Microvasculature of Carotid Atheromatous Plaques: Hemorrhagic Plaques Have Dense Microvessels with Fenestrations to the Arterial Lumen

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Background: Microvessels in atheromatous plaques are well known to play a role in plaque vulnerability associated with intraplaque hemorrhage, but their architecture remains unclear. The morphometry of the microvasculature and hemorrhage of human carotid atheromatous plaques (CAPs) were evaluated, and 3-dimensional (3D) reconstruction of the microvessels was performed. *Methods:* CAPs were obtained by endarterectomy in 42 patients. The specimens were analyzed using light microscopy. Plaque hemorrhage was defined as an area-containing red blood cells (>1 mm^2). To determine the histopathologic features of plaque hemorrhage, the plaque area was divided into 4 regions: cap, shoulder, lipid/necrotic core, and media. Then, the density of microvessels and macrophages in each region was quantified. Two representative lesions with either hemorrhagic or nonhemorrhagic plaque were cut into 90 serial sections. The sections were double stained with anti-CD34 and anti-α smooth muscle actin antibodies, scanned using a digital microscope, and reconstructed using TRI-SRF2 software. *Results:* The hemorrhagic plaques showed a higher density of microvessels than nonhemorrhagic plaques in the shoulder, cap, and lipid/necrotic core ($P = .03$, .009, and .001, respectively), and there was positive correlations between its density and macrophages in each regions ($P < .001$, .001, and .019, respectively). 3D imaging also revealed dense microvessels with a network structure in the cap and shoulder regions of hemorrhagic plaques, and some of the vessels were fenestrated to the arterial lumen. *Conclusions:* The microvasculature of plaques with intraplaque hemorrhage was dense, some of which fenestrated to the arterial lumen. The pathologic 3D imaging revealed precise architecture of microvasculature of

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Received August 26, 2013; revision received October 27, 2013; accepted December 3, 2013.

Conflict of interest: None.

Sources of funding: This work was supported by a Kakenhi grant; a Grant-in-Aid for Young Scientists (B) (24791505) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT); the Japan Society for the Promotion of Science; and a Japan Heart Foundation Research Grant (M.K.).

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1052-3057/$ - see front matter

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http://dx.doi.org/10.1016/j.jstrokecerebrovasdis.2013.12.003

plaques. **Key Words:** Atherosclerosis—intraplaque hemorrhage—microvasculature of plaques—three-dimensional imaging.
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Introduction

Recent studies suggest that the pathogenesis of atherosclerotic disease such as acute myocardial infarction and ischemic stroke is triggered by the instability of atherosclerotic plaques.[1,2] The histopathologic determinants of plaque vulnerability have been characterized by a large lipid/necrotic core, thin fibrous cap, marked inflammation, and intraplaque hemorrhage.[3] Intraplaque hemorrhage is one of the most important phenotypes of plaque vulnerability because it predicts future cardiovascular events.[4] It is well known that the vasa vasorum in the adventitial layer of an artery extends into plaque and is a major source of nutrients to the arterial wall.[5-7] Many studies showed that there is a close relationship between neovascularization and atherosclerosis progression,[6,8] and these new vessels serve as a source of intraplaque hemorrhage.[9] However, the precise structure of the intraplaque microvasculature is still uncertain.

In this study, we examined the microvasculature of carotid atheromatous plaques (CAPs) and its relationship with intraplaque hemorrhage. Recent advances in three-dimensional (3D) imaging technology allow visual inspection and quantification of the architecture of complex biologic tissues and pathologic lesions.[10] Taking advantage of this technology, we analyzed the 3D architecture of microvessels in CAPs. Here, we present evidence that the microvasculature of plaques with intraplaque hemorrhage is dense and fenestrated to the arterial lumen. This important finding may provide new insight into plaque vulnerability because not only the presence of microvessels but also the position of them might be concerned.

