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Angiogenesis in Atherosclerotic Plaque Obtained From Carotid Endarterectomy: Association Between Symptomatology and Plaque Morphology

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Abstract

Carotid plaque with hemorrhage leads to cerebral embolism and ischemic stroke. Plaque angiogenesis and angiogenetic factors such as vascular endothelial growth factor (VEGF) are critical in the progression of atherosclerotic carotid plaque and intraplaque hemorrhage. The correlation between plaque angiogenesis and presence of clinical symptoms was studied in 41 specimens obtained during carotid endarterectomy from 20 symptomatic and 21 asymptomatic patients treated for carotid artery stenosis. Histological findings using hematoxylin-eosin and immunohistochemical staining against von Willebrand factor and VEGF were examined. Intraplaque hemorrhage, calcification, necrosis, and invasion of foam cells were frequently observed in the carotid plaques from symptomatic patients compared with asymptomatic patients. Higher microvessel density was found in the carotid plaques with necrosis and invasion of foam cells compared with plaques without necrosis and/or foam cell invasion, and higher expression of VEGF was found from symptomatic patients compared with asymptomatic patents. These results suggest that plaque angiogenesis and higher level of VEGF expression may enhance the progression of ischemic symptoms in patients with carotid artery stenosis. Invasive macrophages in the plaque of symptomatic patients increase levels of VEGF and might enhance plaque angiogenesis and atherosclerosis progression.

Key words: plaque angiogenesis, vascular endothelial growth factor, carotid artery stenosis, carotid endarterectomy

Introduction

Ischemic stroke remains one of the major issues in public health, and the incidence of carotid artery disease, a major risk factor for ischemic stroke, has increased recently in Japan due to westernization of lifestyle. Atherosclerosis resulting in plaque is a major cause of carotid artery stenosis, and the pathogenesis and risk factors involve hypertension, diabetes mellitus, and hyperlipidemia. Previous pathological studies of carotid artery stenosis have shown that invasion of macrophages into the intimal and smooth muscle layers, associated with adhesion of neutrophils, lymphocytes, and platelets, initiated the pathological status. Production of various cytokines and growth factors resulted in injury of the endothelium.[6,8,11] Macrophages within the carotid plaque migrate into the subendothelial space, show abundant uptake of low density lipoprotein, and transform to foam cells, which produce not only fibrous caps and lipid cores but also cytokines and growth factors, resulting in progression of atherosclerosis.

Plaque angiogenesis may be involved in the proliferation of plaque or thickening of intima, possibly as an effect of angiogenesis factors on progression of the disease.[1,2,4,8,13,14] Such processes are initially induced by shear stress on the vessel walls followed by injury of the endothelium, which accelerated production of various cytokines, and secretion and adhesion of molecules, resulting in invasion of

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macrophages and adhesion of platelets, leukocytes, and lymphocytes to the endothelium.[4,14] Clinical symptoms tend to occur in patients with progressive atherosclerosis, but the pathological conditions and clinical manifestations remain unclear. Endothelial precursor cells derived from the vasa vasorum and systemic circulation may induce invasion and formation of neovessels in the carotid plaque.[10] Several angiogenetic factors such as vascular endothelial growth factor (VEGF) might be involved in this process, as inhibition of VEGF expression reduced progression of advanced atherosclerosis.[1,5,6]

The present study investigated carotid plaque obtained from carotid endarterectomy (CEA) to examine the relationship between plaque angiogenesis and clinical symptoms.

Materials and Methods

Forty-one specimens of carotid plaque were obtained from 41 patients, 36 men and 5 women aged 61 to 83 years (mean 72.3 years), who underwent CEA for treatment of carotid artery stenosis. Degree of stenosis was determined using North American Symptomatic Carotid Endarterectomy Trial criteria. CEA was performed for symptomatic carotid artery stenosis in 20 patients and asymptomatic in 21 patients. The resected carotid plaque was rinsed briefly to remove surface blood and then fixed in formalin. Paraffin-embedded sections, 5-μm thick, were cut through the most stenotic region of the internal carotid artery in the longitudinal direction. Histological features were evaluated by a pathologist (K.K.) and the second author (T.T.) without previous knowledge of the clinical presentation associated with each section.

