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Original article

Mobilization of sca1/flk-1 positive endothelial progenitor cells declines in apolipoprotein E-deficient mice with a high-fat diet

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ABSTRACT

Background: Atherosclerosis features a deterioration of the endothelial layer in all stages. Restoration of the endothelium is associated with circulating stem cell antigen 1 (sca1) and vascular endothelial growth factor receptor type 2 (flk-1) positive endothelial progenitor cells (EPCs). We investigated whether EPC production and/or a mobilization from bone marrow are reduced in severe atherosclerosis.
Methods and results: EPCs in peripheral blood were diminished in ApoE−/− mice with high-fat diet (HFD) whereas bone marrow levels of these cells were not significantly altered compared to controls.

In situ perfusion of the hind limbs demonstrated that EPC mobilization was reduced compared to ApoE−/− mice with normal chow, although increased plasma stromal cell-derived factor (SDF) 1α and responsivity suggested a mobilizing stimulus. The proliferation of sca1/flk-1 positive cells showed no functional impairment.

EPCs could not only be significantly mobilized from the bone marrow through the application of granulocyte colony stimulating factor (GCSF), but also led by trend to a depletion of the bone marrow pool. GCSF levels in plasma were equal in ApoE−/− mice with normal chow or HFD, which excluded a decline in GCSF production.
Conclusion: The capability of the bone marrow pool to adapt the proliferation and mobilization of sca1/flk-1 positive EPCs seems overstrained in ApoE−/− mice with a HFD.

Introduction

Atherosclerosis is a chronic inflammatory disease, which features an accumulation of oxidized lipids and deterioration of the endothelial layer in all disease stages. Damaged, dysfunctional endothelial cells (ECs) lose their capability of vasorelaxation and express adhesion molecules, which lead to the migration of inflammatory cells into the vascular wall [1]. The mismatch of endothelial dysfunction and EC repair is pivotal for the development of atherosclerosis. EC reconstitution is not only generated by proliferation of neighboring ECs, but appears to be influenced by circulating, putatively bone marrow derived endothelial stem and progenitor cells [2]. Interestingly, various subsets of these putative endothelial progenitors are diminished in atherosclerotic disease and are inversely correlated to cardiovascular risk and disease burden [3,4]. Stem cell antigen 1 (sca1) and vascular endothelial growth factor (VEGF) receptor type 2 (flk-1) double positive cells have been considered as circulating endothelial progenitor cells (EPCs) in the past. Previous data by others and us have correlated decreased levels of putative EPCs in peripheral blood with increased atherosclerotic burden and worsened endothelial dysfunction (review see [5]). On the other hand, increased levels of sca1/flk-1 positive EPCs have been associated with improved reendothelialization [6–8] and diminished atherosclerotic plaques [9], suggesting a beneficial effect. However, their cellular identity is questioned in order to understand their biological role and potential. Whereas "early" EPCs most frequently are monocytes that are "contaminated" with thrombocyte microparticles mimicking endothelial features during in vitro culture [10], and endothelial colony forming cells (ECFCs or "late EPCs") resemble ECs in transcriptional profiling [11]. Circulating EPCs finally are

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quantified by flow cytometry, and the most popular and investigated surface marker combinations comprise a heterogeneous composition of CD45 positive and negative cells, with hematopoietic and endothelial potential (also see review [12]). Predominantly circulating EPCs were successfully used as a biomarker. Tracking their routes in the circulation from bone marrow to peripheral blood is relevant to understand their implication in cardiovascular diseases.

We hypothesized, that a reduction in circulating sca1/flk-1 positive EPC production and/or a defect in mobilization from the bone marrow into circulation might be associated with the aggravation of atherosclerotic disease in apolipoprotein E-deficient (ApoE−/−) mice.

The following results illustrate that sca1/flk-1 positive EPCs are diminished in ApoE−/− mice with high-fat diet (HFD), despite mobilizing stimuli. Through the use of an in situ perfusion model we demonstrate for the first time with a direct approach that EPC egress from the bone marrow into the circulation declines, and can be transitorily restored through the application of granulocyte colony stimulating factor (GCSF).

Materials and methods

All animal experiments were performed in accordance with institutional guidelines and the German animal protection law.

