Stereological Study of Neovascularization in Temporal Arteritis

CLAES NORDBORG, KAROLINA LARSSON, and ELISABETH NORDBORG

ABSTRACT. ***Objective***. As giant cell arteritis (GCA) progresses, newly formed microvessels are one of the main sites of leukocyte-endothelial cell interaction. Our aim was to stereologically map the distribution of microvessels in the temporal arterial wall and to assess their relationship to the degree of inflammation in GCA.

Methods. Inflamed temporal arteries from 21 patients who fulfilled the American College of Rheumatology criteria for GCA were analyzed. Paraffin sections, stained with an antibody directed at vascular endothelium, were analyzed stereologically. The degree of inflammation and the surface of microvascular endothelium per volume ($\mu m^2/\mu m^3$) were assessed in 4 different layers of the arterial wall.

Results. The degree of inflammation and of vascularization was greatest in the adventitia, smaller in the media, and smallest in the intima. A significant positive relationship was observed between the degree of inflammation and the degree of vascularization in the media and in the outer and inner layers of the intima. In 8 biopsies, the microvessels formed a prominent plexus in the intima without apparent connection with microvessels in the adventitia/media, and there were no signs of endothelial budding from the arterial lumen.

Conclusion. Our results confirm that inflammation is a major determinant in neovascularization in GCA. Some new microvessels are formed by the budding of the adventitial vasa vasorum. The presence of intimal microvascular networks without apparent connection with microvessels in the media might indicate additional influence on neovascularization. (First Release Aug 15, 2006; J Rheumatol 2006;33:2020–5)

Key Indexing Terms:
GIANT CELL ARTERITIS TEMPORAL ARTERY NEOVASCULARIZATION
STEREOLOGY ENDOTHELIAL PROGENITOR CELL

Giant cell arteritis (GCA) is a vasculitis of large and medium-size arteries that mainly affects elderly individuals. Its immunopathogenesis and morphology have been thoroughly investigated[1-3]. The inflammatory cells are primarily recruited via the vasa vasorum in the arterial adventitia[2,3]. With the development of the process, newly formed microvessels are one of the main sites of leukocyte-endothelial cell interaction, leading to the progress of the inflammatory reaction[4]. Ischemic complications are less common in patients with GCA with prominent neovascularization of the temporal artery and with pronounced tissue and serum angiogenic activity[5,6]. The extent to which this beneficial effect is related to the formation of new vessels in the arterial wall and in peripheral vascular beds, respectively, remains to be investigated.

From the Department of Pathology and Department of Rheumatology, Sahlgrenska University Hospital, Göteborg, Sweden.

Supported by grants from the Göteborg Medical Society, the Göteborg Rheumatism Association, the Swedish Rheumatism Association, and Rune och Ulla Amlöfs Stiftelse.

C. Nordborg, MD, PhD, Department of Pathology; K. Larsson, MD; E. Nordborg, MD, PhD, Department of Rheumatology.

Address reprint requests to Dr. C. Nordborg, Department of Pathology, Sahlgrenska University Hospital, SE-413 45 Göteborg, Sweden. E-mail: claes.nordborg@vgregion.se

Accepted for publication May 23, 2006.

In contrast to previous semiquantitative studies of GCA, our investigation is a stereological quantification of the microvessels in the different layers of the inflamed temporal arterial wall. Instead of counting the number of microvessels, our method provides information about the total endothelial surface per volume tissue, which is more accurate and physiologically relevant. The relationship between neovascularization and the degree of inflammation was investigated.

MATERIALS AND METHODS

Twenty-one positive temporal artery biopsies from 15 women and 6 men, aged 78.8 ± 5.1 years (± SD), were collected from the data files at the Department of Pathology, Sahlgrenska University Hospital, which is the only referral center for temporal artery biopsies in Göteborg. All the patients fulfilled the American College of Rheumatology criteria for GCA[7]. Eight patients were untreated, while 12 patients received corticosteroid treatment before biopsy (mean 11 days; range 2–60). Information on steroid treatment was missing for one patient. The arterial specimens ranged in degree of inflammation from slight to pronounced. The number of cross-sections per case was 3.8 ± 2.1 SD.

