Fewer Circulating Endothelial Progenitor Cells in Newly Diagnosed Neovascular AMD Patients

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Abstract: *Objective*: Patients with age-related macular degeneration (AMD) exhibit pathologic neovascularization under the retina, with choroidal neovascularization (CNV) suggestive of defective angiogenesis. The endothelial progenitor cells (EPCs) present in the peripheral blood contribute to angiogenesis and vasculogenesis, and their regulation is altered in several vascular disorders. We investigated whether the numbers and functional properties of EPCs may be disordered in newly diagnosed neovascular AMD.

Methods Fifteen suitable AMD patients and 10 controls matched for age, risk factors for atherosclerosis and use of medication that could influence the circulating pool of EPCs were studied. Circulating EPCs were assayed by the colony-forming unit (CFU) method. The EPCs' adhesive capacity was studied by evaluating their ability to attach to fibronectin and cultured endothelial cells. Serum levels of vascular endothelial growth factor (VEGF) were studied and correlated with EPC numbers.

Results: The patients had significantly fewer circulating EPCs(16.5±2.8) compared to their controls (31±4.6; p=0.0085). The functional properties of both groups' EPCs were similar.

Conclusions: The peripheral circulating pool of endothelial stem cells is altered in patients with newly diagnosed neovascular AMD, suggesting that pathologic angiogenesis may result from or influence the regulation of endothelial precursor circulation.

INTRODUCTION

Age-related macular degeneration (AMD) is currently the primary cause of severe loss of vision in patients over the age of 50 years in developed countries. Although the exudative form is present in only 15-20% of the AMD population, exudative AMD accounts for much of the important visual impairment [1]. The major cause of severe loss of vision in exudative AMD is pathologic neovascularization under the retina that produces choroidal neovascularization (CNV) [2]. While the pathogenesis of CNV is clearly multifactorial, it is generally considered to be driven by pathological angiogenesis. In angiogenesis, the cellular components of the new vessel complex (endothelial cells, smooth muscle cells and other types) are derived from cells resident within adjacent preexisting capillaries. An alternative process, postnatal vasculogenesis, has been shown to contribute to some forms of neovascularization [3-5]. In vasculogenesis, the cellular components of the new vessel complex originate in part from bone marrow-derived circulating vascular progenitors, which differentiate into mature endothelial cells or vascular smooth muscle cells in situ. The role of bone marrow-derived cells has been studied during the development of ocular vascular ture and retinal neovascularization [6,7], but the contribution of vasculogenesis to the formation of CNV is unknown.

Vascular endothelial cells form a lining for all the blood vessels, providing an essential interface between the lumen and blood-borne elements. In response to specific stimuli, the endothelium provides signals that recruit these blood cells to sites of injury or infection. Endothelial cells are also crucial participants in the formation of new blood vessels as a healing response to tissue ischemia and infarction. A growing body of evidence now suggests that bone marrow-derived endothelial progenitor cells (EPCs), which represent only 0.01% of circulating mononuclear cells, play an important role in the formation of new blood vessels in these pathologic conditions [8,9].

Progenitor cells are primitive bone marrow cells that have the capacity to proliferate, migrate and differentiate into various mature cell types. EPCs, in particular, are capable of maturing into cells that line the lumen of blood vessels, and they play an important role in the maintenance of endothelial integrity. A panel of exogenous and endogenous factors, among which vascular endothelial growth factor (VEGF) is a principal determinant, has been shown to modulate mobilization, recruitment and differentiation of EPCs [10-12].

It was recently shown that reduced levels of EPCs are associated with disorders linked to endothelial dysfunction [13,14]. A decrease in circulating EPCs contributes not only

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to impaired angiogenesis but is also associated with accelerated atherosclerosis [15] and in-stent restenosis [16], whereas ischemia promotes EPC mobilization to the peripheral blood [6,17].

In the present study, we studied peripheral EPC numbers and function in patients with AMD-associated CNV, speculating that their alteration may be associated with the occurrence of CNV. Knowledge of this pathway may elusidate the mechanism of CNV, and if found positive, suggests that targeting stem cell recruitment to the eye may offer a novel therapeutic strategy for ARMD.

