**Age-Dependent Impairment of Endothelial Progenitor Cells Is Corrected by Growth Hormone Mediated Increase of Insulin-Like Growth Factor-1**

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Abstract—Aging is associated with an increased risk for atherosclerosis. A possible cause is low numbers and dysfunction of endothelial progenitor cells (EPC) which insufficiently repair damaged vascular walls. We hypothesized that decreased levels of insulin-like growth factor-1 (IGF-1) during age contribute to dysfunctional EPC. We measured the effect of growth hormone (GH), which increases endogenous IGF-1 levels, on EPC in mice and human subjects. We compared EPC number and function in healthy middle-aged male volunteers (57.4±1.4 years) before and after a 10 day treatment with recombinant GH (0.4 mg/d) with that of younger and elderly male subjects (27.5±0.9 and 74.1±0.9 years). Middle-aged and elderly subjects had lower circulating CD133+/VEGFR-2+ EPC with impaired function and increased senescence. GH treatment in middle-aged subjects elevated IGF-1 levels (126.0±7.2 ng/mL versus 241.1±13.8 ng/mL; P<0.0001), increased circulating EPC with improved colony forming and migratory capacity, enhanced incorporation into tube-like structures, and augmented endothelial nitric oxide synthase expression in EPC comparable to that of the younger group. EPC senescence was attenuated, whereas telomerase activity was increased after GH treatment. Treatment of aged mice with GH (7 days) or IGF-1 increased IGF-1 and EPC levels and improved EPC function, whereas a two day GH treatment did not alter IGF-1 or EPC levels. Ex vivo treatment of EPC from elderly individuals with IGF-1 improved function and attenuated cellular senescence. IGF-1 stimulated EPC differentiation, migratory capacity and the ability to incorporate into forming vascular networks in vitro via the IGF-1 receptor. IGF-1 increased telomerase activity, endothelial nitric oxide synthase expression, phosphorylation and activity in EPC in a phosphoinositide-3-kinase/Akt dependent manner. Small interference RNA-mediated knockdown of endothelial nitric oxide synthase in EPC abolished the IGF-1 effects. Growth hormone-mediated increase in IGF-1 reverses age-related EPC dysfunction and may be a novel therapeutic strategy against vascular disorders with impairment of EPC. (Circ Res. 2007;100:434-443.)

Key Words: endothelial progenitor cells ■ aging ■ insulin-like-growth-factor-1 ■ growth hormone

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Endothelial injury and dysfunction are critical events in the pathogenesis of atherosclerosis. Understanding the mechanisms responsible for the repair of endothelial lesions and restoration of endothelial function will have important clinical implications. As resident endothelial cells infrequently proliferate in the vascular wall, other sources of cellular replenishment have been postulated as mechanisms to repair endothelial lesions. Bone-marrow derived endothelial progenitor cells (EPC) circulate in the blood and contribute to formation of new blood vessels and homeostasis of the vasculature. Patients with reduced EPC levels are at increased risk for cardiovascular events and death. Recent studies suggest augmentation of circulating EPC to result in improved coronary collateral development in coronary artery disease. Increasing age is associated with decreased number and impaired function of EPC, which may facilitate atherosclerotic processes. Regulation of EPC mobilization, differentiation and function is complex, but specific growth hormones and cytokines are explicitly involved. Insulin-like growth factor-1 (IGF-1) enhances migration, tube formation and angiogenesis of mature endothelial cells and increases te-
lomerase activity. The effects of IGF-1 on endothelial cells are mediated at least in part via upregulation of endothelial nitric oxide synthase (eNOS) expression.

Low serum IGF-1 levels, common in the elderly, are associated with an increased risk for ischemic heart disease. Restoration of IGF-1 in elderly individuals by growth hormone therapy may have significant beneficial health effects. In growth hormone deficient patients replacement therapy attenuates the increased tendency to develop endothelial dysfunction and severe atherosclerosis. Recently, Urbanek and coworkers have shown that IGF-1 promotes survival and proliferation of resident cardiac stem cells resulting in improved myocardial regeneration after myocardial infarction.15 Likewise, in the infarcted myocardium, IGF-1 promotes engraftment, differentiation, and function of implanted embryonic stem cells leading to improved myocardial function.16 IGF-1 transgenic mice demonstrate increased telomerase activity and preservation of cardiac resident stem cells.10 The effects of IGF-1 on EPC homeostasis and function are not known.

We investigated whether augmented IGF-1 levels in response to growth hormone treatment may restore the age-dependent decline in EPC levels and function in mice and humans. Ex vivo we tested, whether IGF-1 treatment of EPC of aged individuals would restore function and attenuate cellular senescence. In vitro studies were undertaken to identify underlying molecular mechanisms.

### Materials and Methods

#### Clinical study

The ethical committees of the Universities of Hannover and Würzburg approved the study. Written informed consent was obtained from the volunteers. See the online data supplement available at http://circres.ahajournals.org for details.

