Circulating hematopoietic stem cells in patients with choroidal neovascularization secondary to pathologic myopia

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Abstract

Background Emerging evidences suggest that circulating hematopoietic stem cells (HSCs) affect the pathogenesis of choroidal neovascularization (CNV), however, the roles of HSCs in CNV remain unclear in human population. The current study was designed to investigate the role of HSCs in the pathogenesis of CNV secondary to pathologic myopia (PM).

Methods We clinically documented 78 patients with CNV in PM, and 35 of 78 patients and 28 age-matched controls were experimentally analysed. Functional analyses of HSCs were performed using an *ex vivo* culture system.

Results We disclosed colony-forming units of endothelial cell (CFU-EC) were markedly lower in patients with bilateral CNV compared to those with unilateral CNV (13.8 ± 3.7 vs 45.9 ± 7.8 , $P < 0.001$). Systemic characteristics between both groups showed no significant difference. To identify local ocular factors that may affect the occurrence of CNV, clinical parameters were compared with the following groups in all enrolled subjects: eyes with CNV vs without CNV in unilateral affected patients, and primary affected eyes vs secondary affected eyes in patients with bilateral CNV. However, no statistically significant factors were identified in any of the groups.

Conclusions Circulating HSCs may play a role in the bilateral involvement of CNV in PM patients as one of the systemic factors. Further prospective and longitudinal studies are required.

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Keywords: hematopoietic stem cells; choroidal neovascularization; pathologic myopia

Choroidal neovascularization (CNV) is a common pathological change of various chorioretinal diseases^{1–3} that causes severe vision loss. Recently, several new therapeutic options including photodynamic therapy⁴ and anti-vascular endothelial growth factor (VEGF) drugs⁵ have been developed and many investigators have shown the benefits. However, the pathogenesis of CNV is not fully understood and clinically, CNV is still a formidable adversary.

Because there was little information available about the systemic factors that may affect 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.^{7,8} HSCs/EPCs derived from bone marrow⁹ participate in normal and pathological postnatal angiogenesis¹⁰ and differentiate into a variety of non-endothelial cell (EC) types, including hepatocytes,¹¹ cardiomyocytes,¹² microglia,¹³ and even neurons.¹⁴ It has been suggested that these cells play important roles in repairing injured tissue^{15,16} and disease prognosis.¹⁷ Although HSCs/EPCs might work as a systemic factor, the role of these cells in the formation of CNV remains unclear.

By analysing the circulating HSCs with clinical characteristics of age-related macular degeneration (AMD) patients, we have proposed an important role of these cells in

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AMD; the active CNV could increase the number of HSCs in peripheral circulation and the function of these cells might play a key role in the CNV severity and progression.¹⁸

In this article, we have firstly tried to clarify the roles of HSCs in CNV secondary to pathologic myopia (PM). PM is one of the major leading causes of legal blindness in many countries, especially in Asia including Japan^{19–22} and CNV is the most important factor on visual prognosis in PM.^{23,24} However, little is known about the pathogenesis. In contrast to the AMD, PM have variety of distinct and characteristic chorioretinal changes including posterior staphyloma, lacquer crack, diffuse chorioretinal atrophy, or patchy chorioretinal atrophy.²⁰ Because these local changes have been considered to play important roles in the development of CNV, we considered that analysing those fundus changes with the data of circulating HSCs in PM patients could bring us some important information about the involvement of local and systemic factors in the pathogenesis of CNV. To the best of our knowledge, this is the first report of comprehensive investigation into the local and systemic factors that may affect CNV formation in PM.

