Involvement of circulating endothelial progenitor cells in carotid plaque growth and vulnerability

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OBJECTIVE The roles of endothelial progenitor cells (EPCs) in the development of carotid plaque are still obscure. This study aimed to clarify this by assessing the histological findings of specimens obtained from carotid endarterectomy.

METHODS This study included 34 patients who underwent carotid endarterectomy. MR imaging was performed to semiquantitatively analyze the components of the carotid plaques in all patients. The surgical specimens were subjected to immunohistochemistry. The distributions of the CD34-, CD133-, VEGF-2R–positive cells in the carotid plaques were precisely analyzed, and their number was quantified. Simultaneously, the CD34-positive microvessels were localized.

RESULTS The plaque component was judged as lipid-rich plaque in 19 patients, intraplaque hemorrhage (IPH) in 11 patients, and fibrous plaque in 4 patients. The CD34-positive microvessels were densely distributed in the plaque shoulder and interface-to-media regions. The CD34-, CD133-, and VEGF-2R–positive cells were mainly localized around the CD34-positive microvessels. The number of CD34-positive microvessels significantly correlated with the number of CD34-, CD133-, and VEGF-2R–positive cells (R = 0.308, p = 0.009; R = 0.324, p = 0.006; and R = 0.296, p = 0.013, respectively). Vulnerable plaques (lipid-rich and IPH) had significantly higher numbers of the CD34-positive microvessels (p = 0.007) and CD34-, CD133-, and VEGF-2R–positive cells than fibrous plaques (p = 0.031, p = 0.013, and p = 0.002).

CONCLUSIONS These findings strongly suggest that neovascularization in the plaque shoulder and interface-to-media regions may play a key role in delivering EPCs from the peripheral blood to the carotid plaque, promoting the growth of carotid plaque. Furthermore, the invaded EPCs, especially the CD133-positive immature EPCs, may be related to plaque vulnerability.

http://thejns.org/doi/abs/10.3171/2015.10.JNS151500

KEY WORDS carotid stenosis; endothelial progenitor cells; plaque; neovascularization

Worldwide, carotid stenosis is one of the most frequent causes of ischemic stroke. The annual ischemic stroke risk for patients with carotid stenosis is around 2% to 5%.11,20 Inflammatory processes, arterial remodeling, and proliferation of smooth muscle cells are the dominant features of active atherosclerotic lesions and known to be responsible for plaque rupture and the development of ischemic stroke.17 In particular, plaque neovascularization, intraplaque hemorrhage (IPH), and plaque rupture are highly interrelated events that precipitate the evolution and clinical expression of atherothrombotic diseases.14,17,27,28 These events do not occur in hypercholesterolemic mice—the most commonly used model for atherosclerosis—and thus most relevant, albeit limited, data on IPH come from clinical studies and analyses of human tissues.

During the last decade, neovascularization in plaques has been the target of intense investigations because it is the critical source of IPH and plaque rupture and is ultimately responsible for the sudden onset of stroke.14,17,27,28 Therefore, it is vital to identify new angiogenic factors and signaling pathways that can facilitate the development of diagnostic methods and tailored therapies for plaque stabilization.

Convincing evidence suggests that neovascularization is not solely the result of the proliferation of local endothelial cells (angiogenesis), but also involves bone marrow–derived circulating endothelial progenitor cells (EPCs).16

ABBREVIATIONS EPC = endothelial progenitor cell; FOV = field of view; IPH = intraplaque hemorrhage; TOF = time of flight.