#### Measurement of IGF-1 and Basic Blood Parameters

See the online data supplement available at http://circres.ahajournals.org.

#### Isolation of Peripheral Blood Mononuclear Cells and CD34+, CD117+, CD133+ and CD133+/VEGFR2+ Progenitors

See the online data supplement available at http://circres.ahajournals.org.

#### Determination of Progenitor Cells

A variety of assays was used to determine the number, differentiation, and function of EPC (see online data supplement).

#### Expression of the IGF-1 Receptor on EPC

IGF-1 receptor expression was determined in colony forming units (CFU) and monocytic EPC (see online data supplement).

#### Quantification of eNOS and IGF-1 Receptor Gene Expression of EPC

Total RNA was isolated from EPC of young, middle-aged (before and after GH treatment) and aged individuals according to the manufactures instructions (Qiagen, Germany). Details of the real-time PCR method are described in the supplemental online section.

#### Western Blotting Analysis

See the online data supplement available at http://circres.ahajournals.org.

#### eNOS Activity

eNOS activity was determined as described (18 and online data supplement).

#### Small Interference RNA-Mediated Knockdown of eNOS in EPC

See the online data supplement.

#### Acidic β-Galactosidase Staining of EPC

This was done as described with slight modifications (19; see online data supplement).

#### Telomerase Activity

This was done as previously described (20; see online data supplement).

#### Mouse In Vivo Study

We examined the effects of both GH and IGF-1 treatment in aged mice. We also analyzed GH induced changes in EPC levels and function during interruption of IGF-1 receptor signaling in vivo by a blocking antibody or a small molecule inhibitor (see online data supplement).

#### Statistical Analysis

See the online data supplement available at http://circres.ahajournals.org.

### Results

#### Age-Related Decline in IGF-1 Levels, EPC Number and Function

IGF-1 plasma levels were significantly reduced in the elderly and middle-aged as compared with young men (120.8±10.5 ng/mL and 126.0±7.2 ng/mL versus 223.1±15.7 ng/mL; P<0.0001). Older individuals had significantly lower concentrations of circulating CD133+/VEGFR2+ cells (Figure 1A). EPC function was impaired with age, as determined by reduced colony forming capacity (Figure 1B), and impaired cellular migratory capacity (Figure 1C). Gene expression of eNOS was significantly reduced in EPC from aged subjects, whereas expression of the IGF-1R was unchanged (Figure 1D).

#### Treatment With Recombinant Human Growth Hormone of Middle-Aged Individuals Restored IGF-1 Levels, Increased EPC Number, and Improved EPC Function

A 10 day treatment of middle-aged men with GH increased IGF-1 serum levels from 126.0±7.2 ng/mL to 241.1±13.8 ng/mL (P<0.0001). CD133+/VEGFR2+ cells were 2-fold increased after GH treatment (Figure 1A). Likewise, the number of endothelial CFU was increased in treated individuals (Figure 1B) and both CD133+/VEGFR2+ cells and CFUs correlated with IGF-1 plasma levels (r=0.46, P<0.01 and r=0.60, P<0.0001). Hemoglobin (14.65±0.62g/dL versus 14.46±0.73g/dL), total erythrocyte (4.83±0.73g/dL) or leukocyte numbers (5.01±1.00x10⁶/µL versus 5.02±0.99x10⁶/µL) were unchanged by GH treatment. Migratory capacity of EPC was improved after GH treatment (Figure 1C). eNOS expression in isolated EPC was significantly increased by 64% after 10 day GH treatment, whereas that of the IGF-1R was basically unchanged (Figure 1D).
Figure 1. Circulating EPC number and function of middle-aged subjects before and after treatment with recombinant growth hormone compared with young controls and elderly individuals, as well as in aged mice. Clinical study (A-D): A, Number of circulating CD133⁺/VEGFR2⁺ cells in % of mononuclear cells and (B) number of endothelial colony forming units in young, middle-aged before and after treatment with recombinant human GH and elderly subjects. C, Migratory capacity of EPC from the various study groups. D, Gene expression of IGF-1R and eNOS relative to GAPDH in EPC derived from the various study groups. MA = middle-aged. Mouse study (E-H): E, Number of circulating sca1⁺/flk1⁺ cells in % of mononuclear cells and (F) migratory capacity of monocytic EPC of mice treated with placebo (control), IGF-1 (2 days), GH (2 or 7 days). G, Number of circulating sca1⁺/flk1⁺ cells in % of mononuclear cells and (H) migratory capacity of mononuclear cells and IGF-1 receptor inhibitor II (Calbiochem, Germany). n=4 to 6 per study group.
IGF-1 Mediates the Effects of GH Treatment on EPC Number and Function in Aged Mice

Both treatment of aged male mice with GH (7 days) or IGF-1 (2 days) increased systemic IGF-1 levels [control PBS-treated mice: 230±11006 ng/mL; GH (7d): 520±11006 ng/mL (P<0.001); IGF-1 (2d): 631±142 ng/mL (P<0.0001)] as well as sca1+/flk1+ EPC in peripheral blood and improved EPC function (see Figure 1, E and F). In contrast, a 2 day treatment with GH did not significantly increase IGF-1 levels (GH,2d: 243±6 ng/mL) nor EPC number or function indicating that IGF-1 is the main mediator of the observed effects of GH on EPC. Regression analysis revealed a positive correlation between IGF-1 levels and EPC number (r=0.45; P<0.05) or EPC migratory capacity (r=0.49; P<0.05). When the IGF-1 receptor was blocked either by a neutralizing antibody or a specific IGF-1 receptor inhibitor (see online data supplement), the GH-mediated effects on circulating EPC numbers (Figure 1G) and function (Figure 1H) were abolished.