Materials and Methods

Study Population

Patients with moderate- to high-grade carotid artery stenosis (>70% reduction of the lumen diameter) were prospectively included in this study. All the patients were scheduled to have carotid endarterectomy (CEA) at the University of Ehime Hospital from January 2008 to December 2011. The exclusion criteria were as follows: patients undergoing CEA for restenosis or radiotherapy-induced carotid stenosis, patients with malignant neoplasm, chronic renal failure on hemodialysis or peritoneal dialysis, autoimmune disease, or chronic inflammation. The characteristics of the patients are shown in Supplemental Table 1. Stroke was defined as contralateral cerebrovascular or ocular ischemic symptoms lasting for more than 24 hours, and transient ischemic attack (TIA) was defined as symptoms lasting less than 24 hours. These diagnoses were performed by a neurosurgeon and confirmed by cerebral computed tomography or magnetic resonance imaging. The degree of carotid stenosis was calculated by carotid angiography using the method described in the North American Symptomatic Endarterectomy Trial. All blood tests were performed in a fasting state. This study was approved by the ethics committee of Ehime University Graduate School of Medicine. Written informed consent was obtained from all participating patients.

Preparation of Specimens

Carotid endarterectomy specimens were included in the study without any preselection based on angiography. Samples were divided transversely at the carotid bifurcation, and further sections were taken at 5-mm intervals along the longitudinal axis of the plaque segments. The sections were fixed in formalin and embedded in paraffin using a standardized protocol.[11] Serial 4-μm transverse sections were taken from each paraffin block. The transverse sections were stained with hematoxylin and eosin and elastica Masson to estimate histopathologic morphometry.

Histopathologic Morphometry

Intraplaque hemorrhage was considered to be present if an area of erythrocytes was greater than 1 mm^2.[12] Segments were subjected to histologic examination and classified based on plaque morphology such as early fatty atheroma, late fatty atheroma, thin fibrous cap atheroma, or ruptured plaque (ruptured), as reported previously.[13,14]

Quantification of Vessel Density and Macrophage Density

The area of CAPs was divided into 4 regions (cap, shoulder, lipid/necrotic core, and media), as described in a previous article.[15] The schematic representation of different plaque areas is shown in Supplemental Figure 1. For immunohistochemistry, we used primary antibodies against CD34, alpha smooth muscle actin (αSMA), and CD68 for endothelial cells, vascular smooth muscle cells, and macrophages, respectively (Dako, Copenhagen, Denmark). Staining was visualized using 3,3′-diaminobenzidine tetrachloride (Dako). Sections where the primary antibody was omitted served as specificity controls. Two of the authors (M.K. and M.N.), who are familiar with vascular pathology and blinded to the patient's clinical characteristics, examined the histology

Table 1. *The density of vessels and CD68-positive cells in the different plaque regions*

Plaque region	Nonhemorrhagic plaque (n = 19) Mean ± SE	Hemorrhagic plaque (n = 23) Mean ± SE	*P*
Density of vessels (%/area)			
Shoulder	3.3 ± 1.41	7.39 ± 1.17	.030
Cap	1.38 ± .93	5.61 ± 1.16	.009
Lipid/necrotic core	.42 ± .1	1.47 ± .26	.001
Media	.95 ± .28	.77 ± .21	.613
Density of CD68-positive cells (number/area)			
Shoulder	61.42 ± 10.99	103.65 ± 6.88	.002
Cap	36.79 ± 9.35	78.78 ± 8.79	.002
Lipid/necrotic core	68.37 ± 9.79	99.48 ± 7.7	.015
Media	18.53 ± 3.81	22.39 ± 4.22	.508

P: Student *t* test, nonhemorrhagic plaque vs hemorrhagic plaque.

sections and it showed good agreement between observers. All samples were scanned by digital microscope (Nanozoomer; Hamamatsu Photonics, Hamamatsu, Japan). We used ROI manager and cell counter tool from ImageJ software (NIH, Bethesda, MD) for the manual quantification of vessel densities (sum of each vessel area/counted area, %) and CD-68–positive cells (numbers/counted area).

