Circulating Hematopoietic Stem Cells in Patients with Idiopathic Choroidal Neovascularization

Manabu Sasahara, Atsushi Otani, Yuko Yodoi, and Nagahisa Yoshimura

PURPOSE. In a prior study, the correlation was investigated between circulating hematopoietic stem cells (HSCs) and the activity or severity of choroidal neovascularization (CNV) in patients with age-related macular degeneration (AMD). The present study was designed to explore the role of circulating HSCs in the pathogenesis of CNV in patients with idiopathic (i)CNV.

METHODS. Thirty patients with clinically documented iCNV and 10 age–sex-matched patients without iCNV or systemic disease were enrolled in a case–control study. Circulating HSCs were collected from the patients’ peripheral blood and cultured. Colony-forming capacity, migration activity, and invasion activity of HSCs were experimentally analyzed.

RESULTS. Colony-forming units by Hill’s assay (CFU-Hill) were markedly lower (P < 0.001) in patients with iCNV (12.8 ± 3.2) than in healthy patients (67.6 ± 12.6). Invasion activities of HSCs were reduced significantly in patients with iCNV. CFU-Hill was impaired in the patients (4.0 ± 1.2) with CNV more than 1500 μm compared with the patients (20.3 ± 4.0) with CNV less than 1500 μm (P = 0.006).

CONCLUSIONS. Similar to the previous finding of CNV associated with AMD, impaired functional activities of circulating HSCs were observed in patients with iCNV, which correlated with the size of iCNV. These results in young patients provide evidence to support the role of circulating HSCs in the pathogenesis of CNV. (Invest Ophthalmol Vis Sci. 2009;50:1575–1579) DOI:10.1167/iovs.08-1900

Choroidal neovascularization (CNV) is a common pathologic change that occurs in various chorioretinal diseases1-3 that cause severe vision loss. Recently, several new therapeutic options, including photodynamic therapy4 and anti-VEGF drugs,5 have been developed, and many investigators have shown their benefits. However, the pathogenesis of CNV is still not fully understood. Because little is known about the systemic factors in the pathogenesis of CNV, current therapeutic approaches have been designed to control the local factors.

Recently, circulating hematopoietic stem cells (HSCs) and endothelial progenitor cells (EPCs) have been reported to incorporate into retinal6 and choroidal angiogenic vasculature in murine models.7-9 HSCs and EPCs derived from bone marrow9 participate in normal and pathologic postnatal angiogenesis10 and differentiate into various nonendothelial cell types: hepatocytes,11 cardiomyocytes,12 microglia,13 and even neurons.14 These cells may play important roles in repair of injured tissue15,16 and disease prognosis.17 Although HSCs and EPCs may be systemic factors, the role of these cells in the formation of CNV has remained unclear. After analyzing the correlation of circulating HSCs with clinical characteristics of patients with age-related macular degeneration (AMD), we propose that these cells have an important role in AMD: The active CNV could increase the number of HSCs in peripheral circulation, and the function of the cells may be key in CNV severity and progression.18

In this study, we examined the function of circulating HSCs in patients with idiopathic (i)CNV. In the investigation, we explored whether the functional activities of HSCs have any relation to occurrence, progression, or severity of CNV. Because young patients may have fewer complications that affect the HSC and ocular conditions, the data from this study may reveal more accurately the role of HSCs in CNV pathogenesis.

METHODS

The study protocol was approved by the Ethics Committee of Kyoto University Hospital, and all enrolled subjects gave written informed consent according to the Declaration of Helsinki. We identified 18 patients with iCNV, diagnosed by the doctors at the Center for Macular Diseases in Kyoto University Hospital from September 2005 to August 2007. In all patients, the disease was diagnosed by comprehensive fundus examinations, including slit-lamp biomicroscopy with a contact lens, fluorescein angiography (FA), indocyanine green angiography (IGA), and optical coherence tomography (OCT). FA and ICGA were simultaneously performed with a confocal scanning system (HRA-2; Heidelberg Engineering, Dossenheim, Germany), and two types of OCT were used (Stratus OCT; Carl Zeiss, Dublin, CA, and OCT-ophthalmoscope; Nidek, Gamagori, Japan).