The temporal artery biopsies were fixed in 4% buffered formaldehyde, cut transversely, dehydrated, and embedded in paraffin. Cross-sections 5 μm thick were stained with hematoxylin and eosin. In the cross-sections, the degree of inflammation was semiquantitatively determined in the adventitia, the media, and the peripheral and adluminal halves of the intima. For each layer, two 4-degree scales were used, one to assess the density of the inflammatory infiltrate (0: none; 1: slight; 2: moderate; 3: pronounced) and another to assess the

extension of the infiltrate (0: none; 1: < 30% of circumference; 2: ≥ 30% and < 60% of circumference; 3: ≥ 60% of circumference). For each layer of arterial cross-section, the sum of the 2 scores was calculated. Mean values for the scores for the adventitia, media, and peripheral and adluminal intima were then calculated for each biopsy.

Stereology. Consecutive sections were stained immunocytochemically, using the CD34 antibody (clone QBEnd 10; 1:50; Dako, Glostrup, Denmark), which is directed at human vascular endothelium and human lymphoid and myeloid hematopoietic progenitor cells. Human tonsil was used as a positive control. In the negative controls, the antibody was replaced by a mouse IgG1 (Dako) of corresponding concentration. The negative antibody is directed at aspergillus niger glucose oxidase, an enzyme that is neither present nor inducible in mammalian tissue. The sections were counterstained with hematoxylin.

Photographs of the immunostained cross-sections were taken in a Nikon E400 microscope, using a Nikon Coolpix 990 digital camera. The calibrated pictures were magnified, using an Epson Stylus Photo R800 colour printer. The adventitia, the media, the intima, and the half-distance between the outer and inner border of the intima were delineated on the color prints.

The degree of vascularization was assessed stereologically. By measurements on the photo prints, a surface density value, i.e., endothelial surface per volume tissue ($\mu m^2/\mu m^3$) was determined. A transparent film with a lattice grid, composed of 5 × 5 mm squares, was placed over the calibrated photo print. The number of intersections between horizontal and vertical grid lines and the CD34-positive cells was counted. The surface density was then calculated by multiplying the number of the intersections by 2 and dividing it by the total length in micrometers of the grid lines over the investigated cross-sectional area. The latter was achieved by multiplying the number of grid crosses over the investigated area by the double-calibrated width in micrometers of one of the squares of which the lattice grid is composed[8].

The mean degree of vascularization in the adventitia, media, and peripheral and adluminal intima was calculated for each biopsy. The different wall layers were independently investigated for inflammation and vascularization. The individual assessments and calculations were performed with no knowledge of the other measurements on the same vessels.

Distribution of microvessels in the intima. The material was extended to include temporal artery biopsies from 18 women and 9 men, aged 77.7 ± 5.5 SD years, that displayed neovascularization of the intima. Paraffin sections were stained immunocytochemically, using the CD34 antibody (clone QBEnd 10; 1:50). The sections were counterstained with hematoxylin. Human tonsil was used as a positive control. In the negative controls, the antibody was replaced by a mouse IgG1 (Dako) of corresponding concentration. Arterial cross-sections were examined for the distribution of microvessels in the different layers of the intima.

For further evaluation of intimal layers, 22 arterial cross-sections were stained with the elastin-van Gieson method. The cross-sections were compared with consecutive sections stained immunocytochemically, using the CD34 antibody.

Statistics. The Mann-Whitney rank-sum test was used to investigate differences between the adventitia, media, and peripheral and adluminal intima in terms of the degree of inflammation and degree of vascularization. The relationship between the degree of inflammation and vascularization in the different wall layers was tested, using ANOVA.

RESULTS

Degree of inflammation. Biopsies revealed a varying degree of mononuclear inflammatory cell infiltration in the vessel wall. Giant cells were found in 10 of 21 cases. On average, the degree of inflammation was greatest in the adventitia and decreased gradually towards the lumen (Figure 1, Table 1).

Degree of vascularization. In 10 of 44 cross-sections, microvessels were found only in the adventitia, while in another 5 cross-sections they were confined to the adventitia and the media. In the remaining 29 cross-sections, neovascularization was also seen in the intima. The average degree of vascularization, expressed as endothelial surface per volume tissue ($\mu m^2/\mu m^3$), was greatest in the adventitia and decreased gradually towards the lumen of the vessel (Figure 2, Table 2).

