Impaired Endothelial Repair Capacity of Early Endothelial Progenitor Cells in Prehypertension: Relation to Endothelial Dysfunction

Giovanna Giannotti, Carola Doerries, Pavani S. Mocharla, Maja F. Mueller, Ferdinand H. Bahlmann, Tibor Horvath, Hong Jiang, Sajoscha A. Sorrentino, Nora Steenken, Costantina Manes, Mario Marzilli, K. Lenhard Rudolph, Thomas F. Lüscher, Helmut Drexler, Ulf Landmesser

Abstract—Prehypertension is a highly frequent condition associated with an increased cardiovascular risk. Endothelial dysfunction is thought to promote the development of hypertension and vascular disease; however, underlying mechanisms remain to be further determined. The present study characterizes for the first time the in vivo endothelial repair capacity of early endothelial progenitor cells (EPCs) in patients with prehypertension/hypertension and examines its relation with endothelial function. Early EPCs were isolated from healthy subjects and newly diagnosed prehypertensive and hypertensive patients (n=52). In vivo endothelial repair capacity of EPCs was examined by transplantation into a nude mouse carotid injury model. EPC senescence was determined (RT-PCR of telomere length). NO and superoxide production of EPCs were measured using electron spin resonance spectroscopy analysis. CD34+/KDR+ mononuclear cells and circulating endothelial microparticles were examined by fluorescence-activated cell sorter analysis. Endothelium-dependent and -independent vasodilations were determined by high-resolution ultrasound. In vivo endothelial repair capacity of EPCs was substantially impaired in prehypertensive/hypertensive patients as compared with healthy subjects (re-endothelialized area: 15±3%/13±2% versus 28±3%; P<0.05 versus healthy subjects). Senescence of EPCs in prehypertension/hypertension was substantially increased, and NO production was markedly reduced. Moreover, reduced endothelial repair capacity of early EPCs was significantly related to an accelerated senescence of early EPCs and impaired endothelial function. The present study demonstrates for the first time that in vivo endothelial repair capacity of early EPCs is reduced in patients with prehypertension and hypertension, is related to EPC senescence and impaired endothelial function, and likely represents an early event in the development of hypertension. (Hypertension. 2010;55:1389-1397.)

Key Words: endothelial function ■ prehypertension ■ early endothelial progenitor cells ■ senescence ■ NO

Prehypertension is a highly frequent condition affecting ~30% of the adult US population and has been defined as a systolic blood pressure range between 120 and 139 mm Hg and/or a diastolic blood pressure range between 80 and 89 mm Hg.1-2 Prehypertension is thought to be a precursor of stage 1 hypertension, a concept that has been further supported by the observations of the Trial of Preventing Hypertension, demonstrating that treatment of prehypertension with the angiotensin-receptor blocker candesartan prevented and postponed the development of hypertension even 2 years after the termination of active treatment.3 Importantly, prehypertension is associated with a significantly increased cardiovascular risk.4

Endothelial dysfunction is thought to be critical in the development of vascular disease.5,6 Of note, Taddei et al7 have observed an abnormal endothelium-dependent vasodilation in the offspring of patients with essential hypertension, suggesting that endothelial dysfunction may promote the development of hypertension. Moreover, Schlaich et al8 have demonstrated recently that individuals with a positive family history of hypertension had an abnormal l-arginine uptake, further supporting the concept that an alteration of endothelial function may contribute to the development of essential hypertension. Notably, recent experimental studies have suggested that endothelial progenitor cells (EPCs) promote endothelial in-

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From the Department of Cardiology (G.G., C.D., P.S.M., M.F.M., C.M., T.F.L., U.L.), Cardiovascular Center, University Hospital Zurich, Zurich, Switzerland; Cardiovascular Research (G.G., C.D., P.S.M., M.F.M., C.M., T.F.L., U.L.), Institute of Physiology, University of Zurich, Zurich, Switzerland; Department of Cardiology and Angiology (G.G., C.D., M.F.M., T.H., S.A.S., N.S., C.M., H.D., U.L.), Hannover Medical School, Hannover, Germany; Cardiac and Thoracic Department (G.G., M.M.), University Hospital of Pisa, Pisa, Italy; Department of Medicine IV (F.H.B., H.J.), University of the Saarland, Homburg/Saar, Germany; Institute of Molecular Medicine and Max Planck Research Group on Stem Cell Aging (K.L.R.), University of Zurich, Zurich, Switzerland; Department of Cardiology, Cardiovascular Center, University Hospital Zurich, Rämistr 100 (C-Hof 111), 8091 Zürich, Switzerland. E-mail Ulf.Landmesser@usz.ch
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tegrity.9–11 At present, there are in particular 2 populations of EPCs differentiated based on their appearance in culture, that is, “early” EPCs appearing after 4 to 7 days, similar to those originally described by Asahara et al,12 and “late” EPCs, is, “early” EPCs appearing after 4 to 7 days, similar to those originally described by Asahara et al,12 and “late” EPCs, appearing after 14 to 21 days.13,14 A beneficial effect on endothelial repair after injury has, in particular, been shown for early EPCs.9–11,15,16 Early EPCs have also been termed “circulating angiogenic cells,” and are thought to exert their effects in particular by paracrine mechanisms.14,17 Furthermore, a relation between the number of in vitro formed colony forming units of early EPCs and endothelium-dependent vasodilation has been suggested.18 However, it remains unclear whether in vivo endothelial repair capacity of early EPCs is altered in patients with prehypertension or hypertension and is related to an abnormal endothelium-dependent vasodilation and senescence.