Thirteen patients with iCNV were included in the study. The diagnosis of iCNV was made when there was CNV in patients aged 50 years or younger, without the signs of age-related macular degeneration, intraocular inflammation, angioid streaks, choroidal rupture, pathologic myopia, chorioretinal scars, or chorioretinal dystrophy. Each patient had only one eye involved with CNV. CNV location and size were evaluated with FA and IGA. CNV size was defined as the maximum diameter of fibrovascular tissue, not including chorioretinal atrophy around the CNV membrane. The activity of CNV was judged by (1) significant leakage from CNV lesions in FA and (2) cystoid space or exudative changes in the OCT image. A smoking index was calculated as the sum of the number of cigarettes smoked per day multiplied by years of smoking.

For ex vivo analysis, we collected 20 mL of peripheral blood from the 13 patients with iCNV who met the study criteria and from 10 age-and sex-matched patients served as control subjects. Persons with any ocular diseases or systemic diseases—including systemic hypertension, diabetes mellitus, hyperlipidemia, cardiovascular disease, and cerebrovascular disease—were ineligible to be control subjects. To represent the functional activities of HSCs, we investigated colony-forming units of endothelial cell (Hill assay, CFU-Hill; Fig. 1), migration activity, and invasion activity of HSC-derived cells.

From the Department of Ophthalmology and Visual Sciences, Kyoto University Graduate School of Medicine, Kyoto, Japan.


Submitted for publication February 19, 2008; revised August 4, 2008; accepted February 17, 2009.

Disclosure: M. Sasahara, None; A. Otani, None; Y. Yodoi, None; N. Yoshimura, None

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be marked ‘advertisement’ in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Atsushi Otani, Department of Ophthalmology and Visual Sciences, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8386, Japan; otan@kuhp.kyoto-u.ac.jp.

Investigative Ophthalmology & Visual Science, April 2009, Vol. 50, No. 4
Copyright © Association for Research in Vision and Ophthalmology
Colony-Forming Units Assay

We used a method described by Hill et al.19 to culture HSCs and measure colony forming units. Mononuclear cells from peripheral blood were collected by light-density gradient centrifugation (Ficoll-Paque Plus; GE Healthcare Life Sciences, Piscataway, NJ), and 1 × 10⁷ of mononuclear cells were seeded on six-well human fibronectin-coated plates (BD Biosciences, San Joe, CA) in a 2.5 mL of an endothelial basal medium (EBM; Endocult; StemCell Technologies, Vancouver, BC, Canada) with 20% fetal bovine serum (FBS). After 48 hours, 1 × 10⁶ nonadherent cells were transferred into a new 24-well fibronectin-coated plates in 1 mL of EBM, to avoid contamination with mature endothelial cells and nonprogenitor cells, and incubated for another 3 days. After 5 days’ culture in vitro, endothelial colonies of more than 50 cells in two wells were counted by two independent investigators. Counts of the two wells were scored as the mean colony-forming units. Immunocytochemistry was performed after fixation of 4% paraformaldehyde, and the expression of endothelial marker proteins, kinase insert domain receptor (R&D Systems, Minneapolis, MN), platelet endothelial cell adhesion molecule-1 (R&D Systems), and vascular endothelial-cadherin (Chemicon, Temecula, CA) was confirmed.

Migration and Invasion Assay of HSC-Derived Cells

HSC-derived cells after 5 days’ culture in vitro were placed in the upper chamber (2.5 × 10⁵) of a modified Boyden chamber with a fluorescence blocking system.20 For the migration assay, an angiogenesis/endothelial cell migration system (BD Biocat; BD Biosciences) was used. For the invasion assay, a tumor invasion system (BD Biocat; BD Biosciences) was used. The major difference between the migration and invasion assays was that the modified Boyden chamber was filled with a synthetic matrix (Matrigel; BD Biosciences) in the invasion assay. Each patient’s sample was performed in duplicate. The chamber was placed in a 96-well culture dish containing only EBM (Endocult; StemCell Technologies), and EBM plus 10% FBS or 10% autologous serum from the patients/controls for chemoattractant. Particular chemokine or growth factors were not used as chemoattractants. After 22 hours’ incubation for 37°C, the lower side of the filter was washed, and the migrated cells were stained with a fluorogenic esterase substrate (Calcein AM; Invitrogen-Molecular Probes, Eugene, OR). Migration activity was measured using a fluorescence plate reader with a bottom-reading system (ARVO; PerkinElmer, Fremont, CA). Data were presented as the percentage of relative fluorescence units with chemoattractants (10% FBS or 10% autologous serum) compared with units without chemotactants (EBM only).