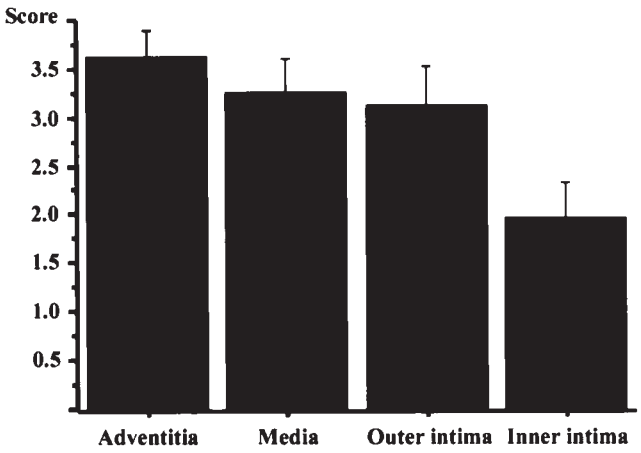

Figure 1. The inflammation score in different layers of the arterial wall (± SEM).

Correlation between vascularization and inflammation. A significant correlation between the degree of inflammation and the degree of vascularization was observed in the media ($p = 0.0003$), the peripheral half of the intima ($p = 0.0113$), and the adluminal half of the intima ($p = 0.0099$) (Figure 3).

There were no significant differences between the glucocorticosteroid treated patients and untreated patients in terms of the degree of inflammation or vascularization in the temporal artery.

Distribution of microvessels in the intima. In sections stained using the elastin-van Gieson method, the peripheral part of the inflamed intima appeared compact, displaying the formation of new elastin and collagen, as well as different degrees of granulomatous inflammation. Remnants of the internal elastic membrane (IEM) were found near the inner border of the media smooth muscle. The original IEM was generally pale, forming regular wavy lines compared to the new elastin, which was found closer to the lumen, and which formed multiple irregular lamellae (Figure 4). In contrast to the peripheral part, the adluminal part of the intima was looser, with sparse collagen, and it was free from elastin. The new intimal microvessels were found mainly in the loose adluminal part, and especially at its border towards the denser outer layer (Figures 4 and 5). In 16 of 27 biopsies, prominent circular microvascular plexa were found in this location. Eight of these displayed no apparent connection with microvessels in the adventitia/media. Another 8 biopsies showed a few single connections with media microvessels. There was no detectable sprouting from the luminal endothelium to the intimal plexa. Eleven biopsies displayed a more diffuse intimal distribution of microvessels.

Table 1. Statistical evaluation (p values) of difference in inflammation score between arterial wall layers (left column) and other layers (score ± SEM).

	Adventitia	Media	Outer Intima	Inner Intima
Adventitia	—	0.4812	0.5050	0.0059
Media	0.4812	—	0.2965	0.0193
Outer intima	0.5050	0.2965	—	0.0200
Inner intima	0.0059	0.0193	0.0200	—
Score	3.6 ± 0.3	3.3 ± 0.4	3.1 ± 0.4	2.0 ± 0.4

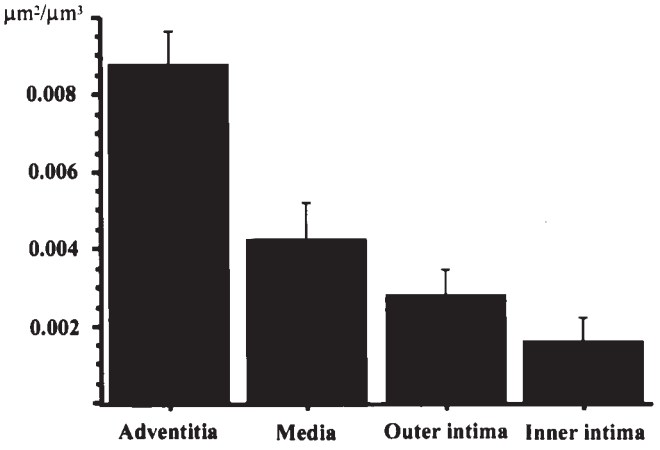

Figure 2. The degree of vascularization ($\mu m^2/\mu m^3$) in different layers of the arterial wall (± SEM).