In the present study, we have, therefore, examined in vivo endothelial repair capacity of early EPCs in prehypertensive and hypertensive patients as compared with healthy subjects (HSs) and analyzed the relationship with endothelium-dependent vasodilation and senescence. Importantly, patients included in the present study had newly diagnosed prehypertension or hypertension as their only cardiovascular risk factor. Moreover, early EPC senescence, as detected by telomere length and senescence-associated β-galactosidase (SA-β-Gal) staining analysis, and NO and superoxide production, as determined by electron spin resonance (ESR) spectroscopy analysis, were examined to understand potential mechanisms leading to an altered early EPC in vivo endothelial repair capacity. In addition, EPC numbers and endothelial apoptotic microparticles (CD31−/Annexin V+ particles) were determined by fluorescence-activated cell sorter (FACS) analysis.

Methods
Written informed consent was obtained from all of the participants, and the study protocol has been approved by the local ethics committee. We screened 130 nonsmoking volunteers, aged between 40 and 70 years, without known cardiovascular disease or ongoing pharmacological therapies. HSs were included when they had no cardiovascular risk factors, a systolic blood pressure <120 mm Hg, and a diastolic blood pressure <80 mm Hg (see below). Patients with prehypertension (systolic blood pressure between 120 and 139 mm Hg) or hypertension (systolic blood pressure ≥140 mm Hg) were included when they had no other cardiovascular risk factors. In particular, subjects with obesity (body mass index: >30), hypercholesterolemia (low-density lipoprotein >160 mg/dL or total cholesterol >240 mg/dL), positive family history for cardiovascular disease, diabetes mellitus, renal impairment, or known cardiovascular disease were excluded. Patients with newly diagnosed hypertension were without pharmacological therapy. Blood pressure measurements were performed according to the Seventh Report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure/European Society of Cardiology 2007 recommendations, that is, all of the subjects underwent ≥3 blood pressure measurements in 2 different visits, after 20 to 30 minutes of rest, and the measurements were spaced by 5- to 10-minute intervals, on both the left and right arm, in the sitting and lying positions. Furthermore, all of the participants underwent a 24-hour blood pressure measurement. Methodological details for the performed analyses, that is, characterization of early EPCs and endothelial function, are provided in the online Data Supplement (please see http://hyper.ahajournals.org).

Results
Characteristics of HSs and Patients With Prehypertension and Hypertension
The characteristics of HSs and patients with prehypertension or hypertension are shown in Table 1. Notably, newly diagnosed prehypertensive and hypertensive patients were included in the present study when they did not have other cardiovascular risk factors. Moreover, no patients had to be excluded because of white coat effects, because in all of the enrolled patients the office blood pressure values were confirmed by 24-hour blood pressure measurements.

Detection of Homing of Early EPCs to Injured Carotid Artery by FACS Analysis
Previous studies have suggested that mesenchymal stem cells have a very limited capacity to reach the carotid artery after

Table 1. Characteristics of the Study Population

<table>
<thead>
<tr>
<th>Clinical Parameters</th>
<th>Healthy (n=16)</th>
<th>Prehypertensive (n=16)</th>
<th>Hypertensive (n=20)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>53±2</td>
<td>57±3</td>
<td>58±2</td>
<td>NS</td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>7/9</td>
<td>10/6</td>
<td>12/8</td>
<td>NS</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>25±0.7</td>
<td>24±0.8</td>
<td>26±0.5*</td>
<td>*P&lt;0.05 vs prehypertension</td>
</tr>
<tr>
<td>Systolic BP, mm Hg</td>
<td>112±2</td>
<td>129±2†</td>
<td>155±4*</td>
<td>*P&lt;0.05 vs prehypertension; †P&lt;0.05 vs healthy</td>
</tr>
<tr>
<td>Diastolic BP, mm Hg</td>
<td>72±2</td>
<td>82±2†</td>
<td>93±2†</td>
<td>*P&lt;0.05 vs prehypertension; †P&lt;0.05 vs healthy</td>
</tr>
<tr>
<td>24-h systolic BP, average; mm Hg</td>
<td>116±1</td>
<td>129±1†</td>
<td>146±3*</td>
<td>*P&lt;0.05 vs prehypertension; †P&lt;0.05 vs healthy</td>
</tr>
<tr>
<td>24-h diastolic BP, average; mm Hg</td>
<td>73±1</td>
<td>81±1†</td>
<td>93±3*</td>
<td>*P&lt;0.05 vs prehypertension; †P&lt;0.05 vs healthy</td>
</tr>
<tr>
<td>Fasting glucose, mg/dL</td>
<td>89±1.5</td>
<td>89±1.5</td>
<td>92±2.5</td>
<td>NS</td>
</tr>
<tr>
<td>HbA1c, %</td>
<td>5.6±0.1</td>
<td>5.7±0.1</td>
<td>5.7±0.1</td>
<td>NS</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dL</td>
<td>124±7</td>
<td>126±4</td>
<td>127±6</td>
<td>NS</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dL</td>
<td>70±3</td>
<td>73±4</td>
<td>68±4</td>
<td>NS</td>
</tr>
<tr>
<td>Creatinine, mg/dL</td>
<td>0.9±0.03</td>
<td>0.9±0.07</td>
<td>0.9±0.02</td>
<td>NS</td>
</tr>
</tbody>
</table>

