Bone Marrow-Derived Endothelial Progenitor Cells Participate in the Initiation of Moyamoya Disease

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Abstract

The mechanisms through which moyamoya disease occurs and progresses remain unknown. Recent studies have indicated the involvement of circulating endothelial progenitor cells (EPCs) in the development of moyamoya disease. This study directly investigated the participation of EPCs in moyamoya disease, using specimens of the supraclinoid internal carotid artery collected from two adult patients. The specimens were stained with primary antibodies against CD34, CD133, and vascular endothelial growth factor receptor-2 (VEGFR2) to localize the circulating EPCs in the thickened intima of occlusive arterial lesion. The CD34- and VEGFR2-positive cells were densely found in the thickened intima of occlusive arterial lesion, particularly clustered in the superficial layer of thickened intima. However, the number of CD34- and CD133-positive cells was very small. The CD34-positive cells also expressed von Willebrand factor on the surface of thickened intima and were also positive for α-smooth muscle actin in the deeper layer. These findings suggest that circulating EPCs may be involved in the development of occlusive arterial lesion in moyamoya disease.

Key words: moyamoya disease, endothelial progenitor cell, intimal thickening, stenosis, pathogenesis

Introduction

Moyamoya disease is an uncommon cerebrovascular disorder that is characterized by progressive occlusion at the supraclinoid internal carotid artery (ICA) and its main branches within the circle of Willis. This occlusion results in the formation of a fine vascular network (the “moyamoya” vessels) at the base of the brain. Moyamoya vessels are the dilated perforating arteries that function as collateral pathways. Moyamoya disease involves both pediatric and adult patients and causes both ischemic and hemorrhagic stroke. Histopathological analyses in the carotid terminations have shown eccentric fibrocellular thickening of the intima, irregular undulation (waving) of the internal elastic lamina, and attenuation of the media.7,13) Occlusive lesion of the supraclinoid ICA is known to progress in both pediatric and adult patients.14) Although numerous studies have suggested the involvements of genetic or infectious factors in the development of moyamoya disease, the underlying mechanism through which moyamoya disease develops and progresses still remains unknown.1,7,13)

Recent laboratory studies have suggested that the endothelial progenitor cells (EPCs) may be important in the pathogenesis of moyamoya disease.11,12,17,25) Significant increase or decrease of the circulating EPCs was demonstrated in the peripheral blood of patients with moyamoya disease, although there were considerable discrepancies. Disturbed function of the circulating EPCs was also identified.11,12) The circulating EPCs are known to be derived from the bone marrow and are critically involved in vascular repair and remodeling.4,10) Accumulating evidence has indicated that the circulating EPCs largely contribute to atherosclerotic intimal thickening.10,16) However, these findings do not directly justify the involvement of the circulating EPCs in the pathogenesis of moyamoya disease, because there is no direct evidence of the involvement of the circulating EPCs in the thickening of the intima at the supraclinoid ICA, i.e., the initiation site of this disease.

The present study investigated if the circulating EPCs are involved in the intimal thickening of the...
supraclinoid ICA in moyamoya disease using an immuno- 
histochemical technique to localize the EPCs in the supraclinoid ICA specimens. The progenitor cell markers were CD34, CD133, and vascular endothelial growth factor receptor-2 (VEGFR2), because these are the most common EPC markers used in previous studies.\(^{9,10}\) CD34 can identify hematopoietic stem cells, EPCs, and endothelial cells (ECs) in small vessels. CD133-expressing cells are a subpopulation of circulating CD34 cells, but cease to express CD34 upon differentiation to ECs. VEGFR2 is expressed during vascular development, and by ECs and their precursor EPCs. Therefore, co-expression of CD34 with VEGFR2 or CD133 indicates that the cell is likely to be an EPC.

**Materials and Methods**

Specimens of the supraclinoid ICA were obtained from two adult patients with moyamoya disease. Patient 1, a 19-year-old female, had suffered sudden onset of headache and vomiting followed by rapid loss of consciousness. Computed tomography (CT) on admission revealed intraventricular hemorrhage. She was diagnosed as moyamoya disease on cerebral angiography (stage 3 on the right side and stage 1 on the left side according to Suzuki's classification). She underwent ventricular drainage immediately after admission, but died of tonsillar herniation due to re-bleeding 4 days later (Fig. 1A–C). Autopsy was performed and the specimen of the supraclinoid ICA was obtained. Patient 2, a 62-year-old female, was admitted to our hospital due to sudden headache and consciousness disturbance. CT on admission demonstrated diffuse subarachnoid hemorrhage. Ruptured basilar artery aneurysm was diagnosed associated with moyamoya disease (stage 6 on the right side and stage 5 on the left side) (Fig. 2A, B). The aneurysm was clipped through the right trans-sylvian approach, but the occluded supraclinoid portion of the right ICA had to be resected for full exposure of the aneurysm. Following surgery, she was transferred to another hospital for rehabilitation. These procedures were approved by the Ethical Committee at Hokkaido University Graduate School of Medicine, and all samples were obtained with agreement of the patients' families. The specimens were immersed in 4% paraformaldehyde and embedded in paraffin for immunohistochemical examinations. The 4-mm thick cross-sections were prepared for subsequent staining.