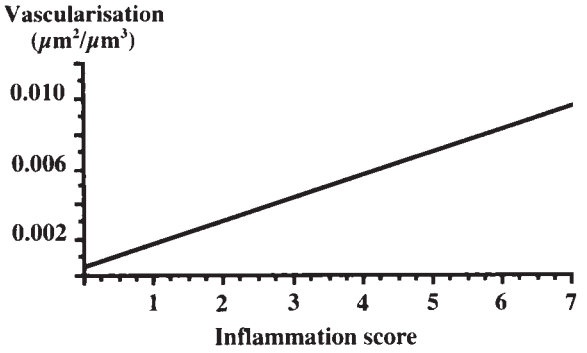

Figure 3. Correlation between vascularization ($\mu m^2/\mu m^3$) and degree of inflammation (inflammation score), based on regression analysis of observations in all arterial wall layers of all biopsies.

The mean biopsy length and number of cross-sections were somewhat greater in arteries with isolated intimal microvessels than in the other 2 categories (Table 3).

DISCUSSION

Our observations support the hypothesis that inflammation is a major inducer of neovascularization in the arterial wall in GCA. A highly significant correlation between the degree of inflammation and the degree of vascularization was found in the media and the outer and inner layers of the intima. However, no significant correlation between the 2 factors was observed in the adventitia, although, on average, the degree of inflammation and vascularization was greatest in this layer. There may be several reasons for this. First, the adventitia is rich in microvessels, even in uninflamed arteries. Not all the vessels are therefore newly formed, which leads to some overestimation of neovascularization. Second, a proportion of the inflammatory cells that enter the artery via adventitial microvessels migrate from the adventitia into the vessel wall. The influence of these cells on formation of new microvessels might therefore be greater at their targets in the media and intima than in the adventitia.

The distribution of new vessels in the different layers of the arterial wall indicates that part of the neovascularization in GCA proceeds from the adventitia towards the arterial lumen through the sprouting of adventitial microvessels (angiogenesis). This is illustrated by the fact that vascularization was greatest in the adventitia and decreased gradually towards the lumen. Further, in 15 of 44 vascular cross-sections, microvessels were found only in the adventitia or in the adventitia and media but not in the intima.

Table 2. Statistical evaluation (p values) of the difference in vascularization between the arterial wall layers (left column) and other layers (surface density: $\mu m^2/\mu m^3 \times 10^{-3}$ ± SEM).

	Adventitia	Media	Outer Intima	Inner Intima
Adventitia	—	0.0004	< 0.0001	< 0.0001
Media	0.0004	—	0.7628	0.0335
Outer intima	< 0.0001	0.7628	—	0.0994
Inner intima	< 0.0001	0.0335	0.0994	—
Surface density	8.8 ± 0.9	4.2 ± 1.0	2.8 ± 0.7	1.6 ± 0.6