BMI indicates body mass index; BP, blood pressure; HbA1c, hemoglobin A1c; LDL, low-density lipoprotein; HDL, high-density lipoprotein; NS, not significant.
intravenous injection, that is, likely a low pulmonary passage. In order to examine whether the intravenously injected early EPCs is home to the injured carotid artery, early EPCs from healthy subjects were stained with carboxyfluorescein-diacetate-succinimidy-ester and injected into the tail vein of nude mice with carotid injury \( (2 \times 10^6 \text{ cells}) \). After 24 hours, the injured and the corresponding sections of the uninjured contralateral carotid artery were homogenized, and the number of labeled EPCs was quantified by using FACS analysis. As shown in Figure 1A, a significant homing of labeled early EPCs was observed in the injured but not in the uninjured carotid artery. To further examine the localization of labeled early EPCs in the carotid artery, confocal laser scanning microscopy was performed.

**Detection of Homing of Early EPCs to Injured Carotid Artery by Confocal Laser Scanning Microscopy Analysis**

Confocal laser scanning microscopy analysis was used to obtain selective images with increasing depth of the carotid arteries. TAMRA (5-carboxytetramethylrhodamine)-labeled early EPCs (red signal) were detected in the endothelial repair zone of the injured carotid artery but not in the uninjured carotid artery after injection into the tail vein of nude mice with carotid injury (Figure 1B). Fluorescence-labeled early EPCs were localized immediately beneath the endothelial layer, as indicated by serial imaging with increasing depth by using confocal laser scanning microscopy analysis of the re-endothelialized zone of the carotid artery (Figure 1B).

**In Vivo Endothelial Repair Capacity of Early EPCs**

Transplantation of early EPCs from HSs markedly accelerated endothelial repair (Figure 2A and 2B). Notably, in vivo endothelial repair capacity of early EPCs from patients with prehypertension and hypertension was markedly reduced as compared with that in HSs (Figure 2A). Representative photographs of repaired endothelium 3 days after transplantation of early EPCs from the 3 different study groups are shown in Figure 2B. The number of early EPCs was similar after 4-day culture in the 3 groups \( (159 \pm 15 \times 10^6/\text{cm}^2 \text{ in HSs versus } 179 \pm 27 \times 10^6/\text{cm}^2 \text{ in prehypertensives versus } 149 \pm 15 \times 10^6/\text{cm}^2 \text{ in hypertensives}; P \text{ value not significant}) \).

**Endothelium-Dependent and Endothelium-Independent Vasodilation**

Flow-dependent, endothelium-mediated vasodilation (FMD) was markedly reduced in prehypertensive patients as compared with HSs (FMD: \( 11.6 \pm 0.7\% \text{ versus } 8.8 \pm 0.7\%; P = 0.01; \) Figure 2C). FMD was further impaired in hypertensive patients \( (6.5 \pm 0.5\%; P < 0.01 \text{ versus prehypertensive}; \) Figure 2C). There was no significant difference in the radial artery diameter between the groups (Table 2). Arterial blood flow measurements were performed to determine whether a reduced increase of arterial blood flow during reactive hyperemia could explain the impaired FMD observed in patients with prehypertension or hypertension. As shown in Table 2, the values for arterial blood flow in response to reactive hyperemia in patients with prehypertension or hypertension were not lower as compared with HSs, thereby rather excluding a reduced arterial blood flow response under reactive hyperemia as a mechanism that could explain the impaired endothelium-dependent vasodilation observed in prehypertensive and hypertensive patients. Moreover, there were no differences in endothelium-independent relaxation of the radial artery among the 3 groups, suggesting a specifically impaired
endothelium-dependent vasodilation in prehypertensive and hypertensive patients (Table 2).

Relation Between Early EPC In Vivo Endothelial Repair Capacity and FMD
In vivo endothelial repair capacity of early EPCs was positively related to FMD ($r=0.38$; $P<0.05$; Figure 2D), suggesting that impaired early EPC-mediated endothelial repair capacity is related to a reduced endothelium-dependent vasodilation.

Early EPC Senescence as Examined by Telomere Length and SA-β-Gal Staining
Senescence of progenitor cells is a potential cause of altered function. We have, therefore, analyzed markers of early EPC

| Table 2. Radial Artery Diameter and Blood Flow at Baseline and During Reactive Hyperemia in Healthy Subjects, Prehypertensive, and Hypertensive Patients |
|---------------------------------|----------------|----------------|----------------|---|
| Endothelium-Dependent and -Independent Vasodilation | Healthy | Prehypertensive | Hypertensive | $P$ |
| Diameter, mm | | | | |
| Baseline | 2.73±0.1 | 2.78±0.1 | 2.99±0.1 | NS |
| Increase under reactive hyperemia, FMD % | 11.6±0.7 | 8.8±0.7 | 6.5±0.5 | $P<0.05$ vs healthy |
| Increase after nitroglycerin, % | 25.9±2.6 | 21.9±3.3 | 21.2±1.5 | NS |
| Blood flow, mL/min | | | | |
| Baseline | 30.6±3.6 | 32.7±2.6 | 37.4±3.0 | NS |
| Reactive hyperemia | 105±4 | 146±15 | 126±17 | NS |

NS indicates not significant.
senescence in prehypertensive and hypertensive patients as compared with HSs. Notably, a significant telomere shortening was observed in early EPCs from prehypertensive patients (HS versus prehypertensive: 11.7±2.1 versus 8.9±0.8 kb; P<0.01) and from hypertensive patients (7.5±1.1 kb; P<0.001 versus HS; Figure 3A) as compared with HSs. As a second marker of early EPC senescence, the acidic SA-β-Gal staining of early EPCs was examined. SA-β-Gal-positive early EPCs were significantly increased in prehypertensive and hypertensive patients as compared with HSs (Figure 3B).