IGF-1 Improves Differentiation and Function of EPC From Young and Elderly Individuals via the IGF-1 Receptor

To test whether increased IGF-1 concentrations may mediate the observed effects of GH treatment, we first investigated whether the IGF-1R is present on EPC. We showed IGF-1R expression on various subtypes of progenitor cells, eg, early outgrowth EPC (CFU assay) and adherent dil-acLDL+/UEA-1+ EPC, which have previously shown to be of monocytic origin, but bind endothelial lectins and express endothelial proteins, such as the von Willebrand factor (vWF) (Figure 2 and 3). To test whether the IGF-1R is functionally active we performed further in vitro studies. IGF-1 treatment enhanced formation of endothelial CFU and increased formation of UEA-1+/dil-acLDL+ cells from cultured PBMC, indicating stimulation of EPC differentiation and function (Figure 4A, B). Migratory capacity of monocytic EPC was likewise improved by IGF-1 treatment (Figure 4A, B). In addition, IGF-1 stimulated migration of CD133+ derived and freshly isolated CD133+/VEGFR2+ EPC, whereas GH was without effect (Figure 5, A and C). The ability to incorporate into forming vascular networks on matrigel was significantly increased in IGF-1 pretreated EPC (Figure 4, A and B). In contrast, GH treatment in vitro, with the exception of minor improvement of cellular migration, did not display significant effects on EPC in the aforementioned assays (Figure 4). Inhibition of the IGF-1R by pretreatment with an inhibitory IGF-1R antibody abolished the effects of IGF-1 (Figure 4). IGF-1 treatment improved migratory capacity of EPC from elderly individuals, as compared with untreated cells (Figure 5B).

To test whether the migratory potential of IGF-1 may apply to other kinds of stem cells we tested the effects of IGF-1 on human mesenchymal stem cells (hMSC). In contrast to the effects on EPC, IGF-1 (100 ng/mL) did not improve the migratory capacity of hMSC (89.5±10.7 versus 78.5±3.2 migrated hMSC/microscopic field; n=8; p=n.s.).
IGF-1 Increases Phosphoinositide 3-Kinase/Akt Mediated Expression and Phosphorylation of eNOS in Cultured EPC

As EPC function and differentiation is in part regulated via eNOS,22 we tested whether GH or IGF-1 treatment would impact phosphoinositide(PI)-3-kinase/Akt/eNOS signaling. Treatment of EPC with IGF-1 induced Akt phosphorylation, as well as expression and phosphorylation (Ser1177) of eNOS (Figure 6A). Functionally, IGF-1 treatment increased eNOS activity (Figure 6B). Inhibition of the PI3-kinase prevented IGF-1 mediated Akt phosphorylation, as well as eNOS expression and phosphorylation. Blocking of the PI3-kinase pathway reduced EPC differentiation and function (Figure 4, A and B). Inhibition of the IGF-1R with an inhibitory antibody completely abolished IGF-1 induced phospho-Akt/eNOS signaling (Figure 6A) and eNOS activity (Figure 6B). In contrast, GH had minor effects on eNOS expression, but did not affect Akt or eNOS phosphorylation or eNOS activity (Figure 6). Functional knockdown of eNOS by small interference RNA abolished the stimulatory IGF-1 effect on cellular migration of EPC, whereas addition of transfection media or scrambled siRNA had no significant effects (Figure 7).

IGF-1 Impacts EPC Cellular Senescence and Telomerase Activity

Vascular endothelial cells with senescence-associated phenotypes are present in human atherosclerotic lesions of elderly patients.23 Acidic β-galactosidase can be detected in cultured EPC as a biochemical marker for the onset of cellular senescence.19,24 Cultured EPC from healthy individuals treated with IGF-1 displayed significantly fewer β-galactosidase positive cells than cells without the addition of IGF-1 (6.5±3.3 versus 21.5±5.3 β-galactosidase positive cells; P<0.001) and showed enhanced telomerase activity, that was attenuated by PI3-kinase inhibition or blocking of the IGF-1R (Figure 8A). Growth hormone was without effect on telomerase activity.

