherosclerose. Hierbij spelen het bevorderen van ontsteking en het veroorzaken van stoornissen in de endotheelfunctie sleutelrollen. In hoofdstuk 4 wordt beschreven dat bij SLE patiënten de circulerende aantallen endotheelvoorlopercellen verlaagd zijn, hetgeen een nieuw geïdentificeerd bijdragend mechanisme vormt bij de versnelde atherosclerose. Daarbij wordt gezien dat ook de bloedcelvoorlopercellen in verminderd getal in het bloed aanwezig zijn en dat een verhoogd aantal in apoptose is. Dit is van belang, omdat SLE-patiënten gevoelig zijn voor het ontwikkelen van tekorten van de diverse soorten bloedcellen. Het verlaagd aantal bloedvoorlopercellen is een indicatie van een beperkte reserve hiervoor. Er zijn twee soorten endotheelvoorlopercellen, waarbij een van de twee soorten een gespecialiseerde vorm van de bloedvoorlopercel is. Opvallend is dat alleen deze laatste soort endotheelvoorlopercel aangedaan is bij de SLE patiënt. Dit suggereert dat het probleem vooraleerst in de versneld apoptoserende bloedvoorlopercel ligt.

Hoofdstukken 5, 6 en 7 richten zich op de effecten van diabetes mellitus (suikerziekte) op voorlopercellen in het bloed en in de vaatwand zelf. HVZ zijn een zeer veelvoorkomende complicatie van diabetes. In hoofdstukken 5 en 6 is gebruik gemaakt van een diermodel voor insulineafhankelijke Type I diabetes middels de injectie van streptozotocine. In hoofdstuk 5 is beschreven dat het dichtgroeien van beschadigde vaten met gladde spiercellen, zogeheten neointimavorming, in versterkte mate optreedt bij muizen met diabetes. Door gebruik te maken van muizen met groen fluorescerende beenmergcellen wordt duidelijk dat een deel van deze cellen in de neointima vanuit het beenmerg komen, waarbij opvalt dat vooral veel beenmergafkomstige cellen rondom het vat gaan zitten. In kweken van cellen van diabetisch muizen rijpen gladde-spiercelvoorlopercellen sneller uit en delen ze ook sneller dan die in kweken van gezonde muizen. Analyse van het RNA in de cel laat zien dat diabetische muizen een verhoogde productie van RNA voor TGF-β en verlaagde productie van RNA voor BMP-6 hebben in hun gladde-spiercelvooropercellen. Van TGF-β is bekend dat het

gladde spiercellen stimuleert tot uitrijping en deling en dat TGF-β-afgifte rondom beschadigde vaten neointima-vorming versterkt. BMP-6 is een natuurlijke remmer van TGF-β en heeft tegenovergestelde effecten. Bovendien zijn zowel TGF- β als BMP-6 eiwitten die uitgescheiden worden door cellen, waarmee ook omliggende cellen beïnvloed kunnen worden. Daarom kan de veranderde verhouding van TGF- β en BMP-6 de versnelde gladde-spiercelvoorlopercel productie waarschijnlijk verklaren en kan het van belang zijn voor zowel de bijdrage van gladde-spiercelvoorlopercellen aan neointimavorming, alsook het gedrag van de lokale gladde spiercellen in hun omgeving. In hoofdstuk 6 wordt aangetoond dat het muismodel voor diabetes ook samengaat met verlaagde aantallen endotheelvoorlopercellen. Diabetes zorgt dus voor een veranderde verhouding van gladde-spiercel- en endotheelvoorlopercellen. Dit komt overeen met bevindingen bij mensen met diabetes.