METHODS

Patient Selection

Informed consent was obtained from all the subjects after explanation of the nature and possible consequences of the study, which was approved by the institutional human experimentation committee. Patients were excluded if they had diseases with a limited survival expectancy (e.g., end-stage cancer, advanced heart disease), alcoholic abuse or drug abuse. Persons were also excluded if they had advanced AMD or had undergone laser photocoagulation for AMD in both eyes, bilateral cataract extraction without signs of AMD, or other eye diseases that could potentially compromise evaluation, or if they used medications known to be toxic to the lens or retina.

Case-control definitions were adopted from a previous AREDS publication [18]. According to their fundus status as graded by clinical examination, patients who were examined at the Tel Aviv Sourasky Medical Center Retina Clinic by a single investigator (AB) were divided into maculopathy groups by size and extent of the drusen in each eye, the presence of geographic atrophy, and evidence of neovascular disease. These groups were numbered serially and defined by increasing severity of drusen or type of AMD as follows.

Group 1 (No Drusen)

Each eye was free of drusen or had non-extensive small drusen, no pigment abnormalities, no advanced AMD, and no disqualifying ocular conditions. Participants in this group had a visual acuity of 20/32 or better in both eyes.

Group 2 (Intermediate Drusen)

At least 1 eye had 1 or more intermediate-sized drusen, extensive small drusen, or pigment abnormalities associated with AMD. Neither eye had large drusen, advanced AMD, or a disqualifying ocular condition. Participants in this group had a visual acuity of 20/32 or better in both eyes.

Group 3 (Large Drusen or Intermediate AMD)

At least 1 eye had either 1 or more large drusen, approximately 20 intermediate-sized soft drusen, or approximately 65 intermediate-sized hard drusen. Neither eye had advanced AMD, a disqualifying ocular condition, or geographic atrophy with a diameter at least one-eighth that of the average disc.

Group 4 (Neovascular AMD–Advanced AMD)

At least 1 eye had neovascular AMD. Neovascular AMD included choroidal neovascularization or retinal pigment epithelial (RPE) detachment in 1 eye (nondrusenoid RPE detachment, serous sensory, or hemorrhagic retinal detachment), subretinal hemorrhage, subretinal pigment epithelial hemorrhage, subretinal fibrosis, or evidence of confluent photocoagulation for neovascular AMD. The term *neovascular* is used collectively for this group of participants because all participants had direct evidence of choroidal neovascularization, according to the assessment of their fundus photographs.

All the patients who showed no evidence of drusens (Group 1) and those with neovascular AMD who presented with a decrease in visual acuity of less than two months (Group 4 - neovascular type) were asked to enroll in the study. Four patients of Group 1 and three of Group 4 declined.

Isolation of EPCs and the Colony-forming Unit (CFU)

Twenty milliliters of blood were drawn and assayed as described before [4]. Briefly, peripheral-blood mononuclear cells were isolated by Ficoll-density gradient centrifugation assay (Amersham Biosciences, Sweden). After being washed with phosphate-buffered saline (PBS, Biological Industries, Israel), isolated cells were re-suspended in growth medium (M199, Biological Industries, Israel) and plated on dishes coated with human fibronectin (Chemicon). They were incubated for seven days in medium that was changed on day three. On day seven, the two investigators independently counted the CFUs.

Confirmation of CFU Phenotype

For phenotyping of the endothelial characteristics of the CFUs, the following antibodies were used for immunofluorescentic and flow-cytometric analysis: rabbit polyclonal anti-Tie-2 (C-20), mouse monoclonal anti-flk-1 (A-3) and goat polyclonal anti-CD31 (PECAM-1, M-20), all from Santa-Cruz Biothecnology, Santa-Cruz, CA, 95060. Endothelial-cell lineage was further confirmed by indirect immunostaining with the use of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate–acetylated low-density lipoprotein (DiI-acLDL) and co-staining with BS-1 lectin (Sigma- Aldrich, Israel) (Fig. **1**).

Fibronectin and Endothelial cell Adhesion Assays

EPCs (day 7) from patients with AMD and those from controls were washed with PBS and gently detached with 0.5 mmol/L EDTA in PBS. After centrifugation and resuspension in basal complete medium supplemented with 5% fetal calf serum (FCS), identical cell numbers were placed onto fibronectin-coated or bovine aortic endothelial cell culture dishes and incubated for 30 minutes at 37°C [7,19]. Adherent cells were counted independently by blinded investigators.

VEGF Plasma Concentrations

Levels of serum VEGF were determined by ELISA according to the manufacturer's instruction (R&D systems).