Elderly individuals displayed significantly increased number of β-galactosidase positive EPC compared with EPC isolated from young subjects, demonstrating increased cellular senescence (Figure 8B), whereas telomerase activity in isolated progenitor cells was reduced (Figure 8C). Treating cultured EPC from aged individuals with IGF-1 reduced cellular senescence (Figure 8B). Likewise, growth hormone mediated IGF-1 increase in middle-aged subjects resulted in reduced EPC senescence (Figure 8D). Telomerase activity of CD133+ enriched progenitor cells was 2.3-fold (P<0.05) increased after treatment with GH and was comparable to that of young individuals (Figure 8C).

Discussion

The results of the current study demonstrate that the age-related decline in EPC number and function can be restored by growth hormone mediated increase of IGF-1 levels. In vitro and in vivo studies extend and support these clinical observations. IGF-1, but not GH enhanced EPC differentiation and function involving PI3-kinase/phosphorylated Akt-mediated increase in eNOS phosphorylation and activity.

The finding of reduced amounts of circulating EPC with increasing age is in line with age-dependent reduction of EPC number in patients with coronary artery disease independent of risk factors for atherosclerosis or of cardiac function.6 Functional impairment of EPC from elderly subjects is related to endothelial dysfunction.7 Experimentally, bone marrow transplantation from young, but not old, nonatherosclerotic mice prevented atherosclerosis progression in apolipoprotein E knockout recipients, suggesting that deficient vascular repair because of increased age is a critical determinant of disease initiation and progression.23 Taken together the data suggest that the age-related decline in progenitor number and function contribute to the progression of atherosclerosis. Recent studies have shown that patients with reduced circulating progenitor cells are at increased risk for cardiovascular events and death independent of other cardiovascular risk factors.3,4

As EPC mobilization and revascularization is in part regulated by NO,26–30 growth hormone-deficient patients with impaired systemic NO formation are expected to be at increased risk for cardiovascular disease.31,32 Treatment of these patients with recombinant human GH results in an IGF-1-related stimulation of endothelial NO formation, decreased peripheral arterial resistance, and reversal of morphological and functional atherosclerotic changes in major arteries.14,31,33 Low circulating IGF-1 levels are independently associated with increased risk for coronary artery disease or fatal ischemic heart disease.12,34–36 In contrast, increased IGF-1 levels are related to reduced mortality in elderly
individuals. Thus, restoration of IGF-1 appears to provide protection from cardiovascular disease progression.

Our data provide a potential mechanistic link between IGF-1 and cardiovascular disease. The age-dependent reduction in IGF-1 levels and circulating EPC numbers, as well as impaired EPC function points to a causal relationship. Indeed, ten days of GH treatment increased IGF-1 levels and circulating EPC in middle-aged subjects to the values observed in the younger group. As impaired function of EPC from older patients with ischemic heart disease may limit their therapeutic potential for clinical cell therapy, reversal of functional impairment of EPC in older individuals by GH treatment has important clinical implications. The opportunity exists to improve function of impaired EPC so as to optimize cell transplantation protocols.

Figure 4. Effects of IGF-1 and recombinant human growth hormone on endothelial progenitor cell function. Colony forming capacity (CFU), differentiation of dil-acLDL+/UEA-1+ cells from peripheral blood mononuclear cells (dil-acLDL/UEA-1), migratory capacity (migration) and incorporation of EPC into vascular structures on matrigel (incorporation) is shown in EPC after 24 hour of treatment with GH (100 ng/mL), IGF-1 (100 ng/mL), and the concomitant treatment of IGF-1 with the PI3-kinase inhibitor wortmannin (100 nM) or an inhibitory IGF-1 receptor antibody (10 μg/mL). Representative pictures (A) and the statistical summary (B) of a minimum of five experiments per study group are shown. * = P < 0.05 vs control; ** = P < 0.001 vs control; † = P < 0.05 vs IGF-1 100 ng/mL; †† = P < 0.01 vs IGF-1 100 ng/mL; ††† = P < 0.001 vs IGF-1 100 ng/mL.
Further evidence for a direct relationship between IGF-1 and EPC comes from the present in vivo and in vitro studies. Treatment of mice with IGF-1 improved number and function of EPC similar to a 7 day treatment with GH that significantly increases systemic IGF-1 levels, whereas a short term GH treatment did not increase IGF-1 nor EPC levels. Additionally, interruption of IGF-1/IGF-1 receptor signaling completely prevented the GH-mediated increase in EPC number and function.

Likewise, IGF-1, but not GH enhanced EPC differentiation, increased colony forming and migratory capacity, and stimulated EPC incorporation into tube-like structures. In addition, telomerase activity in EPC was increased and cellular senescence attenuated. Impaired function of EPC from aged subjects was normalized by IGF-1. In vivo, IGF-1 directly oppose endothelial dysfunction by enhancing NO production (reviewed in 39). IGF-1 increases NOS activity by interacting with a tyrosine kinase membrane receptor that activates phosphoinositide 3-kinase, which in turn activates the serine/threonine kinase Akt signaling pathway.41 In the heart, IGF-1 mediated activation of this pathway promoted cell growth and survival.42 In the present study, we demonstrate stimulation of PI3-kinase-dependent Akt phosphorylation and eNOS activity by IGF-1 in human EPC. Functional knockdown of eNOS using siRNA abolished the IGF-1 effects, underlining the pivotal role of eNOS in the stimulatory effects of IGF-1 on human EPC.

