Carotid plaque instability and ischemic symptoms are linked to immaturity of microvessels within plaques

Benjamin J. Dunmore, PhD,[a] Mark J. McCarthy, PhD, FRCS,[b] A. Ross Naylor MD, FRCS,[b] and **Nicholas PJ Brindle, PhD,[b]** *Cambridge and Leicester, United Kingdom*

Background: **Instability and rupture of carotid atherosclerotic plaques leads to thromboemboli and ischemic symptoms. Angiogenesis occurs within atherosclerotic plaques, and plaque vulnerability and symptomatic carotid disease have been associated with increased numbers of microvessels. In addition to microvessel number, it is possible that the phenotypes of intraplaque vessels could influence plaque stability. To test this, the morphology and maturity of vessels within plaques from symptomatic and asymptomatic patients was determined.**

Methods: **Carotid plaques were collected after endarterectomy from a cohort of 13 asymptomatic patients and 30 symptomatic patients. Plaques were sectioned and immunostained for the presence of endothelial cells, vascular smooth muscle cells, macrophages, and vascular endothelial growth factor. Sections were assessed for microvessel morphology, maturity as judged by smooth muscle cell cover, and the presence of vascular endothelial growth factor and macrophages.**

Results: **Two types of vascular structure were observed within plaques, microvessels and dilated, highly irregular multilobular vessels. These irregular dysmorphic vessels were found almost exclusively in plaques from symptomatic patients. The dysmorphic vessels lacked smooth muscle cells and were highly immature. Plaques also contained vascular endothelial growth factor, and this was observed adjacent to the dysmorphic vessels. This growth factor was found colocalized with macrophages.**

Conclusions: **Symptomatic carotid plaques contain abnormal, immature microvessels similar to those found in tumors and healing wounds. Such vessels could contribute to plaque instability by acting as sites of vascular leakage by inflammatory cell recruitment. The immature vessels within plaques may be therapeutic targets for promoting plaque stabilization. (J Vasc Surg 2007;45:155-9.)**

Clinical Relevance: **This report shows for the first time, to our knowledge, that patients with symptomatic carotid disease, but not asymptomatic patients, have carotid plaques that contain highly immature dysmorphic microvessels. Such microvessels occur in tumors and wound healing. They are recognized as unstable and as sites of vascular leakage and inflammation. These vessels are likely to contribute to the plaque instability associated with symptoms and may be therapeutic targets for promoting plaque stabilization.**

Most strokes associated with carotid artery disease appear to occur because of thromboembolic events.[1] These thromboemboli arise from atherosclerotic plaques, and the frequency of embolization is increased in patients with recent symptoms, including transient ischemic events.[2,3] Indeed, ischemic symptoms are strong predictors of stroke in patients with internal carotid artery stenosis.[4] Importantly, formation of thromboemboli are related not to plaque size but plaque instability, with increased embolization occurring at ulcerated plaques and thrombosis associated with plaque rupture.[2,5] Thinning of the fibrous cap of the plaque, foam cell, macrophage and leukocyte infiltration, and increased inflammation are all more common in symptomatic plaques and are characteristic of plaque vulnerability.[5-7]

Angiogenesis has been noted in atherosclerotic plaques of coronary vessels, where it has been linked with plaque growth, rupture, thrombosis, and intraplaque hemorrhaging.[8-10] The stage in plaque development at which neovascularization occurs has not been precisely defined. Neovessels have been observed in early fatty streaks and appear to become more prominent and numerous as the lesion advances.[11]

The importance of neovessel formation for growth of atherosclerotic plaques has been revealed in studies with the Apo E transgenic mouse model, where inhibition of

From the Department of Obstetrics and Gynaecology, University of Cambridge,[a] and Department of Cardiovascular Sciences, University of Leicester[b].

This work was supported by a grant from the Stroke Association.

Competition of interest: none.

Reprint requests: Mark J McCarthy, PhD, FRCS, Vascular Surgery Group, Department of Cardiovascular Sciences, University of Leicester, RKCSB, PO Box 65, Leicester LE2 7LX, United Kingdom (e-mail: lmcc30@aol.com).