Sections were stained with hematoxylin and eosin to detect the presence of intraplaque hemorrhage, calcification, fibrous caps, necrosis, and invasion of foam cells. Based on the histological classification of atherosclerosis of Stary et al.,[12] specimens were classified into six groups: Type I, lesions consisting of adaptive thickening of intima; type II, lesions consisting primarily of layers of macrophage foam cells; type IV, lesions consisting of dense accumulations of extracellular lipid occupying an extensive but well-defined region of the intima; type III, intermediate stage between type II and type IV consisting of small pools of extracellular lipids; type V, lesions containing prominent new fibrous connective tissue; and type VI, lesions consisting of disruptions of the lesion surface, intraplaque hemorrhage, and thrombotic deposits.

Immunohistochemical staining was performed to detect VEGF and von Willebrand factor (vWF). Microvessel density was measured at the highest density in three tissue sections. Labeling index of VEGF was measured as the ratio of positive cells at the area of highest density as follows: +, less than 50%; ++, more than 50%.

Clinical data, risk factors, and histological and immunocytochemical findings were analyzed with two-way analysis of variance (ANOVA), the Tukey method, and Student's t test. Informed consent for collecting samples was obtained from each patient, and the study was approved by the Ethics Committee of Jikei University School of Medicine.

Results

The risk factors for atherosclerosis did not show any significant difference between symptomatic and asymptomatic patients (Table 1). Patients with or without symptoms who underwent CEA had angiographic evidence of high grade stenosis (73.5 ± 14.7% and 68.7 ± 12.8%, respectively). Symptomatic plaques had higher incidences of plaque hemorrhage, necrosis, extensive inflammatory infiltration consisting of macrophages, and fibrous cap, but no significant difference in the extent of calcification (Table 2).

Endothelial cells positive for vWF were seen in the plaque microvessels consisting of a lumen surrounded by endothelial cells. These vessels were present in the lipid-rich core and were surrounded by necrosis (Fig. 1A, B). Most VEGF-positive cells were smooth

Table 1 Comparison of risk factors in patients with symptomatic and asymptomatic carotid plaques undergoing carotid endarterectomy

Risk factor	Symptomatic (n = 20)	Asymptomatic (n = 21)	p Value
Sex, male:female	18:2	18:3	0.3372
Age, mean ± SD (yrs)	73.4 ± 7.5	71.6 ± 6.1	NS
Hypertension	8 (40.0%)	11 (52.4%)	0.1816
Diabetes mellitus	2 (10.0%)	4 (19.1%)	0.2529
Hypercholesterolemia	3 (15.0%)	4 (19.1%)	0.3035
Stenosis rate, mean ± SD (%)	73.5 ± 14.7	68.7 ± 12.8	NS

NS: not significant, SD: standard deviation.

Table 2 Histological differences between symptomatic and asymptomatic carotid plaques

Histological finding	Symptomatic (n = 20)	Asymptomatic (n = 21)	p Value
Intraplaque hemorrhage	11 (55.0%)	6 (28.6%)	0.060
Calcification	9 (45.0%)	9 (42.9%)	0.244
Necrosis	9 (45.0%)	2 (9.5%)	0.011
Foam cells	15 (75.0%)	13 (61.9%)	0.179
Fibrous cap	10 (50.0%)	6 (28.6%)	0.097

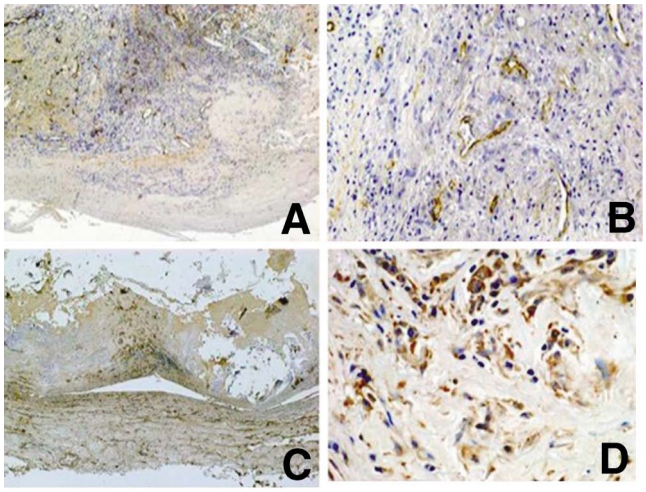

Fig. 1 Photomicrographs demonstrating expression of von Willebrand factor, an endothelial marker in atherosclerotic plaque (A: ×40, B: ×200), and foam cells in atherosclerotic plaque showing positive staining for vascular endothelial growth factor (C: ×40, D: ×200).

