Different Populations of Circulating Endothelial Cells in Patients with Age-Related Macular Degeneration: A Novel Insight into Pathogenesis

Anna Machalińska,*1,2 Krzysztof Safranow;3 Violetta Dziedziejko,3 Katarzyna Mozolewska-Piotrowska,2 Edyta Paczkowska,4 Patrycja Kłos,4 Ewa Pius,4 Katarzyna Grymula,4 Barbara Wiszniewska,1 Danuta Karczewicz,2 and Bogusław Machaliński*1,4

PURPOSE. Circulating endothelial cells (CECs) and endothelial progenitor cells (EPCs) may serve as novel markers of endothelial dysfunction. The presence and clinical implications of CECs and the expression of endothelin (ET)-1, one of the most potent vasoconstrictors, have not been evaluated in patients with the neovascular form of age-related macular degeneration (AMD). This study was conducted to determine the different populations of endothelial cells (ECs) in the peripheral blood of AMD patients and to correlate these findings with the expression of ET-1 and the cytokines and growth factors responsible for EC migration and function.

METHODS. Peripheral blood samples were collected from 29 patients with diagnosed neovascular AMD and from 38 healthy control subjects. CD133+/CD144+ CECs and CD34+/CD133+/CD144+ EPCs were counted and analyzed by flow cytometry. The intracellular expression of ET-1 in peripheral blood nuclear cells (PBNCs) was studied by using qRT-PCR. Western blot, and immunocytostaining assays, and ET-1, IGF-1, VEGF, SDF-1, and HGF plasma concentrations were measured in enzyme-linked immunosorbent assays.

RESULTS. Increased CECs and EPCs were found in the AMD patients compared with the counts in healthy individuals. The expression of intracellular ET-1 was significantly elevated in PBNCs from the AMD patients compared with the control subjects. In addition a significantly higher plasma concentration of IGF-1 was observed, but a lower SDF-1 level in the AMD patients compared with the counts in healthy individuals. The reasons for this generally reflect an increasing prevalence of several known AMD risk factors, such as smoking, hypertension, poor diet, obesity, diabetes, and an increasingly ageing population. Some epidemiologic studies show a clear relationship between ischemic vascular disease and AMD, proposing that the primary insult causing functional deficits in AMD is vascular damage. Hence, there is growing interest in the identification of risk factors or markers, such as those assessing endothelial injury, that may facilitate improved measures for the primary and secondary prevention of AMD.

CONCLUSIONS. These findings suggest that circulating endothelial cells, together with high ET-1 content, may contribute to the development of AMD. Further prospective investigations on the mechanism involved may be relevant to the potential treatment of this disease. (Invest Ophthalmol Vis Sci. 2011;52: 93–100) DOI:10.1167/iovs.10-5756

Age-related macular degeneration (AMD) is the leading cause of irreversible visual impairment and disability among the elderly in developed countries. This progressive degenerative disorder of the central retina, accounting for 54% of blindness in the United States, contributes substantially to the escalating cost of health care. Most cases of vision loss in AMD result from the development of pathologic choroidal neovascularization (CNV) in the neovascular or exudative (wet) form of the disease. The pathogenesis of AMD is still poorly understood, and treatment options remain limited. Prevalence estimates show an exponential increase in the number of AMD patients after the age of 70. The reasons for this generally reflect an increasing prevalence of several known AMD risk factors, such as smoking, hypertension, poor diet, obesity, diabetes, and an increasingly ageing population. Some epidemiologic studies show a clear relationship between ischemic vascular disease and AMD, proposing that the primary insult causing functional deficits in AMD is vascular damage. Hence, there is growing interest in the identification of risk factors or markers, such as those assessing endothelial injury, that may facilitate improved measures for the primary and secondary prevention of AMD.