INCLUDE WHEN CITING Published online February 12, 2016; DOI: 10.3171/2015.10.JNS151500.
These cells are able to proliferate and differentiate into functionally mature endothelial cells, therefore playing an important role in the regeneration of ischemic tissue and maintenance of endothelial integrity.1,2 The number of circulating EPCs increases after ischemic stroke, revealing the importance of EPC-mediated vessel repair as a physiological response of the organism to vascular injury.1,2 EPCs are believed to repair and rejuvenate the arteries even under homeostatic physiological conditions; however, they may also contribute to lesion formation by influencing plaque stability in advanced atherosclerotic plaque under specific pathological conditions.4,29 However, little is known about the effects of EPCs on the course of atherosclerotic disease in humans. Therefore, it is fundamental to understand the regulatory networks that control endothelial progenitor differentiation, as both basic and translational research, in order to develop innovative therapeutic avenues for atherosclerotic disease. Based on these observations, this study aimed to assess the hypothesis that EPCs may also play a crucial role in the development of carotid plaques and trigger the onset of ischemic stroke, in addition to their physiological functions. In this study, the expression of EPC-specific cell surface markers, including CD34, VEGFR-2, and CD133, was confirmed by immunohistochemistry.2,10,22

Methods

Patients

The Institutional Review Board at the Toyama University Hospital approved this study using human carotid plaque samples.

This study included a total of 34 patients who underwent carotid endarterectomy at our hospital between July 2012 and July 2014. There were 30 male and 4 female patients. The mean patient age was 73.1 ± 6.7 years. Of these, 4 patients were asymptomatic, and the other 30 patients developed transient ischemic attack or ischemic stroke. The mean degree of stenosis was 76.2% ± 19.5% (range 52%–99%). The surgical indications were based on the International Guidelines for Symptomatic and Asymptomatic Carotid Stenosis.8 Patient data were obtained via standardized questionnaires and preoperative admission charts. The degree of stenosis was evaluated using 3D CT angiography (GCA9300, Toshiba) and expressed as a percentage according to the North American Symptomatic Carotid Surgery Trial (NASCET) methods.21 In this study, stenosis that was greater than or equal to 70% was defined as high-grade stenosis and less than 70% was defined as low-grade stenosis. In this study, 2 certified neurosurgeons (D.K. and S.K.), who were blinded to all clinical, pathological, and imaging findings, evaluated the clinical data of all patients independently and resolved disagreements by consensus.

Immunohistological Analysis

The specimens were fixed in 4% formaldehyde, embedded in paraffin, and 4-μm-thick axial slices were prepared. The section with the largest plaque burden was classified as the culprit lesion and subjected to subsequent staining. The deparaffinized sections were processed for antigen retrieval for 2 minutes in a pressure pot. Immunohistochemical analysis was used to identify the neovascularized microvessels and EPCs in the carotid plaques. In this study, the expression levels of EPC-specific cell-surface markers, including CD34, VEGFR-2, and CD133, were confirmed by immunohistochemistry.2,10,22 Briefly, each section was treated with the primary antibody against CD34 (mouse monoclonal, dilution 1:100; BD Bioscience Pharmingen), VEGFR-2 (rabbit monoclonal, dilution 1:100; Cell Signaling Technology, Inc.), or CD133 (rat monoclonal, dilution 1:35; Abcam) for 40 minutes at 24°C and then the Envision polymer of DAKO EnVision+Kit (DAKO Cytomation) for 60 minutes. The DAB Chromogen of the DAB Substitute Kit (DAKO Cytomation) was applied for 3 to 4 minutes, and hematoxylin was used for counterstaining.

Double-fluorescence immunohistochemistry was also used to characterize the EPCs in carotid plaques. Briefly, the sections were treated with monoclonal antibodies against CD34 (mouse monoclonal, dilution 1:100; BD Bioscience Pharmingen) and VEGFR-2 (rabbit monoclonal, dilution 1:100; Cell Signaling Technology, Inc.) or against CD34 and CD133 (rat monoclonal, dilution 1:35; Abcam) at 25°C for 1 hour. Goat anti–mouse IgG H&L secondary antibody (Alexa Fluor 555 conjugate) and donkey polyclonal secondary antibody to rabbit IgG H&L (Alexa Fluor 488) were added to identify their immunoreactivities. The fluorescence emitted was observed through each appropriate filter on fluorescence microscopy and digitally photographed using a cooled charge-coupled device camera equipped to the microscope (model BZ-9000, Keyence Co.).