0741-5214/$32.00

doi:10.1016/j.jvs.2006.08.072

plaque neovascularization causes a marked suppression of lesion growth.[12] Neovascularization has also been noted in carotid atherosclerosis, and symptomatic carotid disease is associated with elevated intraplaque neovascularization.[11,13,14] These finding suggest angiogenesis within the carotid plaque could contribute to its vulnerability. In addition to the number of intraplaque vessels, it is also possible that the phenotypes of the neovessels will be important in determining plaque stability. Thus, immature intraplaque vessels could act as sites of inflammatory cell infiltration, leakage, and intraplaque hemorrhaging owing to the activated phenotype and poor integrity of such vessels.[15,16]

In the present study, we examined the maturity of neovessels within carotid plaques that were removed from patients who had undergone carotid endarterectomy for both symptomatic and asymptomatic carotid disease. We also sought to determine whether any relationship existed between intraplaque vessel maturity and symptomatology.

MATERIALS AND METHODS

Patients. This research was approved by the local Ethics Committee. Carotid plaques were collected after endarterectomy from a consecutive cohort of 43 patients. Preoperatively, patients had a >70% internal carotid artery stenosis determined by color-coded duplex ultrasonography scanning.[17] Thirteen patients were asymptomatic, and 30 were symptomatic: 5 had amaurosis fugax, 20 had transient ischemic attacks lasting <24 hours within the previous 6 months, and 5 had a cerebrovascular accident with recovery before surgery. The degree of stenosis between symptomatic and asymptomatic patients, 80% vs 82%, was not significantly different by Mann-Whitney *U* test. No difference was noted between symptomatic and asymptomatic groups with regard to risk factors. Median age was 73 years (range, 60 to 82 years) for symptomatic patients and 70 years (range, 57 to 78 years) for asymptomatic. No significant difference was found with respect to sex between the two groups.

Immunocytochemical staining. Antibodies were from DakoCytomation (Glostrup, Denmark) unless otherwise stated. Carotid endarterectomy samples were removed whole, with minimum trauma to plaques, and placed immediately in 4% formalin. Plaques were decalcified, dehydrated, embedded, and 4-μm sections were cut. Sections were probed with antibodies recognizing endothelial cells (anti-CD34; Novacastra, Newcastle, UK), smooth muscle cell (SMC) actin, macrophages (anti-CD68), and panvascular endothelial growth factor (VEGF) (Santa Cruz Biotechnology, Santa Cruz, Calif). Bound antibody was detected with the Duet/ABC system (DakoCytomation).

For each plaque, at least three sections were viewed by two independent observers blinded to the patient's symptoms. No significant difference was found for interobserver variation ($\kappa = 0.8$). In every section, all vessels were examined and categorized as class I or class II, as indicated in Results. The percentage of plaques containing each class of vessel was recorded. Within each section, all microvessels in each class were scored for extent of SMC coverage as substantial (75% to 100% coverage of vessel perimeter), +3; moderate (50% to 75%), +2; low (25% to 50%), +1; and undetectable, 0. The mean score for each vessel class was recorded.