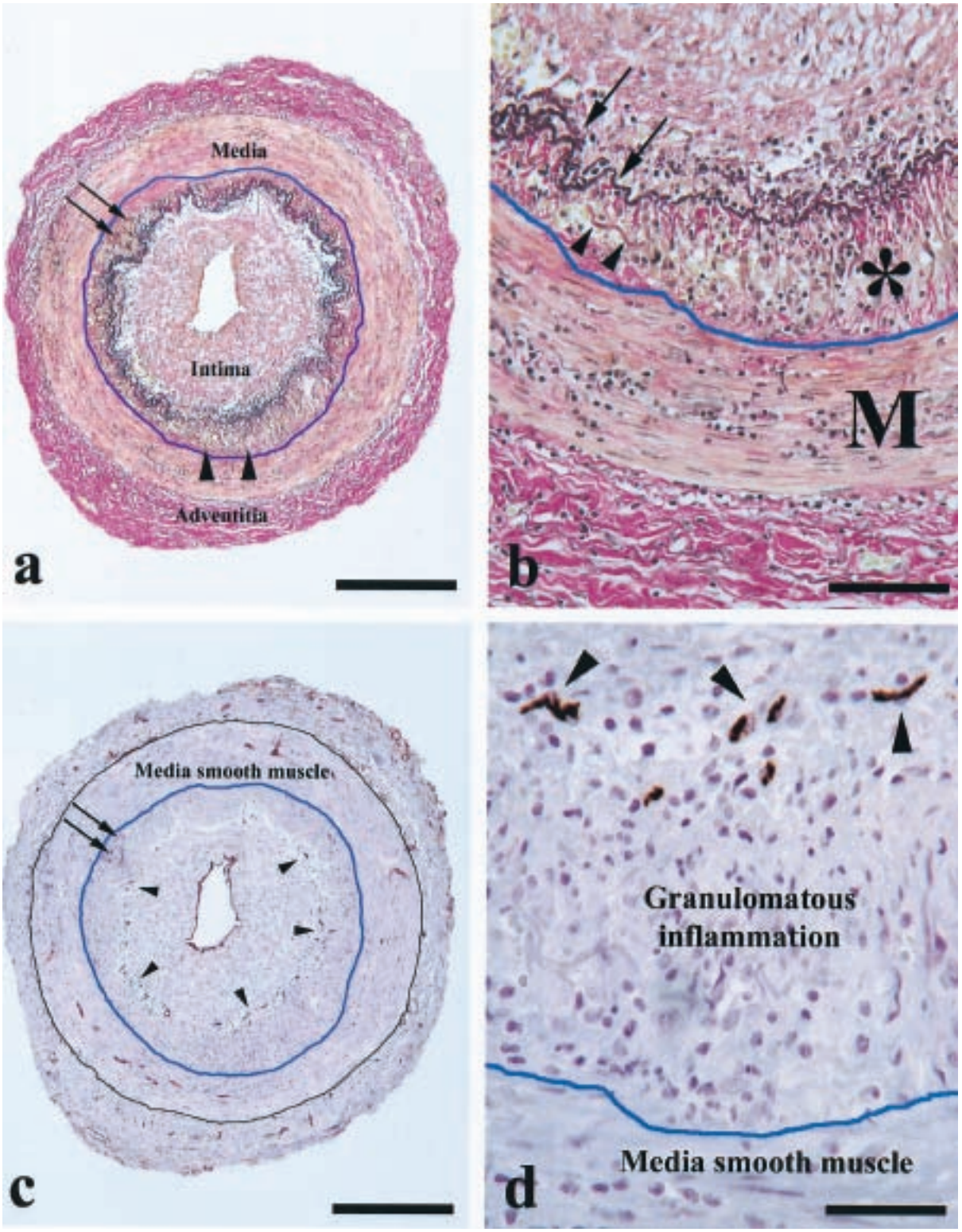

Figure 4. A. Cross-section of a temporal artery displaying diffuse inflammation and severe intimal thickening. Blue line shows the inner demarcation of the media smooth muscle. Note numerous dark elastin lamellae in the outer part of the intima and the lack of elastin in its adluminal part. Arrowheads: granulomatous inflammation. Arrows: giant cells (Elastin-van Gieson stain; bar = 390 μm). B. Detail of A. Blue line shows the inner demarcation of the media smooth muscle. M: media invaded by mononuclear inflammatory cells. Asterisk: granulomatous inflammation. Arrowheads: remnant of the internal elastic membrane. Arrows: new dark elastin lamellae (bar = 130 μm). C. Consecutive section of the same artery. The blue line shows the inner demarcation and the black line the outer border of the media smooth muscle. Note the brown CD34-positive microvessels in the adventitia, media, and intima. There is no apparent connection between microvessels in the loose layer of the intima (arrowheads) and vessels in the media. Arrows: giant cells (anti-CD34; bar = 390 μm). D. Detail of C. Blue line shows the inner border of the media smooth muscle. Arrowheads: early neovascularization on the adluminal side of the granulomatous layer (anti-CD34; bar = 50 μm).