3D Reconstruction of the Intraplaque Vasculature

Among the 42 patients, 2 representative cases with hemorrhagic or nonhemorrhagic CAPs were selected, which were available for preparing serial sections to perform 3D reconstruction because they had no severe structural damage of and calcification in the plaques. Then, we elucidated the difference in structure of the microvasculature between these 2 types of plaques. From the paraffin blocks, 90 serial sections (4-μm thick) were obtained. First, we stained serial sections with CD34 conjugated with aminoethyl carbazole substrate–chromogen (Dako) and scanned the sections using a digital microscope. Then, the sections were decolorized using 100% alcohol. Next, we stained the sections with αSMA conjugated with diaminobenzidine tetrachloride and again scanned the sections using a digital microscope. Finally, the entire series of digital images were stacked, and computer-assisted image reconstruction was performed for more accurate alignment. The 3D reconstruction was performed using TRI-SRF2 software (Ratoc System Eng. Co., Tokyo, Japan).[10]

Statistical Analyses

All patient data were described as the mean ± SD or the number and percentage of patients in each category. The results of the density of vessels and CD68-positive cells were described as the mean ± SEM. Comparisons between hemorrhagic and nonhemorrhagic CAPs were performed using the Student *t* test. Comparisons of multiple groups were performed using analysis of variance or exact tests. The relationships between vascular density and CD68-positive cells were determined using Pearson correlation coefficient. A *P* value less than .05 was considered statistically significant. Analyses were done with SPSS (version 11.0; SPSS Inc., Chicago, IL). Each method for statistical analysis was indicated in the legends of figure and table.

Experimental Results

Patients' Characteristics

Forty-two tissue samples were obtained from 42 patients. The patients were divided into asymptomatic TIA and stroke groups, and the characteristics of each group are shown in Supplemental Table 1. The histologic classification in these 3 patients' groups was shown in Supplemental Figure 2. The majority of patients in each group were men, and there were no differences in the prevalence of hypertension, dyslipidemia, or diabetes among the 3 groups. There were no differences in the plaque types such as early fatty atheroma, late fatty atheroma, thin fibrous cap atheroma, and ruptured plaque among the 3 groups. Concerning the prevalence of intraplaque hemorrhage, densities of vessels and CD68-positive cells were not different as well.

Histopathologic Characteristics of Hemorrhagic Plaques

The density of vessels and CD68-positive cells in different regions of CAPs were summarized in the Table 1. In hemorrhagic plaques, the density of vessels in the shoulder, cap, and lipid/necrotic core regions were greater than that in nonhemorrhagic plaques. Furthermore, hemorrhagic plaques had a higher density of CD68-positive cells than nonhemorrhagic plaques in those 3 regions but not in the media. We also estimated the relationship between vessel density and CD68-positive cell density in each plaque region (Fig 1). In the shoulder,