Within the heart, the IGF-1/IGF-1R system induces division of cardiac stem cells, upregulates telomerase activity thereby counteracting replicative senescence and preserves the pool of functionally active cardiac stem cells.10,15,43 Myocardial regeneration may in part be mediated by IGF-1, and results in delayed onset of heart failure.44 IGF-1 promotes engraftment, differentiation, and functional improvement after transfer of embryonic stem cells for myocardial restoration.16 Urbanek and coworkers found that resident cardiac stem cells express the IGF-1R,15 and our present data demonstrate expression of a functionally active IGF-1R in EPC.
Besides cardiovascular progenitor cells, IGF-1R expression has been observed in erythroid, osteogenic, and neural progenitor cells. Existence of an IGF-1R may be a general feature of progenitor cells. Targeting the IGF-1R on EPC opens a new line of therapeutic possibilities such as the use of GH or IGF-1 to restore progenitor cell function. In contrast, in human mesenchymal stem cells (hMSC) IGF-1 appears to function as a differentiation factor. In line with this finding we found no stimulatory role of IGF-1 on migratory capacity of cultured hMSC.

Transgenic IGF-1 overexpression led to increased telomerase activity and preservation of functional capacity of aging cardiac stem cells. This is of importance as chronic oxidative stress and endogenous NO synthase inhibitors compromise telomere integrity, accelerate the onset of senescence in human endothelial cells and finally are associated with reduced EPC numbers. Recent findings suggest that vascular cell senescence induced by telomere shortening may contribute to atherogenesis. Our finding that augmented IGF-1 levels increase telomerase activity and prevent cellular senescence of aged and dysfunctional progenitor cells may have important clinical implications especially in diseases with increased cellular senescence, such as atherosclerosis.

Although the GH/IGF-I axis is involved in maintenance of normal function and homeostasis of diverse body functions, it also contributes to the progression of a number of common cancers (reviewed in). Although there is no evidence of increased incidence of cancer in GH-deficient- or middle-aged heart failure patients during GH therapy, future clinical trials investigating the role of GH or IGF-1 on stem cell biology should be performed with caution.

The current study identifies IGF-1 as an important regulator of EPC. Correction of age-related decline in number and function of EPC by growth hormone-mediated increase in
IGF-1 may favor endothelial regeneration at sites of tissue damage and finally reduce cardiovascular events. Further prospective studies are needed that determine the effects of growth hormone mediated IGF-1 increase as a novel therapeutic strategy against vascular disorders with impaired number and function of EPC.

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Disclosures

None.

References


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SUPPLEMENTARY INFORMATION

Materials and Methods

Clinical study

Sixteen healthy middle-aged male volunteers between 50 and 69 years (mean age 57.4 ± 1.4 years) were enrolled in the recombinant human growth hormone (GH) treatment group. In comparison, twelve aged male individuals (mean age 74.1 ± 0.9 years) and ten healthy young male volunteers aged 23 to 31 years (mean age 27.5 ± 0.9 years) were investigated. None of the young or middle-aged (GH study group) investigated study subjects smoked or took any drugs. Subjects within the treatment group with a history or sign of hepatic, renal, cardiac, endocrine, metabolic, or malignant diseases were excluded. The GH study group received a fixed dose of 0.4 mg GH (Pharmacia, Karlsruhe, Germany) subcutaneously per day in the evening for 10 days. All measurements were performed directly before and after the 10 day treatment.
Measurement of IGF-1 and basic blood parameters

Blood (10 ml) was drawn from the antecubital vein of patients using syringes containing ethylenediaminetetraacetic acid (EDTA) and immediately put on ice. Blood samples were centrifuged at 1500 g and 4 °C for 15 min. The plasma obtained was used immediately or stored at –80 °C until further analysis. IGF-1 levels were determined using a commercially available IGF-1 detection kit according to the manufacturer's recommendation (human IGF-1: Nichols Institute Diagnostics, San Clemente, USA; mouse IGF-1: Mediagnost IGF-1, Reutlingen, Germany). White and red blood cell counts were determined by standardized techniques.

