End-stage renal disease causes an imbalance between endothelial and smooth muscle progenitor cells

Peter E. Westerweel, Imo E. Hoefer, Peter J. Blankestijn, Petra de Bree, Dafna Groeneveld, Olivia van Oostrom, Branko Braam, Hein A. Koomans, and Marianne C. Verhaar

Departments of 1Vascular Medicine, 2Experimental Cardiology, and 3Nephrology and Hypertension, University Medical Center Utrecht, Utrecht, The Netherlands

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Westerweel PE, Hoefer IE, Blankestijn PJ, de Bree P, Groeneveld D, van Oostrom O, Braam B, Koomans HA, Verhaar MC. End-stage renal disease causes an imbalance between endothelial and smooth muscle progenitor cells. Am J Physiol Renal Physiol 292: F1132–F1140, 2007. First published January 2, 2007; doi:10.1152/ajprenal.00163.2006.—Patients with end-stage renal disease (ESRD) on hemodialysis have an increased risk of cardiovascular disease (CVD). Circulating endothelial progenitor cells (EPC) contribute to vascular regeneration and repair, thereby protecting against CVD. However, circulating smooth muscle progenitor cells (SPC) may contribute to adverse vascular remodeling. We hypothesized that an imbalance occurs between EPC and SPC in ESRD patients and sampled progenitor cells from 45 ESRD patients receiving regular treatment. Our study is the first to show reduced numbers of CD34+/KDR+ hematopoietic stem cell (HSC)-derived EPC (type I EPC). Furthermore, monocyte-derived EPC cultured from mononuclear cells (type II EPC) were reduced in number and had a reduced capacity to stimulate endothelial cell angiogenesis. In contrast, SPC outgrowth was unaffected. In vitro incubation with uremic serum impaired type II EPC outgrowth from healthy donor mononuclear cells and did not influence SPC outgrowth. The hemodialysis procedure itself induced HSC apoptosis and caused an acute depletion of circulating EPC. Taken together, the decreased number and impaired function of EPC are compatible with impaired endogenous vascular repair in hemodialysis patients, whereas the unaffected SPC numbers suggest that the potential of progenitor cells to contribute to adverse remodeling is retained. This EPC-SPC imbalance may contribute to the acceleration of CVD in ESRD patients and could offer novel therapeutic targets.

endothelial progenitor cells; cardiovascular disease; hemodialysis

END-Stage RENAL DISEASE (ESRD) is associated with a marked increase in the incidence of atherosclerotic cardiovascular disease (CVD) (6). Endothelial dysfunction is pivotal in this process. Circulating endothelial progenitor cells (EPC) contribute to endothelial regeneration and repair (1). Reduced EPC numbers may contribute to accelerated atherosclerosis. Several cardiovascular risk factors and the presence of CVD are associated with lower numbers of circulating EPC (10, 39). Furthermore, in patients with coronary artery disease, the number of EPC was recently shown to be an independent predictor of cardiovascular events in two prospective cohort studies (30, 40). In animal models it has been shown that circulating progenitor cells may also differentiate into vascular smooth muscle cells and contribute to atherosclerosis (16, 29, 32). Outgrowth of cells with a vascular smooth muscle/myofibroblast phenotype (SPC) from human blood has been demonstrated (33, 41). SPC numbers were observed to be increased in diabetes mellitus and in coronary artery disease patients (23, 34).

Two types of EPC have been reported. Type I EPC are identified using flow cytometry as CD34+/hematopoietic stem cells (HSC) that coexpress the endothelial marker KDR. CD34+/KDR+ type I EPC are present in the circulation in low numbers and represent a defined subset of true progenitor cells. Type II EPC are identified by in vitro culture of peripheral blood mononuclear cells under conditions facilitating outgrowth of angiogenic cells with an endothelial phenotype. Type II EPC can be obtained from the blood in relatively high numbers and are mostly monocyte-derived cells (25, 28, 37). Although the in vivo importance of incorporation of type II EPC into damaged endothelium has been challenged (24), these cells have been shown to be potent secretors of angiogenic factors, implying an important role in promoting angiogenesis and endothelial repair via paracrine stimulation of the resident endothelium (25, 35, 36). SPC can be cultured from peripheral blood mononuclear cells under culture conditions facilitating smooth muscle cell outgrowth (33, 41). The origin of these cells has not been fully elucidated, but similar to the EPC, both CD34+ HSC (33) and monocytes (34) have been implicated as potential sources of SPC.