Flow cytometry analysis of peripheral blood, bone marrow cells, and venous perfusate

The analysis of peripheral blood, bone marrow cells, and venous perfusate was performed by flow cytometry analysis according to previously published protocols [7]. The lymphocyte population was analyzed for the expression of sca1-APC (R&D Systems, Minneapolis, MN, USA) and flk-1-PE (Becton Dickinson, Franklin Lakes, NJ, USA). Isotype-identical antibodies and unstained samples served as controls (Becton Dickinson). Cell fluorescence was measured immediately after staining using a FACSCalibur instrument (Becton Dickinson). Data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Carotid artery denudation

Carotid artery injury was induced as described previously [7]. Briefly, the common carotid artery was exposed and submitted to an electric injury starting at the bifurcation and continuing to the proximal part of the artery (in total 4 mm denudation). The denuded area of the common carotid artery was determined at day 5 after surgery following intra-arterial injection of 50 μl Evans blue in an enface preparation of the vessel. Evans blue stained denuded areas and the complete vessel area was measured using AxioVision version 4.5.0 software (Zeiss, Jena, Germany). The percentage of reendothelialization 5 days after injury is provided.

In situ perfusion of mouse hind limb

The mice were anesthetized, and the femoral artery and vein of the left hind limb were exposed. The hind limb was functionally isolated by occlusion of the external iliac artery, superficial epigastric branch, and muscular branch (braded silk sutures 6,0, Resorba, Nürnberg, Germany). Heparinized (Heparin-Natrium 25000, Ratiopharm, Ulm, Germany) polyethylene cannulae (Fine Science Tools, 0.2 mm, Heidelberg, Germany) were inserted into the femoral artery and vein. Perfusion buffer (phosphate buffered saline) was infused via the femoral artery and mobilized cells were collected via the femoral vein using a peristaltic pump (Roller Pump Reglo Digital, 4 Channels, Harvard Apparatus, Holliston, MA, USA). The hind limb was perfused for an initial 10 min (0.1 ml/min) to remove remaining blood from the vasculature, and then perfused for a further 60 min to collect mobilized cells. After sample collection, mobilized cells were analyzed through flow cytometry as described above. To exclude mobilized cells that were mechanically mobilized from the bone marrow, the bone marrow of the right and left limb was analyzed separately [13–15].

For further information see *Supplementary methods*.

Results

Circulating sca1/flk-1 positive EPCs and in vitro cultured EPCs decline in ApoE−/− with HFD

ApoE−/− mice developed atherosclerotic plaques, which were aggravated by the addition of a HFD (Fig. S1). We quantified EPC subsets in the blood circuit and bone marrow, with low numbers of those cells indicating an aggravation of disease [16]. As expected, the number of sca1/flk-1 positive EPCs in peripheral blood decreased in ApoE−/− with HFD compared with ApoE−/− receiving normal chow, whereas bone marrow levels of sca1/flk-1 positive EPCs appeared equal between the groups (Fig. 1A). Concordantly, the number of in vitro cultured colony forming units-Hill and late EPC was significantly reduced in ApoE−/− mice with HFD compared to ApoE−/− with normal chow (Fig. 1B).

Low EPC levels have been previously linked to endothelial dysfunction and the diminished capacity of endothelial recovery [7]. Hence, we assessed the impact of HFD on endothelial reendothelialization in ApoE−/− mice after carotid artery denudation. Five days after surgery, ApoE−/− mice with HFD showed significantly reduced reendothelialization (Fig. 1C).

These results raised the question whether EPCs are reduced in atherosclerosis only due to an augmented turnover as previously demonstrated by Foteinos et al. [17], or if less production and mobilization from the bone marrow compartment additionally contribute.

Mobilization of EPCs is diminished in ApoE−/− with HFD

To assess the mobilization from the bone marrow pool in vivo, we used an in situ perfusion model to enumerate sca1/flk-1 positive EPCs (Fig. 2). Since mobilized EPCs might redistribute immediately into different compartments such as liver or spleen, be recruited into atherosclerotic lesions or come back into the bone marrow pool, blood sample analysis of circulating sca1/flk-1 positive EPCs does not necessarily represent their mobilization from the bone marrow but also from other niches. We used in situ perfusion of a functionally isolated (i.e. ligated) hind limb as modified by Pitchford and Rankin [13] to overcome these analytical problems.

In this model, the number of sca1/flk-1 positive EPCs in the perfusate of ApoE−/− with normal chow was significantly higher (Fig. 2A). Bone marrow levels of sca1/flk-1 positive cells of the left (perfused) and right (non-perfused) hind limb did not differ significantly (Fig. 2B).

SDF-1α is increased in ApoE−/− mice with and without HFD

Stromal cell-derived factor 1 alpha (SDF-1α) is pivotal for stem and progenitor homing in tissues [18]. SDF-1α/CXCR4 binding in the bone marrow compartment is responsible for stem cell retention. Elevated levels of circulating SDF-1α represent a strong mobilizing stimulus [19]. We observed equal concentrations of SDF-1α in the plasma samples of ApoE−/− with normal chow or HFD, bone marrow supernates (Fig. 3A), protein or mRNA (Fig. 3B and C). The protein and mRNA expressions of C-X-C motif receptor