In hoofstuk 6 wordt verder onderzocht waarom bij diabetes minder endotheelvoorlopercellen in het bloed zitten, waarbij specifiek de rol van het beenmerg en de vaten in het beenmerg bekeken wordt. Endotheelvoorlopercellen moeten losgemaakt worden uit het beenmerg om in het bloed te komen. Deze zogeheten mobilisatie kan experimenteel getest worden door signaalstofjes te injecteren. Bij gezonde muizen leidt dit inderdaad tot een sterke verhoging van het aantal endotheelvoorlopercellen in het bloed, maar bij de diabetische muizen is deze verhoging beperkt. Dit komt niet doordat de cellen niet in het beenmerg aanwezig zijn, want vergelijkende tellingen van endotheelvoorlopercellen in het beenmerg tonen aan dat diabetische dieren een gelijk of zelfs wat verhoogd aantal endotheelvoorlopercellen in het beenmerg hebben. Het probleem lijkt dus in de mobilisatie zelf te zitten. Bekend is dat bij de mobilisatie van endotheelvoorlopercellen de vaten in het beenmerg van groot belang zijn, maar ook andere steuncellen uit het beenmergstroma en geassocieerde signaalstoffen. Onder de microscoop zien de vaten van de diabetische dieren er onregelmatig uit en de diabetische muizen blijken slecht te kunnen herstellen van een injectie met een chemische stof die het beenmerg verstoort en waarbij het nodig is nieuwe vaten aan te laten groeien voor genezing. In hoofdstuk 6 is onder kweekomstandigheden de interactie tussen cellen in het beenmerg nagebootst. Allereerst blijken in kweek ongeselecteerde beenmergstromacellen van diabetische muizen minder goed in staat voorlopercellen te ondersteunen dan beenmergstromacellen van gezonde muizen. Wanneer alleen endotheel gebruikt wordt in combinatie met voorlopercellen, blijkt dit in aanwezigheid van een verhoogde suikerconcentratie ook gestoord te raken in het vermogen voorlopercellen te ondersteunen in hun overleving en behoud van vermogen om als voorlopercel te functioneren. Remming van stikstofoxide geeft een vergelijkbare stoornis. Tezamen kan hiervanuit de hypothese opgesteld worden dat bij diabetes niet alleen het endotheel in de bloedvaten buiten het beenmerg gestoord raakt (zoals bekend is), maar ook de vaten in het beenmerg een gestoorde functie hebben, hetgeen zich uit in een beperkt vermogen om endotheelvoorlopercellen vanuit het beenmerg naar het bloed te mobiliseren. Zodoende ontstaat er een verlaagd aantal endotheelvoorlopercellen in het bloed, dat leidt tot een gebrekkig herstel van disfunctioneel endotheel.

In hoofdstuk 7 wordt beschreven dat bij diabetes niet alleen de circulerende endotheelvoorlopercellen, maar ook voorlopercellen voor nieuwe vaten in de vaatwand zelf gestoord zijn. Hiervoor werd een model gebruikt waarbij ringetjes van de grote lichaamsslagader, de aorta, van ratten in een groeifactorrijke gel geplaatst worden. Vanuit de aortaringetjes groeien nieuwe vaatjes, waarbij bekend is dat dit gebeurt vanuit een gespecialiseerd soort lokale vaatwandvoorlopercellen die lijken op endotheelvoorlopercellen. De nieuwgevormde vaatjes van de diabetische ratten waren korter en de geïsoleerde vaatwandvoorlopercellen hadden een vertraagde celdeling.

Ook uit studies van anderen is veel bekend geworden over veelal negatieve invloeden van de aanwezigheid van diverse risicofactoren voor HVZ op voorlopercellen, waarbij endotheelvoorlopercellen gestoord raken en gladde-spiercelvoorlopercellen gestimuleerd. Voor een deel wordt dit veroorzaakt door specifieke mechanismen die met een betreffende HVZ-gerelateerde omstandigheid samenhangen, zoals de auto-antistoffen bij SLE, het uremisch milieu bij nierinsufficientie of de mechanische stress van het dialyseren. Deze kunnen aanleiding geven tot verhoogde apoptose of interfereren met een normale uitrijping en celdeling. Voor een deel zijn er waarschijnlijk echter ook mechanismen die bij vrijwel alle HVZ-risicofactoren een rol spelen. Zo treedt een verhoogde beschadiging van het endotheel en daarmee verhoogd verbruik van endotheelvoorlopercellen naar verwachting in het algemeen op. Ook een verlaging van de beschikbaarheid van stikstofoxide is een fenomeen dat bij vrijwel alle HVZ risicofactoren beschreven wordt. Dit kan leiden tot een gestoorde mobilisatie van endotheelvoorlopercellen en anderen hebben gevonden dat stikstofoxide ook voor de uitrijping van endotheelvoorlopercellen van belang is.