Isolation of peripheral blood mononuclear cells (PBMCs) and CD34+\textsuperscript{+}, CD117\textsuperscript{+}, CD133\textsuperscript{+} and CD133\textsuperscript{+}/VEGFR\textsuperscript{+} progenitors

PBMCs were isolated by Ficoll\textsuperscript{®} density centrifugation. For in vitro assays PBMCs were harvested by leukapheresis (Cobe Spectra device, Gombro, Germany) of healthy volunteers (n=5). A portion of PBMCs was incubated with an anti-CD34, anti-CD117 or anti-CD133 antibodies conjugated to magnetic microbeads (Miltenyi, Germany) and then processed using magnetic affinity cell sorting (MACS) according to the manufacturers instructions (Miltenyi, Germany). To isolate CD133\textsuperscript{+}/VEGFR2\textsuperscript{+} EPC, leucapheresis derived cells were first labelled with an APC-coupled monoclonal mouse antihuman CD133 antibody (Miltenyi Biotec, Germany) and APC-CD133-positive cells were magnetically labeled with Anti-APC Multisort Microbeads (Miltenyi, Germany). Then, cells were subjected to MACS procedure (see above). After removing the magnetic beads by a releasing agent (Anti-APC Multisort Kit, Miltenyi, Germany), any remaining magnetically labelled cells were removed by additional MACS procedure. After stopping the release reaction, we used a PE-labelled vascular endothelial growth factor receptor-2 antibody (PE-VEGFR-2; R&D Systems,
Germany) to stain CD133+/VEGFR2+ cells. We finally incubated these cells with anti-PE microbeads (Miltenyi Biotec, Germany) and performed additional MACS.

**Determination of progenitor cells**

A variety of assays was used to determine the number, differentiation and function of EPC. This included the EPC adhesion-related cell culture assay for the identification of monocytic EPC, the detection of CD133+/VEGFR-2+ cells (human study) or sca1+/flk1+ cells (mouse study), Dil-acLDL uptake, von Willebrand factor (vWF) staining, the determination of endothelial colony forming units (CFU), analysis of the migratory capacity of monocytic EPC, CD133+-derived EPC, as well as freshly isolated CD133+/VEGFR2+ EPC. Additionally, incorporation of EPC into endothelial tube like structures was measured (see below for details).

**EPC adhesion-related cell culture assay**

The method has been described previously (1, 2). In brief, 2 x 10^5 PBMCs were cultured on fibronectin-precoated 8-well chamber slides (Lab-Tek, Germany) in EBM-2 culture medium supplemented with EGM SingleQuots except IGF-1 (Cambrex, Belgium) for 4 days. Appropriate amounts of IGF-1 were added. To exclude contamination with mature circulating endothelial cells, we carefully removed culture supernatants 8 h after initial seeding and placed non-adherent cells to new fibronectin-precoated chamber slides. After dilution of 1,1'-dioctadecyl-3,3,3’,3’-tetramethyl-indocarbocyanine perchlorate labeled acetylated LDL (dil-acLDL; Molecular Probes, Eugene, USA) and FITC-conjugated lectin from Ulex europeus (UEA-1; Sigma, Germany) in serum-free EB M2 media, cells were washed twice and incubated for 4 h at 37 °C in EB M2 medium containing 10 µg/ml dil-acLDL and 10 µg/ml UEA-1. After washing, cells were observed by fluorescence microscopy. Only
double positive (dil-acLDL and UEA-1) cells were counted in at least 4 independent randomly selected high-power fields. Additionally, EPC were co-stained with an anti-vWF antibody (Sigma, Deisenhofen, Germany).

Detection of CD133+/VEGFR-2+ cells
PBMCs were isolated by Ficoll® density centrifugation as described previously (2). We incubated a volume of 60 µL (about 2x10^5 PBMCs) with appropriate amounts of vascular endothelial growth factor receptor-2 antibody (VEGFR-2; Reliatech, Braunschweig, Germany) for 15 min at 6°C and subsequently with a FITC-conjugated mouse IgG monoclonal antibody. After washing cells were co-stained with an APC-coupled monoclonal mouse antihuman CD133 antibody (Miltenyi Biotec, Germany). In parallel experiments, we added appropriate isotype controls (APC-conjugated mouse IgG2b and FITC-conjugated mouse IgG monoclonal Ig). We acquired at least 20,000 cells using a FACSCalibur cytometer (Becton Dickinson). The number of progenitor cells was expressed as a percentage of all PBMCs and in relation to total leukocytes. Two investigators independently assessed the number of progenitor cells in blinded experiments. To detect mouse EPC, 100µl of whole blood was stained with a FITC-conjugated rat anti-mouse Ly-6A(Sca-1) and a phycoerythrine (PE)-conjugated rat anti-mouse Flk-1 (VEGF-R2, Ly-73) monoclonal antibody (both BD Pharmingen, Germany), or the respective isotype controls. After an incubation period of 45min at 4°C, erythrocytes were lysed (BD FACS Lysing Solution; BD Pharmingen, Germany) and remaining cells subjected to standard FACS analysis (3).
**Endothelial colony forming units (CFU)**

PBMCs were isolated by Ficoll® density gradient centrifugation and 5x10^6 cells from volunteers were plated on fibronectin-coated 6-well plates in EndoCult™ medium (StemCell Technologies, USA) as described (2). After 48 h, non-adherent cells were collected and plated in replicate fibronectin-coated 24-well plates. Colonies were evaluated and quantified three days later. A colony was defined as a central core of "round" cells with more elongated "sprouting cells" at the periphery and are referred to as early outgrowth colony forming unit - endothelial cell (4). The endothelial lineage of these cells has been confirmed extensively by immunocytochemical staining for vWF, VEGFR-2, and CD31 (4).