Moreover, there was a significant positive relationship between early EPC telomere length and early EPC in vivo endothelial repair capacity (r=0.31; P<0.05; Figure 3C). Similarly, as evaluated by SA-β-Gal staining, early EPC senescence was inversely related to early EPC in vivo endothelial repair capacity (r=−0.30; P<0.05; Figure 3D), suggesting that impaired early EPC senescence is associated with an impaired in vivo endothelial repair capacity of early EPCs. Notably, early EPC telomere length was inversely related to systolic blood pressure values (r=−0.69; P<0.05; Figure 3E). In addition, the analysis of telomerase activity in a subgroup of prehypertensive and hypertensive patients revealed a markedly reduced telomerase activity in early EPCs from these patients as compared with early EPCs from HSs (HSs versus prehypertensive/hypertensive patients: 24.2±3.4% versus 15.8±1.9%; n=12; P<0.05).

**Early EPC NO and Superoxide Production**

Early EPC NO production and oxidative stress have been suggested to play an important role for EPC repair capacity. Early EPC NO and superoxide production were, therefore, examined by ESR spectroscopy analysis. NO production was significantly reduced in early EPCs from prehypertensive/hypertensive patients as compared with HSs (474±28 pmol/60 minutes; P<0.01; n=13 to 18; Figure 4A). There was no significant difference in early EPC superoxide production between prehypertensive/hypertensive patients and HSs (Figure 4B).

**FACS Analysis of the Number of Circulating CD34+/KDR + Mononuclear Cells**

We did not observe a significant difference in the numbers of circulating CD34/KDR double-positive mononuclear cells between newly diagnosed prehypertensive/hypertensive patients and HSs (Figure 5A).

**FACS Analysis of Circulating Endothelial Apoptotic Microparticles**

The number of circulating endothelial apoptotic microparticles was not significantly different between newly diagnosed prehypertensive/hypertensive patients and HSs (Figure 5B).
Discussion

The present study demonstrates for the first time that in vivo endothelial repair capacity of early EPCs is substantially impaired in patients with newly diagnosed prehypertension and hypertension as their only cardiovascular risk factor. Moreover, increased senescence of early EPCs as indicated by telomere shortening and increased SA-β-Gal staining in prehypertensive and hypertensive patients was related to impaired EPC in vivo endothelial repair capacity. In addition, NO production as determined by ESR spectroscopy was substantially reduced in early EPCs derived from prehypertensive and hypertensive patients, providing a further potential underlying mechanism leading to reduced endothelial repair capacity of early EPCs in these patients. Importantly, the present study provides the first evidence that reduced in vivo endothelial repair capacity of early EPCs is related to increased senescence of EPCs and an impaired endothelium-dependent vasodilation, suggesting that a reduced in vivo repair capacity of EPCs is an early event in the development of hypertension.

Endothelial dysfunction is thought to promote the development of vascular disease and hypertension.5,6,20 The present study provides novel evidence indicating that the in vivo endothelial repair capacity of early EPCs, as determined by transplantation of early EPCs into nude mice with vascular injury, is markedly impaired in prehypertensive and hypertensive patients without other cardiovascular risk factors. Moreover, impaired in vivo endothelial repair capacity of early EPCs was related to increased senescence of EPCs and an impaired endothelium-dependent vasodilation, suggesting that a reduced in vivo repair capacity of EPCs is an early event in the development of hypertension and vascular disease. Although these findings do not prove a cause-and-effect relationship, there is evidence to suggest that impaired early EPC repair capacity may contribute to endothelial dysfunction. In a preclinical study, Wassmann et al21 have observed that infusion of circulating early EPCs was able to augment endothelium-dependent vasodilation. Moreover, interventions that augment early EPC function, such as statin therapy or physical exercise, are associated with improved endothelial function.22–24 In addition, Hill et al18 have reported an inverse relation between the in vitro obtained number of EPC colony-forming units from circulating mononuclear cells and the degree of endothelial dysfunction.
Importantly, Murasawa et al\textsuperscript{25} have observed recently that overexpression of human telomerase reverse transcriptase in early EPCs increased their migratory activity and postnatal neovascularization capacity, suggesting that senescence and telomerase activity are important factors regulating EPC function. In the present study, we have characterized EPC senescence in prehypertensive and hypertensive patients and its relation to EPC endothelial repair capacity. Notably, both measurements of telomere length and SA-\(\beta\)-Gal staining indicated an increased senescence of early EPCs from prehypertensive and hypertensive subjects that was related to an impaired in vivo endothelial repair capacity of early EPCs. In addition, our ESR spectroscopy measurements revealed a reduced NO bioavailability in EPCs derived from prehypertensive/hypertensive patients. Notably, several recent studies, including work from our own group, have indicated that endothelial NO synthase is critical for EPC function and that early EPC endothelial repair capacity is reduced as a consequence of reduced NO production in early EPCs.\textsuperscript{10,26} Moreover, lack of endothelial NO synthase has been shown to reduce basal telomerase activity in endothelial cells that was restored by exogenous endothelial NO synthase or an NO donor, thus suggesting that a reduced NO availability may contribute to reduced telomerase activity.\textsuperscript{27}