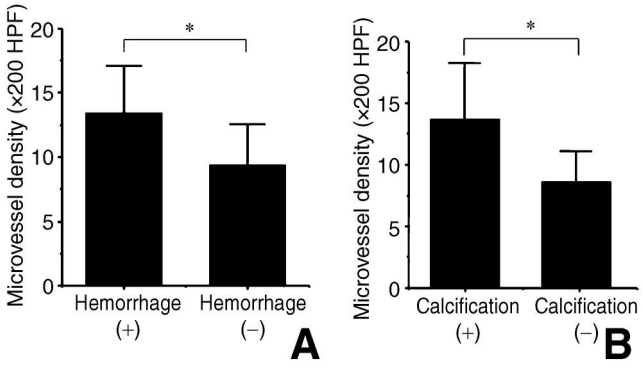

Fig. 2 Comparison of microvessel density (under ×200 magnification) in atherosclerotic plaque with (n = 15) or without (n = 26) intraplaque hemorrhage (A) and with (n = 18) or without (n = 23) calcification (B). Error bars represent standard deviation. *p < 0.001, Student's *t* test. HPF: high power field.

muscle cells. Dense VEGF-positive endothelial cells were also observed surrounding the necrotic areas which consisted of accumulations of foam cells (Fig. 1C, D).

Correlation of vascularization in the plaque with intraplaque hemorrhage or calcification was examined. Carotid plaque with hemorrhage or calcification had significantly higher microvessel density ($p < 0.001$, Student's *t* test) (Fig. 2). To verify the effect of other pathological findings such as necrosis or foam cell invasion on plaque angiogenesis, microvessel density was compared between plaque with or without those findings. Plaques with foam cell invasion had significantly more microvessels

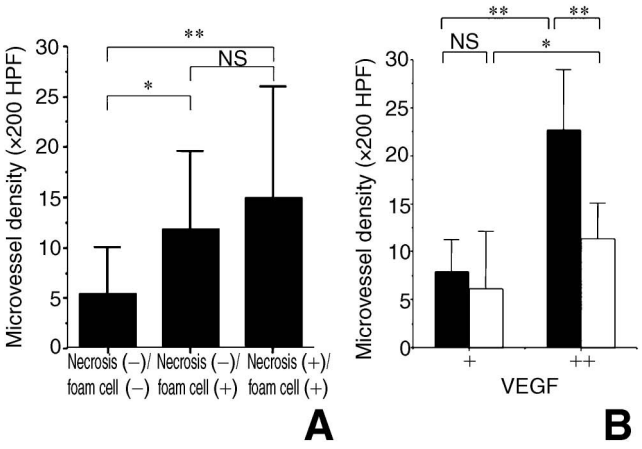

Fig. 3 A: Comparison of microvessel density (under ×200 magnification) in atherosclerotic plaque with or without necrosis and foam cell invasion. Microvessel density in the atheromatous plaque without necrosis and foam cells (n = 12) is significantly lower than that without necrosis and with foam cells (n = 18) and with necrosis and foam cells (n = 11). Error bars represent standard deviation. *p < 0.01, **p < 0.005, Post-hoc analysis by the Tukey method. HPF: high power field, NS: not significant. B: Comparison of microvessel density (under ×200 magnification) and level of vascular endothelial growth factor (VEGF) expression in asymptomatic (*open columns*) and symptomatic (*closed columns*) atherosclerotic plaques. Microvessel density in atherosclerotic plaques with low level of VEGF expression (+) was not significant between asymptomatic (n = 14) and symptomatic patients (n = 15). Microvessel density in atherosclerotic plaques with high level of VEGF expression (++) was statistically significant between asymptomatic (n = 7) and symptomatic patients (n = 5). Microvessel density in asymptomatic plaques with low (n = 14) and high (n = 7) levels of VEGF expression was also statistically significant. Error bars represent standard deviation. *p < 0.05, **p < 0.001, Post-hoc analysis by the Tukey method.