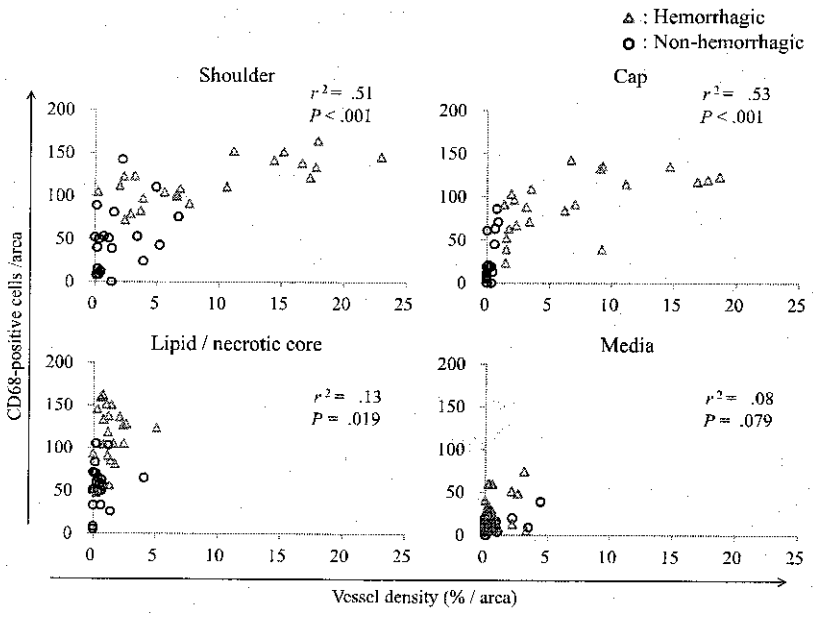

Figure 1. *The relationship between vessel density and CD68 density in each plaque region. The scatter plot shows the quantified data from .22 mm² of each plaque position. At the shoulder and cap regions, there was a positive correlation between vessel density and CD68-positive cells. In the lipid/necrotic core, there were many CD68-positive cells and showed weak positive relationship with vessel density, but its percentage is lower than shoulder and cap. In the media, the macrophage density was low, and there was no relationship with vessel density. Each value was analyzed by Pearson correlation coefficient. (Color version of figure is available online.)*

cap, and lipid/necrotic core regions, the vessel density was positively associated with CD68-positive cells' density but not in the media. It should be noted that the vessel density of the lipid/necrotic core and media is lower than shoulder and cap regions. These findings indicate a more extensive microvasculature in the shoulder and cap regions of hemorrhagic plaques, despite the greater accumulation of macrophages in the lipid/necrotic core.

3D Reconstruction of the Microvasculature in Plaques

3D reconstruction of plaques performed in 2 cases was shown in Figure 2. A nonhemorrhagic plaque had dense collagen and thick elastic fibers extending from the cap to the deep part (Fig 2, A). In contrast, a hemorrhagic plaque showed edematous, had loose collagen matrices under a thin fibrous cap, and had a large hemorrhagic core in the deep part (Fig 2, B).

The 3D reconstructed microvasculature in CAPs revealed a remarkable difference between nonhemorrhagic and hemorrhagic plaques. The nonhemorrhagic plaque had a small CD34-positive lumen that extended from the medial part to the center part of the plaque (Fig 2, C,E). In contrast, a hemorrhagic plaque contained vessels with a remarkably higher density and increased width compared with vessels in a nonhemorrhagic plaque (Fig 2, E vs F). A greater vascularity was clearly observed in the cap and shoulder regions (Fig 2, F), which may correspond to the results as shown in Table 1 and seemed to form a continuous vascular network as importantly able to be observed only by 3D imaging. Moreover, some dilated vessels were bifurcated and elongated coaxially with the arterial lumen, which clearly existed as a true lumen (Fig 2, F).

To examine the possibility that these vessels might be connected to the arterial lumen, we focused on the microvasculature adjacent to the arterial lumen side and reconstructed the vascular lumen (Fig 3). Figure 3, A shows a view from the arterial lumen side that was shown in Figure 2, D, in which CD34-positive vessels were exposed to the arterial lumen as clearly observed in Figure 3, B at a higher magnification of Figure 3, A. Figure 3, C shows a part of the serial microscopic pictures of CD34 immunostaining used to construct Figure 3, B, indicating that a microvessel was surely exposed to the arterial lumen, a small channel of a CD34-positive vessel opened directly into the arterial lumen. We designated these as fenestrated vessels to the arterial lumen.

To determine whether the fenestrated vessels receive blood flow from the arterial lumen, we examined the immunostaining of serial sections with CD34 and αSMA. Figure 3, D showed the same part as that in Figure 3, B without matrix component, indicating that CD34-positive vessels seemed to be surrounded by αSMA-positive cells. In the structure of extracted vessels from Figure 3, D, almost all CD34-positive vessels were covered with αSMA-positive cells as shown in Figure 3, E,F. Thus, these findings may indicate that these vessels were exposed to high internal pressure because of the blood flow from the arterial lumen.