Notably, previous studies have indicated that mesenchymal stem cells have a very limited transpulmonary passage to the carotid artery after intravenous injection.\textsuperscript{19} We have, therefore, performed studies to determine whether human early EPCs can be detected in the injured carotid artery after intravenous injection. After intravenous injection of labeled human early EPCs, we could detect by both (ie, FACS analyses of homogenized carotid arteries and confocal laser scanning microscopy analyses) an increased homing of early EPCs to the injured carotid artery but not to the contralateral uninjured carotid artery. One likely explanation for the observed homing of early EPCs in the injured carotid artery after intravenous injection in the present study is that these cells are substantially smaller in size compared with mesenchymal stem cells as studied by Fischer et al.\textsuperscript{19} Indeed, Fischer et al\textsuperscript{19} have observed recently that bone marrow–derived mononuclear cells, likely in size more similar to early EPCs, have a 30-fold higher pulmonary passage, as detected in the carotid artery after intravenous injection as compared with mesenchymal stem cells.

Furthermore, our confocal laser scanning microscopy analyses suggested that the injected early EPCs can be detected in the subendothelial space of the endothelial repair zone of the injured carotid artery, suggesting that these cells promote the endothelial repair process likely in particular by paracrine mechanisms. These observations are in line with a recent study by Schröeter et al\textsuperscript{6} that detected by fluorescence microscopy a subendothelial homing of intravenously injected early EPCs, promoting the endothelial repair response in another model of carotid injury. Moreover, these observations are consistent with a recent study by Sievking et al\textsuperscript{17} suggesting that early EPCs promote the proangiogenic effects largely in a paracrine fashion. Therefore, the findings of the present study in context with previous findings suggest that the stimulation of the in vivo endothelial repair response by early EPCs is likely largely mediated by paracrine effects.

Endothelial damage is likely characterized by an imbalance of endothelial cell growth/repair and the loss of endothelial cells, that is, by apoptosis.\textsuperscript{28} In the present study we, therefore, determined the number of circulating CD34\(^+\)/KDR\(^+\) and cultured early EPCs, as well as the number of circulating endothelial apoptotic microparticles. We did not observe a significantly reduced number of cultured early EPCs or CD34\(^+\)/KDR\(^+\) cells as determined by FACS analysis. However, an important difference of the present study as compared with previous studies that have examined the number of CD34\(^+\)/KDR\(^+\) cells or cultured early EPCs in patients with established cardiovascular disease and cardiovascular risk factors is that in the present study only patients with newly diagnosed prehypertension/hypertension without other cardiovascular risk factors or known cardiovascular disease were included. Notably, if one looks closely at the results of previous studies, for example, by Vasa et al,\textsuperscript{29} the number of early EPCs (as determined both by the number of CD34\(^+\)/KDR\(^+\) cells or cultured early EPCs) was not different for patients who had only 1 cardiovascular risk factor as compared with control subjects but was substantially reduced in patients with several cardiovascular risk factors. Furthermore, in the study by Vasa et al,\textsuperscript{29} hypertensive patients did not have a lower number of early EPCs as compared with patients without hypertension. In line with these findings are observations from a recent study by Werner et al\textsuperscript{30} that evaluated the prognostic value of CD34\(^+\)/KDR\(^+\) cell numbers for the development of cardiovascular events in patients with coronary artery disease. In a subgroup of 432 coronary artery disease patients with arterial hypertension, there was no association between the number of CD34\(^+\)/KDR\(^+\) cells and arterial hypertension. Moreover, a recent study by Delva et al\textsuperscript{31} examined the number of cultured early EPCs in 36 patients with essential hypertension and 24 control subjects and did not report a reduced number of cultured early EPCs in hypertensive patients. However, in patients with advanced and refractory hypertension, EPC numbers were reduced in a recent study by Oliveras et al.\textsuperscript{32} Therefore, the observations of the present study in the context of previous findings are consistent with the notion that the dysfunction of early EPCs is rather substantially more pronounced and begins earlier as compared with a detectable reduction of the number of circulating early EPCs in patients with hypertension. Importantly, the present study provides for the first time evidence that the in vivo re-endothelialization capacity of early EPCs is already profoundly reduced in patients with prehypertension and is related to impaired endothelium-dependent vasodilation and senescence of early EPCs. Notably, whereas endothelium-dependent vasodilation was more profoundly reduced in patients with hypertension as compared with prehypertension, the endothelial repair capacity of early EPCs was already profoundly impaired in prehypertension, which is in line with the above notion, that the functional impairment of early EPCs is likely an early event in the development of hypertension.