Hematoxylin and eosin staining was used to observe the anatomical structure of the specimens. To localize the circulating EPCs in the specimens, the cross-sections were stained with primary antibodies against CD34 (mouse monoclonal, dilution 1:100; BD Biosciences Pharmingen, San Diego, California, USA), VEGFR2 (rabbit monoclonal, dilution 1:100; Cell Signaling Technology, Inc., Danvers, Massachusetts, USA), and CD133 (rat monoclonal, dilution 1:35; Abcam, Cambridge, UK). To identify the mature endothelial and smooth muscle cells, the cross-sections were also stained with primary antibodies against von Willebrand factor (vWF) (mouse monoclonal, dilution 1:50; Dako A/S, Glostrup, Denmark) and α-smooth muscle actin (α-SMA) (rat monoclonal, dilution 1:100; Abcam), respectively. Briefly, the deparaffinized sections were processed through antigen retrieve for 2 minutes by pressure pot. Each section was then treated with the primary antibodies at 4°C overnight, rinsed three times in phosphate buffered saline and incubated with envision polymer of Dako EnVision + Kit (Dako A/S) for 60 minutes at room temperature. After washing in phosphate buffered saline, the sections were treated with buffered substrate and diaminobenzidine (DAB) chromogen of DAB Substrate Kit (Dako A/S) for 2 to 3 minutes at room temperature. After washed with distilled water, the sections were counterstained with hematoxylin.

To determine whether CD34 was co-localized with other specific markers, double fluorescence immunohistochemistry was also employed. Rhodamine-conjugated goat antibody (dilution 1:200; Chemicon, Temecula, California, USA) and Zenon Alexa Fluor 488 Mouse IgG Labeling Kit (Molecular Probes, Inc., Eugene, Oregon, USA) were used as the secondary antibodies to identify the immunoreactivity for each primary antibody. Finally, the sections were counterstained with 4′,6-diamidino-2-phenylindole. The fluorescence emitted from secondary antibodies was observed through appropriate filters by fluorescence microscopy (Model BZ-9000; Keyence Co., Osaka).

**Results**

**Patient 1:** Histological examination found that the terminal portion of the ICA had severe asymmetrical stenosis of the lumen. The intima was markedly thickened. The internal elastic lamina was also thickened and severely curved. The media showed no significant thickening. These findings were quite typical of moyamoya disease (Fig. 1D).

To localize the circulating EPCs in the thickened intima of moyamoya disease, immunohistochemistry against CD34, VEGFR2, and CD133 was conducted (Fig. 3). The CD34-positive cells were widely distributed in the thickened intima of the supraclinoid ICA. Likewise, the VEGFR2-positive cells were widely observed in the thickened intima of the speci-
Fig. 1 Patient 1. A–C: Computed tomography scan (A), magnetic resonance angiogram (B), and right internal carotid angiogram (C) showing severe intraventricular hemorrhage due to moyamoya disease. D: Photomicrograph disclosing marked intimal thickening that caused severe asymmetrical stenosis of the arterial lumen, thinned media, and doubly and wavelike internal elastic lamina. Scale bar = 200 μm. Hematoxylin and eosin stain, original magnification × 40.

Fig. 2 Patient 2. A, B: Magnetic resonance angiogram (A) and left vertebral angiogram (B) showing the formation of a basilar tip aneurysm associated with advanced moyamoya disease. C: Photomicrograph disclosing marked intimal thickening causing occlusion of the arterial lumen, thinned media, and doubly and wavelike internal elastic lamina. Scale bar = 200 μm. Hematoxylin and eosin stain, original magnification × 20.

Fig. 3 Patient 1. Immunohistochemical staining demonstrating CD34-positive cells (A, B) and vascular endothelial growth factor receptor-2-positive cells (C, D) widely distributed in the thickened intima. The distributions are distinct in the superficial layer of the thickened intima. Few CD133-positive cells are present (E, F). Scale bar = 100 μm. Original magnifications A, C, E: × 40; B, D, F: × 200.

