Neovascularization and Angiogenic Factors in Advanced Human Carotid Artery Stenosis

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Background: Most atherosclerotic lesions are vascularized, so neovessels may also contribute to plaque progression and vulnerability, but their precise role of neovessels in atherosclerosis is still unknown. The aim of this study was to analyze the possible relationships among neovascularization, relevant angiogenic factors, and plaque vulnerability in patients with advanced carotid artery stenosis.

Methods and Results: The study group comprised 56 patients (stable: n=28, unstable: n=28) with advanced carotid artery stenosis (>70%). Immunohistochemistry was performed for smooth muscle, endothelial, and inflammatory cells, macrophages, vascular endothelial growth factor (VEGF), VEGF receptor-2 (VEGFR-2), platelet-derived growth factor (PDGF), and angiopoietin-1,-2 (Ang-1,-2). Furthermore, the concentrations of angiogenic factors were measured in serum. Quantitative expression analysis was performed by SYBR-Green-based real-time polymerase chain reaction. Compared with stable carotid lesions, unstable carotid lesions showed a 1.8-fold increase in neovascularization (P=0.013), which significantly correlated with accumulation of inflammatory cells (factor 1.9, P<0.001). In unstable lesions, compared with stable lesions, VEGF was 1.7-fold increased (P=0.032) and Ang-1 was 1.9-fold reduced (P=0.029). Furthermore, VEGF was higher in the blood of patients with unstable plaques than in stable plaques (0.32±0.22 vs. 0.22±0.16 ng/ml; P=0.002). Significant correlations were observed between plaque vulnerability, VEGF, neovascularization and inflammatory cells.

Conclusions: Our results show a close association between neovascularization, expression of angiogenic factors, inflammation, and plaque vulnerability in patients with advanced carotid stenosis. (Circ J 2012; 76: 1274–1282)

Key Words: Atherosclerosis; Carotid artery; Neovascularization; Plaque vulnerability

The majority of atherosclerotic lesions are vascularized by a large network of small vessels that originate from the adventitial vasa vasorum. These neovessels play an important role in plaque development and progression. Initially, neovascularization is induced by hypoxia and ongoing inflammatory reaction in the atherosclerotic area. Both hypoxia and inflammation activate in turn various angiogenic factors (eg, hypoxia-inducible growth factor, vascular endothelial growth factor (VEGF), fibroblast growth factor, platelet-derived growth factor (PDGF), angiopoietins (Ang), ephrins, and others).

In a healthy organ, the formation of new vessels follows their stabilization by pericytes and smooth muscle cells. Angiogenesis is activated mainly by VEGF and its receptor, VEGFR-2. Following the onset of the first capillaries, pericytes and smooth muscle cells are recruited by PDGF. These cells build the first medial layer and stabilize the vessels. At this stage of vessel development, angiopoietins possess different functions. Ang-1 reduces vascular permeability and stabilizes the vessels, whereas Ang-2, an antagonist of Ang-1, undermines the effect of Ang-1 and leads to vessel destabilization. A possible role of both Ang-1 and -2 in atherosclerotic lesions was mentioned by Lim et al.

Despite the common knowledge that neovessels are present in most atherosclerotic lesions, the contribution of neovascularization to carotid plaque vulnerability is still unknown. Furthermore, with regard to the role of angiogenic factors in atherosclerosis, there is only an indication that VEGF and Ang-2 might contribute to plaque destabilization. The question of the extent to which neovascularization may influence plaque progression towards ischemic stroke has not yet been answered.
The aim of the present study was a detailed analysis of atherosclerotic lesions in patients with advanced carotid artery stenosis and stable or unstable plaques with regard to neovascularization, and the expression and localization of relevant angiogenic factors (VEGF, VEGFR2, PDGF, Ang-1,-2). In addition, the levels of these angiogenic factors were determined in the blood of the corresponding patients.