Discussion

An important aspect of the present study was to compare the histologic characteristics of the plaque types

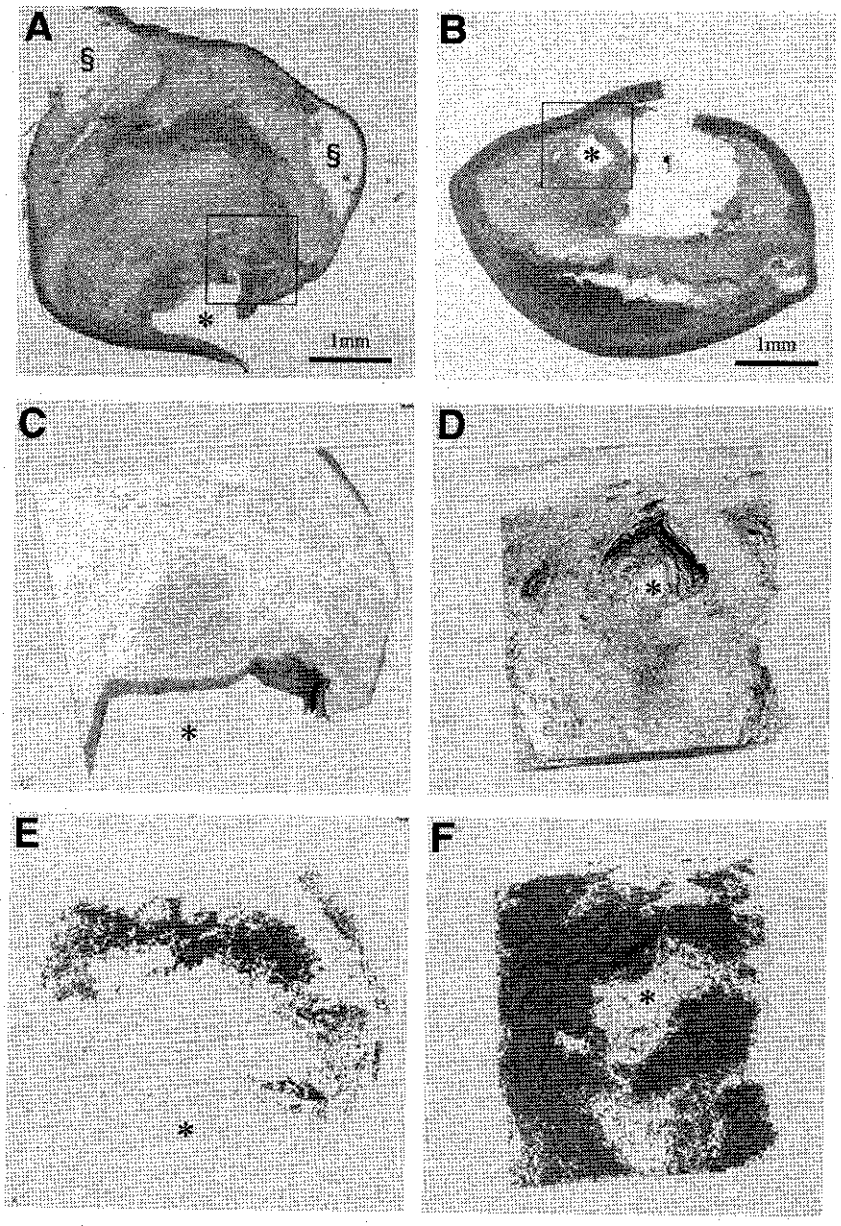

Figure 2. *The 3D reconstruction of nonhemorrhagic and hemorrhagic plaques based on histopathology. Representative cases of nonhemorrhagic plaque (A, C, and E) and hemorrhagic plaque (B, D, and F). In (A) and (B), the tissue sections were stained by elastica Masson. Stacked images of serial sections stained with CD34 with (C and D) or without matrix (E and F) are shown. The vessel density and width are clearly different between the 2 plaque types (see text). "*," Arterial lumen; "§," calcification, and "¶," artificial incision.*

in asymptomatic patients and patients with a TIA or stroke. However, there was no significant difference among them as shown in Supplemental Table 1 and Supplemental Figure 2. Redgrave et al[16] showed that the tendency for plaque inflammation and overall instability decreases with time after a stroke, such as plaques removed in short term after the most recent event were persisted instability in their histologic findings, but those findings were decreased after 180 days. Our results are consistent with their findings.