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mens. More interestingly, these CD34- or VEGFR2-positive cells were densely localized close to the lumen of the arteries, and many fewer CD34- or VEGFR2-positive cells were found in the deeper layer of the thickened intima (Fig. 3). The CD133-positive cells were also identified in the thickened intima, but the immunoreactivities were faint and the number of positive cells was much smaller than that of the CD34-positive cells (Fig. 3).

To determine the phenotypes of EPCs in the thickened intima, double fluorescence immunohistochemistry was also employed. Most CD34-positive cells were densely distributed close to the arterial lumen and were also positive for VEGFR2 in the thickened intima of the specimen (Fig. 4). The CD133-positive cells were also positive for CD34, although the number of such cells was quite small (Fig. 4). Only the CD34-positive cells on the surface of the thickened intima also expressed vWF (Fig. 5). On the other hand, most CD34-positive cells in the deeper layer of thickened intima were also positive for α-SMA (Fig. 5). However, the number of α-SMA- and CD34-positive cells in the media was quite small.

Patient 2: Histological examination showed the terminal portion of the ICA was occluded. The intima was markedly thickened. The media showed no significant thickening. These findings were typical of advanced moyamoya disease (Fig. 2C).

Immunoreactivity for CD34 was less pronounced in Patient 2 than in Patient 1, but the CD34-positive cells were also distributed in the thickened intima of the supraclinoid ICA obtained from Patient 2 (Fig. 6). The VEGFR2-positive cells were also widely ob-
Fig. 4 Patient 1. Double fluorescence immunohistochemical staining showing the majority of CD34-positive cells (A, B; green) are also positive for vascular endothelial growth factor receptor-2 (C, D; red) in the thickened intima (E, F). A few CD34-positive cells (G, H; green) are also positive for CD133 (I, J; red) in the thickened intima (K, L; arrows). IEL: internal elastic lamina. Scale bar = 50 μm. Original magnifications A–F: ×100; G–L: ×400.

Fig. 5 Patient 1. Double fluorescence immunohistochemical staining showing CD34-positive cells on the surface of the thickened intima (A, B; green) are also positive for von Willebrand factor (C, D; red) in the thickened intima (E, F; arrows). CD34-positive cells in the deeper layer of the thickened intima (G, H; green) are also positive for α-smooth muscle actin (I, J; red) in the thickened intima (K, L). IEL: internal elastic lamina. Scale bar = 50 μm. Original magnifications A–F: ×100; G–L: ×400.

Fig. 6 Patient 2. Immunohistochemical staining demonstrating CD34-positive cells (A) and vascular endothelial growth factor receptor-2-positive cells (B) distributed in the thickened intima. CD133- or von Willebrand factor-positive cells are not identified in the specimen (C). The cells in the media are positive for α-smooth muscle actin. Only the cells in the deeper layer of thickened intima are also positive for α-smooth muscle actin, but immunoreactivities are faint (D). Scale bar = 100 μm. Original magnifications ×100.
observed in the thickened intima of the specimen (Fig. 6). However, no CD133-positive cells were identified in the specimen. vWF-positive cells were not identified in the specimen of Patient 2, because the terminal portion of the ICA specimen was already occluded (Fig. 6). Only the cells in the deeper layer of thickened intima were positive for α-SMA (Fig. 6).

**Discussion**

The present study demonstrated that circulating EPCs were closely involved in the intimal thickening of the supraclinoid ICA, which is the initiation site of moyamoya disease. CD34- and VEGFR2-positive cells were widely distributed in the thickened intima in both specimens from two adult patients with moyamoya disease. Therefore, this histopathological analysis directly indicates the involvement of circulating EPCs in the development of moyamoya disease. Interestingly, circulating EPCs were dense in the superficial layer of intima that is close to the lumen of the arteries in the specimen of Patient 1. The number of CD34- and CD133-positive cells was not so large in the thickened intima. Double fluorescence immunohistochemistry in the specimen of Patient 1 revealed that CD34-positive cells also expressed vWF on the surface of the intima and α-SMA in the deep layer of the thickened intima. The histological findings in Patient 2 were a little bit different from those in Patient 1. Thus, cells doubly positive for CD34 and VEGFR2 were not so densely localized in the superficial layer of thickened intima in Patient 2. Furthermore, almost no vWF-positive cells were identified in the thickened intima in Patient 2. The discrepancies between two patients may result from the different disease stage and different age. Patient 1 was aged 19 years and Patient 2 was aged 62 years. Disease stage was grade 3 in Patient 1 and grade 6 in Patient 2. Therefore, the disease stage was much more advanced in Patient 2.