The endothelium, the largest organ in the body, is a major regulator of local vascular homeostasis. In response to various cytokines as well as physical and chemical stimuli, endothelial cells (ECs) synthesize and release various factors that regulate vasomotor function, modulate angiogenesis, trigger inflammatory processes, and affect hemostasis. In recent years, emerging data support the concept of endothelial dysfunction in the course of AMD. There are reports documenting an increased release of plasma markers of vascular injury in this disease, such as von Willebrand factor (vWF). More recently, the measurement of immunologically defined circulating endothelial cells (CECs) in the peripheral blood is gaining ground as an important and novel method of assessing endothelial impairment. CECs are desquamated mature cells that have detached from the intimal monolayer in response to endothelial injury, and their high levels reflect severe endothelial damage. Endothelial damage is ultimately a balance between the magnitude of injury and the capacity for repair. However, ECs are terminally differentiated cells with a very low proliferative potential. Accumulating evidence obtained mainly during the past decade suggests that in adults there is a subpopulation of bone marrow progenitor cells possessing the potential to proliferate and differentiate into...
mature ECs. On stimulation, they can be mobilized into the peripheral circulation, home to ischemic sites, and promote vascular recovery and re-endothelialization of damaged areas. These cells have been termed endothelial progenitor cells (EPCs). Several studies in animal models provide evidence that EPCs may also reveal a major contribution to the formation and growth of CNV.

In the present study, we compared both EPC mobilization and the number of mature CECs in the peripheral blood of patients with the exudative form of AMD and of healthy control subjects. We found an increased number of both CECs and EPCs in the patients with AMD. Moreover, elevated CEC levels were accompanied by elevated IGF-1 plasma level as well as high endothelin (ET)-1 cellular content, suggesting the possibility of their local release at sites of endothelial damage.

METHODS
Characteristics and Selection of the Study Groups
Subjects were recruited from the outpatient population of the Department of Ophthalmology, Pomeranian Medical University, Szczecin, Poland. Twenty-nine subjects with a clinical diagnosis of exudative AMD, with newly diagnosed CNV characterized by serous or hemorrhagic retinal pigment epithelium detachment, subretinal neovascular membrane, subretinal hemorrhage, and fibrous scarring, were included in the study. AMD severity, based on the size and laterality of the lesion, was characterized by fluorescein angiography and by optical coherence tomography. Thirty-eight age- and sex-matched volunteers without AMD (defined as the absence of drusen, pigmentary abnormalities, and neovascularization) were enrolled as a control group. Exclusion criteria included significant chronic systemic conditions, such as collagen or neoplastic disease, diabetes mellitus, renal failure, hepatic dysfunction; any evidence of retinal disease with the exception of AMD (in the AMD group)—that is, glaucoma and intraocular inflammatory diseases; or recent (within 3 months) ocular surgery. All the enrolled subjects underwent a complete ophthalmic examination (i.e., visual acuity, intraocular pressure measurement, and dilated fundus examination using slit lamp biomicroscopy). In all cases, 30° color stereo fundus photographs of the macular region of both eyes were taken. Patients who had undergone laser or intravitreal treatment were excluded from the study. Data regarding medical history and smoking status were precisely collected.

The study adhered to the tenets of the Declaration of Helsinki, and approval was obtained from the Local Research Ethics Committee. Moreover, each patient gave written informed consent for his or her involvement.

Sample Collection
Venous blood samples (~7.5 mL) collected in EDTA tubes were centrifuged (2000 rpm, 4°C, 10 minutes), and the plasma was stored at −20°C to −80°C until assayed. The red blood cells were lysed with the use of lysing buffer (BD Pharm Lyse; BD Biosciences, San Jose, CA) for 15 minutes at room temperature, to isolate peripheral blood nuclear cells (PBNCs).

Flow Cytometry
Two million (2 × 10^6) PBNCs were stained with the monoclonal antibodies anti-CD144 (Soterec, Raleigh, NC), conjugated with fluorescein isothiocyanate (FITC); anti-CD133, conjugated with phycoerythrin (PE; Miltenyi Biotec, Auburn, CA); and anti-CD34 conjugated with allophycocyanin (APC; BD Biosciences) for the analysis of CD34^+ CD133^+ (CECs) and CD133^+ CD144^+ (EPCs) (Figs. 1A, 1B, 2A 2B). Samples stained with the appropriate isotype control (FITC Rabbit IgG; Soterec), PE mouse IgG1 (Miltenyi Biotec), or APC mouse IgG1 (BD Biosciences PharMingen, San Diego, CA), were examined in parallel. The samples were incubated with antibodies for 30 minutes on ice and subsequently stained with the appropriate antibodies.
fixed in 1% formaldehyde.Flow analysis was performed on a cytometer (FACSAria; BD Biosciences). At least 10^5 events were acquired and analyzed (Cell Quest software; BD Biosciences). The number of cells in each population was expressed as the percentage of the total events.