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

METHODS

Subjects. Forty-five ESRD patients (avg. age 55.9 ± 2.1 yr; 67% male) on hemodialysis were included from the dialysis units of the University Medical Center Utrecht and Dianet Dialysis Center Utrecht, The Netherlands. Consecutively attending patients were included, with only current infection and malignancy as exclusion criteria. Patients were maintained on their regular medication. Thirty healthy subjects of comparable age and gender (avg. age 50.2 ± 1.1 yr; 70% male) served as controls. Exclusion criteria for healthy

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Address for reprints and other correspondence: M. C. Verhaar, Dept. of Vascular Medicine, F02.126, Univ. Medical Center Utrecht, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands (e-mail: m.c.verhaar@umcutrecht.nl).
controls were use of medication, hypertension, dyslipidemia, diabetes, renal disease, known cardiovascular disease, current infection, and malignancy. The protocol was approved by the medical ethical committee of the University Medical Center Utrecht.

Type I EPC and HSC flow cytometry. One hundred microliters of EDTA blood were incubated with FITC-conjugated mouse-anti-human CD34 monoclonal antibody (BD Pharmingen, San Diego, CA), phycoerythrin (PE)-conjugated mouse-anti-human KDR (VEGF-R2) monoclonal antibody (R&D Systems), phycoerythrin-cyanine Dye7 (PECy7)-conjugated mouse-anti-human CD45 monoclonal antibody (BD Pharmingen), and 7-amoacytinomyclin D (7AAD; BD Pharmingen) at 4°C. Erythrocytes were lysed in an ammoniumchloride buffer and remaining cells were analyzed by flow cytometry (Beckman Coulter). EPC were identified as CD34+/KDR+ cells and quantified relative to the number of granulocytes in the sample, which were identified as CD45+ cells with a forward/ward scatter pattern typical for granulocytes. Measurements were performed in duplicate, and the results were averaged for further analysis. Isoytop-stained samples served as negative controls. 

Annexin V and TUNEL staining of HSC. One hundred microliters of EDTA blood were incubated with FITC-conjugated mouse-anti-human CD34 monoclonal antibody (BD Pharmingen) and PECy7-conjugated mouse-anti-human CD45 monoclonal antibody (BD Pharmingen) at 4°C. Erythrocytes were lysed in an ammoniumchloride buffer and remaining cells were stained with PE-conjugated mouse-anti-human annexin V (BD Pharmingen) and 7AAD in annexin V Binding Buffer (BD Pharmingen) at room temperature. Cells were washed and analyzed by flow cytometry (Beckman Coulter). Apoptotic HSC were defined as CD34+/annexinV+ cells with a low forward scatter and 7-AADDim staining. Isoytop-stained samples served as negative controls.

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

Type II EPC culture and characterization. Blood samples were collected in EDTA tubes and mononuclear cells (MNC) were isolated using Ficoll density gradient separation (Histopaque 1077, Sigma, St. Louis, MO). MNC were plated on gelatin (Sigma)-coated six-well plates at a density of 10^{5} cells per well in M199 medium (Invitrogen, Breda, The Netherlands) containing 20% fetal calf serum (Invitrogen), 0.05 mg/ml bovine pituitary extract (Invitrogen), 10 U/ml heparin (Leo Pharma, Breda, The Netherlands), and antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml; Invitrogen). Medium was changed after 4 days, washing nonadherent cells away. After 7 days, cells were detached for quanitification using trypsin-EDTA (Invitrogen) and gentile cell scraping. Type II EPC phenotype was confirmed by the binding of FITC-labeled Ulex Europesus Lectin (Vector, Burlingame, CA) and the uptake of DiI-labeled acylated LDL (Molecular Probes, Leiden, The Netherlands). Since >90% of attaching cells obtained an endothelial phenotype, the total cell number was automatically counted using a hemocytometer (1, 20). As culture conditions used to generate type II EPC vary, we confirmed our observations using a second commonly used method for type II EPC culture, which involves growth on high concentrations recombinant VEGF-165. For this, we coated six-well plates with human fibronectin (Becton Dickinson, Alphen aan de Rijn, The Netherlands) and plated 10^{7} cells per well in EGM-2 medium (Cambrex), supplemented with 20% fetal calf serum (Invitrogen), 100 ng/ml recombinant VEGF-165 (R&D systems) additional to that supplied in the Singlequots, and antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml; Invitrogen) (37).