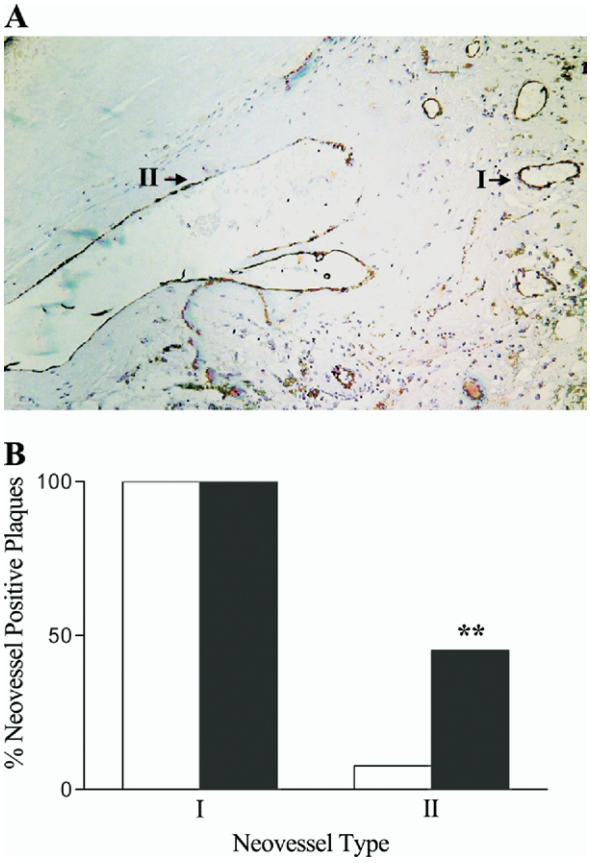

Fig 1. Morphology of neovessels within carotid atherosclerotic plaques. **A**, Representative section of plaque from symptomatic patient illustrates neovessel morphologies designated classes I and II. **B**, Percentage of plaques positive for each class of neovessel for plaques from asymptomatic *(white bars)* and symptomatic *(black bars)* patients. **Significant difference between asymptomatic and symptomatic group ($P < .02$).

Statistical analysis. Nonparametric continuous variables were compared by using the Mann-Whitney *U* test. Categoric data were analyzed with the Fisher exact test. Statistical significance was taken as $P < .05$.

RESULTS

Symptomatic lesions contain aberrant immature microvessels. It has been previously reported that symptomatic carotid plaques contain increased numbers of microvessels compared with asymptomatic lesions.[11,13,14] In the present study, we examined the nature of the microvessels within these plaques. Microvessels were visualized within plaques by immunostaining, and Fig 1 shows representative images of intraplaque vessels. Two distinct types

of vascular structure were observed: apparently normal microvessels and dilated, highly irregular multilobular vessels (Fig 1). For the purposes of this study, we designated microvessels as class I, and the highly irregular vessels, as class II. The criteria for identifying the irregular vessels was their multilobular morphology, although the class II vessels were larger than class I vessels, with a median cross-section area of 0.125 mm^2 for class II and 0.0025 mm^2 for class I. The occurrence of the vessel types was assessed in symptomatic and asymptomatic plaques (Fig 1, *B*). A significant association was found between symptomatology and the presence of the irregular vessels within plaques (Fig 1, *B*).

The irregular enlarged vessels found in symptomatic lesions are reminiscent of the neovessels observed in a number of pathologic situations, including growth of some tumors and wound healing.[18,19] Such neovessels are often referred to as immature because they have not acquired the coating of smooth muscle cells associated with mature vessels.[16] It was of interest therefore to assess the maturation state of intraplaque vessels by immunostaining for the presence of smooth muscle cells. As shown in representative images in Fig 2, class I microvessels possessed a tight coat of smooth muscle cells. The class II irregular vessels appeared to be deficient in smooth muscle cells, however, with areas entirely lacking in SMA staining, and where smooth muscle cells were detected, they were poorly organized (Fig 2, *A*). The intensity of smooth muscle cell coverage was assessed in both symptomatic and asymptomatic plaques (Fig 2, *B*). Almost no smooth muscle cells were associated with the irregular vessels that were present only in symptomatic plaques.

Immature microvessels are associated with local VEGF and macrophage accumulation. A key function of perivascular cells is to support the underlying endothelium by providing survival signals.[20,21] In the absence of perivascular support, cells microvessels undergo regression unless maintained by growth factors within the microenvironment. We therefore examined plaques for the presence of VEGF. VEGF was observed within plaques and, interestingly, could be seen localized both adjacent to vessels and at distant sites (Fig 3, *A*). VEGF immunoreactivity was observed adjacent to class I and class II vessels, although class II vessels had more intense staining than class I (Fig 3, *B*).