Materials and methods

The study protocol was approved by Ethical Committee of Kyoto University Hospital and all the enrolled subjects gave their written informed consent according to the Declaration of Helsinki. From November 2004 to May 2006, consecutive 101 patients with CNV secondary to PM were diagnosed by comprehensive fundus examinations, including slit-lamp biomicroscopy with a contact lens, fluorescein angiography (FA), indocyaninegreen angiography (ICGA), and optical coherence tomography (OCT) at Center for Macular Diseases in Kyoto University Hospital. FA and ICGA were simultaneously performed using confocal scanning system (HRA-2, Heidelberg Engineering, Dossenheim, Germany) and two types of OCT were used (Stratus OCT: Carl Zeiss, Dublin, CA, USA; OCT-ophthalmoscope; Nidek, Gamagori, Japan). Inclusion criteria of this study were as follows; (1) Both eyes were high myopia defined as more than -6.5 D or axial length of 26.5 mm. (2) Intensive clinical examinations including FA, ICGA and OCT were performed in both eyes. (3) Patients having CNV at least in one eye. Seventy-eight of 101 patients satisfied the criteria. The current study is cross-sectional. CNV location and size was evaluated using fluorescein angiogram and ICG angiogram. In particular, CNV size was defined as the maximal diameter of fibrovascular tissue, and did not include chorioretinal atrophy around CNV membrane. Fundus changes of PM such as diffuse chorioretinal atrophy, patchy chorioretinal atrophy, or

chorioretinal atrophy of the macula were evaluated by fundus photograph according to Tokoro's classification.²⁰ Diffuse chorioretinal atrophy appears in the posterior pole as a yellowish-white fundus change (Supplementary Figure 1A). In this paper, diffuse chorioretinal atrophy includes spotty or linear lesions with the choroidal vessels. Lesions across choroidal vessels are excluded, because we consider that lesions across choroidal vessels are similar pathologic changes to lacquer crack. Patchy chorioretinal atrophy was subdivided into two groups (P_1 and P_2). P_1 was defined as spotty lesion of the patchy atrophy, 0.6–0.8 mm, less than 0.5 optic disc diameter. P_2 was defined as patchy lesion of the patchy atrophy, a well-defined round or oval lesion larger than 0.5 optic disc diameter (Supplementary Figure 1B). Posterior staphyloma was observed by indirect ophthalmoscopy (Supplementary Figure 1C). Lacquer crack was observed as hypofluorescent linear lesion imaged by the late phase of ICGA, because ICGA reveals the size, number, and extent of lacquer cracks more clearly than slit-lamp biomicroscopy or FA (Supplementary Figure 1D).²⁵

For *ex vivo* analysis, we randomly collected 20 ml of peripheral blood from 35 of 78 patients who were included in the criteria described above and from 28 age-matched controls. The person who had high myopia, macular diseases, other ocular diseases except senile cataract, or systemic diseases such as, diabetes mellitus, cardiovascular diseases, or cerebrovascular diseases was not included in the controls. We mainly investigated colony-forming units of EC (CFU-EC, Hill's assay, Figure 1) and migration activity of HSC-derived cells as the representatives of functional activities of HSCs,

Figure 1 Endothelial colony derived from circulating hematopoietic stem cells.

because most important pathological event of CNV formation is angiogenesis. We also analysed the number of circulating HSCs (CD34⁺ cells) using flow cytometry.

Colony-forming units assay

Culture of HSCs and measuring CFUs were performed using an established method previously described by Hill *et al.*²⁶ Mononuclear cells from peripheral blood were collected by light-density gradient centrifugation (Ficoll-Paque Plus, Amersham Biosciences) and 1×10^7 of mononuclear cells were seeded on 6-well human fibronectin-coated plates (BD Biosciences) in a 2.5 ml of an endothelial basal medium (EBM) (Endocult, StemCell Technologies) with 20% fetal bovine serum (FBS). After 48 h, 1×10^6 non-adherent cells were transferred into a new 24-well fibronectin coated plates in 1 ml of EBM to avoid contamination with mature ECs and non-progenitor 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. Mean value of three wells was scored as CFUs. Immunocytochemistry was performed after fixation of 4% paraformaldehyde, and the expression of endothelial marker proteins, kinase insert domain receptor (R&D Systems), platelet EC adhesion molecule-1 (R&D Systems), vascular endothelial-cadherin (Chemicon) was confirmed.