SPC culture. MNC were plated in six-well plates coated with human fibronectin (Sigma) at a density of 5 × 10^{6} cells per well and cultured in low-glucose DMEM, supplemented with 20% fetal calf serum, t-glutamine (2 mM), and antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml; Invitrogen). Medium was changed after 4 days, washing nonadherent cells away. Seven days after the original plating, cells were detached using trypsin-EDTA and a cell scraper. Since the vast majority of cells have a SPC phenotype, identified by the coexpression of α-smooth muscle actin and collagen-1, the total adherent cell number was automatically counted using a hemocytometer (23).

In vitro angiogenesis assay. The capacity of type II EPC to excrete paracrine angiogenic factors was assessed by testing the effect of type II EPC conditioned medium on mature endothelial cells. Seven-day type II EPC were cultured in serum-free endothelial medium [Endothelial Basal Medium-2 supplemented with selected EGM-2 aliquots: hEGF, porcine insulin, GA-1000, R²-IGF-1, ascorbic acid and heparin (Cambrex)] for 20 h. Conditioned media were stored at −80°C until further processing after removal of nonadherent cells by centrifugation. For further experiments, conditioned media were diluted to correct for the type II EPC cell number in the original culture; 7.5 × 10³ early passage human umbilical vein endothelial cells (HUVEC) were suspended in conditioned media and placed on matrigel (Chemicon). HUVEC suspended in blank medium (the serum-free medium with selected EGM-2 aliquots was used to make the conditioned medium) and regular EGM-2 supplemented with 100 ng/ml additional VEGF were used as negative and positive controls. After 20 h, cells were labeled with calcine-AM (Molecular Probes), fixed in 4% paraformaldehyde for 30 min, and photographed at ×50 magnification using an inverted fluorescence microscope. The formation of tubular structures was quantified based on tube length using Scion Image software (Scion, Frederick, MD) and expressed in arbitrary units.

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

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

RESULTS

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

Type I EPC and total HSC are reduced in ESRD patients. On flow cytometry, CD34+ HSC were readily identifiable and contained a subfraction of cells staining positive for the EPC marker KDR. The total number of circulating CD34+ HSC was lower in ESRD patients [43.2 (7.0) vs. 90.0 (9.4)/10^{5} granulocytes, 52% reduction; P = 0.0003; Fig. 1A]. The percentage of HSC coexpressing KDR was higher in ESRD patients than in controls [31 (3) vs. 20 (2)%; P = 0.023]. The
The absolute number of type I EPC in the peripheral blood was significantly lower [10.6 (1.1) vs. 16.1 (1.4)/10^5 granulocytes, 34% reduction; \( P = 0.0046 \); Fig. 1B].

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

**Type II EPC outgrowth is impaired in ESRD, whereas SPC outgrowth is unaffected.** After 7 days of culture, type II EPC expressed KDR, took up DiI-labeled acetylated LDL, and bound FITC-labeled lectin, while SPC did not display these endothelial characteristics. Conversely, SPC expressed α-SMA, while type II EPC did not. Type II EPC did express some collagen-1 intracellularly near the plasma membrane, although not as abundant as the SPC, which also secreted it (Fig. 3). Quantification of type II EPC after 7 days of culture revealed that type II EPC outgrowth from the MNC was reduced by 58% in ESRD patients vs. controls \([3.3 (0.6) vs. 7.9 (1.9)/1,000 MNC; \ P = 0.030; \) Fig. 3]. Because type II EPC culturing conditions are not uniform, we confirmed our observations using a second commonly used protocol \((37)\) in a subgroup of patients. We observed a similar reduction using this method, although with higher efficacy of EPC outgrowth \([8.6 (2.7) vs. 15.7 (5.1) \) type II EPC/1,000 MNC corresponding with a 45% reduction, \( n = 5 \); our standard culture method in this subgroup showed a 42% reduction with 5.9 (0.9) vs. 10.1 (3.5) type II EPC/1,000 MNC]. In contrast to the reduction in type II EPC, SPC outgrowth was not affected in ESRD patients \([5.2 (0.7) vs. 5.8 (1.2) SPC/1,000 MNC; \ P = 0.706; \) Fig. 3].