In the present study, no increase of circulating endothelial apoptotic microparticles was observed in prehypertensive or...
hypertensive patients. Notably, Preston et al.\(^3\) have observed an increased number of circulating endothelial apoptotic microparticles in severely hypertensive patients. Werner et al.\(^4\) have recently reported a relation between endothelial dysfunction and the degree of endothelial cell apoptosis, as measured by circulating CD31/Annexin V\(^+\) endothelial apoptotic microparticles in patients with established coronary disease. Therefore, the results of the present study, together with previous observations, suggest that a detectable increase of circulating endothelial apoptotic microparticles is likely occurring in a more advanced stage of hypertension or in patients with established cardiovascular disease.

**Limitations of the Study**

Different methods are in use to isolate and culture EPCs.\(^4\) At least 2 populations have been commonly differentiated, that is, early EPCs and late outgrowth EPCs obtained after several weeks of culture, as pointed out recently.\(^1,13,17\) We have examined in the present study the endothelial repair capacity of early EPCs. Given that early EPCs are rather more frequent in number, they may play a particularly important role for the stimulation of endothelial repair processes. Furthermore, because of a substantial variation in the FACS-determined number of CD34\(^+/\)KDR\(^+\) cells and with respect to the group sizes of the present study, conclusions with respect to the numbers of EPCs as determined by FACS analysis need to be interpreted with caution. Given that there was, however, rather a trend for increased numbers of CD34\(^+/\)KDR\(^+\) cells in prehypertension/hypertension, the present data suggest that the number of these cells is likely not reduced in newly diagnosed hypertensive/hypertensive patients without other cardiovascular risk factors or known cardiovascular disease and that the functional impairment of early EPCs with respect to their endothelial repair capacity is likely substantially more pronounced in this early stage of the disease.

**Perspectives**

Endothelial dysfunction is thought to contribute to the development of hypertension and atherosclerotic vascular disease, however, the underlying mechanisms are incompletely understood. The present study provides novel evidence suggesting that in vivo endothelial repair capacity of early EPCs, likely largely mediated by paracrine effects, is markedly reduced in patients with prehypertension and hypertension and is related to the impairment of endothelial function. These findings indicate that an impaired endothelial repair capacity of EPCs is an early event in the development of hypertension and vascular disease and raise the possibility that interventions to preserve endothelial repair capacity of EPCs, for example, by inhibition of their accelerated senescence and reduced NO availability, may represent an attractive novel approach to stimulate endogenous endothelial repair responses and potentially to prevent endothelial dysfunction and the development and progression of vascular disease.

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**Disclosures**

None.

**References**


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Impaired Endothelial Repair Capacity of Early Endothelial Progenitor Cells in Prehypertension – Relation to Endothelial Dysfunction

Giannotti et al, Early EPC Repair Capacity in Prehypertension

Giovanna Giannotti, MD1-3, Carola Doerries, MD1,2, Pavani S Mocharla, BS1; Maja F Mueller, BS1,2, Ferdinand H. Bahlmann, MD, PhD4; Tibor Horváth, BS2, Hong Jiang, MD4, Sajoscha A Sorrentino, MD2, Nora Steenken, MD2, Costantina Manes, MD1,2, Mario Marzilli, MD3, K Lenhard Rudolph, MD5, Thomas F Lüscher, MD1, Helmut Drexler, MD2, Ulf Landmesser, MD1,2

1Department of Cardiology, Cardiovascular Center, University Hospital Zurich, Switzerland; and Cardiovascular Research, Institute of Physiology, University of Zurich, Switzerland.

2Department of Cardiology and Angiology, Hannover Medical School, Hannover, Germany.

3Cardiac and Thoracic Department, University Hospital of Pisa, Pisa, Italy.

4Department of Medicine IV, University of the Saarland, Homburg/Saar, Germany.

5Institute of Molecular Medicine and Max Planck Research Group on Stem Cell Aging, University of Ulm, Germany.

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Address for correspondence:

Ulf Landmesser, MD
Department of Cardiology, Cardiovascular Center
University Hospital Zürich
Rämistr. 100 (C-Hof 111)
8091 Zürich
Switzerland
Tel.: +41-(0)44-255-9595
Fax: +41-(0)44-255-4401
E-mail: Ulf.Landmesser@usz.ch
**METHODS**

**Isolation and Cultivation of Early EPCs:** Early EPCs were isolated and cultured as described in detail previously. In brief, peripheral blood mononuclear cells were isolated by density gradient centrifugation, and 5x10⁵ cells/cm² were cultured on fibronectin-coated plates in endothelial cell basal medium-2 supplemented with endothelial growth medium-SingleQuots as indicated by the manufacturer except for hydrocortisone (Lonza). After 4-day culture, non-adherent cells were removed by washing plates with PBS, and the attached cells were harvested for analysis. In previous studies we and others have defined the cells deriving from this culture method as early endothelial progenitor cells, characterized by endothelial cell marker expression (>80% cells expressing endothelial markers, such as von Willebrand factor and CD31), as well as by dual staining for acetylated low-density lipoprotein and lectin, i.e. FITC-labelled Ulex europaeus agglutinin-1 lectin (UEA-1; Sigma; 1:15). Early EPCs were counted in >4 randomly selected high-power fields as double stained cells for both, UEA-1 and acLDL-DiI by using an inverted fluorescent microscope (Leica) as described in detail previously.