In the immunohistological analysis, CD34-positive tube-like formation was defined as a microvessel, as reported before.30,32

The carotid plaque was divided into 4 regions (shoulder, bottom, core, and interface-to-media region), as described previously.12 The 4 regions are represented in Fig. 1. The CD34-positive microvessels and the CD34-, CD133-, and VEGF-2R–positive cells were counted in each region using the cell counter tool of ImageJ software (National Institutes of Health). In this study, the positive cell count was performed by a certified neurosurgeon (D.K.).

MR Imaging of Carotid Plaques

In all patients, the plaque components were precisely evaluated using a 1.5-T MR apparatus, as described previously.13 For plaque characterization, long-axis and axial images of the carotid artery were obtained from the 3D gradient-echo sequence and the area with the highest stenotic degree. 3D time-of-flight (TOF) MR angiography was acquired through dual carotid bifurcations in the axial plane. The imaging sequences were as follows. For 3D TOF, the field of view (FOV) was 220 mm/87.5%, TR was 23 msec, and TE was 7 msec. For the T1-weighted images, FOV was 200 mm/100%, TR was 500 msec, and TE was 11 msec. The slice thickness was 1.2 mm for 3D TOF and 4 mm for the T1-weighted images. The plaque component was defined as highly intense when the plaque displayed a signal intensity > 200% of muscle intensity at any section in the plaque; otherwise, the plaque was categorized as isointense. The plaque was considered fibrous when it
was isointense on both T1-weighted and TOF images. The plaque was considered lipid-rich when they had high signal intensity on T1-weighted images but were isointense on TOF images. The plaque was considered to show IPH when the intensity was high on both T1-weighted and TOF images.\textsuperscript{23,26,31} In the present study, plaques with IPH and lipid-rich plaque were defined as vulnerable plaques, whereas fibrous plaques were defined as stable plaques.

### Statistical Analysis

Continuous data were statistically analyzed, using 1-factor ANOVA followed by post hoc Tukey-Kramer test, Pearson correlation coefficient, or Mann-Whitney U-test, as appropriate. Values of $p < 0.05$ were considered statistically significant.

### Results

#### Distribution of CD34-Positive Microvessels

Immunohistochemistry revealed that the CD34-positive microvessels were distributed within the plaque, although their density widely varied among patients. They were not contiguous with the lumina, strongly suggesting that a majority of them originated from the adventitial vasa vasorum (Fig. 2).

The total number of CD34-positive microvessels widely varied among patients, ranging from 12 to 381 with a mean value of $181.2 \pm 81.9$. In addition, their local distribution also differed among the 4 regions. Their numbers were $2.1 \pm 1.1$ in necrotic core, $102 \pm 61.3$ in shoulder, $20.0 \pm 21.7$ in bottom, and $77.1 \pm 31.7$ in the interface-to-media region. The number of CD34-positive microvessels was significantly larger in the shoulder than in the core and bottom ($p = 0.002$ and $p = 0.005$, respectively). Moreover, the numbers of CD34-positive microvessels were also significantly larger in the interface-to-media than in the core and bottom ($p = 0.003$ and $p = 0.008$, respectively). Thus, plaque neovascularization was more distinct in the shoulder and interface-to-media region.

#### Distribution of EPCs

Immunohistochemistry revealed that the CD34-positive cells were densely localized around the CD34-positive microvessels in the carotid plaques (Fig. 2A). Likewise, CD133- and VEGFR-2-positive cells were also distributed in the same manner (Fig. 2B and C). Furthermore, double-fluorescence immunohistochemistry demonstrated that the CD34-positive cells were also positive for either VEGFR-2 or CD133, suggesting that they are EPCs (Fig. 3).

The total number of CD34-, CD133-, and VEGF-2R-positive cells varied widely among patients. The number of CD34-positive cells ranged from 88 to 501 (mean $349.5 \pm 144.8$), that of CD133-positive cells ranged from 12 to 228 mean ($118.2 \pm 42.1$), and that of VEGFR-2-positive cells ranged from 79 to 471 (mean $312.1 \pm 122.4$).