Migration assay of hematopoietic stem cell-derived cells

HSC-derived cells after 5 days culture *in vitro* were placed in the upper chamber (2.5×10^4) of a modified Boyden chamber with a fluorescence block system²⁷ (BD Biocoat™ Angiogenesis System: Endothelial Cell Migration, BD Biosciences) in duplicate for each patient's sample. The chamber was placed in a 96-well culture dish containing EBM (Endocult, StemCell Technologies) and 10% FBS. After 22 h 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, Molecular Probes). Migration activity was measured using a fluorescent plate reader with a bottom-reading system (ARVO, PerkinElmer). Data were presented as percentage of relative fluorescence units with chemoattractants to without chemoattractant (EBM only).

The number of circulating hematopoietic stem cells

The number of circulating CD34⁺ HSCs was counted using flow cytometric analysis.¹⁸ Circulating CD34⁺ mononuclear cells in the peripheral blood were stained using a reagent kit (ProCOUNT, BD Biosciences, Bedford, MA, USA) and measured with a flow cytometer

(FACSCalibur, BD). The known amount of stained and lysed, but not washed, blood added to a known amount of microbeads allows, in combination with the observed ratio between the number of flow cytometrically counted beads and CD34⁺ cells, the calculation of absolute CD34⁺ cell count. The data were analysed using Cell Quest software (BD Biosciences). In each blood sample, more than 60 000 cell events were obtained. An isotype-negative control optimized the settings of the fluorescence detectors for each subject.

Statistical analysis

All results for continuous variables in the experimental data are expressed as means $\pm$ SEM. Comparisons between groups were analysed by paired or unpaired Student's *t*-test (two-sided) for parametric analysis. For non-parametric analysis, Mann-Whitney's *U*-test was used. Categorical analysis was calculated by χ^2 test and Fisher's exact test. Multiple regression analysis was performed to correlate CFU-EC with the systemic risk factors or clinical conditions. $P < 0.05$ was considered statistically significant. All analyses were performed with SPSS 13.0 software.

Results

Patient backgrounds

Seventy eight patients were included in this study. The average age was 64.8 ± 11.5 years (range 22–84 years). Eight patients (10%) were less than 50 years old; 14 (18%) were male and 64 (82%) were female.

Majority of the subjects had active CNV lesion, which was demonstrated by significant fluorescein leakage on angiogram, haemorrhage, and cystoid macular oedema observed by OCT. Patients who underwent photodynamic therapy or intravenous steroid injection within 6 months and within 3 months were 89% ($n = 31$) and 74% ($n = 26$), respectively.

Clinical evaluation

To find a local ocular factor, which may affect the development of CNV, we compared the clinically documented fundus changes in each eye of an individual patient. The first investigation was the comparison of fundus appearance between the affected eye and non-affected eye in unilateral CNV patients. In this investigation, we have focused on a question whether the affected eye had severer pathologic changes than the non-affected eye. The second investigation was in the bilateral affected patients and the severity of PM changes was compared between a primary affected eye and a

secondary affected eye. To achieve these investigations, the all enrolled patients ($n = 78$) were divided into unilateral (57 patients, 73%) and bilateral (21 patients, 27%) CNV affected groups. Table 1 shows the comparison of clinical parameters between an affected eye and non-affected eye in patients of unilateral involvement. As expected, visual acuity (VA) of the eyes with CNV was significantly worse than the eyes without CNV. There was no significant difference in many of the clinical parameters examined, which may reflect the severity of PM, such as axial length, posterior

staphyloma, patchy chorioretinal atrophy, or lacquer cracks. But, diffuse chorioretinal atrophy was more frequently seen in the eyes with CNV (89%) compared to the contra lateral eyes without CNV (74%) with borderline significance ($P = 0.051$). Table 2 shows the comparison of clinical parameters between a primary affected eye and a secondary affected eye in patients of bilateral involvement. The size of CNV was significantly larger in primary affected eyes than the secondary affected eyes ($P = 0.02$). Similar to the analysis of unilateral CNV affected cases, many of the fundus

Table 1 Comparison of clinical characteristics between eyes with and without choroidal neovascularization with unilateral involvement in pathologic myopia