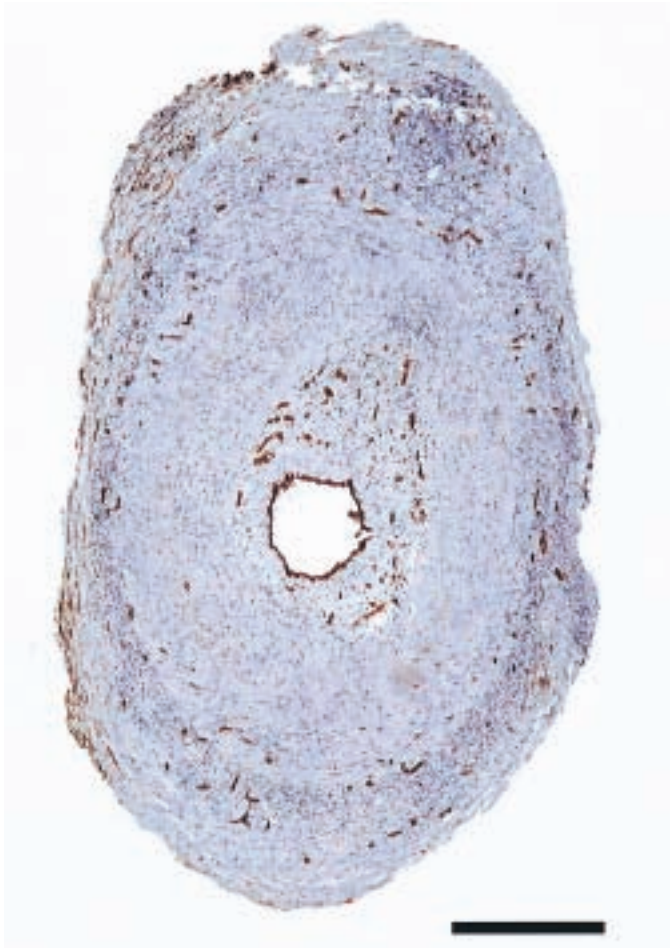

Figure 5. Advanced intimal neovascularization in a diffusely inflamed artery. There is no apparent connection between the intimal plexus and microvessels in the peripheral layers of the vessel wall (anti-CD34; bar = 650 μm).

Previous studies have adressed the intimal neovascularization in GCA[4,5]. According to our observations, the formation of new microvessels shows a striking preference for the loose adluminal part of the intima. In 8 of 27 biopsies that displayed intimal neovascularization, the microvessels formed an isolated circular plexus in this inner layer, with no apparent connection with vascular sprouts in the adventitia/media (Figures 4 and 5). Nor was there any detectable sprouting from the luminal endothelium of the temporal artery. In another 8 biopsies, single microvascular sprouts were found between the intimal plexus and the media. The latter observation may indicate that the intimal microvascular plexa are formed by sprouting from the media. Conversely, the presence of isolated intimal microvascular plexa in almost one-third of the biopsies might indicate that microvessels are formed in the intima, later to be connected to a peripheral microvascular plexus by sprouting from one or both plexa. Theoretically, the observations may thus indicate an alternative form of neovascularization, via the recruitment and migration of stem cells. There is growing evidence that hematopoietic stem cells as well as marrow-derived and non-marrow-derived mesenchymal stem cells play important roles in vascular biology because of their ability to differentiate into both endothelial and smooth muscle cells[9]. Endothelial progenitor cells (EPC) may be considered to occupy the middle of the differentiation spectrum between hematopoietic stem cells and endothelial cells. EPC, which are localized in the postnatal bone marrow, may be mobilized to induce the formation of microvessels in peripheral tissues. Observations in experimental animals and humans indicate that the recruitment and homing of marrow-derived EPC is a natural response to tissue ischemia; EPC are mobilized following burns and myocardial infarction. Proinflammatory cytokines, growth factors such as erythropoietin and vascular endothelial growth factor (VEGF), estrogen, and physical activity have been reported to stimulate the recruitment of EPC[10-14].

A recent study[15] addressed the possible role of VEGF-634 G → C polymorphism in GCA clinical manifestations. The G allele was significantly increased in patients with biopsy-proven GCA with ischemic complications, and additionally, a higher risk of developing severe ischemic complications was observed for –634 GG homozygous individuals. Interestingly, functional studies have demonstrated that VEGF –634 G allele is associated with lower circulating VEGF levels *in vivo*, reduced VEGF transcription, and less expression of internal ribosomal entry site-mediated VEGF[16]. Thus, it is plausible that patients with GCA carrying the VEGF –634 G allele might undergo inhibition of compensatory mechanisms of neoangiogenesis, and therefore an exacerbation of the ischemic phenomena. Accordingly, Cid, *et al*[5,6] showed that a high serum angiogenic activity and prominent neovascularization of the temporal arterial wall are associated with a low prevalence of ischemic complications in GCA. It remains to be determined whether this effect is related to the mobilization of EPC. Further efforts should be made to elucidate this question morphologically as well as by using other methods; cir-

Table 3. Arteries and cross-sections with various distribution of microvessels in the intima.