Flow Cytometric Analysis

We performed flow cytometric analysis to characterize the cultured HSCs, by Hill assay. More than 5.0 × 10⁷ cells were collected and washed by flow cytometry buffer (2% FBS, 10%NaN₃ in PBS) followed by analysis (FACS Calibur and CellQuest Pro software; BD biosciences). Antibodies for CD11b, CD14, CD31, CD34, CD38, CD44, CD45, CD117, CD114, and Lineage (BD Biosciences) and CD105, CD115, and CD144 (eBioscience, San Diego, CA) and the isotype controls were used. For each sample, data were collected from 20,000 events.

Statistical Analysis

All results are expressed as the mean ± SEM. Comparisons between group means were analyzed by paired or unpaired Student’s t-test (two-sided) for parametric analysis and the Mann-Whitney U test for nonparametric analysis. Statistical significance was set at P < 0.05. All analyses were performed with commercial software (SPSS 13.0 software; SPSS, Chicago, IL).

RESULTS

Patient Backgrounds

The average age of the 13 patients with iCNV was 34.2 ± 8.8 years (Table 1), and 69% (9/13) were women. Among the 10 healthy control subjects, the average age was 31.6 ± 6.3 years, and 70% (7/10) were women. The corrected visual acuity in logMAR ranged from 0.15 to 1.05 with a mean of 0.50. Eleven (85%) of the 13 patients had subfoveal CNV. The mean size of the CNVs was 1528 ± 883 µm. Active CNV lesions were present in 31% (4/13) of patients. The most frequent previous ocular treatment was photodynamic therapy, used in 38% (5/13) of patients. None of 13 patients had systemic hypertension, diabetes mellitus, hyperlipidemia, or cardiovascular disease, and one patient had a history of meningitis. Five of 13 (38%) patients smoked currently or formerly.

Functional Evaluation of HSCs

Regarding the functional activities of HSCs, the size of each colony formed in the cells was smaller in patients with iCNV than in the control subjects (Fig. 1), and the number of colony-forming cells in each colony was smaller in the patients than in the control subjects (data not shown).

The average CFU-Hill was markedly lower in patients with iCNV than in control patients (12.8 ± 3.2 vs. 67.6 ± 12.6, P < 0.001, Fig. 2A), but migration activity of those cells to 10% FBS showed no statistically significant difference between the two patient groups (171.5% ± 15.1% vs. 168.2% ± 9.6%; Fig. 2B). Migration activity to 10% autologous serum also showed no significant difference between the groups (312.1% ± 19.2% vs. 291.4% ± 34.8%, Fig. 2C).

In contrast to the similar migration activity findings, invasion activity to 10% FBS of the cells was significantly lower in patients with iCNV than in control subjects (149.9% ± 13.3% vs. 191.5% ± 12.4%; P = 0.04, Fig. 2D). The invasion activity to 10% autologous serum was also lower in patients with iCNV than in control subjects (213.6% ± 30.4% vs. 358.7% ± 30.5%, P = 0.01, Fig. 2E).

Among the various combinations we examined between clinical characteristics and the data on circulating HSCs in our patients with iCNV, CFU-Hill and the size of CNV showed a
possible correlation. Patients with larger CNV had markedly lower CFU-Hill than did patients with smaller CNV (mean, 4.0 ± 1.2 vs. 20.3 ± 4.0, P = 0.006, Fig. 2F). Activity of CNV showed no correlations to the HSCs functions.

Cell Surface Markers of HSCs

We performed flow cytometric analysis to identify the surface-expressed markers at culture days 0 and 5 of the Hill assay. The expression of CD38, CD105, CD115, CD117, CD144, and CD146 in the whole mononuclear cells (Fig. 3A) were slightly increased by culture day 5. On the contrary, CD11b expression was decreased by day 5 in the whole mononuclear cells. To further investigate the changes in cell surface markers, we removed and analyzed the lymphoid cells (Fig. 3B). In those cell populations, the number of CD14- or CD34-positive cells were decreased, but the number of cells positive for CD115, CD144, CD146, or CD117 were significantly increased at day 5 compared with day 0 (Table 2). Especially, the increase in expression of CD144 and CD146 were dramatic (Table 2).

Those data suggest that the condition of CFU-Hill culture induce HSCs into endothelial lineage but does not diminish the myeloid lineage. The cells we analyzed for function in this study expressed the mixed markers of endothelial and myeloid.

**Discussion**

Although the incidence of iCNV is not as high as that of CNV in AMD, the disease often results in permanent, severe central vision loss similar to AMD. Thus, it is important to clarify the pathogenesis of iCNV, and because CNV of several origins is considered to share many pathologic mechanisms, analyzing the factors that affect iCNV has potential benefits for AMD research.