**Animals and in vivo Re-endothelialization Assay:** Male NRMInu/nu athymic nude mice, aged 7 to 10 weeks, were used to allow injection of human EPCs. Animals were anesthetized with ketamine (100 mg/kg IP) and xylazine (5 mg/kg IP). Carotid artery injury was performed as described in detail previously. Briefly, the left common carotid artery was injured with a bipolar microregulator (ICC50, ERBE Elektromedizin, Tuebingen, Germany). An electric current of 2 W was applied for 2 seconds to each millimetre of carotid artery over a total length of exactly 4 mm with the use of a size marker parallel to the carotid artery. Early EPCs (5 x 10⁵ cells) were resuspended in 100 μl of prewarmed PBS (37°C) and transplanted 3 hours after carotid injury via tail vein injection with a 27-gauge needle. Three days after carotid injury, endothelial repair was evaluated by staining denuded areas with 50 μl of solution containing 5% Evans blue dye via tail vein injection as described in detail previously. The re-endothelialized area (REA) was calculated as the difference between the blue-stained area and the injured-area by computer-assisted morphometric analysis. Early EPCs from each prehypertensive/hypertensive patient or healthy subject were transplanted into 2 nude mice with carotid injury, and the mean values of the re-endothelialized area were used for analysis, i.e. 104 nude mice were used in total for the comparison of in vivo endothelial repair capacity of EPCs from prehypertensive/hypertensive patients and healthy subjects. The local committee on animal research approved all procedures involving animals.

**Detection of Homing of Early EPCs to Carotid Artery by FACS Analysis:** Early EPCs from healthy subjects (n=3) were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE; CellTrace™ CFSE Cell Proliferation Kit; C34554; Invitrogen) according to the manufacturer's instructions and injected into the tail vein of nude mice with carotid injury (see above) to examine the homing to the injured and uninjured carotid artery. After 24 hours the animals were sacrificed, blood was immediately removed and the injured section of the carotid artery and the corresponding uninjured section of the contralateral carotid artery were dissected. The carotid arteries were then opened and incubated with 0.2% collagenase for 30 min at 37 °C, and then flushed with precooled washing buffer (10mM HEPES, 0.1% BSA in HBSS). The cell suspension was filtered through a 100 μmeter mesh and centrifuged at 1200rpm for 5 min. The cells were resuspended in 2 ml of FACS buffer and analysed by the BD FACSCanto II system.

**Detection of Homing of Early EPCs to Carotid Artery by Confocal Laser Scanning Microscopy Analysis:** For the confocal laser scanning microscopy analysis the cell-tracker 5-carboxytetramethyl-rhodamine (TAMRA; red signal) was used to label early EPCs from healthy
subjects that were subsequently injected into the tail vein of nude mice with carotid injury.
Confocal laser scanning microscopy analysis was used to obtain selective images with increasing depth (magnification X200). First, the endothelial layer was visualized by whole mount CD31 immunostaining (green signal) of the injured (endothelial repair zone) and uninjured contralateral carotid artery, and DAPI staining (mounting media) was used for nuclei staining (blue signal). Serial images with increasing depth were obtained by confocal laser scanning microscopy analysis of the re-endothelialized zone of the injured carotid artery and the corresponding section of the uninjured carotid artery.

**Measurement of Endothelium-dependent and Endothelium-independent Vasodilation:**
Endothelium-dependent and –independent vasodilation of the radial artery were measured using a high-resolution A-mode ultrasonic echo-tracking device (ASULAB) as described in detail previously.1,3,6,7 In brief, radial artery diameter measurements were obtained with a 10-MHz transducer positioned perpendicular to the vessel without direct skin contact, with ultrasonic gel as the transmitting medium. Stereo Doppler guidance was used to ensure a correct vertical position of the probe over the artery. Blood flow velocity was recorded continuously by an 8-MHz Doppler probe (Vasoscope III), radial artery diameter was determined every 30 seconds until stable baseline conditions were obtained. A wrist arterial occlusion (8 minutes) was performed, and flow-dependent, endothelium-mediated vasodilation (FMD) of the radial artery in response to reactive hyperaemic blood flow was measured. Nitroglycerine was administered (400 mcg, s.l.) to assess endothelium-independent vasodilation. Arterial blood flow was determined as described previously.1,3,6,7 This method is well established in our laboratory, has an excellent reproducibility and variability and has been described in detail previously.1,3,6,7

**Early EPC Senescence Analysis:**
**Early EPC Telomere Length Measurements:** TaqTM Syber Green supermix with ROX (Bio-RAD) was used as master mix. The total reaction volume was 25 μl containing 25 ng of genomic DNA. Measurements were performed on the ABI 7300 Real time PCR system (Applied Biosystems, Foster City, CA). As a standard a reference DNA sample (BJ26.5 cells) was included for each measurement to control the day-to-day variations. Individual DNA samples were measured in duplicates.
The composition of T and S PCR reaction mixes were identical except for the oligonucleotide primers. The final telomere primer concentrations were as follows: for telomere amplification (T): tel1, 100nM; tel2, 900nM. For amplification of the single copy gene (S) (human β-globin): hbg1, 300nM; hbg2, 700nM. The primer sequences (written 5’ to 3’) were tel 1b, (CGGTTTGGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTTGTT); tel 2b (GGCTTGCCTTACCCCTACCCCTACCCCTACCCCTACCCCT); hbg1, (GCTTTCGTACACAACCTGTGTTCACTAGC), hbg2 (CACCAACTTCATCCACGTTCACC).
The thermal cycling profile for both amplicons began with 95°C incubation for 10 minutes. For telomere PCR, there followed 30 cycles of 95°C for 15 seconds, 56°C for 1 minute. For hbg PCR, there followed 35 cycles of 95°C for 15 seconds, 54°C for 1 minute.
The relative T/S ratios were converted to telomere length in base pairs using the correlation curve of the relative T/S ratio determined by qPCR with TRF length determined by Southern blot analysis of the above described set of 11 standard cell lines.

