Uremia causes endothelial progenitor cell deficiency

KIRSTEN DE GROOT, FERDINAND HERMANN BAHLMANN, JOHANNA SOWA, JANA KOENIG, JAN MENNE, HERMANN HALLER, and DANilo FLISER

Division of Nephrology, Department of Internal Medicine Hannover Medical School, Hannover, Germany

Uremia causes endothelial progenitor cell deficiency.

Background. Circulating bone marrow–derived endothelial progenitor cells (EPCs) promote vascular repair. Their number in peripheral blood correlates with endothelial function and cardiovascular risk in humans. We explored whether uremia influences the number of EPCs.

Methods. We assessed circulating CD34+ hematopoietic progenitor cells in whole blood using flow cytometry and EPCs (in vitro assay) in 46 patients with advanced renal failure and in 46 age- and gender-matched healthy subjects. Further, the effect of uremia on EPC differentiation was studied in vitro and in vivo.

Results. Both in renal patients (r = 0.34, P < 0.02) and in healthy subjects (r = 0.32, P = 0.04) the number of EPCs was significantly correlated to the absolute number of CD34+ hematopoietic progenitor cells. Renal patients had significantly fewer EPCs than healthy subjects, however (167 ± 15 cells/high power field vs. 235 ± 17 cells/high power field; P < 0.05). Uremic serum significantly (P < 0.05) inhibited EPC differentiation and functional activity in vitro. Amelioration of uremia after institution of renal replacement therapy in patients with terminal renal failure also significantly (P < 0.05) increased the number of EPCs.

Conclusion. Uremia inhibits differentiation of EPCs. This may impair cardiovascular repair mechanisms in patients with renal failure.

Patients with renal failure are characterized by high cardiovascular morbidity and mortality, and most die of complications related to atherosclerosis, namely myocardial infarction and stroke [1]. Several traditional and non-traditional cardiovascular risk factors are thought to play a certain role, but the idea that impaired vascular repair mechanisms may contribute to the problem has not been pursued so far. In this respect current research has focused on bone marrow–derived endothelial progenitor cells (EPCs), because these cells mediate reparative processes in the cardiovascular system [2–4]. EPCs are considered to originate from CD34+ hematopoietic stem cells, which differentiate via separate pathways into erythrocytes, thrombocytes, various lineages of leukocytes, and endothelial cells. EPCs circulate in the vasculature where they home and incorporate into sites of active neovascularization [5, 6]. In patients with coronary artery disease the number of EPCs correlates strongly with the number of cardiovascular risk factors [7]. This correlation exists even in subjects without manifest atherosclerosis. In the latter population the number of EPCs also correlates with the degree of endothelial dysfunction [8].

We could recently demonstrate that administration of recombinant human erythropoietin (rhEPO) or its analogue darbepoetin enhances EPC differentiation in vitro and in vivo [9, 10]. Furthermore, in laboratory animals rhEPO causes a significant mobilization of EPCs from the bone marrow [11]. Thus, a reduced number and/or impaired function of EPCs due to EPO deficiency could be a potential cardiovascular risk factor contributing to morbidity and mortality in patients with advanced renal failure. In the present study we therefore tested the hypothesis that the number EPCs is reduced in uremia. For this purpose we assessed EPCs in uremic patients and in age- and gender-matched control subjects. In addition, we studied patients with end-stage renal disease (ESRD) who started renal replacement therapy but did not yet require rhEPO. Finally, we cultivated EPCs in the presence of serum from uremic and healthy subjects.

METHODS

Participants and protocol

The study protocol was approved by the Hannover Medical School Ethics Committee. We assessed the number EPCs in patients with advanced renal failure and in age- and gender-matched control subjects after obtaining informed consent (Table 1). Patients with concomitant chronic inflammatory diseases or clinically manifest acute infections, malignant diseases, manifest or occult bleeding conditions, or recent cardiovascular events were
were acquired. Two blinded investigators independently assessed the number of HPCs. Day-to-day variability of the absolute HPC number was below 12% as assessed by flow cytometry of HPCs in eight healthy subjects on four consecutive days. Interassay variability (N = 10) was below 5%.