In seeking the risk factors of CNV, both local ocular factors and systemic factors should be considered. Although few reports about risk factors for iCNV exist, myopia has been proposed as a local ocular risk factor. Spitznas and Boker21 studied 151 eyes with iCNV and reported that the probability of development of neovascularization was proportional to the degree of myopia. Machida et al.22 reported high prevalence of myopia in Japanese patients with iCNV. Inflammatory response has been proposed as another local ocular risk factor because

**Table 1. Characteristics of Patients with Idiopathic CNV**

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (y)</th>
<th>Sex</th>
<th>VA (logMAR)</th>
<th>CNV Location</th>
<th>CNV Size (Jum)</th>
<th>CNV Activity</th>
<th>Therapy</th>
<th>Systemic Diseases</th>
<th>Smoking Index</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>46</td>
<td>M</td>
<td>0.40</td>
<td>Subfoveal</td>
<td>3170</td>
<td>Inactive</td>
<td>TAIV, PDT × 2</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>38</td>
<td>F</td>
<td>1.05</td>
<td>Juxtafoveal</td>
<td>750</td>
<td>Active</td>
<td>None</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>37</td>
<td>F</td>
<td>0.15</td>
<td>Subfoveal</td>
<td>2700</td>
<td>Inactive</td>
<td>PDT × 3</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>38</td>
<td>F</td>
<td>0.22</td>
<td>Subfoveal</td>
<td>2880</td>
<td>Active</td>
<td>None</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>43</td>
<td>F</td>
<td>0.22</td>
<td>Subfoveal</td>
<td>1400</td>
<td>Inactive</td>
<td>PDT × 3</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>31</td>
<td>F</td>
<td>0.70</td>
<td>Subfoveal</td>
<td>1420</td>
<td>Inactive</td>
<td>PDT × 1</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>39</td>
<td>M</td>
<td>1.00</td>
<td>Subfoveal</td>
<td>1540</td>
<td>Inactive</td>
<td>None</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>39</td>
<td>F</td>
<td>1.00</td>
<td>Subfoveal</td>
<td>800</td>
<td>Inactive</td>
<td>None</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>9</td>
<td>26</td>
<td>F</td>
<td>0.30</td>
<td>Subfoveal</td>
<td>500</td>
<td>Inactive</td>
<td>None</td>
<td>None</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>42</td>
<td>F</td>
<td>0.30</td>
<td>Subfoveal</td>
<td>890</td>
<td>Active</td>
<td>None</td>
<td>Meningitis</td>
<td>200</td>
</tr>
<tr>
<td>11</td>
<td>24</td>
<td>M</td>
<td>0.40</td>
<td>Juxtafoveal</td>
<td>1920</td>
<td>Inactive</td>
<td>None</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>12</td>
<td>26</td>
<td>M</td>
<td>0.40</td>
<td>Juxtafoveal</td>
<td>870</td>
<td>Inactive</td>
<td>Bevacizumab</td>
<td>None</td>
<td>200</td>
</tr>
</tbody>
</table>

The smoking index was calculated as the sum of the number of cigarettes smoked per day multiplied by years of smoking. TAIV, intravitreal injection of triamcinolone acetonide; PDT, photodynamic therapy; Bevacizumab, intravitreal injection of bevacizumab.

**Figure 2.** Functional analyses of circulating hematopoietic stem cells in the patients with idiopathic CNV. (A) CFU-Hill in the patients with iCNV and control patients. (B, C) Migration activity in 10% FBS and 10% autologous serum. (D, E) Invasion activity in 10% FBS and 10% autologous serum. (F) Comparison of CFU-Hill in CNV size. Mean ± SEM. FBS; fetal bovine serum, AS; autologous serum.
Figure 3. Flow cytometric analyses of the cultured cells. (A) Gated region including whole mononuclear cells. (B) Gated region excluding lymphocytes. FSC, forward scatter.

anti-inflammatory therapies such as systemic steroid or local injection of steroid hormone often show a stabilizing effect on iCNV.23,24 Derosa et al. reported that the combination of HLA-B7 and HLA-DR2 markers was significantly increased in patients with iCNV, and those can be considered as a possible systemic risk factor. Previously, we showed functional impairments of HSCs in patients with AMD with severe CNV and proposed that HSCs may have a protective role against CNV progression.18 Based on the results from the study reported in this article, we believe that decreased functional activities of circulating HSCs may be a systemic risk factor for iCNV formation.