**Immunocytofluorescence Staining of Peripheral Blood–Derived Cells**

Populations of CD144^+ and CD144^- cells were sorted by multiparameter live sterile cell sorting (FACSAria Cell-Sorting System; BD Biosciences) from peripheral blood circulating cells. Cell staining for the CD144 antigen was performed according to the protocol described for peripheral blood cell phenotyping. The sorted cells were subsequently fixed (3.7% paraformaldehyde, 15 minutes), permeabilized (0.1% Triton X-100), and incubated with rabbit anti-ET-1/2/3 primary antibody (Santa Cruz Biotechnology) and goat anti-rabbit IgG with horseradish peroxidase as a secondary antibody binding. For the same reason, the cells were stained solely with isotype control antibody (FITC-conjugated). Staining was performed analogously, to identify any unspecific antibody binding. For the same reason, the cells were stained solely with anti-rabbit secondary antibody conjugated with Alexa Fluor 350 (Invitrogen, Paisley, UK) for 1 hour on ice in the dark. Isotype control antibody (FITC-conjugated) staining was performed analogously, to identify any unspecific antibody binding. For the same reason, the cells were stained solely with anti-rabbit secondary antibody (Alexa Fluor 350-conjugated), omitting the primary antibody. The nuclei were stained with 7-AAD (BD PharMingen) for 10 minutes in the dark. For fluorescence images, a bioimager with a spinning disc-based confocal excitation system (Pathway 855; BD Biosciences) was used.

**Real-Time Reverse Transcription–Polymerase Chain Reaction**

Total mRNA was isolated both from PBNCs (2 x 10^6; RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany), as well as from the sorted CD144^+ and CD144^- cell populations (5 x 10^4; RNeasy Plus Micro Kit; Qiagen GmbH). The RNA was reverse-transcribed with Moloney murine leukemia virus reverse transcriptase and oligo dt primers (Fermentas International Inc., Burlington, ON, Canada). Quantitative assessment of ET-1 mRNA levels was performed by real-time RT-PCR (CFX96 Real-Time PCR Detection System; Bio-Rad Inc., Philadelphia, PA). A 25-μL reaction mixture contained 12.5 μL of SYBR Green reagent (IQ Super Mix; Invitrogen), 10 ng of cDNA template, and one pair of the primers: 5'-GGC CCT GAT GGA TAA AGA GTG TG-3' (forward) and 5'-GAA CGG TCT GTT GCC TCC TTC GT-3' (reverse) primers for ET-1 or 5'-AAC GGG GGA TCT TCA AAC CT-3' (forward) and 5'-TGA CTT TG CAC AGC CCA AGA TA-3' (reverse) primers for B2-microglobulin (B2M), designed on computer (Primer Express; Applied Biosystems, Foster City, CA). The real-time cycling conditions were: 1 cycle at 95°C for 10 minutes, followed by 40 cycles at 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 15 seconds. Relative quantification of ET-1 mRNA expression was performed with the comparative Ct method. The relative quantitation value of the target, normalized to an endogenous control B2M (housekeeping) gene and relative to a calibrator, was expressed as 2^{ΔΔCt}, where ∆Ct = [Ct of the endogenous control gene (B2M)] - [Ct of the target gene (ET1)].

**Western Blot**

PBNCs (4 x 10^6) were lysed for 10 minutes on ice in lysing buffer (M-Per; Pierce, Rockford, IL) containing protease and phosphatase inhibitors (Sigma-Aldrich, St. Louis, MO). The total protein concentrations were determined using the Bradford protein assay (Sigma-Aldrich). Subsequently the extracted proteins (20 μg/well) were separated on 14% gel (SDS-PAGE; Mini Protein Tetra Cell System; Bio-Rad). Fractionated proteins were transferred onto a 0.2-μm PVDF membrane (Bio-Rad) and the membranes were blocked with 3% bovine serum albumin (BSA) in buffer for 1 hour at RT. Endothelin protein was detected using rabbit polyclonal IgG antibody (Santa Cruz Biotechnology) and goat anti-rabbit IgG with horseradish peroxidase as a secondary antibody (Santa Cruz Biotechnology). The membranes were developed with Western blot analysis (ECL Advance Western Blotting.
Detection Kit; Amersham Life Sciences, Buckinghamshire, UK) and bands were subsequently visualized (Gel Doc-It Imaging system; Bio-Rad, Hercules, CA). Equal loading in the lanes was evaluated by stripping the blots for 2 hours at 37°C and then overnight at RT (IgG Elution Buffer; Thermo Scientific, Rockford, IL) and reprobing with GAPDH (goat polyclonal IgG) HRP-conjugated antibody.