Potential sources of angiogenic growth factors include inflammatory cells; thus, so sections were stained for the presence of macrophages. Macrophage infiltration was evident adjacent to vessels (Fig 3). Furthermore, these areas of macrophage accumulation correlated with areas of VEGF expression, suggesting the macrophages as a possible source of this growth factor within the plaques. There was slightly more staining for macrophages adjacent to the irregular vessels compared with class I vessels, although this did not reach statistical significance (Fig 3, *C*).

DISCUSSION

Here we provide the first evidence, to our knowledge, for dysmorphic, highly dilated, immature microvessels within carotid plaques and their association with symptomatology. Because symptomatology likely reflects thromboembolic activity,[2,3] it suggests that the aberrant intraplaque vessels are linked to events at the plaque leading to neurologic events. It should be noted, however, that these data are correlative, and further work will be required to establish whether a causal link exists between the presence of these intraplaque vessels and neurologic events.

An important observation of the study was that the aberrant intraplaque vessels were largely devoid of a smooth muscle cell covering. Lack of mural cell coating is typically taken as an indication that vessels have not matured into fully functional patent vessels, and they are usually referred to as "immature" for this reason.[16] The timescale over which such vessels appear in plaques has yet to be deter-

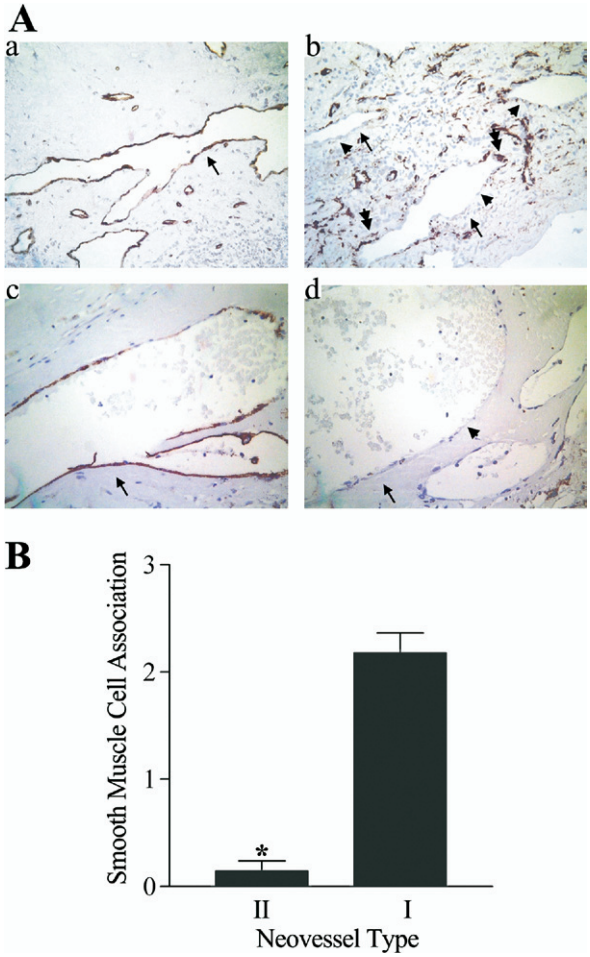

Fig 2. Dysmorphic vessels within symptomatic plaques have poor smooth muscle cell (SMC) coverage. **A**, Representative photomicrographs illustrate paucity of SMC on class II vessels. Areas of negative SMC staining *(arrowheads)* and disorganized SMC *(double arrowheads)* on class II vessels *(arrows)* are illustrated. Panels *a* and *c* are stained for endothelial cells; *b* and *d* are stained for SMC actin. (immunostain, magnification = 100×) **B**, SMC coverage of intraplaque vessels. Values are mean and SEM. $*P < .05$ vs class I vessels.