Modulatie van voorlopercellen als therapie voor HVZ of nierziekten

In dit proefschrift wordt het effect van enkele medicamenten op endotheelvoorlopercellen beschreven. In de patiëntenstudies beschreven in hoofdstukken 2 en 4 viel op dat er associaties bestonden tussen de hoogte van de gebruikte dosis erythropoietine en het circulerend aantal endotheelvoorlopercellen bij dialysepatiënten en een associatie met de gebruikte dosis hydroxychloroquine bij SLE patiënten. Vanwege de studieopzet waarbij patiënten eenmalig gezien werden, kan hiermee echter nog niet geconcludeerd worden dat dit ook inderdaad een causaal verband hield en is vervolgonderzoek nodig om over een eventuele stimulerende werking duidelijke conclusies te trekken.

In hoofdstuk 8 wordt aangetoond dat behandeling met een remmer van de signaalstof RANTES, bedoeld als nieuwe therapie tegen atherosclerose, de nieuwgroei van vaten remt na vaatafsluiting. Bekend is dat deze nieuwgroei deels afhangt van endotheelvoorlopercellen en ook bekend is dat RANTES betrokken is bij het aantrekken van endotheelvoorlopercellen door beschadigd weefsel. Een ongewenst gevolg van de bescherming tegen atherosclerose door RANTES-remming is dus dat vaatnieuwvorming bij vaatafsluitingen gestoord raakt.

Hoofdstuk 9 beschrijft een dierexperiment waarbij gekeken werd of behandeling met PPAR-γ-agonist rosiglitazon (een bloedsuikerverlagende middel) het herstel van een beschadigde nier kon bevorderen en wat de rol van endotheelvoorlopercellen hierbij was. Hierbij werd weer gebruik gemaakt van proefdieren waarbij de beenmergcellen te traceren waren op basis van een genetisch verschil. De nierschade werd veroorzaakt door injectie van een antilichaam gericht tegen de steuncellen in de glomeruli. Als reactie hierop treedt een ontsteking in de nier op, een zogeheten glomerulonefritis, waarbij ook het endotheel beschadigd raakt. Hoewel rosiglitazon wel beschermend werkte, was dit niet het gevolg van een versterkt herstel van endotheel door beenmergafkomstige voorlopercellen.

In hoofdstuk 10 worden de resultaten gegeven van een gerandomiseerde dubbelblinde cross-over studie waarbij mannen met overgewicht en het zogeheten metabool syndroom behandeld werden met cholesterolverlagende medicatie. Het metabool syndroom is een combinatie van overgewicht, hoge bloeddruk, veranderde vetstofwisseling en insulineresistentie. Dit gaat samen met een belangrijk verhoogd risico op HVZ. Allereerst werd vanuit een vergelijk met gezonde mannen duidelijk dat de mannen met overgewicht en het metabool syndroom een verlaagd aantal endotheelvoorlopercellen hebben. Met cholesterolverlagende medicatie was dit volledig tot normaal niveau te herstellen. Hierbij maakte het niet uit of deze medicatie opgebouwd was uit een hoge dosis van de

cholesterolaanmaakremmer simvastatine of een gecombineerde behandeling van een lage dosis simvastatine in combinatie met cholesterolopnameremmer ezetimibe. Als nevenbevinding werd gezien dat het eiwit SCF-sR een redelijke goede weerspiegeling gaf van het aantal endotheelvoorlopercellen in het bloed, zowel met als zonder behandeling. Dit betekent dat het mogelijk toepasbaar is als vervangende meting voor de gecompliceerde en tijdrovende meting van het daadwerkelijke celaantal.

De onderliggende mechanismen van deze medicamenteuze behandelingen behoeven nog verdere verduidelijking. Om middels een effect op endotheelvoorlopercellen een beschermend effect op HVZ of nierziekte te bewerkstelligen, zou een interventie theoretisch kunnen werken door 1) de mobilisatie van endotheelvoorlopercellen te stimuleren; 2) de celdeling en overleving van endotheel-voorlopercellen te stimuleren, of te beschermen tegen apoptose; en/of 3) de sturing naar en hechting aan de plek van schade te verbeteren.

Tot slot

De studies in het huidig proefschrift en die van anderen tonen aan dat beenmergafkomstige voorlopercellen voor endotheel en gladde spiercellen in het bloed circuleren. Deze dragen bij aan fysiologische en pathofysiologische remodellering van bloedvaten en nieren. Risicofactoren voor HVZ beïnvloeden de mobilisatie, uitrijping en functie van voorlopercellen via diverse mechanismen. Circulerende voorlopercellen vormen een nieuw therapeutisch aangrijpingspunt voor HVZ en nierziekten.