**Incorporation into endothelial tube like structures**

We measured incorporation of EPC during endothelial tube formation as previously described (2). Briefly, dil-acLDL-prelabeled EPC (2 x 10^4 cells) were mixed with human umbilical vein endothelial cells (HUVECs; 4 x 10^4) on an 8-well glass slide precoated with 100 µL Matrigel (BD Bioscience, Germany) in 200 µL EBM-2 medium with supplements (except IGF-1 and FCS, Cambrex, Belgium) with the addition of 0 – 100 ng/ml IGF-1 (Sigma-Aldrich, Deisenhofen, Germany) or GH (Pharmacia, Erlangen, Germany, 10-100 ng/ml). Further, the 100ng/ml IGF-1 group was pre-treated (30min) with an inhibitory IGF-1 receptor (IGF-1R) antibody (10µg/ml) or a phosphoinositide 3 (PI-3)-kinase inhibitor (wortmannin, 100 nM, Sigma-Aldrich, Deisenhofen, Germany). After 24 h of incubation in 5% CO₂ humidified atmosphere at 37 °C, cells were co-stained with UEA-1 and examined under a fluorescence microscope. The amount of incorporated dil-acLDL-labeled EPC in formed endothelial tubes was determined. Two investigators in blinded experiments
examined at least four randomly selected high-power fields. At least five experiments were done per study group.

**Cellular migration assay**

Migratory capacity of monocytic, CD133⁺ derived or freshly isolated CD133⁺/VEGFR2⁺ EPC was investigated using the modified Boyden chamber assay as described previously (5). In brief, IGF-1 (10-100 ng/ml) or GH (10-100 ng/ml) pre-treated (24h) monocytic (1 x 10⁵), CD133⁺-derived (1 x 10⁴) or CD133⁺/VEGFR2⁺ EPC (1 x 10⁴) were cultured in inlets (Falcon HTS Fluoro Blok insert, 8-µm pore size), which were placed in 24-well culture dishes containing endothelial basal medium (Clonetics, Germany) and 50 ng/mL VEGF, and 100 ng/mL stromal cell-derived factor (SDF)-1 to measure the migratory capacity of EPC. After 24h, migrated cells on the bottom of the membrane were stained with dil-acLDL and counted by fluorescence based microscopic evaluation of the bottom side of the membrane. Additional migration assays were performed with human mesenchymal stem cells (hMSC) obtained from Cambrex (Belgium), that were cultured and expanded in mesenchymal stem cell medium (MSCGM with Singlequots; Cambrex, Belgium). hMSC were treated with or without IGF-1 (100ng/ml) for 24h before starting the migration assay (see above).

**Western Blotting analysis**

EPC were cultured for 4 days in the presence of endothelial growth media (EBM-2 supplemented with EBM SingleQuots (Clonetics, Germany) and 20% FCS). Then, medium was switched to FCS- and IGF-free EBM-2 medium and cells were treated with ascending concentrations of IGF-1 or GH for 8h. Protein content of cell lysates was determined by Smith assay. Cell lysates were mixed with sample loading buffer
and separated under reducing conditions on 12% SDS-polyacrylamide gel. Proteins were electro-transferred onto PVDF membranes (Immun-Blot® 0.2 µm, Bio-Rad), incubated for 1 hour in Tris buffered saline-Tween (TBS-T) with 5% blocking agent (Amersham), and followed by overnight incubation at 4°C with primary antibodies. The bands were detected using a chemiluminescence assay (ECL+Plus, Amersham). Primary antibodies used included anti-eNOS, anti-phospho-eNOS (Ser 1177) (BD Bioscience, Heidelberg, Germany), anti-Akt, anti-phospho-Akt (Cell Signaling Technologies, Beverly, USA) and anti-GAPDH (Abcam, Cambridge, United Kingdom).

Quantification of eNOS and IGF-1 receptor expression of EPC by real-time RT PCR
mRNA expression of human eNOS and IGF-IR were quantified by real-time PCR (iCycler; Bio-Rad Laboratories). PCR amplification was performed for 45 cycles using SYBR Green at a primer annealing temperature of 60°C. Oligonucleotide sequences were: eNOS-sense: 5´-CGG CAT CAC CAG GAA GAA GA-3´ and antisense: 5´-CAT GAG CGA GGC GGA GAT-3´. IGF-IR- sense: 5´-AAG GCT GTG ACC CTC ACC AT-3´ and antisense: 5´-CGA TGC TGA AAG AAC GTC CAA-3´. Gene expressions were normalized to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH): sense primer: 5´-CCA CAT CGC TCA GAC ACC AT-3´ antisense primer: 5´-CCA GGC GCC CAA TAC G-3´. In conventional RT-PCR, all primers generated only one amplification band visualized by agarose gel electrophoresis, demonstrating specificity. For each gene, a standard was constructed by cloning the RT-PCR product into a pCR2.1-TOPO vector (Invitrogen). The identity of the insert was confirmed by sequencing (T7 promoter). Serial 10-fold dilutions of the generated plasmid were used as standard curve for quantifications. Results were given as gene of interest relative to GAPDH.
Small interference RNA-mediated knockdown of eNOS in EPC