We analyzed colony-forming capacity of circulating HSCs (CFU-Hill) and found impaired CFU-Hill in the patients with iCNV. CFU-Hill was originally reported by Hill et al.19 as one of the functional activities of circulating EPCs and was later redefined by Yoder et al.20 as a functional capacity of myeloid progenitors. Hill et al. suggested that levels of CFU-Hill may be a biomarker for vascular function and cumulative cardiovascular risk because reduced CFU-Hill was observed in more progressed patients. Similar results using CFU of HSCs were reported in patients with diabetes mellitus.27 These reports suggest that CFU of circulating HSCs may be a novel biomarker for vascular-related diseases.

In the present study, patients with iCNV had impaired CFU-Hill, which worsened with age in accordance with knowledge that CFU decreases as age increases.18 However, we found no systemic or cardiovascular disease history in our patients, so we could not find the reason for decreased HSC function in patients with iCNV in relation to systemic diseases. Since it is unlikely that CNV causes functional impairment of HSCs, it is easier to understand that patients with impaired function of HSCs have increased susceptibility to iCNV. In contrast to the patients with iCNV, those with AMD showed no significant difference in CFU-Hill compared with the age-matched control patients.18 We suspect that this difference may have originated from the difference in possible systemic and ocular factors. Although our data in this article showed a significant difference in HSC function, we do not think functionally impaired HSC is the only risk factor for iCNV. As stated, there are several local ocular risk factors—myopia, inflammatory response, and other unidentified factors—for iCNV. According to many animal studies, circulating progenitor cells patrol in a nondisease state but they can be recruited to the injury site if disease occurs (e.g., angiogenesis,6,7,26 ischemia,9 and atherosclerosis29). It may be reasonable to consider that circulating HSCs is a modifier of those local ocular events and that both local and systemic factors are related to the incidence of iCNV.

Our results support the possibility that HSC functions play a role, not only in the occurrence of CNV, but also in iCNV progression or severity. In our previous study about AMD, patients with severe CNV such as larger CNV or bilateral involvement showed impaired HSC function compared with those with milder cases.18 These findings are consistent with the results of the present study (Fig. 2F). A recent animal study of a laser-induced CNV model reported that macrophages control the fate of pathologic angiogenesis and senescent macrophages lose their effectiveness in controlling pathologic angiogenesis, resulting in increasing CNV severity.50 This study suggests that circulating bone marrow–derived cells, including myeloid progenitors, have a role in the progression of CNV. In a recent study, Yoder et al.26 suggest that CFU-Hill represents the capacity of myeloid progenitor rather than that of endothelial progenitors. These findings may indicate that the functional activities of myeloid progenitors play a key role in controlling the fate of pathologic angiogenesis, in clinical words, CNV progression or severity.

Invasion activity is an important capacity for HSCs to home to the injured tissue site, and impaired invasion ability causes reduced functional recovery in ischemic tissue.51 The migration and invasion assays differ by whether or not a modified Boyden chamber is filled with the synthetic matrix (Matrigel; BD Biosciences). A solubilized basement membrane preparation from mouse sarcoma includes laminin, collagen type IV, heparin sulfate proteoglycans, and many growth factors that enhance angiogenesis. Therefore, the invasion assay is more angiogenic than the migration assay. Thus, our data indicate that circulating HSCs in iCNV may show more distinct impaired functions in angiogenic conditions. In the actual CNV lesion,
recruited HSCs in iCNV may fail to display their own ability and may result in increasing CNV severity. Molecular mechanisms to explain the loss of functionality, such as some changes in proteases or cell surface molecules, were not elucidated in the present study, and identifying them is of interest.

In conclusion, we found that some of the functional activities of circulating HSCs were reduced in the patients with iCNV, especially in those with progressing lesions. Because circulating HSCs may relate to CNV progression or severity in iCNV, the functional activities of circulating HSCs may become a novel biomarker for iCNV. In addition, maintenance, or if possible, gain of function of circulating HSCs may become a new therapeutic strategy for inhibiting CNV progression. Last, an interesting unsolved question remains: Why are circulating HSC functions in patients with iCNV reduced? Cellular senescence and oxidative stress are reportedly related to the impaired functional activities of HSCs. We are now investigating to determine the cellular and molecular mechanisms of this reduction of function.

Acknowledgments

The authors thank colleagues and laboratory members Takanori Kameda, Hiroko Hizaki, Yuko Sashara, Kaori Asamoto, Chiharu Sengoku, and Yukiko Makiyama for helpful advice and technical support and, especially, Kayo Nishida for technical support in culturing the HSCs.

References