**Isolation of EPCs**

We isolated peripheral blood mononuclear cells from 14 mL blood in order to cultivate EPCs as described elsewhere [2, 13, 14]. We used density gradient centrifugation with Bicoll (Biochrome, Berlin, Germany), and seeded 10⁷ cells on 6-well plates coated with human fibronectin (Sigma Chemical Co., St. Louis, MO, USA) in endothelial basal medium (EBM-2) (Clonetics, Walkersville, MD, USA). The medium was supplemented with endothelial growth medium-2 (EGM-2) Single Quots containing fetal bovine serum (FBS), human vascular endothelial growth factor (VEGF-A), human fibroblast growth factor-B (FGF-B), human epidermal growth factor (EGF), insulin-like growth factor-1 (IGF-1), and ascorbic acid in appropriate amounts. After 4 days in culture, we removed nonadherent cells by washing the plates with phosphate-buffered saline (PBS). We trypsinated the remaining adherent cells and reseeded 10⁶ cells on fibronectin-coated 6-well plates. New media was applied and the cell culture was maintained through day 7. We further performed fluorescent chemical detection in order to determine the cell type of the attached human peripheral blood mononuclear cells after 7 days in culture. To detect the uptake of 1,1′-dioctadecyl-3,3′,3′-tetramethylindocarbocyanine–labeled acetylated low-density lipoprotein (acLDL-DiI) (Molecular Probes, Eugene, OR, USA), we incubated the cells with acLDL-DiI (6 µg/mL) at 37°C for 2 hours. Cells were then fixed with 1% paraformaldehyde for 10 minutes and incubated with FITC-labeled Ulex europaeus agglutinin-1 (UEA-1) (Sigma) for 1 hour. After the staining, we viewed the samples with an inverted fluorescent microscope (Leica, Wetzlar, Germany). We counted double stained cells for both UEA-1 and acLDL-DiI as EPCs. Two blinded investigators counted at least four randomly selected high power fields. Using such a protocol we also cultivated EPCs from eight healthy subjects in four experiments in the presence of uremic and normal serum.

**Flow cytometry of circulating HPCs**

In all participants we analyzed the total number of circulating HPCs using flow cytometry (Epics XL Cytometer; Coulter Beckman, Krefeld, Germany). We adopted a gating strategy for flow cytometry on the basis of the International Society of Hematology and Graft Engineering (ISHAGE) guidelines, and used the CD34 and CD45 expression patterns as well as the morphologic qualities of progenitor cells for their detection (Fig. 1) [12]. For this purpose we stained whole ethylenediaminetetraacetic acid (EDTA) blood within 6 hours after drawing the blood. Thereafter, we incubated a volume of 100 µL with an appropriate amount of fluorescein isothiocyanate (FITC)-labeled monoclonal mouse antihuman-CD45 antibody (Coulter Beckman) for 20 minutes. For detection of HPCs we added phycoerythrin-labeled monoclonal mouse anti-human-CD34 antibody (Coulter Beckman) to the sample after titration of the optimal antibody concentration. In addition, we added a phycoerythrin-labeled mouse IgG1-antibody (Coulter Beckman) to a second anti-CD45 stained blood sample as the isotype control. Subsequent lysis was done with ammonium chloride, and at least 200,000 CD45+ cells were acquired. Two blinded investigators independently

---

**Table 1. Clinical and laboratory data of patients with advanced renal failure and age- and gender-matched healthy subjects**

<table>
<thead>
<tr>
<th>Renal patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>46</td>
</tr>
<tr>
<td>Age years</td>
<td>58.6 ± 2.51</td>
</tr>
<tr>
<td>Gender male/female</td>
<td>29/17</td>
</tr>
<tr>
<td>Serum creatinine mg/dL</td>
<td>5.67 ± 0.39</td>
</tr>
<tr>
<td>CD34+ hematopoietic progenitor cells per µL</td>
<td>1.62 ± 0.19</td>
</tr>
<tr>
<td>Endothelial progenitor cells per high power field</td>
<td>167 ± 15</td>
</tr>
<tr>
<td>Haematocrit %</td>
<td>31.9 ± 0.79</td>
</tr>
<tr>
<td>Erythropoietin U/L</td>
<td>8.6 ± 0.56</td>
</tr>
<tr>
<td>High sensitive C-reactive protein mg/L</td>
<td>6.2 ± 1.18</td>
</tr>
<tr>
<td>White blood cell count 10⁹/µL</td>
<td>8.0 ± 0.3</td>
</tr>
<tr>
<td>Lymphocyte count % of white blood cells</td>
<td>16.8 ± 1.2</td>
</tr>
<tr>
<td>Monocyte count % of white blood cells</td>
<td>7.5 ± 0.4</td>
</tr>
</tbody>
</table>

*aP < 0.05 comparison between renal patients and healthy controls.