Methods

Patients

The study group consisted of 56 patients with high-grade carotid artery stenosis (>70%) intended for carotid thromboendarterectomy (CEA). The degree of stenosis was evaluated by color-coded duplex sonography, following the ECST-criteria in all patients. Patients underwent a detailed examination by...
a neurologist within 2 days before and after the procedure. All clinical data available were recorded for each patient, including age, sex, neurological symptoms, blood pressure, hypertension, hyperlipidemia, hypercholesterolemia, chronic kidney disease, diabetes, coronary artery disease, and smoking within the preceding 6 months. Patients with atrial fibrillation, a possible risk factor for cardioembolic stroke, were excluded from the study. A stroke or transient ischemic attack was defined as a cerebral or retinal ischemic symptom persisting >24 h. All patients with neurological symptoms underwent additional magnetic resonance imaging (T1, T2, diffusion) to confirm the occurrence of stroke. Carotid plaques of all patients were analyzed by histology and characterized for lesion stability as described previously, resulting in 28 patients with stable and 28 individuals with unstable plaques (a more detailed description of the plaque characterization is provided below).

The study was performed according to the Guidelines of the World Medical Association Declaration of Helsinki. The Ethics Committee of Klinikum rechts der Isar, Technical University of Munich approved the study and written informed consent was given by all patients.

### Carotid Artery Classification and Determination of Plaque Stability

Immediately following CEA, the tissue specimens were fixed in formalin for 24 h. According to the morphological consistency of the whole plaque, the first cut (for specimen segmentation) was done at the site of the most advanced atherosclerosis and this area was defined as the “clinically relevant section”. Both segments adjacent to the clinically relevant section were excluded from the study. A stroke or transient ischemic attack was defined as a cerebral or retinal ischemic symptom persisting >24 h. All patients with neurological symptoms underwent additional magnetic resonance imaging (T1, T2, diffusion) to confirm the occurrence of stroke. Carotid plaques of all patients were analyzed by histology and characterized for lesion stability as described previously, resulting in 28 patients with stable and 28 individuals with unstable plaques (a more detailed description of the plaque characterization is provided below).

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### Histology and Immunohistochemistry

For cell characterization, carotid artery samples were treated with antibodies against vascular smooth muscle cells (SMCs; anti-SMA, Dako, Glostrup, Denmark), endothelial cells (ECs; anti-von Willebrand factor VIII, Dako), macrophages/monocytes (anti-CD68, Dako), and inflammatory cells (anti-CD45, Dako). Angiogenic factors were detected with the following primary antibodies: VEGF, PDGF (LabVision, Wiesbaden, Germany), VEGFR2, Ang-1 and -2 (R&D Systems, Wiesbaden, Germany). Following primary antibody incubation, the individual factors were visualized using either the APAAP ChemMate Detection Kit (Dako) or LSAB ChemMate Detection Kit (Dako) according to the manufacturer’s instructions.

### Analysis of Immunohistochemistry

The analysis of stained carotid specimens was performed by light microscopy (Axioplan2; Carl Zeiss, Germany). Digital photographs were taken of each stained sample, analyzed by Vision Software 4.8 (Zeiss, Goettingen, Germany), and the mean±SD was calculated. The soft and collagenous areas were labeled, and their content displayed as a percentage of the total plaque area without the lumen. Calcium, collagen and elastic fibers were stained in the same manner and defined as follows: calcified areas appeared dark violet with EVG staining (Figures 1A, B); collagen fibers were pink/violet areas, elastin blue/black. These areas were labeled using the abovementioned software and the percentage of the whole plaque area (without the lumen) was calculated. Cellularity, infiltrates, and macrophages were analyzed in the same manner and stable plaque was set as 100%.

The staining for angiogenic factors was analyzed as follows: (−) no staining, (+) positive staining detected in the majority of cells and all specimens, (±) not all cells in all specimens were positive for the staining.
For determination of the expression of angiogenic factors in the individual cells within the carotid atherosclerotic lesions, consecutive slides from each specimen were prepared and incubated with the appropriate antibodies. In all cases 1 slide was stained with an antibody to detect the specific cell type and a consecutive slide was stained with the antibody against the individual angiogenic factors of interest.