**Enzyme-Linked Immunosorbent Assays**

Plasma concentrations of vascular endothelial growth factor (VEGF), stromal derived factor-1 (SDF-1), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), and ET-1 (Big ET-1; Biomedica Medizinprodukte GmbH & Co., KG, Vienna, Austria) were measured by using commercially available enzyme-linked immunosorbent assays (ELISAs; Quantikine kits; R&D Systems, Minneapolis, MN), according to the manufacturer’s protocol. Absorbance was read at 450 nm (ELX 808u, automated Microplate Reader; Bio-Tek Instruments, Inc.). The results were analyzed by using a quadratic or 4PL algorithm curve fit.

**Statistics**

Quantitative variables were compared between groups with the Mann-Whitney test. Fisher’s exact test was used for nominal variables. Correlations between quantitative variables were measured with Spearman’s rank correlation coefficient. Multivariate analyses were performed with the general linear model (GLM) after logarithmic transformation of the EPC and CEC counts, with distributions significantly different from normal (Shapiro-Wilk test). Results with P < 0.05 were considered statistically significant.

**Results**

**Characteristics of Study Subjects**

The characteristics of the patients and control subjects are summarized in Table 1. The AMD and control groups were matched for age and sex, and selected well-known AMD risk factors, including smoking, hypertension, history of ischemic heart disease, and stroke.

**Differences in the Number of Circulating Endothelial and Progenitor Cells between the Study Groups**

Both EPCs and CECs play an important role in neovascularization. EPCs are a heterogeneous group of cells that are characterized by the expression of surface markers such as CD34, CD133, VE-cadherin (CD144), VEGFR-2, and von Willebrand factor. In the transition from bone marrow toward the peripheral blood, the EPCs gradually lose CD133 and more slowly, CD34 antigens. Mature ECs may appear in the circulation by detaching from activated or damaged vessels. CECs express also CD144, and VEGFR-2, but unlike the EPCs, they are negative for the hematopoietic marker CD45 and CD133. Of note, it has been demonstrated that blockade of VE-cadherin (CD144), a circulation marker exclusively expressed by ECs and EPCs, can successfully reduce the contribution of these cells to the choroidal neovascular lesion. Based on the data, the surface phenotypes for EPCs and CECs were defined in our study as CD34+/CD133−/CD144+ and CD144−/CD133−, respectively.

First, we analyzed the presence of mature CECs in the peripheral blood of the patients with exudative form of AMD and healthy control subjects. We found a statistically significant (P = 0.0023) elevation in the percentage of CECs, defined as CD144+/CD133−, in the AMD patients compared with that in the control subjects (median, 0.140% and 0.089%, respectively, Fig. 1). Next, we focused on the number of circulating early EPCs. We noted a significant (P = 0.029) elevation in the percentage of CD34+/CD133−/CD144+ cells in peripheral blood of the AMD patients compared with the control group (medians 0.0040% and 0.0023%, respectively; Fig. 2). The number of circulating EPCs and CECs was unaffected by size or laterality of CNV (P > 0.2). Representative studies of circulating CECs and EPCs are shown in Figures 1A, 1B, 2A, and 2B. Furthermore, within the AMD (but not the control) group we observed a lower EPC number in the peripheral blood of the patients with a history of smoking (medians, 0.002% for current and past smokers and 0.010% for nonsmokers, P = 0.014). No correlation between EPCs and CECs was observed in any of the groups (P > 0.3).