**Paracrine angiogenic function of type II EPC outgrowth is impaired in ESRD.** An important mode of action for type II EPC is the secretion of paracrine acting angiogenic factors. To test the angiogenic capacity of type II EPC, HUVEC were taken up in type II EPC-conditioned medium and plated on matrigel. Under these conditions, HUVEC formed tubular outgrowths on type II EPC-conditioned medium but not on SPC-conditioned medium (Fig. 3). Quantification of type II EPC outgrowth using a second commonly used protocol \((37)\) in a subgroup of patients. We observed a similar reduction using this method, although with higher efficacy of EPC outgrowth \([8.6 (2.7) vs. 15.7 (5.1) \) type II EPC/1,000 MNC corresponding with a 45% reduction, \( n = 5 \); our standard culture method in this subgroup showed a 42% reduction with 5.9 (0.9) vs. 10.1 (3.5) type II EPC/1,000 MNC]. In contrast to the reduction in type II EPC, SPC outgrowth was not affected in ESRD patients \([5.2 (0.7) vs. 5.8 (1.2) SPC/1,000 MNC; \ P = 0.706; \) Fig. 3].

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**Table 1. Patient characteristics**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>ESRD Patients (n = 45)</th>
</tr>
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<tr>
<td>Age, yr</td>
<td>55.9 (2.1)</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>30/15</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>74 (5)</td>
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<tr>
<td>Blood pressure, mmHg</td>
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<tr>
<td>Diastolic</td>
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<tr>
<td>Systolic</td>
<td>141 (5)</td>
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<tr>
<td>White blood cells (*10^6/ml)</td>
<td>8.3 (0.6)</td>
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<tr>
<td>Mononuclear cells (*10^6/ml)</td>
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<tr>
<td>Granulocytes (*10^9/ml)</td>
<td>6.0 (0.5)</td>
</tr>
<tr>
<td>Hemoglobin, mmol/l</td>
<td>7.1 (0.3)</td>
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<tr>
<td>Glucose, mmol/l</td>
<td>6.4 (0.4)</td>
</tr>
<tr>
<td>Total cholesterol, mmol/l</td>
<td>3.40 (0.13)</td>
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<tr>
<td>LDL, mmol/l</td>
<td>1.47 (0.11)</td>
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<tr>
<td>HDL, mmol/l</td>
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<tr>
<td>Triglycerides, mmol/l</td>
<td>1.63 (0.22)</td>
</tr>
<tr>
<td>Time on dialysis, mo</td>
<td>19.3 (9.3)</td>
</tr>
<tr>
<td>Dialysis session duration, min</td>
<td>195 (5)</td>
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<tr>
<td>Dialysis Kt/V</td>
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<tr>
<td>Type of access: Fistel</td>
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<tr>
<td>History of CVD</td>
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<td>Hypertension (RR &gt; 145/95 or drug use)</td>
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<td>Diabetes</td>
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<td>Medication</td>
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<td>Antihypertensive drugs†</td>
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<td>Statin</td>
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<tr>
<td>Folic acid</td>
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<tr>
<td>Erythropoietin (darbepoetin alpha)</td>
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</tr>
<tr>
<td>Avg. erythropoietin dose, µg/wk</td>
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</tr>
<tr>
<td>Principle causes of ESRD</td>
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<td>Glomerulonephritis</td>
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<tr>
<td>Hypertension</td>
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<tr>
<td>Polycystic kidney disease</td>
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<td>Intoxication</td>
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<td>Diabetes mellitus</td>
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<tr>
<td>Pyelonephritis</td>
<td>10%</td>
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<tr>
<td>Unknown/other</td>
<td>16%</td>
</tr>
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</table>

*Predialysis; ESRD, end-stage renal disease; CVD, cardiovascular disease. †Mainly ACE inhibitors.