	Eyes with CNV ($n = 57$)	Eyes without CNV ($n = 57$)	P-value
Refraction (D)			
Mean $\pm$ SD	-11.48 ± 4.63	-11.35 ± 4.09	0.074
Range	-4.0 to -22.0	-4.25 to -20.0	
Keratometry (mm)			
Mean $\pm$ SD	7.60 ± 0.27	7.62 ± 0.27	0.83
Range	6.82–8.09	6.85–8.25	
Axial length (mm)			
Mean $\pm$ SD	28.86 ± 1.31	28.60 ± 1.63	0.35
Range	26.35–32.50	24.43–31.25	
Visual acuity			
20/200–20/40	31 (54%)	11 (19%)	<0.001
<20/200	14 (25%)	5 (8.8%)	0.04
CNV location			
Subfoveal	31 (54%)	—	
Juxtafoveal	24 (42%)	—	
Extrafoveal	2 (3.5%)	—	
CNV size (μm)			
Mean $\pm$ SD	1492 ± 811	—	
Range	350–4130	—	
Posterior staphyloma	24/26 (92%)	20/26 (77%)	0.25
Diffuse atrophy	51 (89%)	42 (74%)	0.051
Patchy atrophy			
P_1	9 (16%)	11 (19%)	0.81
P_2	6 (11%)	8 (14%)	0.78
MA	6 (11%)	4 (7.0%)	0.74
Lacquer crack			
Detected in ICGA ^a	50/55 (91%)	44/54 (81%)	0.18
Not applicable	2 (3.5%)	3 (5.3%)	

CNV = choroidal neovascularization; ICGA = indocyaninegreen angiography; MA = chorioretinal atrophy of the macula; P_1 = spotty lesion of the patchy atrophy; P_2 = patchy lesion of the patchy atrophy.

^aNot applicable eyes were excluded.

Table 2 Clinical characteristics of patients with bilateral myopic choroidal neovascularization: comparison of primary and secondary affected eyes

	Primary affected eyes ($n = 21$)	Secondary affected eyes ($n = 21$)	P-value
Refraction (D)			
Mean $\pm$ SD	-10.88 ± 3.32	-10.40 ± 2.95	0.14
Range	-4.25 to -17.0	-5.25 to -10.50	
Keratometry (mm)			
Mean $\pm$ SD	7.50 ± 0.33	7.38 ± 0.38	0.64
Range	7.05–8.11	6.59–7.96	
Axial length (mm)			
Mean $\pm$ SD	28.53 ± 1.42	28.59 ± 1.65	0.91
Range	26.38–32.63	24.66–32.58	
Visual acuity			
20/200–20/40	9 (43%)	14 (67%)	0.35
<20/200	9 (43%)	2 (9.5%)	0.053
CNV location			
Subfoveal	18 (86%)	13 (62%)	0.16
Juxtafoveal	3 (14%)	7 (33%)	0.28
Extrafoveal	0 (0%)	1 (4.8%)	—
CNV size (μm)			
Mean $\pm$ SD	2444 ± 1029	1772 ± 817	0.02
Range	1160–5220	610–3930	
Posterior staphyloma	11/11 (100%)	11/11 (100%)	1.0
Diffuse atrophy	21 (100%)	21 (100%)	1.0
Patchy atrophy			
P_1	4 (19%)	7 (33%)	0.48
P_2	5 (24%)	5 (24%)	1.0
MA	15 (63%)	0 (0%)	<0.001
Lacquer crack			
Detected in ICGA ^a	11/12 (92%)	20/21 (95%)	0.38
Not applicable	9 (43%)	0 (0%)	

CNV = choroidal neovascularization; ICGA = indocyaninegreen angiography; MA = chorioretinal atrophy of the macula; P_1 = spotty lesion of the patchy atrophy; P_2 = patchy lesion of the patchy atrophy.

^aNot applicable eyes were excluded.

changes did not show significant difference between the primary and secondary CNV eyes; however, the chorioretinal atrophy of the macula ($P < 0.001$) is significantly frequently seen in primary affected eyes.