---

excluded from the study. None of the patients received a therapy with rhEPO or analogues, and blood transfusions were not administered for at least 3 months before study entry. In addition, we studied six patients with ESRD (five males and one female, aged 58.4 ± 9.0 years) who started renal replacement therapy, but did not yet require rhEPO treatment nor blood transfusion before and during the first 2 weeks of hemodialysis. We assessed the absolute number of EPCs and CD34+ hematopoietic progenitor cells (HPCs) in these patients before and after 2 weeks of treatment. All routine laboratory measurements were done using certified assay methods.
After 4 hours of incubation in 5% CO₂ humidified atmosphere at 37°C, the three-dimensional organization of the cells was examined under an inverted phase-contrast photomicroscope using following grades: 0, individual cells, well separated; 1, cells begin to migrate and align themselves; 2, capillary tubes visible, no sprouting; 3, sprouting of new capillary tubes visible; 4, closed polygons begin to form; and 5, complex mesh-like structures develop. A blinded investigator examined at least ten randomly selected high power fields.

Further, we performed a Transwell migration assay in order to study EPC migration. The migratory capacity of EPCs was assessed by their ability to cross the 8 μm pores of migration chambers represented by transwells fitted with polycarbonate membranes (10 mm filters, 8 μm pore size) (Nunc A/S, Roskilde, Denmark). For this purpose 5 × 10⁵ day 7 EPCs of eight healthy volunteers, previously starved for 24 hours, were plated in the upper wells of transwell chambers containing either EGM-2 with a 10% addition of serum from a healthy or a uremic person. The migration apparatus was assembled and incubated for 6 hours in a humidified environment (5% CO₂) at 37°C. After incubation, the upper wells of the migration chamber were removed, and the migrated cells were counted by flow cytometry.

**Statistical analysis**

We compared baseline characteristics of patients with renal failure and control subjects using a χ² test for categorical variables and an unpaired t test for continuous variables (SPSS, version 10.0.7 for Windows; Chicago, IL, USA). Results were corrected for multiple comparisons. The statistical significance was set at P < 0.05. Data are shown as mean ± SEM. Further, in both groups we performed Pearson's correlation analysis between the numbers of EPCs, on the one hand, and age, the number of CD34+ HPCs, and blood hematocrit, EPO, and high sensitive C-reactive protein (hsCRP) levels on the other hand. In addition, independent predictors of EPC number were evaluated using a stepwise multiple regression analysis with combined data of renal patients and healthy subjects. The difference in EPC number in patients with ESRD before and after renal replacement therapy was analyzed using a Mann-Whitney U test, and data obtained in vitro experiments were compared using a t test for random data.

**RESULTS**

Patients with advanced renal failure and healthy controls were well matched with respect to age and gender (Table 1). Renal patients had significantly fewer EPCs per high power field than healthy subjects, however. Individual data on the total number of EPCs are shown in Figure 2. Cell culture plates from a representative patient and a matched control subject are shown in Figure 3 (upper panel). The absolute number of circulating CD34+ HPCs was lower in renal patients as well, but the difference was not significant. In addition, renal patients had...
significantly lower blood EPO concentrations (Fig. 2) and significantly higher hsCRP levels.

In patients with advanced renal failure the number of EPCs did not correlate with age ($r = 0.11, P = 0.46$), hematocrit ($r = 0.10, P = 0.49$), or blood EPO ($r = 0.06, P = 0.73$), and hsCRP ($r = -0.07, P = 0.64$) concentrations. In contrast, the correlation between EPCs and the absolute number of CD34$^+$ HPCs was significant ($r = 0.34, P < 0.02$). In healthy subjects this correlation was significant ($r = 0.32, P < 0.04$) as well, whereas EPC number did not correlate with age ($r = -0.20, P = 0.19$), hematocrit ($r = -0.19, P = 0.20$), or blood EPO ($r = 0.10, P = 0.54$), and hsCRP ($r = -0.05, P = 0.75$) concentrations. The stepwise multiple regression analysis revealed that the absolute number of CD34$^+$ HPCs ($r = 0.31; P < 0.004$) and plasma EPO levels ($r = 0.22; P < 0.041$) were independent predictors of the total number of EPCs in our study cohort.

In patients with ESRD the number of EPCs increased significantly ($P < 0.05$) from $187 \pm 45$ cells/high power field to $275 \pm 30$ cells/high power field after institution of renal replacement therapy. The number of CD34$^+$ HPCs increased as well, but the difference did not reach statistical significance ($1.75 \pm 0.29$ vs. $1.92 \pm 0.31; NS$). In contrast, the hematocrit level remained unchanged ($33.4 \pm 2.6$ vs. $32.9 \pm 2.3; NS$).