13

ACKNOWLEDGEMENTS

PUBLICATIONS

CURRICULUM VITAE

Acknowledgements (dankwoord)

Wetenschappelijk onderzoek is in de loop van de tijd voor mijn carrière en persoon zeer belangrijk geworden. Wetenschap bedrijven is samenwerken en bij mijn onderzoek hebben vele mensen een rol gespeeld. Ik dank hen voor de instructie, inspiratie en inzet.

Ik heb mij gelukkig mogen prijzen met een aantal bijzondere personen die ik als belangrijke mentoren ervaren heb. Mijn eerste ervaringen met wetenschappelijk onderzoek deed ik op bij professor Ton Rabelink, eerst als student tijdens mijn wetenschappelijk stage en later als promovendus. Beste Ton, jouw enthousiasme voor de wetenschap heeft me geweldig geïnspireerd. Ik kreeg bij jou de vrijheid om mij wetenschappelijk te ontplooien met een voortdurende stimulans hiervan maximaal gebruik te maken. Ik heb veel van je geleerd en heb me niet aflatend gesteund gevoeld. Je leerde mij de schoonheid van de translationele geneeskunde, het belang van mooie plaatjes en het afdwingen in plaats van afwachten van 'geluk'.

Met het voortijdige vertrek van professor Rabelink kwam het laboratorium Vasculaire Geneeskunde onder de hoede van professor Hein Koomans. Beste Hein, ik heb me telkens weer verbaasd over jouw geweldige analytisch vermogen. In enkele minuten gesprek over een onderwerp buiten je directe aandachtsgebied lukte het je telkens precies de juiste vraag te stellen waarmee ik weer maanden aan de slag kon. Ik dank je voor de deur die altijd openstond en ook jouw inspirerende bevlogenheid met de wetenschap. Je leerde mij de schoonheid van de basale fysiologie, de nadelen van vlees en zout en te streven naar de kern in elke analyse.

Omdat vaten gelukkig niet alleen in de nier, maar rond het hart krullen, kwam ik in aanraking met mijn tweede promotor professor Pieter Doevendans. Beste Pieter, de inbreng van jou en jouw onderzoeksgroep heeft ons onderzoek niet alleen een extra anatomische, maar ook wetenschappelijke dimensie gegeven. Jouw kritische blik en goed strategisch inzicht hebben mijn onderzoek focus helpen vinden. Ik dank je in het bijzonder voor de stimulans een eigen projectvoorstel te schrijven en de steun die je daarbij gegeven hebt. Je leerde mij hoe succesvol onderzoek ook sexy moet zijn, de relatie tussen wielrennen en onderzoek, en hypothesen te beperken tot één zin.

Vanuit de interne geneeskunde werd ik begeleid door professor Elsken van der Wall en later mijn promotor professor Douwe Biesma. Beste Elsken, dank voor je vertrouwen in mij als wetenschapper en als arts. Ik heb de vrijheid en steun die je me gegeven hebt zeer gewaardeerd en je hebt belangrijke ervaringen zoals mijn periode in de VS helpen mogelijk te maken. Beste Douwe, met mijn onderzoek op het letterlijk en figuurlijk grensgebied tussen bloed en vaten was ik zeer verheugd met een hematoloog / vasculair-geneeskundige als promotor. Ik zag je vertrekken uit het Sint Antonius Ziekenhuis in Nieuwegein, maar ben blij dat ik op jou als promotor en hoofdopleider in het UMC Utrecht terug mag blijven vallen.

I was extremely fortunate to be given the chance to perform part of my research project in the lab of professor Shahin Rafii at Weill Cornell Medical Center in New York, USA. Dear Shahin, I thank you for providing me with the opportunity to participate in the phenomenal science performed at your lab. Your unlimited devotion to science and unstoppable enthusiasm have been a great inspiration. You taught me that there is always a mechanism underlying the mechanism and how good science can only be achieved through hard work and relentless verification.