**Analysis of Neovascularization**

Quantitative analysis of neovessels within the carotid atherosclerotic lesions was performed by light microscopy. Specimens were stained with factor VIII and all positive neovessels within the carotid plaques were counted. Regarding the comparison between stable and unstable lesions, the clinically relevant part of the individual plaques was selected each time.
Table 3. Cells Within Atherosclerotic Carotid Lesions Positive for Angiogenic Factors VEGF, VEGFR-2, PDGF, Ang-1, and -2 Detected by Immunohistochemistry

<table>
<thead>
<tr>
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<th>Neovessel ECs</th>
<th>Luminal ECs</th>
<th>SMCs</th>
<th>Infiltrates</th>
<th>Macrophages</th>
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<tr>
<td>VEGF</td>
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<td>Ang-2</td>
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+, most cells in all specimens were positive; ±, not all cells were positive; −, all cells were negative for the corresponding staining. Consecutive immunostaining was performed for all specimens.

VEGF, vascular endothelial growth factor; VEGFR-2, VEGF receptor-2; PDGF, platelet-derived growth factor; Ang-1, -2, angiopeptin-1, -2; EC, endothelial cell; SMC, smooth muscle cell.

(see section “Carotid Artery Classification and Determination of Plaque Stability”).

Enzyme-Linked Immunosorbent Assay (ELISA) Analyses
Angiogenic factors were quantified in serum samples using appropriate ELISA assays from R&D Systems (MN, USA) according to the manufacturer’s protocols. Measurement of clinical and chemical parameters was performed in our routine clinical chemistry laboratories.

Quantitative Real-Time Polymerase Chain Reaction (PCR)
Quantitative PCR was performed using the same specimens as described, but adjacent to the histological samples (20 μm each). The RNA was isolated by High Pure RNA Paraffin Kit according to the manufacturer’s instructions (Roche, Mannheim, Germany). The isolated RNA was transcribed into complementary DNA using the cDNA Synthesis Kit RevertAid (Fermentas, St. Leon-Rot, Germany). Quantitative real-time PCR was performed using SYBR-Green fluorescence dye (pegLab, Erlangen, Germany) and SYBR-Green-Cycler iQ5 (Bio-Rad, Hercules, CA, USA). All results were normalized for the expression of GAPDH and confirmed by gel electrophoresis. The following primers were used: GAPDH-F2 (forward) 5’-cactgccaacagtgtgag-3’, GAPDH-R2 (reverse) 5’-tgagccagcatcgcttgag-3’, 122 base pairs (bp); VEGF-F2 5’-atgaggacacgtgctgaca-3’, VEGF-R2 5’-agctgacacatttgccgcttgg-3’, 120 bp; PDGF-F4 5’-tcagtgctgttgagatggtg-3’, PDGF-R4 5’-gaagagccaccagagaggtg-3’, 126 bp; VEGFR2-F2 5’-agtgttgagaactctccagggag-3’, VEGFR2-R2 5’-agctgacacatttgccgcttgg-3’, 122 base pairs (bp); Ang1-F3 5’-gaggacaccggctctgacca-3’, Ang1-R1 5’-cgccgtctgtgctgacccca-3’, 101 bp; Ang2-F1 5’-aaaatgtagacagacctcctcca-3’, Ang2-R3 5’-actgtctgtgtcttcagggc-3’, 90 bp.

Statistical Analysis
All data were analyzed using SPSS for Windows version 17.0. (SPSS Inc, Chicago, IL, USA). First, normal distribution was evaluated by Kolmogorov-Smirnov test. Continuous variables were compared by either the parametric t-test for unpaired samples or the non-parametric Mann-Whitney U-test. The data were monitored using either standard bar graphs or a box plot diagram showing median and 25th/75th percentiles. Correlations between continuous variables were quantified using Pearson’s correlation coefficient for normally distributed samples or by Spearman’s rank correlation coefficient for non-parametric values. All statistical comparisons were 2-sided in the sense of an exploratory data analysis using P<0.05 as the level of significance.