Functional evaluation of HSCs

Thirty-five PM patients with CNV were enrolled to the *ex vivo* analysis of circulating HSCs. Table 3 shows systemic characteristics of these patients and age-matched non-PM controls. Details of analysed data are shown in Table 4. The average CFU-EC or migration activity of myopic CNV patients ($n = 35$) did not significantly different to that of age-matched controls ($n = 28$, Figure 2a and b). The number of circulating CD34+ HSCs also

Table 3 Systemic characteristics of subjects used for HSC analysis: myopic CNV vs control

	Myopic CNV (n = 35)	Controls (n = 28)	P-value
Age (years)			
Mean $\pm$ SD	66.3 $\pm$ 13.3	65.6 $\pm$ 3.6	0.86
Range	27–82	26–87	
Gender (female)	30 (86%)	21 (75%)	0.45
Hypertension	9 (26%)	6 (21%)	0.92
Diabetes mellitus	2 (5.7%)	4 (14%)	0.39
Hyperlipidemia	11 (31%)	7 (25%)	0.78
Statin medication	6 (17%)	3 (11%)	0.72
Cardiovascular disease	7 (20%)	4 (14%)	0.74
Cerebrovascular disease	0 (0%)	1 (3.6%)	0.44
History of smoking	8 (23%)	5 (18%)	0.86

CNV = choroidal neovascularization, HSC = hematopoietic stem cell.

Table 4 Data of HSC analysis

	<i>Controls</i> (n = 28)	<i>Myopic CNV</i>		
		<i>Overall</i> (n = 35)	<i>Unilateral</i> (n = 27)	<i>Bilateral</i> (n = 8)
<i>CFU-EC</i>				
Mean ± SEM	39.4 ± 5.1	41.3 ± 5.7	49.5 ± 7.8	13.8 ± 3.7
Range	0.5–115.0	1.0–138.0	1.0–138.0	1.0–33.5
Median	39.0	33.5	46.5	13.3
<i>Migration (percentage of basal)</i>				
Mean ± SEM	155.1 ± 5.4	147.9 ± 4.4	150.7 ± 8.3	138.3 ± 7.8
Range	112–216	105–207	112–207	105–175
Median	155.0	144.0	146.0	136.0
<i>CD34+ (cell/μl)</i>				
Mean ± SEM	3.95 ± 0.43	4.55 ± 0.37	4.56 ± 0.47	4.53 ± 0.61
Range	1.49–8.67	1.67–8.61	1.66–8.61	1.92–6.70
Median	3.95	4.53	4.59	4.41

CFU-EC = colony-forming units of endothelial cell; HSC = hematopoietic stem cell.

showed no difference between the patients and controls (Figure 2c). We then analysed whether there are any association between clinical characteristics and functional activities of circulating HSCs. Examined clinical parameters were refraction, axial length, corrected VA, CNV location, CNV size, laterality of CNV, diffuse chorioretinal atrophy, patchy atrophy, lacquer cracks, and posterior staphyloma. Among the various combinations examined, we found that CFU-EC of bilateral CNV patients showed markedly lower than those of unilateral CNV patients ($P < 0.001$, Figure 2d). In addition, the migration activity of HSCs was also reduced by 24% in bilateral patients compared to unilateral patients (Figure 2e). No difference was observed in systemic characteristics between the bilateral and unilateral groups (Table 5). Multiple regression analysis also showed that this result in function may not be brought by these systemic factors (Table 6). In contrast, the number of circulating CD34+ HSCs also showed no difference between the unilateral and bilateral patients (Figure 2f).

Discussion

CNV is the most common cause of severe vision loss in PM same as in AMD. Although there are several differences in the clinical characteristics between AMD and PM the pathogenesis of CNV are considered same; neovascularization originate from choroidal vasculature that grown through the break of Bruch's membrane. Studies on the histopathology of CNV also suggested that there are no significant differences in the constituent cells and in the expression of several important cytokines including VEGF-A.^{28,29} One of the characteristic features of PM patients that differ from AMD patients is the progressive elongation of the eyeball that results in posterior staphyloma. Within the posterior staphyloma, many characteristic fundus changes including diffuse atrophy of retina and choroid, patchy chorioretinal atrophy, and lacquer cracks are observed. Because these pathologic changes possibly result in the breaks of Bruch's membrane, it is reasonable that the development of CNV have correlation with these changes.^{20,30,31} Another characteristic of PM patients is the younger age. In contrast to AMD patients, CNV can be developed in the younger patients in PM and it is reported that PM is the most major cause of CNV development in the young generation.² This fact may also be explained by the chorioretinal changes of PM. Indeed, as the possible developmental factors of myopic CNV, increasing age,²⁰ axial length,²⁰ lacquer crack,^{30,31} and patchy atrophy³¹ have been reported.