	No. of Biopsies	Total No. of Cross-sections	Biopsy Length, mm ± SEM	No. of Sections per Biopsy, ± SEM
Isolated plexus	8	26	15.9 ± 1.8	3.3 ± 0.4
Plexus with minor connection with media microvessels	8	21	15.0 ± 3.4	2.6 ± 0.5
Diffusely spread microvessels	11	31	13.3 ± 2.0	2.8 ± 0.4

culating human EPC may be isolated, quantified, and analyzed[10,11].

Stem/progenitor cells could not be immunocytochemically identified in our study (data not shown). The reasons for this may be manifold. First, if present, they are likely to constitute a very small part of the inflammatory infiltrate. Second, CD34-positive stem/progenitor cells lose their CD133 expression when transforming into more mature endothelial-like cells; it is unclear whether this takes place during their transmigration from the bone marrow into the circulation or later during their circulation or migration[10,11]. For this reason, as the maturing EPC expresses CD34, the possibility that some of the new microvessels in the inflamed arteries are of progenitor cell origin could be neither proved nor excluded.

REFERENCES

1. Nordborg E, Nordborg C. Giant cell arteritis: epidemiological clues to its pathogenesis and an update on its treatment. Rheumatology Oxford 2003;42:413-21.
2. Weyand CM, Goronzy JJ. Giant cell arteritis and polymyalgia rheumatica. Ann Intern Med 2003;139:505-15.
3. Weyand CM, Ma-Krupa W, Goronzy JJ. Immunopathways in giant cell arteritis and polymyalgia rheumatica. Autoimmun Rev 2004;3:46-53.
4. Cid MC, Cebrián M, Font C, et al. Cell adhesion molecules in the development of inflammatory infiltrates in giant cell arteritis. Arthritis Rheum 2000;43:184-94.
5. Cid MC, Hernández-Rodríguez J, Esteban M-J, et al. Tissue and serum angiogenetic activity is associated with low prevalence of ischemic complications in patients with giant-cell arteritis. Circulation 2002;106:1664-71.
6. Hernandez-Rodriguez J, Segarra M, Vilardell C, et al. Elevated production of interleukin-6 is associated with a lower incidence of disease-related ischemic events in patients with giant-cell arteritis: Angiogenic activity of interleukin-6 as a potential protective mechanism. Circulation 2003;107:2428-34.
7. Hunder GG, Bloch DA, Michel BA, et al. The American College of Rheumatology 1990 criteria for the classification of giant cell arteritis. Arthritis Rheum 1990;33:1122-8.
8. Weibel ER. Stereological methods. I. Practical methods for biological morphometry. London: Academic Press; 1979.
9. Riha GM, Lin PH, Lumsden AB, Yao Q, Chen C. Application of stem cells for vascular tissue engineering. Tissue Eng 2005;11:1535-52.
10. Masuda H, Asahara T. Post-natal endothelial progenitor cells for neovascularisation in tissue regeneration. Cardiovasc Res 2003;58:390-8.
11. Hristov M, Erl W, Weber PC. Endothelial progenitor cells. Mobilization, differentiation, and homing. Arterioscler Thromb Vasc Biol 2003;23:1185-9.
12. Hill JM, Zalos G, Halcox JPJ, et al. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. N Engl J Med 2003;348:593-600.
13. Asahara T, Kawamoto A. Endothelial progenitor cells for postnatal vasculogenesis. Am J Physiol Cell Physiol 2004;287:C572-C579.
14. Aicher A, Zeiher AM, Dimmeler S. Mobilizing endothelial progenitor cells. Hypertension 2005;45:321-5.
15. Rueda B, Lopez-Nevot MA, Lopez-Diaz MJ, Garcia-Porrua C, Martin J, Gonzalez-Gay MA. A functional variant of vascular endothelial growth factor is associated with severe ischemic complications in giant cell arteritis. J Rheumatol 2005;32:1737-41.
16. Lambrechts D, Storkebaum E, Morimoto M, et al. VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. Nat Genet 2003;34:383-94.