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**Fig. 1.** Circulating levels of hematopoietic stem cell (HSC) and type I endothelial progenitor cells (EPC). Circulating levels of HSC \((A)\) and type I EPC \((B)\) are reduced in blood samples taken immediately before a hemodialysis session in end-stage renal disease (ESRD) patients \((n = 30)\) compared with healthy controls \((n = 15)\). *P < 0.05.*
Healthy controls. Uremic serum reduced type II EPC outgrowth by 46% [13.4 (1.8) vs. 24.2 (2.1)/10⁵ granulocytes; P = 0.004; Fig. 5A]. SPC outgrowth was unaffected by the uremic serum [28.1 (5.9) vs. 29.3 (7.9)/10⁵ MNC; P = 0.708; Fig. 5B].

Hemodialysis causes a depletion of type I EPC and HSC from the circulation and induces HSC apoptosis. We investigated whether a dialysis session would directly increase EPC numbers. However, type I EPC levels were reduced at the end of the dialysis session compared with levels before the start of dialysis [11.1 (1.5) vs. 7.5 (1.2)/10⁵ granulocytes; P = 0.0054; Fig. 6A]. To investigate a possible involvement of apoptosis in this process, we measured the fraction of CD34+ HSC that bound annexin V. Annexin V binds to phosphatidylserine, which is normally located on the inner side of the cell membrane, but translocates to the external side in an early phase during apoptosis, enabling extracellular annexin V binding. This is distinctive from nonprogrammed causes of cell death. During dialysis, the fraction of annexin V-positive HSC increased [27.1 (3.4) vs. 37.1 (4.4); P = 0.04; Fig. 6B], confirming a role for apoptosis in the observed HSC depletion. HSC apoptosis rates before dialysis were comparable to control levels. Additional analysis showed that less than 2% of HSC were TUNEL positive (data not shown), indicating that circulating apoptotic HSC are in an early phase of apoptosis as they in majority do not display signs of late apoptosis, such as TUNEL staining of DNA strand breaks. Binding of annexin V to type I EPC could not be studied because the necessary combination of fluorescent label conjugated antibodies is not available.

VEGF plasma levels are not significantly decreased in ESRD patients. VEGF induces mobilization of EPC and is present in the circulation (2). We measured plasma VEGF levels, to evaluate whether decreased levels of mobilizing stimuli might underlie the reduced EPC levels. However, plasma VEGF was not significantly reduced in ESRD patients [63.4 (16.1) vs. 68.0 (34.2) pg/ml; P = 0.891].

DISCUSSION

The present data show that in our population of ESRD patients on regular medication levels of total HSC, HSC-derived type I EPC and monocyte-derived type II EPC are markedly reduced compared with healthy controls, whereas SPC levels are not affected. Furthermore, type II EPC capacity for paracrine angiogenic stimulation was impaired. Our observations suggest that the uremic environment causes a relative imbalance in vascular progenitor cell differentiation with impaired regenerative potential and enhanced proatherosclerotic tendency as a consequence. Progenitor cell imbalance may have a pathophysiological role in the development of CVD in ESRD patients. In patients with preterminal renal insufficiency, EPC and SPC may also influence the progression of renal disease, since EPC have been shown to incorporate in damaged glomerular endothelium (26, 27), whereas glomerulosclerosis may be initiated by bone marrow-derived circulating cells (8).

In our population the marked progenitor cell impairments were observed despite standard medical treatment, indicating that additional therapies aimed at enhancing EPC and/or inhibiting SPC may be of added benefit for ESRD patients.