As shown in Fig. 4, the local distributions of the CD34-, CD133-, and VEGF-2R-positive cells also differed among the 4 regions. The number of CD34-positive cells was $4.2 \pm 5.1$ in the core, $221.1 \pm 98.1$ in shoulder, $44.8 \pm 31.3$ in bottom, and $131.4 \pm 73.3$ in interface-to-media region. Thus, the number of CD34-positive cells was significantly larger in the interface-to-media region than in the core and bottom ($p = 0.001$ and $p = 0.005$, respectively). Likewise, the number of CD34-positive cells was significantly larger in the interface-to-media region than in the core and bottom ($p = 0.002$ and $p = 0.004$, respectively). Furthermore, the number of VEGFR-2-positive cells was $1.8 \pm 2.1$ in the necrotic core, $211.4 \pm 130.4$ in the shoulder, $28.9 \pm 11.4$ in the bottom, and $108.2 \pm 81.2$ in the interface-to-media region. The number of VEGF-2R–positive cells was significantly larger in the shoulder than in core and bottom ($p = 0.002$ and $p = 0.005$, respectively). The number of VEGF-2R–positive cells was significantly larger in the interface-to-media region than in the core and bottom ($p = 0.003$ and $p = 0.007$, respectively).

The number of CD133-positive cells was $0.6 \pm 1.8$ in the core, $66.8 \pm 30.3$ in the shoulder, $8.1 \pm 11.7$ in the bottom, and $40.3 \pm 33.1$ in the interface-to-media region. The number of CD133-positive cells was significantly larger in the shoulder than in the core and bottom ($p = 0.001$ and $p = 0.004$, respectively). Moreover, the number of CD133-positive cells was significantly larger in the interface-to-media region than in the core and bottom ($p = 0.003$ and $p = 0.008$, respectively). Thus, the EPCs were primarily distributed in the shoulder and interface-to-media regions of carotid plaques.

#### Relationship Between Microvessels and EPCs

As shown in Fig. 5, there were significant correlations between the number of the CD34-positive microvessels and CD34-, CD133-, and VEGF-2R–positive cells in the carotid plaques ($R = 0.308$, $p = 0.009$; $R = 0.324$, $p = 0.006$; and $R = 0.296$, $p = 0.013$, respectively). Thus, the EPCs were densely identified in neovascularization-rich carotid plaques.
Preoperative MR imaging revealed lipid-rich plaques in 19 patients, IPH in 11 patients, and fibrous plaque in 4 patients. Thus, 30 plaques were categorized as vulnerable plaques and the remaining 4 as stable plaques. Of these, 3 stable plaques (75.0%) and 1 vulnerable plaque (3.3%) were asymptomatic.

Statistical analysis showed that the vulnerable plaques had a significantly larger number of CD34-positive microvessels than stable plaques: 320.9 ± 122.9 and 120.3 ± 91.1, respectively (p = 0.007; Fig. 6). The numbers of CD34-, VEGFR-2–, and CD133-positive cells were also significantly larger in vulnerable plaques than in stable plaques (p = 0.031 for CD34, p = 0.013 for VEGFR-2, and p = 0.002 for CD133). Thus, the number of EPCs correlated very well with plaque vulnerability in the carotid artery. Of these, CD133 was the most reliable predictor for plaque vulnerability.

On the basis of the assessment of symptomatology, there were significant differences in the numbers of CD34-, VEGFR-2–, and CD133-positive cells and CD34-positive microvessels between high symptomatic stenosis and asymptomatic stenosis (p = 0.021 for CD34-positive cells, p = 0.011 for VEGFR-2–positive cells, p = 0.003 for CD133-positive cells, and p = 0.019 for CD34-positive microvessels).

On the basis of the assessment of stenosis degree, there were no significant differences in the numbers of CD34-, VEGFR-2–, and CD133-positive cells and CD34-positive microvessels between high-grade stenosis and low-grade stenosis.

**Plaque Vulnerability**

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stenosis (p = 0.099 for CD34-positive cells, p = 0.155 for VEGFR-2–positive cells, p = 0.103 for CD133-positive cells, and p = 0.211 for CD34-positive microvessels).