Tijdens mijn onderzoeksperiode is er één persoon geweest in het UMCU die mij voortdurend terzijde heeft gestaan: mijn co-promotor Marianne Verhaar. Beste Marianne, we hebben turbulente tijden meegemaakt in het laboratorium vasculaire geneeskunde. Dankzij jou is ons onderzoek hierbij voortdurend op koers gebleven. Je hebt me op vele vlakken begeleid en ik heb ontzettend veel van je geleerd. Ik kon altijd bij je terecht en kon altijd vertrouwen op je steun. Je hebt me geremd waar nodig en gestimuleerd waar mogelijk. Je leerde me precies en kritisch te zijn, het belang van menselijk leiderschap en ik hoop dat je me geleerd hebt hoe je patiëntenzorg, wetenschap, onderwijs, management en ouderschap succesvol kunt combineren.

Ik wil verder vooral mijn directe collega's bedanken in het laboratorium Vasculaire geneeskunde. Allereerst de analisten die zeer belangrijk zijn geweest voor het slagen van de diverse projecten. Krista den Ouden, heel erg bedankt voor je geweldige inzet en kritische beschouwing van elke punt en komma in mijn concept-manuscripten. Je hebt iedere muis of rat (levend, in celsuspensie of als 5µm plakje) perfect verzorgd en hiermee gewaarborgd dat tegenvallende resultaten echt alleen te wijten konden zijn aan mijn proefopzet.
Karin van 't Wout, zoals je leest hebben we met Krista het onmogelijke mogelijk gemaakt door jou te vervangen zonder dat de boel in het honderd is gelopen. Ik kon altijd van je op aan en benijdt nog altijd het GDL dat ze zo'n organisatorisch talent hebben kunnen aannemen.
Petra de Bree, ook jij heel erg bedankt voor je inzet en hulp bij het tot stand komen van het werk in (en buiten) dit boekje. Je bent het onbetwiste gezicht van ons lab en hoeksteen van expertise. Ik heb veel van je geleerd over de jaren.
Ben van Middelaar, hoewel je inmiddels natuurlijk een nieuwe plek bij de buren gevonden hebt, wil ik je nogmaals heel erg bedanken voor je hulp bij mijn eerste experimenten. Ik begon als student bij jou met twee linkerhanden en je hebt geweldig geholpen hier verandering in te brengen. Ik heb ook veel plezier gehad bij onze badder-sessies van de ratten met de ischemische achterpootjes en aan onze gedeelde passie voor theater.
Ook alle anderen in het lab wil ik danken voor de samenwerking. Cindy Loomans, voor het delen van de haat-liefde verhouding met de endotheelprogenitorcel en het prachtige uitzicht over Boston. Despina Xanthakis, voor het opzetten van de uiteindelijk toch nuttige eNOS sandwich ELISA en eindeloze positiviteit in het lab. Lonneke Bevers, voor je hulp bij het eNOS-werk en het vele borrel-initiatief. Maarten 'fakkelbrenger' Rookmaaker, voor je uitstekende voorwerk en vermakelijke cabareteske beschouwingen die vaak nog hout sneden ook. Jeroen van Wijk, voor het helpen ontdekken van de onzin van de polypil en het opvangen van Karin bij haar vertrek uit het lab. Arno van Oostrom, voor het op gang helpen houden van koffie-stroom en gezelligheid in de laagtijdagen van het lab. Olivia van Oostrom, voor de medisch-biologische inbreng en de diabetische verdieping. Kim Jie, voor het vinden van bewijs voor EPC als cardiorenale connector, het waarborgen van mijn veiligheid onderwater in het Grevelingenmeer en de inbreng van nieuwe 'schwung' in het lab. Ralf Sprengers, voor de translatie naar de vasculair bedreigde patiënt en overnemen van de beenmergblik. Michelle Goossens, voor het ontfutselen van bloed van kleine kindertjes en de bereidheid de reanimatie-achterwacht te vormen tijdens mijn eerste diensten. En ook mijn dank aan de vele anderen: Dafna Groeneveld, Marloes Bergevoet, Fabrice Martens, Anton-Jan van Zonneveld, Hetty de Boer, Caroline Verseyden, Livio Kleij.
Onze collega's van de 'klinische kant' van het vasculaire geneeskundig onderzoek eveneens heel hartelijk dank. Frank Visseren, voor de lessen in statistiek, het invallen voor televisie-interviews en natuurlijk de mooie gelegenheid om samen te werken op jouw studies. Gideon Hajer, voor de goede koffiegesprekken, uitstekende organisatie van de Rome-reis en de vele buisjes obesen-bloed. Ook Jobien Olijhoek, Annemieke Sloeserwij en Corina Joosten bedankt.