than plaque without invasion ($p = 0.01$, ANOVA). Microvessel density in the atheromatous plaque without necrosis and foam cells was significantly lower than in plaque without necrosis and with foam cells, and plaque with necrosis and foam cells ($p < 0.01$ and $p < 0.005$, respectively, Post-hoc analysis by the Tukey method) (Fig. 3A).

Finally, the correlations between level of immunohistochemical VEGF expression and microvessel density in symptomatic and asymptomatic patients were examined, since VEGF is known to be a potent angiogenetic factor which is upregulated under hypoxic conditions. Microvessel density between low (+) and high (++) levels of VEGF expression was statistically significant by two-way ANOVA. Moreover, microvessel density was not significant

in atherosclerotic plaques with low levels of VEGF expression between asymptomatic (n = 14) and symptomatic patients (n = 15). Microvessel density was statistically significant in atherosclerotic plaques with high levels of VEGF expression between asymptomatic (n = 7) and symptomatic patients (n = 5) ($p < 0.001$, Post-hoc analysis by the Tukey method). Microvessel density was also statistically significant in asymptomatic plaques between low (n = 14) and high (n = 7) levels of VEGF expression ($p < 0.05$, Post-hoc analysis by the Tukey method). Therefore, high level of VEGF expression was correlated with high microvessel density in the plaque. Symptomatic patients tended to have significantly higher microvessel density in plaque than asymptomatic patients by Post-hoc analysis (Fig. 3B). Proliferation and migration of foam cells were frequently observed in the vicinity of necrosis. VEGF expression is observed in not only smooth muscle and endothelial cells of microvessels in the plaque, but also in the foam cells coexisting with the expanding necrosis. The highest expression of

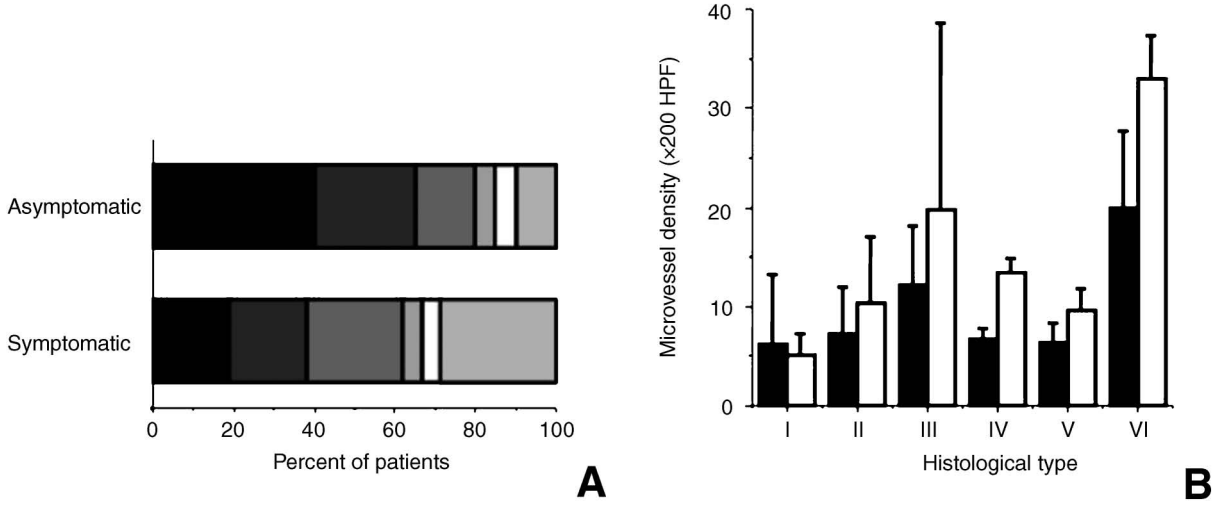

Fig. 4 A: Distribution of histological types of symptomatic and asymptomatic carotid plaques. ■: Type I, ■: type II, ■: type III, ■: type IV, □: type V, ■: type VI. B: Correlation between microvessel density and histological classification of the plaque in symptomatic (*closed columns*) and asymptomatic (*open columns*) patients. Error bars represent standard deviation. HPF: high power field.