Our study is the first to show that patients with ESRD have reduced levels of circulating type I EPC, defined as CD34+ KDR+ cells. Such reductions in type I EPC are particularly relevant as, at least in patients with coronary artery disease, reduced type I EPC numbers predicted future cardiovascular events (30, 40). Previous studies reported decreased levels of the whole CD34+ HSC population in ESRD, consistent with our data (9, 11, 15). However, our observations contrast with the single study that has reported on type I EPC in ESRD, by Herbrig et al. (15), who showed no difference despite a (nonsignificant) decrease in HSC levels. Of note, in our population the reductions in type I EPC levels were also less conspicuous than the reductions in total HSC numbers. Our observations of reduced numeric outgrowth of type II EPC in our population of ESRD patients on hemodialysis are in line with several previous reports (7, 9, 11). Again, contrasting findings were reported by Herbrig et al. (15), who observed an increased number of type II EPC in ESRD patients on hemodialysis. Differences in study methods may play a role. Culture conditions to obtain type II EPC are not uniform and the methods used by various groups differ on several points. We confirmed the observations using our standard culture conditions (1, 20) in a subset of samples using a second commonly used culture method (37). In a recent review, Herbrig et al. (14) suggested that the apparent discrepancies of their study with those of others might be due to the patient selection, the majority of patients receiving EPO treatment, or the high VEGF levels in their population, which were found to be twofold higher than their controls. Although we did find an association between EPC levels and the EPO dose, overall a majority of our patients received EPO also (80%). The VEGF levels in our study were not higher than in controls, but we did not observe a correlation between EPC and VEGF levels, making a determining role for VEGF less likely.
discrepancy of Herbrig’s study with our observations on type I and type II EPC and with all previous studies on type II EPC may be due to their very specific patient selection. They included a highly selected ESRD population without concomitant CVD risk factors and use of various cardiovascular drugs, thereby excluding over 80% of their initial population. Our population consisted of patients on regular treatment with minimal exclusions and thus more representative for the ESRD population as a whole, but heterogeneous in its composition and influencing factors. We found no major statistically
significant contributing effects of concomitant individual cardiovascular risk factors or drugs on EPC numbers; however, the statistical power to detect such differences was limited. Cardiovascular risk factors were absent in our control population, where the majority of the ESRD patients in our study had hypertension, diabetes, a history of CVD, dyslipidemia, or a combination of factors. These cardiovascular risk factors such as hypertension can be both a cause and consequence of ESRD. An important limitation of our study is therefore that we can altogether not dissociate the effect of renal impairment per se with the presence of cardiovascular risk factors. Of note, levels of cardiovascular risk factors may correlate differently with the manifestation of CVD in ESRD patients than in the general population, further complicating any such interpretation (17).

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

We observed that apart from altered numeric outgrowth, the paracrine actions of type II EPC were reduced in ESRD patients compared with controls. Type II EPC from ESRD patients have previously been shown to exhibit an impaired capacity to migrate, adhere to matrix molecules or mature endothelial cells, and to incorporate into endothelial cell vascular structures (7, 9, 15). However, the particular importance of the paracrine actions of type II EPC is increasingly recognized, whereas their role of active participation in the newly formed endothelium is under debate (25, 35). We found that type II EPC-conditioned medium of ESRD patients was less capable of stimulating HUVEC to form vascular structures than that of healthy controls. Hence, in ESRD patients, not only less type II EPC are available to home to sites of endothelial damage, but they are also less capable of stimulating resident endothelial angiogenesis. The aberrant outgrowth of EPC was not accompanied by changes in SPC outgrowth. We therefore speculate that the regenerative capacity of pro-

Fig. 4. Paracrine effect of type II EPC-conditioned medium on HUVEC angiogenesis. When taken up in conditioned medium from healthy control type II EPC, mature endothelial cells (HUVEC) were stimulated to form angiogenic structures when placed on Matrigel (A). Formation of these structures was significantly hampered when HUVEC were incubated with conditioned medium from ESRD patient type II EPC (B). As a positive control, 100 ng/ml recombinant VEGF-165 was used (C). The average length of the formed tubular structures was lower in the assays from ESRD patients than in those from healthy controls and even (nonsignificantly) lower than those with control basal medium (D). $^{*}P < 0.05$, $n = 10$ patients vs. 10 controls.