As shown in Figure 3 (lower panel) serum from uremic patients significantly ($110 \pm 14$ vs. $168 \pm 28, P < 0.05$) inhibited EPC differentiation in vitro in comparison to serum from healthy individuals. Further, the ability of EPCs to contribute to tube formation was significantly reduced in uremic patients as compared to healthy subjects (tube formation index: uremic serum $3.7 \pm 0.2$, healthy serum: $4.3 \pm 0.2; P < 0.05$). Finally, the migration capacity of EPC cultured in the presence of uremic serum was reduced to $72\%$ of the migration capacity of EPCs cultured in the presence of serum obtained from healthy subjects.

**DISCUSSION**

The results of the present study document that the number of EPCs is significantly reduced in patients with advanced renal failure as compared with age- and gender-matched healthy subjects. In addition, we found a significant correlation between CD34$^+$ HPCs and EPCs both in healthy subjects as well as in renal patients. Taken together, these findings point to a problem of differentiation of precursor cells to EPCs or to reduced mobilization of EPCs from the bone marrow, or both, in uremia. The former assumption is supported by the observation of a
significant inhibitory effect of uremic serum on the differentiation capacity of EPCs in vitro. However, we cannot rule out the possibility that uremia hampers the attachment of EPCs to the extracellular matrix (fibronectin). Furthermore, functional properties such as the capability of EPCs to migrate and to form tube-like structures were impaired. In addition, a significant increase in the number of EPCs goes in parallel with amelioration of uremia after institution of renal replacement therapy in patients with terminal renal failure. We have studied a relatively small number of patients with ESRD, as it is difficult to find patients in the terminal phase of their renal disease with stable hemocrit without requiring rhEPO replacement. In order to more convincingly prove the adverse effect of uremia on EPCs we have also explored the direct effect of uremic serum on EPC development in vitro. The results of these experiments confirmed the hypothesis that uremia hinders EPC differentiation. The reason(s) for this inhibitory action have to be unfolded yet, but the finding is reminiscent of the well-known defects of cellular function caused by uremic intoxication [16, 17].

One important factor contributing to EPC deficiency in patients with advanced renal failure could be lack of EPO, because we could recently demonstrate that administration of rhEPO to renal patients and to healthy subjects stimulates EPCs in vitro and in vivo via the AKT pathway. Furthermore, we and others were able to demonstrate that EPO directly enhances EPC mobilization from the bone marrow [10, 11]. Hence, one would expect that EPC numbers are, at least in part, correlated to EPO blood levels. EPCs in uremic patients were not correlated to EPO blood levels, and the same was true also for healthy subjects. This finding is contradictory only at the first glance. In the regression analysis independent predictors of EPC levels in our cohort were the number of CD34+ HPCs and plasma EPO levels, however. Further, the dose of rhEPO which already markedly stimulated EPCs in vivo was below that required to achieve a significant increase in hemocrit levels. Thus, it is conceivable that EPC number and the number of red blood cells are regulated by independent mechanisms and/or at different EPO blood concentrations. Patients examined in the present study had advanced renal failure and uniformly low EPO blood concentrations. Studies exploring the relationship between blood EPO levels and EPCs in patients at different stages of renal failure will clarify this issue.

In addition to EPO deficiency, a number of cardiovascular risk factors are known to be present in uremic patients, which all theoretically can influence EPC number and function (e.g., dyslipidemia and hypertension) [7, 8, 18]. It is almost impossible to clearly distinguish the impact of these factors singularly on EPCs in renal patients, however. EPCs have come into focus of cardiovascular research recently, because they govern endothelial and hence vascular repair [2–4]. This has been shown in several experimental studies using different animal models of cardiovascular injury [6, 19, 20]. Even more intriguing were data obtained in human studies, showing that the number of circulating EPCs predicts outcome after myocardial infarction. Thus, studies exploring such a relationship are warranted also in renal patients (i.e., a population characterized by high cardiovascular morbidity and mortality due to vascular complications) [1].

CONCLUSION

Differentiation of EPCs is inhibited in uremia. This may impair cardiovascular repair mechanisms in patients with renal failure. Since EPO stimulates EPCs, treatment with rhEPO might be indicated at an earlier stage of renal failure as currently recommended. ACKNOWLEDGMENTS

We thank Dr. Cinkilic, Dr. Hafer, and Dr. Hiss for referring patients to the study, and E. Niemczyk, E. Bahllmann, as well as B. Hertel for excellent technical support. The study was supported by an unrestricted research grant from Hoffman-La Roche AG.

Reprint requests to Kirsten de Groot, M.D., Division of Nephrology, Department of Internal Medicine, Hannover Medical School Carl-Neuburg-Straße 1, 30625 Hannover, Germany. E-mail: groot.kirsten.de@mh-hannover.de

REFERENCES