**Early EPC Telomerase Activity Measurement:** Quantitative analysis of telomerase activity was performed using a TeloTAGGG PCR ElisaPLUS Kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer’s instructions and as previously described.8

**Beta Galactosidase staining in Early EPCs:** The positivity of early EPCs for SA-β-gal staining was examined as previously described Briefly, 1 x 10^6 cells were washed twice in buffered PBS, fixed for three minutes in 2% paraformaldehyde, washed, and incubated for 24h at 37°C (no CO2)
with freshly prepared SA-ß-gal stain solution (1 mg/ml 5-bromo-4-chloro-3-indyl ß-d-galactopyranoside, 5 mmol/l potassium ferrocyanide, 5 mmol/l potassium ferricyanide, 150 mmol/l NaCl, 2 mmol/l MgCl2, 0.01% sodium deoxycholate, and 0.02% Nonidet-40). Early EPCs were counterstained with 4',6-diamino-phenylindole (0.2 μg/ml in 10 mmol/l Tris-HCl, pH 7.0, 10 mmol/l ethylenediamine tetra-acetic acid, 100 mmol/l NaCl) for 10 min, and the number of blue-stained cells was counted in three-random fields.

**Electron Spin Resonance (ESR) Spectroscopy Analysis of Nitric Oxide (NO) Production of Early EPCs**: NO production was measured by ESR spectroscopy analysis using the spin trap colloid Fe(DETC)2 as described previously.1,5,9,10 In brief, 5x10^5 early EPCs were resuspended in 750 μl of Krebs-Hepes buffer (37°C) and 250 μl of colloid Fe(DETC)2 (final concentration 285 μM) was added to each sample and incubated at 37°C for 60 min. ESR spectra were recorded in liquid nitrogen using a MiniScope ESR spectrometer (Magnettech). ESR instrumental settings were as follows: center-field (B0) 331.3G, field sweep 80G, sweep time 180 sec, modulation amplitude 10000 mG, 4dB.

**ESR Spectroscopy Analysis of Early EPC Superoxide Production**: Superoxide (O2−.) production of early EPCs was measured by ESR spectroscopy analysis using the spin trap 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CM-H; Noxygen, Germany) as described previously.1,5 In brief, 2.5x10^5 EPCs were resuspended in 46μl solution containing diethyldithiocarbamic acid sodium salt (DETC), Deferoxamine and Krebs-Hepes-buffer. ESR measurements were performed after CM-H addition in 50 μl glass capillaries (Brand, Germany). ESR spectra were recorded using a MiniScope ESR spectrometer (Magnettech; Berlin, Germany). Early EPC O2−. production was determined by following the oxidation of CM-H by O2−. to paramagnetic CM*. ESR instrumental settings were as follows: B0 field 335G, field sweep 50G, sweep time 60sec, modulation amplitude 2500mG.

**FACS Analysis of Circulating CD34+/KDR+ Mononuclear Cells**: The percentage of double-positive CD34+/KDR+ mononuclear cells was determined by FACS Analysis. MNC were isolated from EDTA whole blood by Ficoll Hypaque. MNCs (3-5 x 10^5) were incubated for 15 min at 4°C in the dark with fluorescein conjugated KDR (R&D Systems, Germany), CD34 PE (Immunotools, Friesoyte, Germany) and corresponding isotype control antibodies (IgG), respectively. After incubation, cells were washed twice and resuspended in PBS BSA 0.1%.

**Measurement of Circulating Endothelial Apoptotic Microparticles (CD31+/Annexin V+)**: As previously described,11 to determine the amount of circulating endothelium-derived apoptotic microparticles, plasma derived from 10 mL citrate-buffered blood was immediately centrifuged at 10 000 g for 2 minutes to generate platelet-poor plasma. 50 μL of platelet-poor plasma were incubated with 6μl of CD31 FITC conjugated (Immunotools, Friesoyte, Germany) and 6μl AnnexinV PE conjugated (by Pharmingen). The antibodies used for our FACS analysis were the following: CD34 PE conjugated (by Immunotools, Friesoyte, Germany; Clone 4H11[APG]); VEGF R2/KDR Fluorescein conjugated (by R&D Systems, Germany).

**Statistical Analysis**: Data in the text, table and figures are expressed as means ± SEM. Comparisons of continuous variables were performed by a one-way-analysis-of-variance (ANOVA). Relations between variables were analysed by linear regression analysis. A P value <0.05 was considered as statistically significant.

The primary end point of the present study was the in vivo endothelial repair capacity of early EPCs, which was therefore used to determine the study size. The relevant alternative was a 20 % difference of re-endothelialized area between the groups. With the assumption of a common SD of
15%, a sample size of 16 patients was needed to have a power of >90% to reject the null hypothesis in favour of the alternative hypothesis with a 0.05 type I error.

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

REFERENCES