Statistical Analysis

Clinical variables between groups were compared by the Mann-Whitney test. Student's t-test was employed for comparison of valued numbers. A *p* value lower than 0.05 was considered statistically significant.

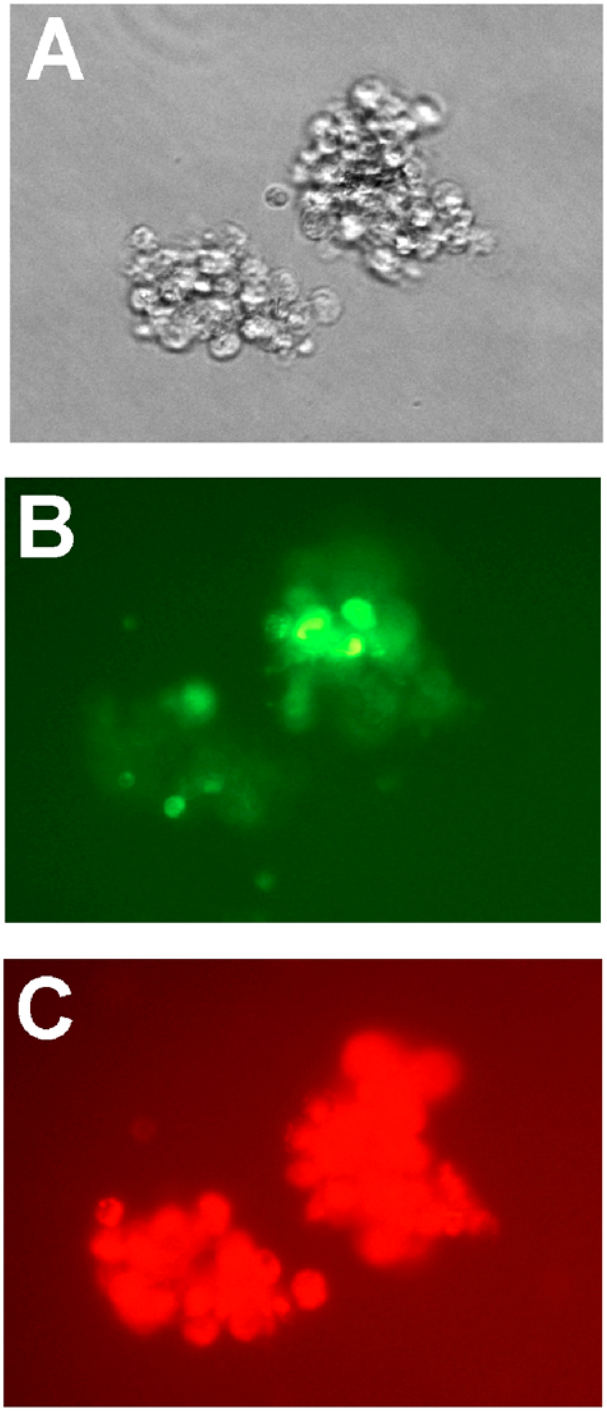

Fig. (1). Confirmation of endothelial phenotype of endothelial progenitor cells (EPCs).
A: Phase contrast
B: Staining with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-acetylated low-density lipoprotein (DiI-acLDL)
C: Staining with BS–1 lectin

RESULTS

A total of 107 patients which were reffered for the Tel Aviv Sourasky medical Center Retina clinic during a two months period (March - April 2004) were examined and graded according to the ARDES classification. Out of these patients, 14 patents were graded as grade 1, 35 as grade 2, 30 as grade 3, 18 as grade 4- neovascular and 10 as grade 4- atrophic. Fifteen patients (age range at enrollment 55-80 years) with neovascular AMD were enrolled according to the local institutional protocol. Ten other patients from the same clinic with no drusens (Group 1) formed the control group. There were 8 males (53.3%) and 7 females (46.7%) whose average age was 76.5 years in the neovascular AMD group and seven males and three females whose average age was 74.5 years in the control group (p = NS for gender and age). There were no statistically significant differences in the general medical profile and use of medications, except for the presence of hypertension, which was significantly higher in the neovascular AMD group: hypertension is not known to influence EPCs assays (Table **1**).