To investigate whether and how these local ocular factors are involved in the development of myopic CNV,

Figure 2 Analyses of the functions and numbers of circulating hematopoietic stem cells (HSCs) in patients with choroidal neovascularization (CNV) secondary to pathologic myopia (PM). (a) Comparison of colony-forming units of endothelial cell (CFU-EC) between the age-matched healthy controls ($n=28$) and patients with CNV secondary to PM ($n=35$). (b) Comparison of migration activity between the controls ($n=28$) and patients ($n=35$). (c) Comparison of the number of circulating CD34+ HSCs between the age-matched healthy controls ($n=28$) and the patient with CNV secondary to PM ($n=35$). (d) Impaired CFU-EC in patients with bilateral CNV ($n=8$) compared to those with unilateral CNV ($n=27$). (e) Reduced migration activity of endothelial progenitor cells in patients with bilateral CNV ($n=8$) compared to those with unilateral CNV ($n=27$). (f) No significant difference in the number of circulating CD34+ HSCs between patients with unilateral ($n=27$) and bilateral ($n=8$) CNV.

Table 5 Systemic characteristics of subjects used for HSC analysis: unilateral vs bilateral

	Unilateral (n = 27)	Bilateral (n = 8)	P-value
Age (years)			
Mean ± SD	65.3 ± 14.3	69.9 ± 8.7	0.40
Range	27–82	56–78	
Gender (female)	22 (81%)	8 (100%)	0.28
Hypertension	9 (33%)	0 (0%)	0.081
Diabetes mellitus	2 (5.7%)	0 (0%)	1.0
Hyperlipidemia	9 (26%)	2 (25%)	1.0
Statin medication	3 (11%)	1 (13%)	1.0
Cardiovascular disease	6 (17%)	1 (13%)	1.0
Cerebrovascular disease	0 (0%)	0 (0%)	1.0
History of smoking	6 (17%)	2 (25%)	1.0

HSC = hematopoietic stem cell.

we first examined the fundoscopic differences between the eyes of an individual patient. By comparing each eye of individual patient, it was possible to ignore the influences of systemic factors and we thought that the local ocular factors that affect the pathogenesis of CNV might be brought into relief. However, unexpectedly, there were no fundoscopic differences between the

affected eyes and the non-affected eyes in unilateral CNV patients. In addition, we could find no significant difference except diffuse chorioretinal atrophy of the macula in those between the primary affected eyes and the secondary affected eyes of bilateral CNV affected patients. These data do not deny the importance of pathologic changes of PM in the development of CNV; however, our results may indicate that these characteristic pathological changes in PM is a necessary condition, but not a sufficient condition, in CNV development. Other conditions may be required to develop CNV in the eyes of PM.

Circulating HSCs have been shown to involve in the pathogenesis of CNV in murine laser-induced CNV model.^{7,8} From the previous functional analysis of circulating HSCs in the neovascular AMD patients, we disclosed functional impairments of HSCs in patients with severer CNV, and revealed that HSCs may have a protective role against CNV progression.¹⁸ Similar to the data of AMD patients, our data on functional analysis of HSCs suggest that circulating bone marrow-derived stem cells may contribute to the pathogenesis of myopic CNV as one of the systemic factors. Among several combinations, we found that the laterality of CNV is

Table 6 Multiple regression analysis between CFU-EC and clinical conditions

Independent variable	β coefficient ($R = 0.91$)	P-value
<i>HSC analysis</i>		
Migration	0.51	0.04
CD34+ cells	0.28	0.27
<i>Clinical characteristics</i>		
Age	0.05	0.85
Sex (male)	-0.29	0.31
Refraction	-0.17	0.47
Keratometry	0.00	1.00
Axial length	-0.26	0.30
Corrected visual acuity	0.07	0.70
Laterality of CNV (bilateral)	-0.14	0.76
CNV location (subfoveal)	0.38	0.08
CNV size	-0.38	0.32
Posterior staphyloma	-0.01	0.97
Diffuse atrophy	-0.21	0.29
Patchy atrophy	-0.14	0.50
<i>Systemic characteristics</i>		
Hypertension	0.27	0.38
Diabetes mellitus	-0.11	0.54
Hyperlipidemia	-0.12	0.57
Heart disease	0.45	0.09
Medication of anticoagulants	0.30	0.19
History of smoking	-0.33	0.23