**Increased Intracellular ET-1 Level in AMD Patients**

Endothelin is a powerful vasoconstrictor peptide that also acts as a modulator of vasomotor tone and cell proliferation. Thus, taking into consideration its biological and pathophysiological significance, we measured the plasma concentration of ET-1. We did not notice any significant differences between the groups (0.568 vs. 0.625 fmol/mL; P > 0.05). Hence, we performed a highly sensitive laboratory assay (i.e., quantitative real-time RT-PCR), to analyze the intracellular expression of mRNA for ET-1 in PBNCs. We found increased expression of ET-1 on the mRNA level in the PBNCs of the patients with exudative AMD, compared with the healthy control subjects (median, 0.189 and 0.134, respectively, P = 0.04; Fig. 3A). In the control group, we observed significantly higher mRNA expression in the current or past-smoker population (0.23 vs. 0.10, P = 0.011). Moreover, we found increased expression of intracellular ET-1 in PBNCs from the AMD patients at the protein level, determined with the Western blot technique, which additionally confirmed our findings obtained with qRT-PCR (Fig. 3B). Finally, to visualize the cell population with high ET-1 content among peripheral blood cells, we performed an immunocytofluorescence analysis. For this, we sorted from among the PBNCs a population of endothelial CD144− and nonendothelial CD144+ cells. Next, we found that endothelial CD144+ cells sorted from the peripheral blood simultaneously expressed ET-1, as revealed by immunofluorescence staining (Fig. 4). To provide more specific evidence that ET-1 is predominantly expressed in CD144− cells, we performed qRT-PCR analysis on the sorted cell populations. We found that ET-1 expression on mRNA level was significantly up-regulated within CD144+ population compared with the CD144− fraction (Fig. 3C).

### Table 1. Clinical Characteristics of the Study Groups

<table>
<thead>
<tr>
<th></th>
<th>AMD Group</th>
<th>Control Group</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subjects, n</td>
<td>29</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Sex (male/female)</td>
<td>11/18</td>
<td>11/27</td>
<td>0.60</td>
</tr>
<tr>
<td>Patient’s mean age in years ± SD</td>
<td>71.8 ± 9.5</td>
<td>73.0 ± 8.0</td>
<td>0.94</td>
</tr>
<tr>
<td>Bilateral CNV, %</td>
<td>43</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Size of lesion &gt;3000 μm, %</td>
<td>63</td>
<td>—</td>
<td></td>
</tr>
<tr>
<td>Smoking status, %</td>
<td>Current</td>
<td>14</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Past or current</td>
<td>50</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Hypertension</td>
<td>46</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>History of ischemic heart disease</td>
<td>12</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>History of stroke</td>
<td>4</td>
<td>6</td>
</tr>
</tbody>
</table>
Measurements of Endothelial Cells

The amount of the transcript is expressed in arbitrary units relative to the control gene. LQ-UQ, lower and upper quartiles. 

**FIGURE 3.** (A) Expression of ET-1 mRNAs in isolated PBNCs collected from patients enrolled in the study. The amount of the transcript is expressed in arbitrary units relative to the control gene. LQ-UQ, lower and upper quartiles. (B) Representative Western blot analyses of ET protein levels in PBNCs lysates collected from AMD subjects (lanes 1–4) compared with healthy volunteers (lanes 5–8). ET-1 protein was highly expressed in samples from AMD patients. GAPDH levels are shown as the protein loading control. (C) Expression of ET-1 mRNA in sorted PB CD144⁺ vs. CD144⁻ cells collected from AMD patients enrolled in the study (n = 18). The amount of the transcript is expressed in arbitrary units relative to the control gene B2M (2^ΔΔCt, where Ct represents the difference in threshold cycle between the control and target gene). LQ-UQ, lower and upper quartiles.

Evaluation of the Level of Cytokines/Growth Factors Responsible for Migration and Function of ECs

A variety of cytokines and growth factors influence and strictly regulate angiogenesis. Since angiogenesis is not mediated by only one growth factor, but results rather from the integrated activities of many regulatory molecules that function synergistically, we studied changes in concentrations of the most important growth and chemotactic factors critical for endothelial and progenitor cell homing, mobilization, and differentiation. We analyzed IGF-1, VEGF, SDF-1, and HGF plasma concentrations in the patients with the exudative form of AMD and the healthy subjects (Table 2). We found a significantly higher plasma concentration of IGF-1 in the AMD patients compared with that in the control group (P = 0.001). In contrast, SDF-1 concentration was evidently lower in the AMD patients than in the healthy control subjects (P < 0.0001). Of note, comparison of the healthy subjects and the patients with AMD revealed that both VEGF and HGF levels did not differ significantly. Moreover, we observed that the plasma HGF level was significantly elevated in the subjects with hypertension (median, 1127 vs. 791 pg/mL, P = 0.0044) and the control (median, 1228 vs. 828 pg/mL, P = 0.0024) groups. In addition, we found within the control (but not the AMD) group that the plasma SDF-1 level was higher in the subjects with hypertension (median, 3282 vs. 2523 pg/mL, P < 0.0001, and 1453 vs. 927 pg/mL, P = 0.0007, respectively). We also observed that SDF-1 strongly positively correlated with HGF plasma concentration in both the AMD and control groups (Rs = +0.50, P = 0.0050 and Rs = +0.68, P < 0.0001, respectively).