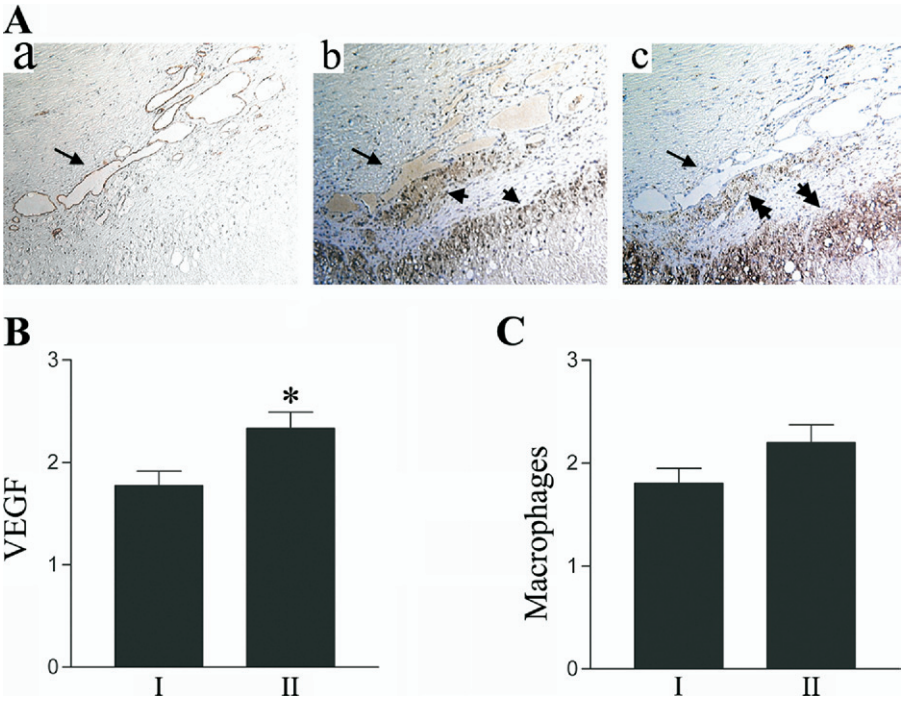

Fig 3. Vascular endothelial growth factor (VEGF) expression associated with neovessels within carotid atherosclerotic plaques. **A**, Representative photomicrographs illustrate VEGF expression within symptomatic plaques containing class II vessels *(arrows,* and colocalization of VEGF *(arrowheads)* and macrophages *(double arrowheads)*. Panel *a* endothelial staining, *(b)* immunolocalization of macrophages and *(c)* immunolocalization of VEGF. (immunostain, magnification = 100×) **B**, VEGF immunostaining adjacent to vessels as mean and SEM, **P* < .05 vs class I vessels. **C**, Macrophage immunostaining adjacent to vessels as mean and SEM.

mined, although it is certainly possible that apparently immature vessels could form late in plaque evolution in response to an increasingly proangiogenic microenvironment within the plaque. In this respect, the similarly abnormal vessels seen in some tumors are thought to arise, at least in part, due to the angiogenic milieu within the tumor.[16]

Interaction of smooth muscle cells with microvessels is important for suppressing leakage and promoting stability and quiescence of the vessels.[16,22] The absence of perivascular support in the aberrant plaque vessels suggests they may be sites where vascular leakage of blood into the plaque could occur. This could contribute to plaque friability by allowing intraplaque accumulation of red blood cells, which can act to expand the necrotic core of the plaque.[23] Furthermore, platelets and red blood cells leaked within the plaque can enhance macrophage activation and foam cell formation after their phagocytosis.[24,25] It should also be noted that immature microvessels with a proangiogenic phenotype are likely to provide entry points for intraplaque infiltration with lymphocytes and macrophages.[23] Intraplaque macrophages and lymphocytes are rich sources of growth and inflammatory factors as well as matrix metalloproteases, which have been implicated in promoting plaque instability.[5,7] It is possible, therefore, that these immature intraplaque vessels contribute to symptomatology by promoting plaque instability. In future studies, it will be of considerable interest to determine the inflammatory activation state of the intraplaque vessel types and the relationship of this to plaque stability.