Results

Patients’ Characteristics
The patients’ characteristics are summarized in Table 1. The average age of the study population was 69.1 ± 8.5 years. In the group with unstable carotid plaques, 59.0% had previous neurological symptoms (non-disabling stroke, transitory ischemic attack). In contrast, only 32.0% of the patients with stable carotid lesions had neurological symptoms (P=0.023). None of the other characteristics, including medication and associated diseases, was statistically significant between the groups (Table 1). Regarding medication, almost all patients received either ASS (aspirin) or clopidogrel (plavix). Furthermore, approximately 70% of patients were on statins.

Histological Plaque Characterization
The results of the semiquantitative analysis of the various histological features of the carotid plaques are summarized in Table 2. Regarding cellularity, calcification, and the content of elastin, SMCs or macrophages, no differences were observed between the study groups. In contrast, a significant increase in neovascularization (P=0.013) and inflammatory cells served between the study groups. In contrast, a significant increase in neovascularization (P=0.013) and inflammatory cells served between the study groups. In contrast, a significant increase in neovascularization (P=0.013) and inflammatory cells served between the study groups. In contrast, a significant increase in neovascularization (P=0.013) and inflammatory cells served between the study groups. In contrast, a significant increase in neovascularization (P=0.013) and inflammatory cells served between the study groups. In contrast, a significant increase in neovascularization (P=0.013) and inflammatory cells served between the study groups. In contrast, a significant increase in neovascularization (P=0.013) and inflammatory cells served between the study groups. In contrast, a significant increase in neovascularization (P=0.013) and inflammatory cells served between the study groups.

Histological Characterization of Neovascularization
The visualization of neovascularization within carotid lesions demonstrated that 93.8% of all plaques contained neovessels. Closer characterization revealed that 97.1% of these microvessels were immature, containing only ECs without the stabilizing layer of SMCs, independent of the size of the neovessel (Figures 1C,D). The amount of stable/mature microvessels (containing SMCs) was extremely low at 2.9% (Figure 1E). Furthermore, the number of neovessels within the plaques differed between the study groups. Unstable plaques had a 1.8-fold increase in the number of neovessels (av. 166±82) compared with stable plaques with 91±55 neovessels (P=0.013) (Figure 1F).

Analysis of Angiogenic Factors in Carotid Plaques by Immunohistochemistry
The immunostaining of all angiogenic factors tested in our study is summarized in Figure 2 and Table 3. No differences were observed between stable and unstable carotid plaques regarding the localization of expression of angiogenic factors (Figure 2). The expression of VEGF was detected in all cells within the carotid plaques, with the exception of inflammatory
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In contrast, a markedly lower extent of immunostaining was observed for VEGFR-2 compared with VEGF (Figures 2B,G). Only ECs, macrophages and macrophage-derived foam cells were positive for this VEGF receptor. The expression pattern of PDGF was similar to that of VEGF (Figures 2C,H). The staining of Ang-1 was the weakest of all angiogenic factors tested (Figures 2D,I). SMCs and luminal ECs were negative for this staining. A stronger positive reaction was observed only for macrophages and foam cells. The staining of Ang-2 was more intense than for Ang-1 and all cells with the exception of infiltrates were positive (Figures 2E,J). Neovessels showed similar expression pattern to ECs of the lumen (Table 3). The only difference was observed for VEGFR-2, which was expressed only in the neovessels.

Quantitative Real-Time PCR Analysis of Angiogenic Factors

Because immunohistochemistry allows only semiquantitative analysis of gene expression, we also performed quantitative real-time PCR. The highest expression was observed for VEGF...
in both stable and unstable carotid plaques (Figure 3). In contrast, the expression of VEGFR-2 was approximately 10-fold lower compared with VEGF and Ang-1 expression was 3- to 5-fold lower than for Ang-2.

Regarding the comparison between the study groups, significant differences were observed for the expression of VEGF and Ang-1. The expression of VEGF was increased in unstable carotid plaques (1.7-fold, P=0.032), and the expression of Ang-1 was reduced (1.9-fold, P=0.029).