The only significant association observed between the number of circulating cells and the concentration of cytokines or growth factors was negative correlation of the SDF-1 level with the number of EPCs in the control group (Rs = −0.38, P = 0.020). Multivariate analyses of patients and control subjects adjusted for age, sex, presence of ischemic heart disease, and hypertension showed that exudative AMD was an independent factor associated with higher number of EPCs (β = +0.27, P = 0.042) and CECs (β = +0.41, P = 0.0021), lower concentration of SDF-1 (β = −0.48, P < 0.00001), and higher concentration of IGF-1 (β = +0.46, P < 0.0018). Of interest, when the analysis was also adjusted for SDF-1 concentration, the number of EPCs and CECs was no longer significantly associated with the presence of AMD (P = 0.55 and P = 0.24, respectively), but the only significant predictor of the higher number of these

**FIGURE 4.** Immunocytofluorescence of peripheral blood-derived ECs. Nuclei are visualized with 7-AAD staining. Individual images depict the expression of CD144 and ET-1 antigens, and the lack of CD133 molecule on the CECs, respectively. The isotype control confirms that no unspecific binding of antibodies occurs. A pseudocolor is assigned to each staining as follows: anti-ET-1 (red), anti-CD144 (green), nuclei (blue). Representative data are shown. Scale bar, 10 μm.
cells was lower SDF-1 concentration ($\beta = -0.41$, $P = 0.046$ for EPCs and $\beta = -0.49$, $P = 0.014$ for CECs).

**Discussion**

The measurement of immunologically defined CECs in venous blood is now an important and novel method for assessing vascular alterations. In healthy persons, ECs line the vascular tree and adhere to the basement membrane. Any pathologic processes that cause damage to the endothelium may also cause endothelial cell detachment, resulting in an increased number of CECs circulating in the blood. The mechanism of CEC detachment is complex and involves many factors, such as mechanical injury, the alteration of endothelial and subendothelial cellular adhesion molecules, defective binding to anchoring matrix proteins, and cellular apoptosis in response to oxidative stress. In humans, elevated levels of CECs have been detected in diverse conditions, including cardiovascular disorders, stroke, diabetes mellitus, inflammatory and connective tissue diseases, cancer, and many others. Herein, we present the first data to demonstrate elevated levels of CECs, defined as CD144$$^+$CD133$$^-$, in the peripheral circulation of patients with AMD. Since circulating cells work in concert with extracellular factors, we then turned our special attention to potential associations between ECs and the release of proteins regulating vasoconstriction. The vasoactive factors produced by ECs include ET-1, which is one of the most potent vasoconstricting peptides. However, ET-1 normally is present in blood at low concentrations, even its slight changes can lead to noticeable local or systemic effects. Since, ET-1 receptors are expressed in human retina and optic nerve head, it can suggest that ET-1 plays a pathophysiologic role in the eye.

In this study, we demonstrated for the first time an increased mRNA and protein level of ET-1 in PBNCs of patients with exudative AMD. Furthermore, we revealed that ET-1 mRNA level is higher in CD144$$^+$ (endothelial) cells than in the CD144$$^-$ (nonendothelial) cell population. Thus, ET-1 overexpression in PBNCs of AMD patients may result from an increase in the number of CECs expressing ET-1 (shown in immunofluorescence testing, Fig. 4). On the other hand, the upregulation of ET-1 mRNA synthesis within CECs derived from AMD patients may also be responsible for such an effect, since we did not find correlation between the ET-1 level and the number of CECs. We assume that damaged ECs may release a variety of proinflammatory and vasoconstricting molecules, including ET-1, leading to derangement between endothelium-derived relaxing and contracting factors. Local retinal ischemia consequently develops and this promotes pathologic blood vessel formation in the course of AMD.