In het bijzonder ook wil ik 'mijn' medisch en medisch-biologische studenten noemen voor hun bijdragen aan het tot stand komen van dit boekje en werk dat hier niet in opgenomen is, maar ook voor hun inbreng van enthousiasme in het lab. Ik heb jullie met veel plezier begeleid en heb op mijn beurt ook veel van jullie geleerd. John Janssen, die zich op imposante wijze vastgebeten heeft in eNOS-modulatie van geïsoleerde CD34+ progenitorcellen en daarbij getalenteerd biljarter bleek te zijn. Cindy van Velthoven, die geweldig werk verricht heeft aan SPC en neointimavorming in het diabetische muismodel en zich daarbij begiftigd schilderes toonde. Janneke Jaspers, die de invloed van diabetes op EPC-mobilisatie onderzocht in muizen en daarbij mij geweldig gezellig kwam opzoeken in New York. Remco Luijten, die in korte tijd 30 vrouwen (waarvan de helft met SLE) wist te verleiden om deel te nemen aan ons onderzoek en daarbij in de weekenden nog aan voetbal toekwam ook. Jan van Ramshorst, die zich razendsnel heeft weten te bekwamen in het hanteren van humaan beenmerg, uiteindelijk nog de meeste indruk maakte met zijn studie naar NO/ROS in EPC van dialysepatiënten en natuurlijk met zijn piano-optreden op nationale televisie. Stella Brouwer, die middels PCR uitgebreide genexpressieprofielen heeft samengesteld voor patiënten met het metabool syndroom en daarbij een veel bereisde vrouw bleek. Caroline van Boheemen, die de differentiatie van progenitorcellen na 'capture' op anti-CD34 glaasjes in soms een bloedbad onderzocht en daarbij met mij (studenten)politieke aspiraties bleek te delen. Hannah Visser, die het de stromale cel/progenitorcel interactie bij nierfalen uitzocht en dit de Spaanse post-doc vloeiend in haar eigen taal kon uitleggen.

Verder dank aan onze buren en belangrijke samenwerkende groepen: de experimentele cardiologie, nefrologie en pathologie. Hierbij van de experimentele cardiologie onder andere kamergenoten Anke Smits, Piet van Vliet en Sophie Bekkers, en verder Arjan Schoneveld, Bernard te Boekhorst, Chaylendra Strijder, Cor Seinen, Corina Metz, Dik Versteeg, Dominique de Kleijn, Jan-Willem Leeuwis, Joost Sluijter, Joris Rotmans, Karlijn van Keulen, Leo Timmers, Manon Oude-Nijhuis, Pieter Bot, Sebastian Grundmann, Simone Post, Tom Korfage en Willem Hellings. En van de nefrologie onder andere Adele van Dijk, Arjan van Zanten, Branko Braam, Brigitte van Jaarsveld, David Ishola, Helena Chon, Lars Penne, Lennart Bongartz, Maarten Koeners, Mehdi Rastmanesh, Nel Willekes, Paula Martens, Peter Blankenstijn, Simona Racasan, Ria de Winter, Richard Huijbrechts, Ronald Hene, Sebastiaan Wesseling, Walter Boer en Walter Brummelhuis.
Van de pathologie Roel Goldschmeding Tri-Quang Nguyen, Frans van Nieuwenhoven en Peggy Roestenberg. Roel en Tri, ik heb het geweldig leuk gevonden dat we de interesse voor de SPC deelden (door sommigen toch echt liever MFPC genoemd…); jullie specifieke expertise is van groot belang voor het welslagen van deze onderzoekslijn in het UMCU. Ook de hulp bij kleuringen, PCR en beoordelingen van histologie heb ik zeer gewaardeerd.
In het bijzonder wil ik verder bedanken Gerard Pasterkamp, voor de steun in de rug ten tijde van organisatorische onduidelijkheden en altijd goede vragen bij clustermeetings; Imo Hoefer, voor veel meer dan de hulp bij de flowcytometrie want uiteindelijk vooral voor het bieden van een impuls aan onderzoekskwaliteit en collegialiteit in het lab; Marie-Jose Goumans, voor het beantwoorden van de vele vragen over celbiologische obstakels en het initiëren van een verbreding van het progenitorcelwerk in het UMCU en de onovertroffen naamgeving van haar eerste zoon. Verder Jaap Joles, die mij onafgebroken heeft gestimuleerd het beste te maken van mijn dierexperimentele studies en die ons spelenderwijs in de nefrobibliotheek de wetenschappelijke literatuur leert doorgronden.