The origin of the aberrant intraplaque vessels is not clear. One possibility is that localized elevations in concentrations of angiogenic growth factors within the plaque may result in remodelling of nearby vessels. Such remodelling could involve expansion of microvessels without, or preceding, a concomitant increase in smooth muscle cover. Indeed, highly enlarged irregular vessels, such as those seen in the symptomatic plaques, are often observed in conditions of elevated VEGF.[26-28] A potential source of the angiogenic factors could be infiltrating inflammatory cells. The remodelled vessels, if immature, could act as sites of further recruitment of inflammatory cells. This could lead to additional increases in VEGF concentration and further vessel remodelling and result in the highly dilated irregular vessels observed. The localization of macrophages and VEGF expression seen adjacent to these vessels in the current study may therefore have resulted from the phenotype of the immature vessels and contribute to their maintenance and even expansion.

Although further work is required, the findings of the present study may have clinical relevance. Thus, the characteristics of the irregular intraplaque vessels may allow them to be detected and distinguished from other in-

traplaque vessels by one of the current imaging methodologies, such as magnetic resonance or contrast ultrasound imaging.[29,30] Such information may be valuable for detection of vulnerable plaques. Furthermore, if the appearance of irregular vessels is found to contribute to plaque instability, these vessels could be attractive therapeutic targets. For example, it may be possible to suppress transition to symptomatic plaques, or even stabilize already symptomatic plaques, by inhibiting formation or inducing regression of these intraplaque vessels. In this context, it would be interesting to test the effects of current antiangiogenic compounds on growth and regression of the class II vessels.

CONCLUSION

This study has shown that symptomatic atherosclerotic plaques are associated with the presence of abnormal intraplaque vessels. These vessels are immature and have adjacent macrophage infiltration and elevated VEGF. Such vessels could contribute to plaque friability by acting as sites of vascular leakage and inflammatory cell recruitment within the plaque.

AUTHOR CONTRIBUTIONS

Conception and design: MM, AN, NB
Analysis and interpretation: BD, MM, NB
Data collection: BD, MM
Writing the article: BD, MM, AN, NB
Critical revision of the article: BD, MM, AN, NB
Final approval of the article: BD, MM, AN, NB
Statistical analysis: BD, MM, NB
Obtained funding: AN, NB
Overall responsibility: NB