In the present study, we showed that hemorrhagic plaques from the carotid artery were characterized by a microvasculature with a high vascular density converging on the cap and shoulder regions, where the vascular density was positively associated with the CD68-positive cell density. Moreover, we demonstrated the evidence that some of the dilated vessels in a case connected directly to the arterial lumen in 3D reconstruction and we designated these vessels as "fenestrated."

Previous histopathologic studies of intraplaque vessels have shown that these vessels are responsible for the development of plaque with a fragile and leaky structure.[17] Intraplaque vessels have been thought to originate from the vasa vasorum in the adventitia and have the potential to cause intraplaque hemorrhage, recruit inflammatory cells, and accelerate the proliferation of smooth muscle cells.[9,18] However, the hemodynamics in the vessels fenestrated to the arterial lumen should be completely different from

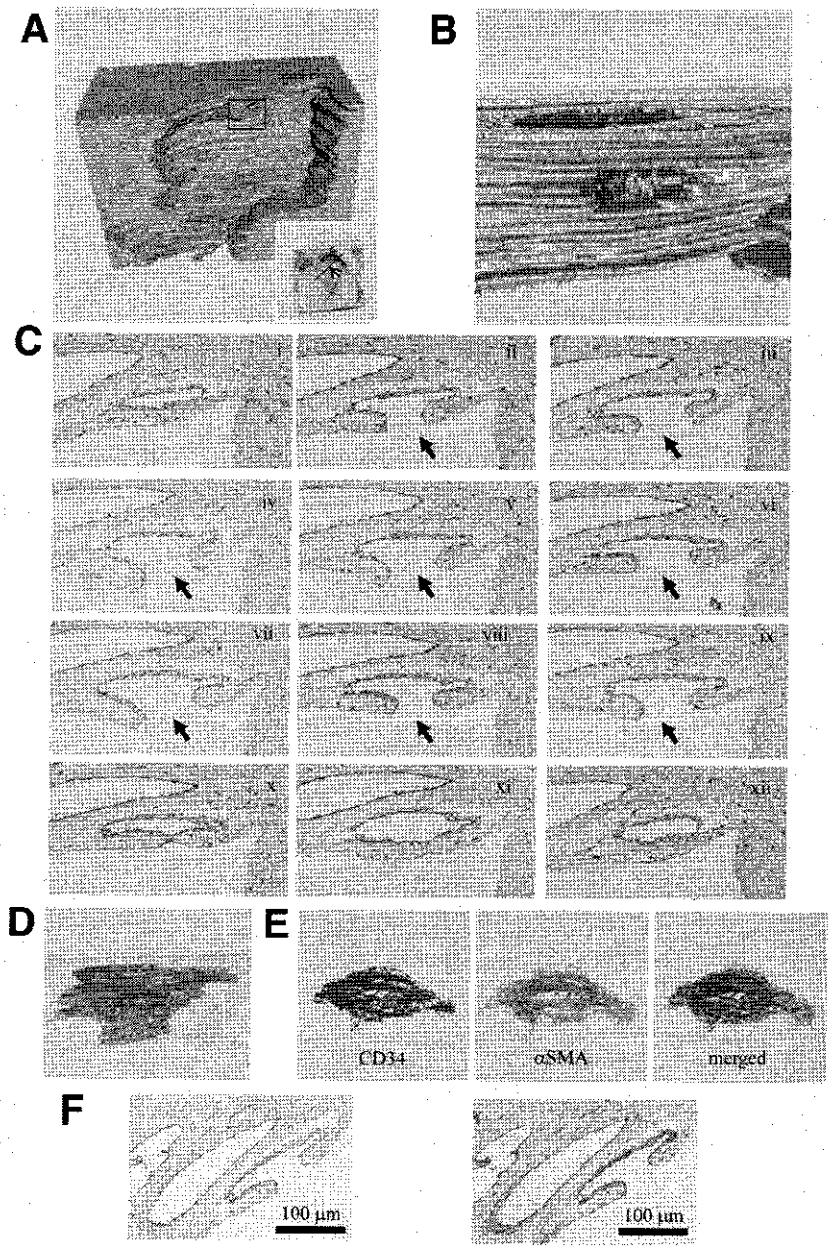

Figure 3. *The microvasculature of hemorrhagic plaque with fenestration to the arterial lumen. (A) Stacked images of serial sections stained with CD34 in the view from the arterial lumen as shown in the inset. (B) Higher magnification of (A), showing that some vessels seem to be exposed to the arterial lumen. (C, i-xii) Serial sections of a part of (B) at the face of the arterial lumen show a small channel opened (arrow). (D and E) Extracted vasculature from (B) stained with CD34 (red) and alpha smooth muscle actin (αSMA) (yellow). The exposed vessels to the arterial lumen are associated with αSMA-positive smooth muscle cells (D), and as shown in E, a view from the top of (D), these vessels form a complex network with αSMA-positive cells in the plaque. (F) A part of microscopic pictures of (D) shows that αSMA-positive cells actually cover the small channel.*

that in the vessels derived from adventitia in atheromatous plaques. The vessels fenestrated to the arterial lumen are exposed to higher blood pressure, and this should lead to greater blood flow into the plaque than the blood flow through vessels from the adventitia. Actually, the vascular wall of the fenestrated vessels had well-developed smooth muscle. These vessels may accelerate the development of inflammatory lesions and massive hemorrhage. Thus, based on their morphometry, these vessels have the potential to make plaques more vulnerable.