Binnen het UMCU heb ik advies gevonden bij velen en heb ik op diverse manieren kunnen samenwerken. Van de afdeling anatomie Ronald Bleijs, Willem van Wolferen en Simon Plomp. Van de longziekten Laurien Ulfman. Van de hematologie/immunologie Henk Rozemuller, Anton Martens,

Henk-Jan Prins, Christina Olivo en Ineke Slaper. Van de orthopedie Laura Creemers, Natalja Fedorovich, en Rene Haverslag. Van de reumatologie Ron Derksen. In het GDL Cees Brandt, dierverzorgers en ondersteunend personeel.

Buiten het UMCU hebben we meerdere vruchtbare samenwerkingen op kunnen zetten. Voor mij waren de 'kijkjes in de keuken' elders een groot plezier en uitermate leerzaam. In het Hubrecht laboratorium Linda van Laake. In het AMC de Hebe-trial coördinatie: Alexander Hirsch, Joost Haeck, Anja van der Laan en Jan Piek. In het AMC tevens Peter Bonta en Carlie de Vries, alsook Geurt Stokman en Jaklien Leemans. In het UMCG Groningen Jan-Luuk Hillebrands, Jan Rozing, Geanina Onuta en Heleen Rienstra. Bij de Sanquin bloedbank Rachel van Beem, Willy van Noort en Jaap-Jan Zwaginga. In het VUMC Robert Verloop en Victor van Hinsbergh.

Also many thanks to my international collaborators: my colleagues at the Rafii lab at Weill Cornell Medical College in New York. Working with you has been a great inspiration and fantastic experience: Andrea, George, Ilaria, Isabelle, Jason, JiYeon, Kilang, Koji, Koos, Li, Marco, Mariana, Masaya, Sergei, Sina, Tricia, Yuki and in particular Ian, whose comradeship in science and life has been a blessing.
Also in Wurzburg, Germany: Thomas Thum and colleagues: thank you for my time at your laboratory.

Dank ook aan mijn vrienden en in het bijzonder mijn paranimfen Joost Hopman en Alvin Schadenberg.

Verder wil ik mijn ouders noemen voor hun steun en stimulans bij mijn ontplooiing.

Tot slot dank aan mijn vrouw Saskia, op wie ik altijd kan rekenen voor liefde, steun en begrip. Samen met de rest van ons gezin-in-wording ben je mijn bron van inspiratie en geluk.

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Curriculum Vitae

Peter Edwin Westerweel was born on the 21st of November 1978 in Wijk-bij-Duurstede, The Netherlands, as the son of doctor Ron Westerweel and Arianne Sterk. After graduating high school at "'t Hooghe Landt College" in Amersfoort in 1996, he was admitted to the University of Utrecht, the Netherlands, to study Medicine. As a medical student, he was introduced to basic science at the department of Vascular Medicine of the division of Internal Medicine at the University Medical Center Utrecht, which was headed by professor Ton Rabelink at the time. He spent a year at the laboratory under supervision of Dr. Marianne Verhaar. After obtaining his degree in Medicine in 2003, he returned to work there as a research-physician under supervision of again Dr. Marianne Verhaar and professor Hein Koomans of the department of Nephrology & Hypertension. In 2004 he was awarded the Dr. E. Dekker Fellowship by the Dutch Heart Foundation. This project brought him in close cooperation with professor Doevendans and the Interuniversity Cardiological Institute Netherlands (ICIN). In 2007, supported by ZonMw with an AGIKO grant and by the Dutch Heart Foundation, he spent 5 months at the Ansary Stem Cell Center for Regenerative Medicine at Weill Cornell Medical College in New York, USA, headed by professor Shahin Rafii. Upon return, he continued his work at the department of Vascular Medicine in the UMC Utrecht. In September 2007, he also started his residency in Internal Medicine at the St. Antonius Hospital in Nieuwegein, the Netherlands, supervised by Dr. Geers in the St. Antonius Hospital and Professor Elsken van der Wall and Professor Douwe Biesma in the UMC Utrecht. Peter Westerweel married Saskia Holtslag in 2007 and they are currently expecting their first child.