Table 1: Characteristics of Patients with Newly Diagnosed Neovascular Age-Related Macular Degeneration and Controls

General data profile	AMD	Control	p value*
Number of patients enrolled, total	15	10	
Gender, M/F	8/7	7/3	NS
Age, years, mean±SEM	76.5±1.8	74.5±2.1	NS
Smoking, %	16.7	0	NS
Hyperlipidemia, %	42.9	30	NS
Hypertension, %	71.4	10	p=0.0115
Diabetes mellitus, %	14.3	0	NS
Medications			
Aspirin, %	57.1	30	NS
Beta blockers, %	57.1	10	NS
Calcium blockers, %	35.7	20	NS
ACE inhibitors	46.7	20	NS
Nitrates, %	14.3	20	NS
Statins, %	42.9	30	NS
Diuretics, %	7.1	0	NS
Digoxin, %	7.1	0	NS
Warfarin, %	7.1	0	NS
Hypoglycemics, %	14.3	0	NS
Serum markers			
VEGF, pg/ml±SEM	22.9±5.6	33.2±19.4	NS

AMD, Age related macular degeneration; *VEGF*, vascular endothelial growth factor; *ACE*, angiotensin-converting enzyme; *NS*, non-significant
Mann-Whitney test. *A p value lower than 0.05 was considered statistically significant.

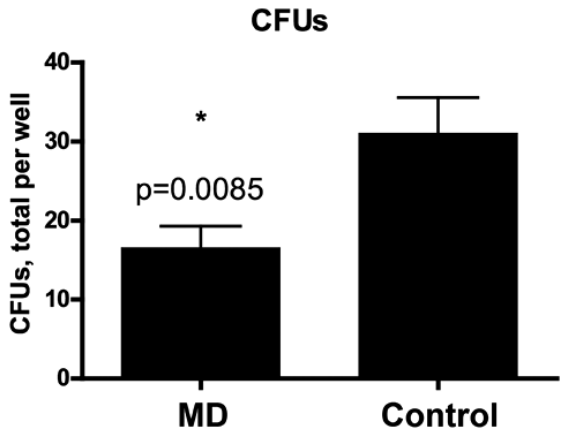

Fig. (2). Colony-forming units (CFUs) in patients with neovascular age-related macular degeneration (AMD). The number of CFUs was determined as described in Materials and Methods.

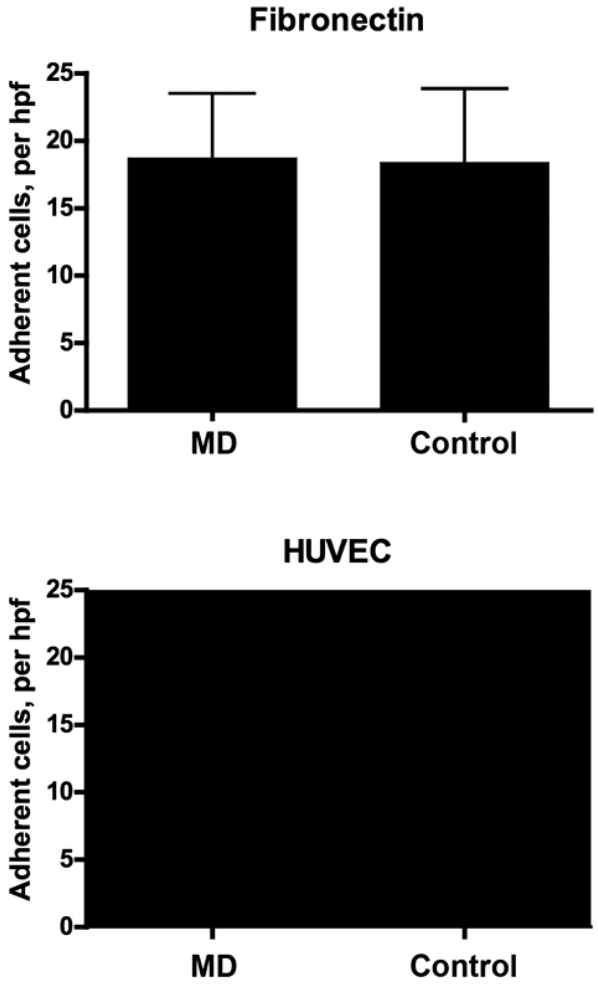

Fig. (3). Adhesive properties of endothelial progenitor cells (EPCs) from neovascular age-related macular degeneration (AMD) patients and controls. **A**. Binding of EPCs to fibronectin. **B**. Binding of EPCs to endothelial cells. *Hpf*, high power field; *HUVEC*, human umbilical vein endothelial cells.