Where these fenestrated vessels came from? It is well known that the vasa vasorum from the adventitia supplies only the outer third of the arterial wall because of compressive forces generated from systemic blood pressure within the arterial lumen.[19] Gossl et al[20] showed that normal porcine coronary arteries have 2 different types of vasa vasorum: vasa vasorum externae originating from branches of the main artery to enter the vessel wall, and vasa vasorum internae originating directly from the arterial lumen to enter the vessel wall. Kumamoto et al[21] also showed that newly formed intimal vessels originated mainly from the adventitial vasa vasorum and also partly from the coronary lumen. Our observation of fenestrated intraplaque vessels might originate from the arterial lumen side, as well.

We supposed these fenestrated intraplaque vessels might develop in response to intraplaque remodeling. That is, an initial event such as intraplaque hemorrhage and/or plaque rupture may result in a prolonged inflammatory response, macrophage accumulation, and neovascularization. Because CEA was performed at least 3 months after ischemic symptoms in this study, there might be enough time for remodeling to occur in a ruptured plaque.

Regarding our results, we propose a hypothesis for the relationship between vulnerable plaque and its microvasculature. That is, in the early phase of CAPs, proteoglycans and lipid are deposited in the medial layer of the carotid artery,[21] and they induce SMC proliferation and macrophage infiltration[18] followed by vasa vasorum proliferation from the adventitial side. These vessels play a causative role in small hemorrhage and bring inflammatory cells into the plaque.[17,22] After an event such as plaque rupture or thrombus formation, small and large vessels develop progressively from the arterial lumen in response the plaque rupture or thrombus formation.

Conclusions

We found that microvessels at the cap and shoulder region had a remarkably higher density in hemorrhagic than nonhemorrhagic plaques. Moreover, 3D reconstruction based on histopathology showed the evidence that some of these vessels was fenestrated to the arterial lumen.

Acknowledgment: We thank Naoko Takahira, Norimasa Arita, and Tatsuhiko Miyazaki for supporting immunohistochemistry. A part of this work was presented at the 2012 ACC meeting in Chicago, IL.

Supplementary Data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jstrokecerebrovasdis.2013.12.003.

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