Analysis of Selected Angiogenic Factors in Blood Serum

Regarding our evaluation of the expression and localization of angiogenic factors in carotid plaques, our results demonstrated the appearance of all angiogenic factors tested and especially increased expression of VEGF in unstable plaques. The next step was therefore to analyze these angiogenic factors in the blood of the study patients. We excluded VEGFR-2, because in contrast to the other angiogenic factors, VEGFR-2 is a surface marker expressed mainly on ECs and thus the probability of attaining a level in blood is low.

The results of the blood analysis are shown in Figure 4. The values of Ang-1 and PDGF did not differ between the study groups. In contrast, the serum level of VEGF was significantly increased in patients with unstable carotid plaques (P=0.002). Interestingly, in contrast to VEGF, the serum level of Ang-2 was decreased in patients with unstable lesions (P=0.001).
Correlation Analysis of Blood Factors and Carotid Artery Plaque Features

The main aim of the study was to ascertain the role of angiogenic factors in plaque progression and rupture. Therefore, correlations analysis was performed to reveal possible relationships among neovascularization, angiogenic factors, and other clinical parameters. No significant relationships were observed between the number of neovessels, expression angiogenic factors, and associated diseases.

Regarding the inter-relationship of individual angiogenic factors, correlations were found for the occurrence of neovessels, and the expression of VEGF, Ang-1, and PDGF within atherosclerotic carotid lesion (Figure 5). Whereas the correlation between neovessels and the expression of VEGF and Ang-1 was positive, showing a concomitant increase in the number of neovessels and the expression of both angiogenic factors (r=0.295 and 0.458, P=0.037 and <0.001, respectively). For PDGF the correlation was negative, with high expression of PDGF associated with low number of neovessels (r=-0.590, P<0.001) (Figure 5). Furthermore, regarding plaque morphology and angiogenic factors, correlations were observed between neovascularization and cellularity (r=0.239, P<0.026) and between the occurrence of neovessels and inflammation (r=0.416, P<0.001) (Figure 5).

Discussion

Based on our present data, we conclude that in our study group neovascularization was associated with carotid plaque stability and that some angiogenic factors, such as VEGF or Ang-1, might correspond to an increased risk of ischemic stroke.

As already stated in our previous studies, there are significant differences in the morphology of unstable and stable carotid plaques. In the context of this study, differences in the number of neovessels in the clinically relevant cross-sections were observed between unstable and stable lesions. Interestingly, the number of infiltrates was also increased in unstable plaques and correlated with the extent of neovascularization. Furthermore, the majority of neovessels in our study were immature (97.1%), without any stabilizing layer of SMCs, independent of vessel size. Such microvessels are unstable and highly permeable, which allows inflammatory cells to pass easily through the layer of ECs and accumulate deep within the plaque. Inflammatory cells in turn release a plethora of cytokines and matrix metalloproteinases. Consequently, neovascularization and accumulation of inflammatory cells may contribute to plaque progression and destabilization.

All relevant angiogenic factors tested in our study were detected in advanced carotid lesions. The highest expression was observed for VEGF. Furthermore, VEGF expression was correlated with the density of neovascularization. VEGF is one of the basic angiogenic factors essential for growth and development of new vessels. In addition, VEGF increases vessel permeability and thus promotes the accumulation of inflammatory cells. In this manner, both neovessels and VEGF expression can markedly contribute to plaque progression and vulnerability. There is evidence that progression in atherosclerosis leads to hypoxia. Oxygen deficiency in turn activates the expression of VEGF. These facts may explain the presence of VEGF in all cells located in carotid lesions. In contrast, the expression of VEGFR-2 was 10-fold lower than that of VEGF. Furthermore, as expected, only ECs were positive for this angiogenic factor. Macrophages and foam cells were expressing VEGFR-2 as well. However, these cells are positive for the majority of antibodies used for immunostaining. Therefore, the specificity of the expression of VEGFR-2 in these cells is questionable.