Discussion

The principal findings reported here are, first, that EPCs are densely distributed around the microvessels in carotid plaques. Second, both the microvessels and surrounding EPCs are unevenly localized in the shoulder and interface-to-media regions. Microvessels are not contiguous with the arterial lumen and, therefore, are considered primarily derived from the vasa vasorum during the development of carotid plaques. In addition, the homing pathway of the microvessels under pathological conditions appears completely different from that under healthy physiological conditions. Third, vulnerable plaques contain more EPCs and microvessels than stable or fibrous plaques. Finally, CD133-positive cells may be the most reliable predictor of vulnerable plaques in the carotid artery.

The Roles of EPCs and Microvessels in Carotid Plaque

EPCs are known to repair and rejuvenate the arteries under physiological conditions; however, under specific pathological conditions, they may also contribute to lesion formation by influencing plaque stability in advanced atherosclerosis. Recently, EPCs have been described as a subpopulation of pluripotent cells within the peripheral blood from the bone marrow, capable of differentiating into endothelial cells. Under physiological conditions, the integrity of the endothelial monolayer can be maintained by the replication of adjacent cells. In particular, progenitor cell homing prevented damage to the endothelium's integrity in aging-induced oxidative stress. Under pathological conditions such as atherosclerosis and cancer, however, EPCs are known to play an important role in disease progression. In fact, the present study demonstrated that EPCs coexisting with microvessels were mainly observed in the shoulder and interface-to-media regions. Moreover, the number of EPCs identified in the carotid plaque strongly depends on the quantity of the microvessels, suggesting that EPCs might be supplied via the newly developed microvessels from the vasa vasorum. The vasa vasorum acts not only as the blood conduit tube but also as a major EPC reservoir in advanced plaques. Since the EPCs play an important role in neovascularization, increasing quantities of these cells may promote neovascularization in the plaque and negatively impact plaque stability. These negative interactions may result in plaque growth and/or IPH and provoke ischemic stroke. Kumamoto et al. reported that adventitia-derived intimal vessels occurred approximately 28 times more frequently than lumen-derived vessels in coronary plaques. These data agree with the present findings that adventitia-derived angiogenesis in conjunction with the EPCs' ability to home to plaques via the microvessels from the vasa vasorum may be a key mechanism in the development of carotid plaques. Under normal conditions, the vasa vasorum forms a network of microvasculature on the surfaces of large arteries and supplies oxygen and nutrients to the outer layers of the arterial wall. Under pathological conditions, however, the role of EPCs is dramatically changed. Therefore, the vasa vasorum may be one of the major therapeutic targets to preventing the growth of carotid plaque. According to Moulton et al., it may be valuable to block angiogenesis via the vasa vasorum with angiostatins in order to reduce the accumulation of macrophages in plaques and prevent the progression of atherosclerosis. Taken together, EPC-derived angiogenesis may play a key role in plaque growth and instability by promoting the accumulation of both EPCs and macrophages.

In the present study, the majority of the EPCs and microvessels were found in the shoulder and interface-to-media regions. The data strongly suggest that the shoulder and interface-to-media regions are the responsible sites for importing EPCs from the peripheral blood and advancing...
plaque growth. These regions, may be novel therapeutic and diagnostic targets for carotid plaque treatment, although further studies are warranted prior to clinical application.

**Plaque Components and EPCs Levels**

In the current study, vulnerable plaques had a significantly larger number of CD34–, VEGFR-2–, and CD133–positive cells, as well as the CD34–positive microvessels, than stable plaques. This finding suggests that the increase in EPCs in carotid plaques was closely associated with plaque vulnerability. Angiogenesis is a pivotal defense mechanism for counteracting hypoxia and necessary for plaque regression. However, the neovessels in the plaque are also known to be susceptible to IPH. In this scenario, EPCs may play an important role in the development of plaque instability. Using flow cytometry, some investigators have analyzed the circulating EPCs in the peripheral blood and reached the conclusion that the circulating EPC level is reduced in patients with advanced carotid atherosclerosis. Therefore, the circulating EPCs may accumulate in carotid plaques, contribute to neovascularization, and decrease stability. Finally, the circulating EPC level may be decreased in patients at higher risk for ischemic stroke.