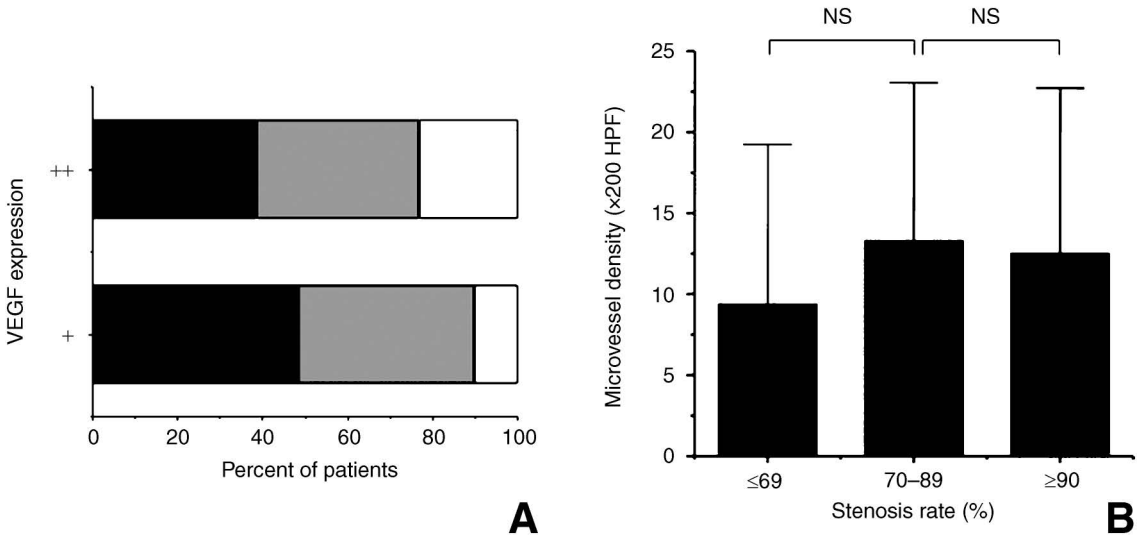

Fig. 5 A: Comparison of stenosis rate of carotid arteries and level of vascular endothelial growth factor (VEGF) expression in the plaque obtained from carotid endarterectomy (CEA). ■: ≤69%, ■: 70–89%, □: ≥90%. B: Comparison of microvessel density in the plaque obtained from CEA (under ×200 magnification) and stenosis rate of carotid arteries. Error bars represent standard deviation. NS: not significant.

VEGF was demonstrated in the foam cell-rich plaque in the vicinity of the necrosis. Some foam cell aggregates also showed extensive, diffuse, and intracellular staining.

Morphological findings according to Stary's classification[12] are demonstrated in Fig. 4. Type I, II, and III lesions, consisting primarily of layers of foam cells without lipid accumulation, were frequently observed in asymptomatic patients. Type VI lesions, containing thick layers of fibrous connective tissue with thrombus, hemorrhage, and necrosis, were mainly observed in symptomatic patients (Fig. 4A). Advanced histological types of atherosclerotic lesion were also associated with higher numbers of microvessels in the plaque (Fig. 4B).

Finally, the associations between stenosis rate, level of VEGF expression, and microvessel density were investigated, but there were no significant findings (Fig. 5).