Expression of PDGF was high and comparable with that of VEGF. In contrast to VEGF, however, no differences were found between the study groups. PDGF is normally released from ECs to recruit pericytes and SMCs to stabilize newly developed vessels. These circumstances are in discord with our results showing that most of the neovessels in the carotid atherosclerotic plaques were immature, containing only the EC layer. This discrepancy can be explained by the fact that PDGF is also released during injury to facilitate wound healing. Atherosclerosis is a type of chronic injury, leading consequently to the increased expression of PDGF. In this context, however, another issue needs to be addressed. Why does the increased expression of PDGF not lead to the recruitment of SMCs? There may be several reasons for this discrepancy. First, SMCs do not possess the desired phenotype required for recruitment compared with healthy tissue. Second, although chronic inflammation in the atherosclerotic plaque leads to the release of a plethora of cytokines and growth factors, these signal molecules are not in balance, leading often to apoptosis and death of SMCs. Third, following the development of new vessels, pericytes are preferentially recruited, not the already fully differentiated SMCs. The role of pericytes in atherosclerotic lesions is, however, still unknown.

Regarding Ang-1 and -2, our results demonstrated low expression of Ang-1 compared with Ang-2. In addition, a significant decrease in Ang-1 was found in stable plaques. These results confirm the crucial role of angiopoietins in angiogenesis. Ang-1 leads to the recruitment of pericytes and vessel stabilization, whereas Ang-2 inhibits the corresponding signal transduction. Both the low level of Ang-1 and the high expression of VEGF lead to an increase in neovessel permeability and accumulation of inflammatory cells. These features may again contribute to plaque instability. Furthermore, the absence of Ang-1 as a team-player with PDGF may be an additional bottleneck to stabilization of the neovessels. This might be another explanation for the occurrence of immature neovessels within atherosclerotic plaques.

Based on our results we can summarize as follows: (1) the majority of carotid plaques are positive for neovessels; (2) a significant increase in the number of neovessels is found in unstable plaques; (3) all relevant angiogenic factors are expressed in atherosclerotic lesions; (4) there is high expression of VEGF, especially in unstable plaques, which may increase permeability and consequently the accumulation of inflammatory cells; and (5) the low expression of Ang-1 together with a high level of Ang-2 might further contribute to the instability and permeability of neovessels. In summary, neovascularization seems to be closely associated with carotid plaque progression and lesion vulnerability. Our results also confirm the assumption of Moreno et al of the progressive role of neovessels in human atherosclerosis.

Reliable biomarkers of plaque vulnerability could markedly contribute to the prediction of patients at risk of stroke. Thus, we also analyzed the levels of the angiogenic factors in blood serum. Our results demonstrated a significantly increased concentration of VEGF in the blood of patients with unstable carotid artery lesions. These results correlated well with the increased expression of VEGF and occurrence of neovessels in the unstable carotid plaques. It seems reasonable that the VEGF level in blood is associated with plaque progression and/or vulnerability. Interestingly, we found discrepancies between the expression of Ang-1 and -2 in carotid lesions and their concentration in blood of the corresponding
patients. In contrast to the significant reduction of expression of Ang-1 in unstable carotid plaques, we did not observe any changes in the blood levels in these patients. The reason for the missing expected correlation might be the low expression of Ang-1 in carotid atherosclerotic plaques. Thus, diffusion of Ang-1 into blood from these lesions may not have been sufficient to detect any changes in the concentration of this factor in patients’ serum samples.

**Study Limitations**
Independent of the careful and detailed characterization of all carotid plaques regarding plaque stability, some patients could still have escaped our attention. However, because of the exclusion of patients without well-defined plaque morphology and analysis of all the histological data by an experienced pathologist, such mistakes were kept to a minimum. Furthermore, our study is limited by the relatively small sample size. In addition, our conclusion regarding the association of neovascularization with carotid plaque instability is limited by the fact that our patients do not suffer exclusively carotid artery stenosis. Some patients did have coronary heart disease concomitant chronic kidney disease or diabetes. These accompanying diseases can also contribute to the differences in neovascularization between stable and unstable carotid lesions.

**Conclusions**
In summary, our present study assumes an important role of neovascularization in plaque progression and vulnerability. Significant correlations were observed between neovascularization, unstable carotid plaques, and expression of angiogenic factors, especially VEGF. Consequently, neovascularization might be a promising target for new diagnostic and therapeutic strategies.

**Acknowledgments**
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**References**