CNV = choroidal neovascularization; CFU-EC = colony-forming units of endothelial cell; HSC = hematopoietic stem cell.

related to the functional capacities of HSCs. In bilateral affected patients, functional activities of HSCs were significantly reduced compared to the unilateral affected patients. These data are the first clinical evidence to demonstrate the roles of circulating HSCs for CNV in PM as one of the systemic factors.

Recent study on CNV model reports that circulating HSCs regenerate the injured retinal pigment epithelial (RPE) cells or other type of cells (astrocyte, macrophage/microglia, mural cells, and vascular ECs).³² The study also suggests that circulating HSCs have a repairing capacity for chronic injured RPE or neurosensory retina. Similar paradigm was already proposed in the studies dealing with atherosclerosis.^{15,33} EPCs derived from HSCs are considered to have the capacity to repair vascular injuries. The injured endothelial monolayer is regenerated by circulating EPCs, accelerating reendothelialization and limiting atherosclerotic lesion formation. Moreover, EPC impairment by risk factors, such as age and diabetes, may contribute to atherogenesis and atherosclerotic disease progression. Thus, a possible role of circulating HSCs may be a tissue repair for tissue injuries in highly myopic eyes, and we speculate that impaired functional HSCs cannot maintain

subclinical tissue injuries, resulting in having high risks for CNV development and bilateral involvement. To strengthen this hypothesis, it is necessary to correct the blood samples from myopic without CNV persons. Our result was that CFU-EC showed no significant difference between the non-myopic control subjects and the PM patients with CNV (Figure 2a and b). If CFU-EC or the migration activity of myopic without CNV people is higher than unilateral CNV patients, the functional capacities of HSCs might be a risk factor for CNV in PM. Another limitation of this study is the small number of bilateral patients who were experimentally analysed ($n = 8$). A larger population study is required. In addition, CNV size was not related to HSC functions in PM patients. Contrastively, AMD patients with larger CNV had reduced functions of HSCs.¹⁸ This difference may be originated from the small number in this study or may show the different role of circulating HSCs between the development and the progression of CNV in two different ocular environments (AMD and PM).

We have recently reported that the number of HSCs was significantly increased with the activity of CNV in neovascular AMD patients and showed a possibility that active CNV lesion can mobilize HSCs from bone marrow.¹⁸ To investigate the role of HSCs, the number of cells involved is another important factor that should be considered in addition to the function of cells. Although the number of HSCs in myopic CNV patients was higher than that of healthy controls, statistically significant difference was not present in our study population (Figure 2c). Furthermore, probably because the majority of both unilateral and bilateral patients had active CNV lesion, the number of HSCs showed no difference between the two groups in contrast to the function of HSCs. Thus, the functional difference rather than the number of BM-derived HSCs may be a clue in making the clinical differences in our myopic CNV cases; however, requirement of further study is beyond doubt.

In conclusion, we have investigated the funduscopy choriorretinal changes as local factors and circulating BM-derived stem cells as systemic factors whether they have any role in the pathogenesis of CNV in PM patients. Although we could not find local ocular factors that can affect the laterality of myopic CNV, impaired function of circulating BM-derived stem cells were observed in patients of bilateral CNV involvement. Because CNV is known to grow through a break of Bruch's membrane, ocular changes including choriorretinal atrophy in PM patients may play an important role in the pathogenesis of CNV. However, the reason why not all the PM patients develop CNV in both eyes in spite of the presence of choriorretinal changes is an unsolved question. Our

results of functional differences of circulating BM-derived stem cells in PM patients may suggest a key to solve this question. Further longitudinal clinical studies and experimental studies are expected to clarify the underlying mechanism of pathogenesis of CNV in PM patients.

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