REFERENCES

1. Golledge J, Greenhalgh RM, Davies AH. The symptomatic carotid plaque. Stroke 2000;31:774-81.
2. Sitzer M, Muller W, Siebler M, Hort W, Kniemeyer HW, Jancke L, et al. Plaque ulceration and lumen thrombus are the main sources of cerebral microemboli in high-grade internal carotid artery stenosis. Stroke 1995; 26:1231-3.
3. Markus HS, Thomson ND, Brown MM. Asymptomatic cerebral embolic signals in symptomatic and asymptomatic carotid artery disease. Brain 1995;118:1005-11.
4. NASCET. Beneficial effect of carotid endarterectomy in symptomatic patients with high-grade carotid stenosis. North American Symptomatic Carotid Endarterectomy Trial Collaborators. N Engl J Med 1991;325: 445-53.
5. Carr S, Farb A, Pearce WH, Virmani R, Yao JS. Atherosclerotic plaque rupture in symptomatic carotid artery stenosis. J Vasc Surg 1996;23: 755-65.
6. Bassiouny HS, Sakaguchi Y, Mikucki SA, McKinsey JF, Piano G, Gewertz BL, et al. Juxtalumenal location of plaque necrosis and neoformation in symptomatic carotid stenosis. J Vasc Surg 1997;26:585-94.
7. Jander S, Sitzer M, Schumann R, Schroeter M, Siebler M, Steinmetz H, et al. Inflammation in high-grade carotid stenosis: A possible role for macrophages and T cells in plaque destabilization. Stroke 1998;29: 1625-30.
8. Zhang Y, Cliff WJ, Schoefl GI, Higgins G. Immunohistochemical study of intimal microvessels in coronary atherosclerosis. Am J Pathol 1993;143:164-72.
9. O'Brien ER, Garvin MR, Dev R, Stewart DK, Hinohara T, Simpson JB, et al. Angiogenesis in human coronary atherosclerotic plaques. Am J Pathol 1994;145:883-94.
10. Kumamoto M, Nakashima Y, Sueishi K. Intimal neovascularization in human coronary atherosclerosis: Its origin and pathophysiological significance. Hum Pathol 1995;4:450-6.
11. Jeziorska M, Woolley DE. Local neovascularization and cellular composition within vulnerable regions of atherosclerotic plaques of human carotid arteries. J Pathol 1999;188:189-96.
12. Moulton KS, Vakili K, Zurakowski D, Soliman M, Butterfield C, Sylvin E, et al. Inhibition of plaque neovascularization reduces macrophage accumulation and progression of advanced atherosclerosis. Proc Natl Acad Sci USA 2003;100:4736-41.
13. McCarthy MJ, Loftus IM, Thompson MM, Jones L, London NJM, Bell PRF, et al. Angiogenesis and the atherosclerotic plaque: An association between symptomatology and plaque morphology. J Vasc Surg 1999;30:261-8.
14. Mofidi R, Crotty TB, McCarthy P, Sheehan SJ, Mehigan D, Keaveny TV. Association between plaque instability, angiogenesis and symptomatic carotid occlusive disease. Br J Surg 2001;88:945-50.
15. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. Nat Med 2000;6:389-95.
16. Jain RK. Molecular regulation of vessel maturation. Nat Med 2003;9: 685-93.
17. Grant EG, Benson CB, Moneta GL, Alexandrov AV, Baker JD, Bluth EI, et al. Carotid artery stenosis: gray-scale and Doppler US diagnosis—Society of Radiologists in Ultrasound Consensus Conference. Radiology 2003;229:340-6.
18. Paku S, Paweletz N. First steps of tumor-related angiogenesis. Lab Invest 1991;65:334-46.
19. Ren G, Michael LH, Entman ML, Frangogiannis NG. Morphological characteristics of the microvasculature in healing myocardial infarcts. J Histochem Cytochem 2002;50:71-80.
20. Benjamin LE, Hemo I, Keshet E. A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. Development 1998;125:1591-8.
21. Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E. Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. J Clin Invest 1999;103:159-65.
22. Armulik A, Abramsson A, Betsholtz C. Endothelial/pericyte interactions. Circ Res 2005;97:512-23.
23. Virmani R, Kolodgie FD, Burke AP, Finn AV, Gold HK, Tulenko TN, et al. Atherosclerotic plaque progression and vulnerability to rupture: Angiogenesis as a source of intraplaque hemorrhage. Arterioscler Thromb Vasc Biol 2005;25:2054-61.
24. Kockx MM, Cromheeke KM, Knaapen MWM, Bosmans JM, De Meyer GRY, Herman AG, et al. Phagocytosis and macrophage activation associated with hemorrhagic microvessels in human atherosclerosis. Arterioscler Thromb Vasc Biol 2003;23:440-6.
25. Kolodgie FD, Gold HK, Burke AP, Fowler DR, Kruth HS, Weber DK, et al. Intraplaque hemorrhage and progression of coronary atheroma. N Engl J Med 2003;349:2316-25.
26. Rosenstein JM, Mani N, Silverman WF, Krum JM. Patterns of brain angiogenesis after vascular endothelial growth factor administration in vitro and in vivo. Proc Natl Acad Sci (USA) 1998;95:7086-91.
27. Drake CJ, Little CD. VEGF and vascular fusion: Implications for normal and pathological vessels. J Histochem Cytochem 1999;47: 1351-6.
28. Pettersson A, Nagy JA, Brown LF, Sundberg C, Morgan E, Jungles S, et al. Heterogeneity of the angiogenic response induced in different normal adult tissues by vascular permeability factor/vascular endothelial growth factor. Lab Invest 2000;80:99-115.
29. Kerwin W, Hooker A, Spilker M, Vicini P, Ferguson M, Hatsukami T, et al. Quantitative magnetic resonance imaging analysis of neovasculature volume in carotid atherosclerotic plaque. Circulation 2003;107: 851-6.
30. Feinstein SB. Contrast ultrasound imaging of the carotid artery vasa vasorum and atherosclerotic plaque neovascularization. J Am Coll Cardiol 2006;48:236-243.

Submitted Jul 5, 2006; accepted Aug 27, 2006.