Fig. 5. Effect of uremic or control serum on type II EPC and SPC outgrowth from healthy mononuclear cells. Serum from uremic patients impaired type II EPC outgrowth (A), without affecting SPC outgrowth (B) from healthy mononuclear cell cultures compared with serum from healthy controls. $^{*}P < 0.05$, $n = 7$ patients vs. 7 controls in paired analysis of 7 independent experiments.
Fig. 6. Effect of dialysis on progenitor cell levels and apoptosis. During dialysis, circulating levels of type I EPC decreased (A), while inducing progenitor cell apoptosis as evidenced by annexin V staining of CD34+ HSC (B). *P < 0.05, n = 17 patients before and after dialysis in paired analysis.

genitor cells may be impaired in ESRD, while the capacity of progenitor cells to contribute to fibrosis and adverse remodeling of vascular lesions is unaffected.

Interestingly, a dialysis session did not increase but markedly reduced circulating progenitor cells in the circulation. This observation cannot be attributed to changes in circulating blood volume as cell numbers were expressed relative to the number of granulocytes, which moreover did not change significantly during the procedure. Another possibility is EPC sequestration. Leukocyte subpopulations, particularly monocytes but also neutrophils and to some extent lymphocytes, are known to sequestrate during hemodialysis, probably due to complement activation as a result of contact between the blood and the dialysis membrane. For all leukocytes, however, sequestration is a rapidly occurring process that is maximal after ∼10 min, after which circulating leukocyte levels start to rise again and fully recover well before the end of the dialysis session (31). We cannot exclude the possibility of sequestration of EPC during dialysis, but since we measured decreased progenitor cell levels at the very end of the dialysis session at a time point when other leukocyte subpopulations are no longer sequestered, this does not seem likely. Increased apoptosis could also be responsible for the depletion of EPC during hemodialysis. Therefore, we investigated the binding of apoptosis marker annexin V to circulating HSC, which indeed increased during dialysis, suggesting increased apoptosis to be a causative mechanism. This is in line with other studies showing an induction of apoptosis in the general leukocyte population by hemodialysis (3).

Repetitive depletion of type I EPC during dialysis sessions may exhaust the available progenitor cell pools. However, this may only in part explain the decreased progenitor cell levels at baseline. We and others (9) showed that uremia itself has a profound effect on progenitor cells. Furthermore, in patients with severe renal insufficiency but not yet on dialysis, HSC and type II EPC were decreased compared with controls and increased after the initiation of dialysis (9). Finally, a recent study demonstrated fully normalized HSC and type II EPC levels in nocturnal hemodialysis, which is a very intensive dialysis regimen both in frequency and duration resulting in extensive contact of blood with the dialysis membrane, which is highly effective in clearing uremic toxins (7). Interestingly, previous studies showed unexpected impairment of endothelial function occurring during hemodialysis (22). This appeared to be related to the type of dialysis filter (18). It remains to be established whether particular hemodialysis methods or materials may ameliorate the induction of progenitor cell apoptosis and depletion during dialysis sessions. All our patients dialyzed with a polysulphone filter, which was previously shown not to impair endothelial function (18).

We investigated patients receiving standard treatment, including antihypertensives and statins, which are known to increase EPC numbers in other populations (21, 38). However, we did not find differences in progenitor cell levels associated with the use of these drugs in our study. Interestingly, erythropoietin was dose dependently associated with higher numbers of circulating HSC, and to a lesser extent type I EPC. Although this observation is likely to be confounded by our cross-sectional study design, it could reflect a beneficial effect of recombinant erythropoietin on EPC levels. Of note, we did not find any correlation with the hemoglobin or erythrocyte levels, suggesting that modulation of EPC numbers by erythropoietin may be unrelated to effects on erythropoiesis. Erythropoietin administration has indeed been shown to upregulate HSC and type II EPC numbers in patients with advanced renal failure and renal anemia, but also in nonrenal conditions, such as congestive heart failure, myocardial infarction and even in healthy controls in vivo, as well as ex vivo in the absence of the erythroid system (4, 5, 12, 19). These data support a direct stimulating effect on EPC of exogenous erythropoietin.

A well-known endogenous EPC-mobilizing factor is VEGF (2). We measured VEGF plasma levels, which were not different between patients and controls. This is consistent with the work of others who measured similar or even increased levels (7, 13, 15). The reduced EPC levels are therefore not due to a lack of VEGF, although other unidentified mobilizing factors may be deficient.

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

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