CFU numbers representing the EPC numbers were significantly higher in the control group (31 per well vs. 16.5 per well, respectively, p=0.0085; Fig. **2**). The adhesive ca pacity to fibronectin and to human umbilical vein epithelial cells (HUVEC) was not significantly different (18.7 vs. 18.4 and 17.8 vs. 20.8 adherent cells per high power field, respectively) (Fig. **3**).

DISCUSSION

The concept of endothelial progenitor cells recruitment into choroidal neovascularization is not a new one, but rather a natural evolution of the work of Espinosa-Heidmann *et al*. and others who utilized the laser model of CNV which has now become an accepted model for AMD [20-22]. To the best of our knowledge, our results are the first to describe altered numbers of EPC in choroidal neovascular patients. The far-reaching consequences of this finding are that by pharmacologically altering the number of EPCs we may be able to offer new treatment for CNV in AMD.

Pathologic neovascularization under the retina that produces CNV is a major cause of vision loss in AMD patients. It has been suggested that defective regulation of angiogenic processes may underlie the development of this disorder. Since EPCs are known to develop into mature endothelial cells and to acquire their mature functional properties [8], we hypothesized that the pool of EPCs in these patients may be reduced and that this could be associated with an excessive angiogenic process. Our findings indicate that the number of circulating EPCs established by a validated CFU assay [6,13,16] was significantly reduced in patients with newly diagnosed CNV compared with their controls. Since age and risk factors for atherosclerotic heart disease have been shown to be associated with reduced number of EPCs [13,14], we took care to match the newly diagnosed CNV patients with controls according to these parameters. It emerged that the number of EPCs was significantly reduced after normalization of both groups. These results are supportive of the idea that altered regulation of the endothelial stem cell pool that results in pathological revascularization may contribute to the histopathological findings of AMD. We can not, however, exclude the possibility that the reduced numbers of EPCs in these patients are a result of augmented angiogenesis caused by other factors by which the peripheral endothelial stem cells are "consumed" and their numbers are reduced. Unlike other studies [3,23], we did not find a correlation between VEGF serum levels and EPC numbers to suggest that other cytokines or growth factors may be responsible for the reduced EPC pool in these patients.

The importance of the number of EPCs as well as their functional properties in terms of regulation of endothelial repair, which, in turn, may contribute to endothelial dysfunction in this particular group of patients [24] has been well demonstrated over recent years. The ability of EPCs to adhere to endothelial cell and matrix surfaces and their migrating properties may be equally important in determining their angiogenic potential. We found that the functional properties of EPCs from newly diagnosed CNV patients and controls did not differ in their ability to adhere to cultured endothelial cells and to fibronectin-coated plates. In conclusion, we have demonstrated dysregulation in the pool of peripheral endothelial stem cells in patients with newly diagnosed AMD-associated CNV, findings that may be either causal or consequential to the histopathological findings in these patients. Whether measurement of circulating EPCs can serve

as a surrogate marker of AMD or provide prognostic information for individual patients awaits further studies.

Our manuscript has several limitations and drawbacks: We were able to document lower number of CFUs for EPCs generated from blood of patients with AMD who had CVN than control patients without the disease. Yet , there is no evidence that the proliferation, differentiation, and/or apoptosis (death) of circulating EPCs in AMD patients were different from those of circulating EPCs in control patients. More over, we could not detect differences in the adhesive ability of EPCs to fibronectin or HUVECs. Therefore, while it may be reasonable to speculate that the lower number EPC CFUs produced by blood cells of patients with AMD compared to those by blood cells of control patients was due to some sorts of yet-to-be-defined problems in the regulation of the peripheral EPC pool, the exact mechanism which cause the difference detected is yet to be found. Another major drawback of our manuscript is the finding that we found that AMD patients with CVN would have lower, rather than higher, levels of EPCs.A potential explanation for the finding is the presence of enhanced oxidative stress or other humoral factors in these patients that cause peripheral depletion of EPCs. Another limitation of our work is the comparison of only advanced AMD patients with CVN (group 4) to the control patient group that has no Drusen (group 1) in this study. It would be interesting and important to also include patients in groups 2 and 3 who had moderate AMD but without CVN in this study for comparison. Another significant weakness of the experimental design is the relatively small number of patients in each of the test group. Although the authors conclude that a number of measured parameters were not statistically significantly different among the two test groups, The small sample size of the two test groups cannot allow them to conclude with sufficient confidence that the lack of significant differences were not due to insufficient power of the study design.

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