In this study, primary antibodies against CD34, CD133, and VEGFR2 were used to identify the EPCs in the thickened intima of moyamoya disease vessels. Most previous studies have also employed hematopoietic, progenitor, and endothelial markers such as CD34, CD133, and VEGFR2 to characterize the circulating EPCs. However, recent studies have disclosed that functional endothelial outgrowth is derived from the circulating CD34-positive mononuclear cells that are also positive for VEGFR2, but negative for CD133. A recent review concluded that assessment of CD34- and VEGFR2-positive mononuclear cells may be the most accurate method to identify circulating EPCs at present. In fact, CD34- and VEGFR2-positive cells, but not CD34- and CD133-positive cells, were widely distributed in the thickened intima of specimens obtained from our present patients with moyamoya disease. Similar findings were reported in atherosclerotic lesions of the coronary artery. More interestingly, the distribution of CD34- and VEGFR2-positive cells in the thickened intima was not homogeneous, and these cells were densely engrafted in the superficial layer of the thickened intima, that is close to the arterial lumen, in Patient 1. These findings strongly suggest that CD34- and VEGFR2-positive cells in the thickened intima may migrate from the circulating blood and be derived from the circulating EPCs in moyamoya disease. On the other hand, these findings were not observed in Patient 2, probably because the disease stage in Patient 2 was more advanced and the ICA had already been occluded. In this situation, the pathological changes in the thickened intima might be modified because of the cessation of blood flow.

The biological features of EPCs in the bone marrow and peripheral blood have been intensively studied. According to recent studies, the circulating EPCs largely contribute to vascular repair and remodeling under physiological and pathological conditions. Thus, the EPCs are mobilized from bone marrow that is known as a major store of adult progenitor cells, and migrate onto the site of arterial injury in response to homing signals mediated by several cytokines such as VEGF, nitric oxide, and stromal cell-derived factor-1 that are released from the vessel walls and activated platelets. Then, the EPCs are retained on the vascular surface through binding to adhesion molecules such as P/E-selectin and intercellular adhesion molecule-1 and promote differentiation into ECs. The EPCs are considered to contribute to endothelial repair through direct patch and indirect angiogenic factor release in severely damaged vessels, and are also involved in neovascularization of ischemic tissue through these two mechanisms.

Recent studies have proven that cardiovascular risk factors such as hypertension, diabetes mellitus, hypercholesterolemia, and smoking impair the number and function of circulating EPCs. Reduced number of circulating EPCs is independently related to atherosclerosis disease progression, predicting the occurrence of increased incidence of cardiovascular events and death. EPC counts significantly differed between stroke patients and control subjects. Therefore, EPCs are considered as a cellular biomarker for atherosclerosis to reflect the degree of endothelial damage and turnover and the associated burden of cardiovascular risk. Furthermore, administration of bone marrow-derived cells or cul-

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tured EPCs in the acute stage of myocardial infarction may reduce infarct size and improve cardiac contractile function.\textsuperscript{10} On the other hand, there is increasing evidence that circulating EPCs are important in the complex pathophysiology of atherosclerosis, including the development and progression of atherosclerotic plaque.\textsuperscript{10,16} Immunohistochemical studies have proven that EPCs positive for CD34 and VEGFR2 are more extensively distributed within the atherosclerotic lesions of the coronary artery, compared with controls.\textsuperscript{5,22}

This study also found the different phenotypes of the CD34-positive cells in the thickened intima. The CD34-positive cells on the surface of intima expressed vWF, but not $\alpha$-SMA, whereas the cells in the deeper layer of the thickened intima expressed $\alpha$-SMA, but not vWF, indicating that the EPCs may differentiate along different pathways in response to the engrafted site within the thickened intima. Previous observations support this finding. The local microenvironment such as blood flow largely affects the directions of EPC differentiation.\textsuperscript{24} Previously, intracranial arterial lesions in moyamoya disease were reported to consist predominantly of cells with the phenotype of smooth muscle cells.\textsuperscript{15} However, bone marrow-derived EPCs give rise to most of the smooth muscle cells that contribute to arterial remodeling in models of post-angioplasty restenosis, graft vasculopathy, and hyperlipidemia-induced atherosclerosis.\textsuperscript{16}

In conclusion, this study provides the first evidence that circulating EPCs are present within the thickened intima of occlusive arterial lesions in moyamoya disease. In this study, the intracranial artery specimens obtained from only two patients with moyamoya disease were reported to consist predominantly of cells with the phenotype of smooth muscle cells.\textsuperscript{15} However, bone marrow-derived EPCs give rise to most of the smooth muscle cells that contribute to arterial remodeling in models of post-angioplasty restenosis, graft vasculopathy, and hyperlipidemia-induced atherosclerosis.\textsuperscript{16}

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