EPC cultured for 4 days were transfected with eNOS-siRNA using the BLOCK-iT™ Transfection Kit (Invitrogen, Germany). We incubated EPC with the siRNA oligonucleotide HSS107238 (150nM) from the NOS3 Stealth™ Select RNAi Kit (Invitrogen, Germany) for 48h to downregulate eNOS expression. FITC-labeled scrambled siRNA (Control-FITC block-it fluorescent Oligo #2013, Invitrogen, Germany) was used as a negative and transfection control. eNOS expression was monitored by Western blotting (see upper section).

eNOS activity

eNOS activity was determined by assessing the conversion of L-[guanidino-\textsuperscript{15}N\textsubscript{2}]arginine to \textsuperscript{15}N-nitrite with gas chromatography/mass spectrometry. EPC were incubated for 24h at 37°C with 5mM L-[guanidino-\textsuperscript{15}N\textsubscript{2}]arginine). The ratio of formed \textsuperscript{15}N-nitrite/\textsuperscript{14}N-nitrite was then calculated.

Expression of the IGF-1 receptor on EPC

IGF-1 receptor (IGF-1R) expression was determined after addition of an anti-IGF-1R antibody (R&D Systems, Wiesbaden, Germany) for 12h. Thereafter cells were washed and a rhodamine-labeled mouse IgG antibody was added for 4h. In addition, cells were stained with FITC-labeled UEA-1 as described above. After magnetic affinity cell sorting (MACS; see above), CD133\textsuperscript{+} progenitor cells were cultured for 14 days on fibronectin coated dishes with EBM-2 medium, supplements (Cambrex, Belgium) and 20% FCS to promote endothelial differentiation as described (6). Additional migration assays of IGF-1 treated CD133\textsuperscript{+}-derived EPC were performed (Fig. 5A).
Acidic beta-galactosidase staining of EPC

After culturing EPC for 4 days and subsequent treatment, cells were fixed with a glutaraldehyde/formaldehyde solution and stained with beta-galactosidase staining solution for 48h. In addition the total cell number was assessed. The absolute numbers of beta-galactosidase-positive cells were counted out of 500 cells.

Telomerase activity

We isolated CD34+, CD117+ and CD133+ positive cell fractions by magnetic cell sorting and found the highest telomerase activity in CD133+ cells (data not shown). Telomerase activity was measured in isolated CD133+ progenitors and cultured EPC using a commercially available PCR-based assay according to the manufacturer’s protocol. Telomeric repeat amplification protocol (TRAP) assays were performed using biotin-labeled TS primers.

Mouse in vivo studies

To test whether GH directly or via increase of IGF-1 improves EPC number and function, we treated aged male mice (6-8 months old, Harlan-Winkelmann, Germany) intraperitoneally either with placebo (150µL PBS; n=6), GH (Sigma-Aldrich, Germany; 2.5µg GH/g/day solved in 150µl PBS once per day for 2 or 7 days; each n=6) or mouse IGF-1 (Sigma-Aldrich, Germany; 1.5µg mouse IGF-1/g body weight solved in 150µl PBS for three times per day for 2 days; n=5) (7, 8). We then determined mouse EPC number (sca1+/flk1+ cells by FACS analysis; see above or 3), as well as EPC function (cellular migration assay; see above).
In a further in vivo study we treated aged mice with GH (2.5µg GH/g/day solved in 150µl PBS once per day for 7 days) and systemically blocked the IGF-1 receptor either with a neutralizing IGF-1 receptor antibody (#MAB391, R&D Systems, Germany; 50µg/day/animal for 7 days) or a diaryl urea compound (N-(2-methoxy-5-chlorophenyl)-N'-(2-methylquinolin-4-yl)-urea; IGF-1R Inhibitor II, Cat. No. 407248, Calbiochem, Germany; 100mg/kg every second day for 7 days), which recently was described to be a selective and highly potent inhibitor of the IGF-1 receptor by blocking autophosphorylation (both in human cell lines and in mice in vivo studies; see 9). After treatment we again determined mouse EPC number (sca1+/flk1+ cells by FACS analysis; see above), as well as EPC function (cellular migration assay; see above).

Statistical analysis

Data are expressed as mean±SEM. Statistical analysis was performed by one-way ANOVA followed by multiple comparisons using Fisher's protected least-significant difference test. To analyze relationships between variables simple regression analyses were performed. Statistical analysis was performed using StatView 5.0 statistic program (Abacus Concepts, Berkley, CA, USA). Statistical significance was assumed at P<0.05.
References