**Biological Properties of EPCs in Carotid Plaque**

In the present study, there were strongly significant differences in the number of CD133–positive cells between stable and vulnerable plaques. Asahara et al. first reported that EPCs are considered to express CD34 and VEGFR-2. The subsequent discovery of another marker, CD133, allowed further categorization of the EPC population into 2 subpopulations: CD133–positive/CD34–positive/VEGFR-2–positive cells and CD133–negative/CD34–pos-

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**Fig. 5.** The scatter plots demonstrate the significant correlations between CD34–positive microvessels and CD34–positive, VEGFR-2–positive, and CD133–positive cells (R = 0.308, p = 0.009; R = 0.324, p = 0.006; R = 0.296, p = 0.013, respectively). Figure is available in color online only.
As a heterogeneous population of progenitors, the development and severity of atherosclerosis at the cellular level. The present data strongly demonstrate that vulnerable plaques include more immature EPCs and CD133-positive/CD34-positive/VEGFR-2-positive cells, suggesting that their homing may be related to plaque instability. Therefore, immature EPCs can be a therapeutic target for unstable plaques at higher risk for the onset of ischemic stroke. As the EPCs mature toward endothelial cells, they gradually lose CD133 expression but upregulate the expression of adhesion molecules and acquire endothelial functions such as low-density lipoprotein uptake and nitric oxide synthesis. In the present study, both CD133-negative/CD34-positive/VEGFR-2-positive cells and CD133-positive/CD34-positive/VEGFR-2-positive cells coexisted in unstable plaques with high vascular density, whereas only CD133-negative/CD34-positive/VEGFR-2-positive cells existed in stable plaque with low vascular density. Thus, premature CD133-expressing EPCs are most likely mobilized to unstable plaques from the peripheral blood via the neovessels in plaques. Recently, Herrmann et al. suggested that CD34-positive/CD133-positive EPCs are enriched in the bone marrow, promote neovascularization, and have high angiogenic potential. Chi et al. reported that the CD34-positive/CD133-positive cells may predict the development and severity of atherosclerosis at the cellular level. Taken together, these individual reports may explain the positive relationship between the number of CD133-positive EPCs and the number of microvessels in carotid plaques.

As limitations, despite the limited number of samples, this study accurately reflects and encapsulates the histological characteristics of carotid plaques. Future studies should encompass larger populations and focus on the therapeutic control of EPCs in these patients.

Conclusions

Using carotid plaque specimens, the findings provide strong evidence that EPCs travel preferentially to vasa vasorum–derived microvessels and promote angiogenesis. Despite the limited sample size, this study accurately reflects and encapsulates the histological characteristics of carotid plaques. These findings strongly suggest that neovascularization in the plaque shoulder and interface-to-media regions may play a key role in delivering EPCs from the peripheral blood to the carotid plaque, promoting the growth of carotid plaques. Furthermore, the invaded EPCs, especially CD133-positive immature EPCs, may be related to plaque vulnerability. In light of the observed higher expression of EPCs in plaques from patients with symptomatic carotid stenosis, it is logical to speculate that EPCs may also contribute to the onset of ischemic stroke. Future studies should encompass larger populations and focus on therapeutic control of EPCs in these patients.

References


FIG. 6. Bar graph shows the number of CD34-positive, VEGFR-2-positive, and CD133-positive cells and CD34-positive microvessels in stable and vulnerable carotid plaques. *p < 0.05, **p < 0.01 between 2 groups. The whiskers indicate the standard deviation.
Disclosures
The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions
Conception and design: Kashiwazaki. Acquisition of data: Kashiwazaki, Noguchi. Analysis and interpretation of data: Kashiwazaki, Hayashi, Noguchi, Tanaka. Drafting the article: Kashiwazaki. Critically revising the article: Kuwayama, Hayashi, Tanaka. Reviewed submitted version of manuscript: Akioka, Kuwayama, Tanaka, Kuroda. Approved the final version of the manuscript on behalf of all authors: Kashiwazaki. Statistical analysis: Kashiwazaki, Akioka, Kuwayama, Hayashi. Administrative/technical/material support: Kuwayama, Kuroda. Study supervision: Kuroda.

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