Discussion

The present study investigated the correlation between histological findings including angiogenesis of the plaque obtained from CEA and the clinical symptoms. Histologically, symptomatic plaques had significantly more necrosis and intraplaque hemorrhage than asymptomatic plaques. Microvessel density associated with invasion of foam cells in the vicinity of necrosis was greater in hemorrhagic plaque. Angiogenesis was found in some cases of non-hemorrhagic plaque, possibly because intervention occurred just before bleeding in the plaque. According to the histological classification of atherosclerosis,[12] advanced types of lesions were mainly observed in symptomatic plaques, whereas less advanced lesions were mainly observed in asymptomatic plaques. However, asymptomatic plaques contained significantly more microvessels than symptomatic ones. These findings suggest that plaque angiogenesis in asymptomatic patients might trigger intraplaque hemorrhage and lead to symptomatic plaque. Chronological investigation is necessary to determine whether or not asymptomatic plaque with higher neovessel density become symptomatic plaque. Plaque angiogenesis might be the factor of formation of unstable plaque.

The role of plaque angiogenesis in the progression of atherosclerosis remains unknown. Microvessel numbers and expression of angiogenesis factors such as VEGF in the tumor tissues are correlated with malignancy and progression of the tumors.[3] Intraplaque hemorrhage caused by rupture of neovessels in the arterial walls results in formation of emboli and dissection of the vascular walls.[4,7–9,14] The mechanism of intraplaque hemorrhage is considered to be rupture of the neovasculature, which is poorly differentiated and anatomically immature. Increased pressure in the stenotic lesion might cause rupture of the soft plaque and occlusion of major vessels.[4,7,9,11,14] VEGF is one of the most important indicators.[1,2,14] VEGF expression is induced by hypoxia inducible factor (HIF) and hypoxic conditions.[2] The deep layer in the carotid plaque is speculated to be hypoxic, so can easily induce VEGF. VEGF induces vascular endothelial proliferation, migration, and neovascularization in the atherosclerotic plaque. The neovessels are anatomically fragile, resulting in unstable plaque or intraplaque hemorrhage.

The present study focused on the numerous neovessels in the intima, which are thought to be involved in the pathogenesis of intimal thickening and proliferation in the carotid artery stenosis. Immunohistochemical staining showed strong positive reaction for VEGF in the foam cells, which indicated that invasion of foam cells might have a potent effect on plaque angiogenesis. Histochemical findings showed endothelial cells and smooth muscle cells of the microvessels were occasionally positive for VEGF. In contrast, areas of foam cell invasion with extending necrosis consisted of clusters of VEGF-positive foam cells. These results suggest that accumulation of foam cells surrounding necrosis may be the source of VEGF resulting in plaque angiogenesis, especially in the symptomatic lesions. The source of VEGF was considered to be macrophages and smooth muscle cells.

Interestingly, the numerous neovessels observed in plaque with extending necrosis reflected the higher expression of VEGF in the pseudopalisading area surrounding the necrosis in malignant gliomas. This pathological finding suggested that the microenvironment of the unstable plaque might have been exposed to hypoxia, since VEGF is known to be induced by HIF. VEGF might have an indirect effect on the pathogenesis of intra-plaque hemorrhage caused by ruptured neovessels, which are fragile and anatomically undifferentiated. The present study also investigated whether other factors have effects on plaque angiogenesis. Risk factors for atherosclerosis, serum level of cholesterol and the value of high sensitive C-reactive protein, did not have any effect on VEGF expression and plaque angiogenesis (data not shown).

Stenosis rate in the carotid artery showed no significant difference between symptomatic and asymptomatic patients. In addition, stenosis rate and plaque angiogenesis or expression of VEGF showed no significant differences. Symptoms of stroke can

be more frequently caused by artery-to-artery embolism induced by biological changes on the surface of intima, rather than hemodynamic stress. Our findings were different from previous studies indicating the possibility of plaque angiogenesis inducing progression of symptomatic plaque, but expression of VEGF and plaque angiogenesis might show chronological discrepancies.

Our data demonstrated that induction of VEGF and plaque angiogenesis might result in unstable plaque, involving foam cell proliferation, necrosis, intra-plaque hemorrhage, and thrombosis. In particular, symptomatic patients had significantly more necrosis and plaque hemorrhage associated with VEGF expression than asymptomatic patients. Plaques with necrosis and foam cells were associated with increased number of plaque neovessels. VEGF might be secreted from foam cells surrounding necrosis and induced plaque angiogenesis may enhance progression of clinical symptoms. Further prospective studies are required to determine the effect of plaque angiogenesis on the progression of plaque formation and stroke.