In this study, we demonstrated an elevated number of CECs together with high ET-1 content in the exudative AMD group, suggesting that endothelial dysfunction along with excessive local ET-1 release plays a role in the progression of AMD. We hypothesize that an increase in local ET-1 release from damaged endothelium may promote the CNV development in advanced AMD. This hypothesis is based on recent evidence suggesting that ET-1 not only stimulates the contraction of ocular blood vessels, but also affects the growth of vascular cells. It has been demonstrated that ET-1 serves as the general paracrine mitogen for pericytes, inducing their proliferation. The presence of ET receptors has been documented in different sites of mouse retina, including retinal pigment epithelium, outer plexiform layer, ganglion cell layer, retinal astrocytes, and vascular endothelia. However, after light-induced retinal degeneration, a significant overexpression of ET receptors in astrocytes and its processes has been observed, suggesting the important role of ET in reactive gliosis in response to retinal injury. We hypothesize that the local release of ET-1 from injured ECs may contribute to the formation of fibrous scarring, along with CNV development in the late stage of AMD. However, we were not able to measure intravitally the ET-1 concentration in close proximity to AMD lesions in the group of investigated patients. Nevertheless, the elevated level of ET-1 in peripheral blood cells may reflect an increase in its local expression in the affected retinal or choroidal vessels since there are available data providing evidence of the abnormal ET-1 local synthesis in patients with proliferative diabetic retinopathy or proliferative vitreoretinopathy. Of note, we did not find any increase in ET-1 plasma level in the AMD patients. This observation may suggest that ET-1 is released locally in the area of ischemia and injury. Furthermore, its half-life in plasma is very short, hence evaluation of ET-1 in plasma may not reflect its excessive release.

To further evaluate vascular function, we analyzed the number of EPCs recruited from the bone marrow into the circulation. Until recently, CNV was thought to arise exclusively from resident ECs of the choriocapillaris. However, it has been demonstrated in animal models that bone marrow-derived cells are recruited from the circulation to participate in CNV formation and provide up to 50% of the vascular cells. We demonstrated an elevated plasma level of CD34$$^+$CD133$$^+$CD144$$^-$ cells, which were referred to as early EPCs in patients with the exudative form of AMD. Our results are comparable with those in previous reports documenting a significantly elevated level of CD34$$^+$ hematopoietic stem cells in patients with active CNV.

The nature and exact role of mobilized EPCs in CNV development has not yet been entirely elucidated. The question remains whether the high EPC level is the cause or the consequence of CNV development. Furthermore, little is known about the interactions between EPCs and mature ECs at vascular injury/angiogenic sites. In a healthy organism, the injured and damaged ECs are rapidly removed by macrophages and neighboring cells before their inflammatory potential develops. In disease, however, EPCs are present in a high number in the circulation, overwhelm the clearance mechanisms, and trigger

---

**Table 2. The Plasma Concentration of VEGF, SDF-1, HGF (pg/mL), and IGF-1 (ng/mL) in Patients with AMD and Control Subjects**

<table>
<thead>
<tr>
<th></th>
<th>Control Group</th>
<th>AMD Group</th>
<th>$P$†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Median (Q1–Q3)*</td>
<td></td>
</tr>
<tr>
<td>VEGF, pg/mL</td>
<td>207 ± 149</td>
<td>181 (237)</td>
<td></td>
</tr>
<tr>
<td>SDF-1, pg/mL</td>
<td>2654 ± 559</td>
<td>2748 (1005)</td>
<td></td>
</tr>
<tr>
<td>HGF, pg/mL</td>
<td>1099 ± 330</td>
<td>1043 (461)</td>
<td></td>
</tr>
<tr>
<td>IGF-1, ng/mL</td>
<td>65.5 ± 15.4</td>
<td>68.8 (24.8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>195 ± 157</td>
<td>159 (176)</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>2058 ± 539</td>
<td>1960 (728)</td>
<td>0.0001</td>
</tr>
<tr>
<td></td>
<td>1024 ± 274</td>
<td>975 (393)</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>84.6 ± 19.1</td>
<td>82.8 (23.0)</td>
<td>0.001</td>
</tr>
</tbody>
</table>

* Q1–Q3, interquartile range. † Mann-Whitney test.
proinflammatory pathways, thereby gaining pathogenic importance.\(^{32}\) It can be assumed that EPCs exposed to the proinflammatory microenvironment at the injury sites may exert unwanted effects, such as rapid and uncontrolled immature vessel proliferation leading to CNV development and visual loss. Our hypothesis can be compared with previous reports documenting impaired function of circulating EPCs in the exudative form of AMD, suggesting that this may contribute to reduced vascular repair and enables CNV development and progress.\(^{33}\) Nevertheless, the relationship between the extent of vascular damage and circulating EPCs may be difficult to interpret due to the fact that their number may depend on the delicate and poorly understood balance between the speed/frequency of EPC mobilization from bone marrow, their increased engraftment, and their turnover at the sites of vascular injury.

In light of the results, the functional association between the concentration of CECs and soluble regulator proteins in peripheral blood seems to be of significant importance. We found that the increase in early EPCs and CECs numbers was accompanied by a higher plasma concentration of IGF-1. This cytokine has been described as a potent stimulator of retinal EC growth and proliferation.\(^{34}\) An elevated level of IGF-1 in the serum and vitreous of patients with proliferative diabetic retinopathy has been associated with the development of retinal neovascularization.\(^{35,36}\) Furthermore, knockout mice lacking IGF-1 have been shown to have arrested vascular development.\(^{57}\)

The increased serum level of IGF-1 in the AMD patients shown in the present study may be related to the process of angiogenesis under hypoxic conditions. Of note, in our study, the serum levels of other proangiogenic cytokines, including VEGF and HGF, did not vary between the patients with AMD and the control group. Moreover, the SDF-1 plasma level in the AMD group was significantly lower than in the healthy subjects. These observations are consistent with recently published reports documenting decreased plasma levels of VEGF and SDF-1 in the active stage of AMD.\(^{34,35}\) Furthermore, some reports have shown that SDF-1 immunoreactivity was significantly reduced in both retinal pigment epithelium and choroidal stroma in human eyes obtained from patients with late AMD. What is more, the immunoreactivity became very weak or nearly absent in disciform scars in patients with CNV compared with healthy control subjects.\(^{50}\) Based on these findings as well as on the results of our study, we suggest a disturbed local synthesis of the cytokines and growth factors under conditions of chronic vascular endothelial injury. Our hypothesis can also be supported by reports showing that SDF-1 and VEGF levels are elevated in acute ischemia but low in chronically ischemic tissue.\(^{51}\) Our findings also revealed a negative correlation between the number of EPCs circulating in peripheral blood and plasma SDF-1 level. Low SDF-1 was an independent predictor of a high number of EPCs, as evidenced by the multivariate analysis. The association of higher EPC and CEC counts with AMD became nonsignificant when the multivariate analysis was adjusted for SDF-1 concentration, suggesting that reduction of SDF-1 level may be a pathophysiological link between AMD and elevated CECs. Of note, the results are consistent with recent extensive studies showing that SDF-1 plays an important role in the egress of EPCs to peripheral blood and their homing, and its level is inversely associated with the number of circulating EPCs.\(^{40,41}\) The concentration of SDF-1 in the peripheral circulation reflects proportionally the level of locally released chemokine, for example at sites of injury. Once secreted, SDF-1 is at least partially responsible for attracting and homing the cells essential for tissue repair or local proliferation.\(^{42}\) Since EPCs express CXCR4, they can also be attracted in the SDF-1/CXCR4 axis.\(^{44}\) Thus, we hypothesize that a lower SDF-1 level enables EPCs to remain in the circulation. However, it has also been reported that stem/progenitor cells are mobilized and home to the ischemic tissue because of the gradient of SDF-1 level between the area of injury and the bone marrow.\(^{44}\) We measured SDF-1 peripheral blood concentration, which does not reflect precisely the site of injury/bone marrow gradient. The precise elucidation of the whole configuration of various cellular and humoral factors involved in such regulation requires further investigation, including measurements of their tissue concentrations.

In conclusion, our results suggest that an endothelial alteration accompanies AMD. This reinforces the hypothesis of a key role of vascular injury in the pathogenesis of this condition and also advances our understanding of the pathophysiology of this disease. Moreover, our data support the concept that assessment of CECs along with endothelial progenitor cells may be a useful biomarker for exudative AMD. An increased number of CECs and EPCs in the neovascular form of AMD is associated with lower SDF-1 concentrations, reflect a severe vascular disturbance, and may contribute to the disease process. However, the interactions of CECs with damaged endothelium remain rather speculative at present. We suggest that ET-1 released locally from damaged ECs may contribute to the induction of local ischemia and may play a role in the development of CNV. These findings can help expand our knowledge of the pathogenic mechanisms of AMD and may be relevant to the potential treatment of this disease. The evaluation of different subpopulations of blood ECs opens new directions for the study of endothelial injury and repair in AMD.

References