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References

1) Celletti FL, Waugh JM, Amabile PG, Brendolan A, Hilfiker PR, Dake MD: Vascular endothelial growth factor enhances atherosclerotic plaque progression. *Nat Med* 7: 425–429, 2001
2) Kanno H, Kubo N, Yamamoto I: [Role of VEGF and its regulatory molecular mechanism in cervical carotid plaques]. *Surgery for Cerebral Stroke* 32: 320–324, 2004 (Japanese)
3) Lamszus K, Ulbricht U, Matschke J, Brockmann MA, Fillbrandt R, Westphal M: Levels of soluble vascular endothelial growth factor (VEGF) receptor 1 in astrocytic tumors and its relation to malignancy, vascularity, and VEGF-A. *Clin Cancer Res* 9: 1399–1405, 2003
4) Lusis AJ: Atherosclerosis. *Nature* 407: 233–241, 2000
5) Luttun A, Tjwa M, Moons L, Wu Y, Angelillo-Scherrer A, Liao F, Nagy JA, Hooper A, Priller J, De Klerck B, Compernolle V, Daci E, Bohlen P, Dewerchin M, Herbert JM, Fava R, Matthys P, Carmeliet G, Collen D, Dvorak HF, Hicklin DJ, Carmeliet P: Revascularization of ischemic tissues by PlGF treatment, and inhibition of tumor angiogenesis, arthritis and atherosclerosis by anti-Flt1. *Nat Med* 8: 831–840, 2002
6) Moulton KS, Vakili K, Zurakowski D, Soliman M, Butterfield C, Sylvin E, Lo KM, Gillies S, Javaherian K, Folkman J: Inhibition of plaque neovascularization reduces macrophage accumulation and progression of advanced atherosclerosis. *Proc Natl Acad Sci U S A* 100: 4736–4741, 2003
7) Nakaoka T, Ikeshima H, Okada T, Itou K: [Neovascularization noted in the carotid artery plaque: Contrast sonographic study with pulse inversion harmonic imaging method]. *Surgery for Cerebral Stroke* 35: 167–173, 2007 (Japanese)
8) Nakatomi H, Segawa H, Kurata A, Shiokawa Y, Nagata K, Kamiyama H, Ueki K, Kirino T: Clinicopathological study of intracranial fusiform and dolichoectatic aneurysms. Insight on the mechanism of growth. *Stroke* 31: 896–900, 2000
9) Nishikawa M, Nishio A, Takami T, Goto T, Ueda M, Hara M: [Characterization of plaque in the internal carotid artery: comparison neuroradiological findings with pathological findings]. *Surgery for Cerebral Stroke* 34: 199–205, 2006 (Japanese)
10) Sata M, Saiura A, Kunisato A, Tojo A, Okada S, Tokuhisa T, Hirai H, Makuuchi M, Hirata Y, Nagai R: Hematopoietic stem cells differentiate into vascular cells that participate in the pathogenesis of atherosclerosis. *Nat Med* 8: 403–409, 2002
11) Shimokawa H: Function of adventitia of artery: Overview. *Vessel and Endothelium* 9: 245–248, 1999
12) Stary HC, Chandler AB, Dinsmore RE, Fuster V, Glagov S, Insull W Jr, Rosenfield ME, Schwartz CJ, Wagner WD, Wissler RW: A definition of advanced types of atherosclerotic lesions and a histological classification of atherosclerosis. A report from the Committee on Vascular Lesions of the Council on Arteriosclerosis, American Heart Association. *Circulation* 92: 1355–1374, 1995
13) Sueishi K, Yonemitsu Y, Nakagawa K, Kaneda Y, Kumamoto M, Nakashima Y: Atherosclerosis and angiogenesis. Its pathophysiological significance in humans as well as in an animal model induced by the gene transfer of vascular endothelial growth factor. *Ann N Y Acad Sci* 811: 311–324, 1997
14) Yutani C: [Histopathological aspects of carotid atherosclerosis form autopsy specimens and restenosis after carotid endarterectomy from biopsy specimens]. *No Shinkei Geka Journal* 12: 459–465, 2003 (Japanese)

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