The Relevance of Vascular Endothelial Growth Factor, Hypoxia Inducible Factor-1 Alpha, and Clusterin in Carotid Plaque Instability

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Background: Stroke is one of the leading causes of morbidity and mortality. Thromboembolism, as a major cause of carotid artery-related stroke, can be caused by plaque rupture which is associated with neoangiogenesis within the carotid plaque. *Aim:* We sought to investigate a possible correlation between angiogenesis-related factors and preoperative neurological manifestations in patients with internal carotid artery stenosis, for a better understanding of thromboembolism in internal carotid artery stenosis-related stroke. *Methods:* This study included 54 patients (asymptomatic, n = 20 and symptomatic, n = 34) undergoing carotid endarterectomy for high-grade internal carotid artery stenosis. In the retrieved carotid plaques, angiogenesis-related factors (vascular endothelial growth factor [VEGF], hypoxia inducible factor-1 alpha [HIF-1α], and Clusterin) were measured by immunohistochemistry and quantified by real-time polymerase chain reaction. *Results:* We demonstrated the expression of VEGF, HIF-1α, and Clusterin by endothelial cells and smooth muscle cells in the carotid plaques. Noteworthy, mRNA VEGF levels were .7-fold higher in symptomatic patients (P = .017) compared to asymptomatic patients. In contrast, mRNA Clusterin levels were 1.8-fold lower (P = .021). Levels of mRNA HIF-1α were 1.5-fold higher in asymptomatic patients, but no statistical significance was reached between the 2 groups. *Conclusions:* Our results show an association between VEGF and Clusterin and neurological symptoms of patients with high-grade carotid artery stenosis.

Key Words: Clusterin—VEGF—HIF1-alpha—carotid artery

Introduction

Stroke is one of the leading causes of disability and death in adults. Fifteen to 20% of ischemic strokes result from internal carotid artery stenosis (ICAS).[1] Thromboembolism, the predominant mechanism of ischemic stroke, is caused by plaque rupture, which is the result of excessive microvessel formation (neoangiogenesis) and subsequent intraplaque haemorrhage.[2] Intraplaque hypoxia, which is increased with the growth of the atherosclerotic plaque, is a strong activator of neoangiogenesis in atherosclerosis.[3,4]

Angiogenesis is regulated by a delicate balance between pro - and antiangiogenic factors.[3,5,6] In hypoxic conditions within the plaque, hypoxia inducible factor-1 (HIF-1) furthers vascular endothelial growth factor (VEGF)-driven

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angiogenesis in order to increase the delivery of oxygen and nutrients.[7] Likewise, Clusterin (Clu), besides numerous other biological functions, has been associated with angiogenesis.[8] An association of these angiogenic factors (serum levels of VEGF) has already been reported with the degree of carotid artery stenosis[9] or with unstable atherosclerotic plaques[10] in patients with acute coronary syndrome.[11]

The aim of our study was to investigate the relationship between the angiogenic factors Clusterin, VEGF, and HIF-1α in carotid plaques and clinical symptoms in patients undergoing surgery for high-grade internal carotid stenosis, for a better understanding of thromboembolic complications in ICAS-related stroke.

Methods

Study Design

This study was conducted as a single-center, nonrandomized, prospective cohort study in a large tertiary care medical center, evaluating the association of carotid plaque ribonucleic acid (RNA) levels of angiogenic factors with clinical presentation in patients undergoing carotid endarterectomy (CEA). All patients provided written informed consent. The study was approved by the local Ethics Committee (EC nr: 11-208-VK).

Study Population

Plaques from 54 consecutive patients undergoing carotid surgery for high-grade ICAS according to international guidelines were recruited.[12,13] Patients were classified by their clinical presentation, that is, as either asymptomatic (n = 20) or symptomatic (within 6 months prior to surgery, n = 34). Magnetic resonance or computed tomography angiography in addition to duplex ultrasonography was performed in all patients prior to CEA according to NASCET criteria.[14,15] Diagnosis of stroke was provided by independent neurologists according to the TOAST classification.[16,17] Patients who gave their written informed consent underwent standardized vascular screening, including a health questionnaire and laboratory assessment. Exclusion criteria comprised age 18 years or younger, pregnancy, acute peripheral vascular occlusion and symptomatic coronary artery disease, myocardial infarction, or acute coronary syndrome in the previous 3 months. Surgery was standardized by performing eversion endarterectomy under local anesthesia with the systemic administration of 5000 IU of Heparin before clamping of carotid arteries.

ICAS Assessment

Duplex grading of ICAS and initial screening was performed by duplex ultrasound according to previously published criteria and current guidelines.[14,15,18] A computed tomography or magnetic resonance-angiogram (CTA or MRA) of carotid vessels was performed in order to assess surgical feasibility and distal or intracerebral lesions.

Total RNA Purification and cDNA Preparation

Human carotid atherosclerotic plaques were collected from patients with ICAS undergoing CEA. Frozen tissue was homogenized using a ball mill (Retsch, Haan, Germany), and mRNA was isolated using High Pure RNA Tissue Kit (Roche) according to manufacturer's instruction. Reverse transcription was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche).

Real-Time Polymerase Chain Reaction

Real-time polymerase chain reaction (rt-PCR) was performed using LightCycler TaqMan Master (Roche) according to the manufacturer's instructions. Primers were designed using the Roche Universal ProbeLibrary Assay Design Centre (http://www.universalprobelibrary.com): GAPDH (forward primer: 5′-agccacatcgctcagacac-3′, reverse primer: 5′-gcccaatacgaccaaatcc-3′, UPLprobe #60; Amplicon Size [bp] 66); VEGF (forward primer: 5′-ctacctccaccatgccaagt-3′, reverse primer: 5′-ccacttcgtgatgattctgc-3′, UPLprobe #29; Amplicon Size [bp] 74); HIF-1α (forward primer: 5′-cagctatttgcgtgtgagga-3′, reverse primer: 5′-ttcatctgtgctttcatgtcatc-3′, UPLprobe #89; Amplicon Size [bp] 75); Clusterin (forward primer: 5′-gagcagagcgctataaatacgg-3′, reverse primer: 5′-ccaattctggagtctttgcac-3′, UPLprobe #63; Amplicon Size [bp] 113). The amplification conditions consisted of an initial incubation at 95°C for 10 minutes, followed by 45 cycles of 95°C for 10 seconds, 63°C for 20 seconds, 2°C for 6 seconds, and a final cooling to 40°C. Data were analyzed using LightCycler Software Version 3.5 (Roche).

Immunofluorescence

Paraffin-embedded sections were deparaffinized and then boiled for antigen retrieval in citrate buffer (Dako).

The following primary antibodies were used: either goat polyclonal antibody anti-Clusterin (used at 10 μg/mL; R&D System), mouse monoclonal antibody anti-HIF-1α (used at 13.3 μg/mL; R&D System), or mouse monoclonal antibody anti-VEGF (used at 15.6 μg/mL; R&D), combined with rabbit polyclonal antibody anti-Von Willebrand factor (1:500 dilution; Dako). After blocking for 30 minutes with 3% BSA (bovine serum albumine) + .3% Triton X-100 in PBS (phosphate-buffered saline), primary antibodies were incubated overnight at 4°C. After extensive washing in PBS, slides were incubated with matching secondary antibodies for 1 hour at room temperature in the dark—Alexa Fluor-488 goat antimouse IgG or Alexa Fluor-488 donkey antigoat IgG and Alexa Fluor-633 goat antirabbit IgG (Invitrogen-Molecular Probes). Finally, mouse monoclonal antibody antismooth muscle actin directly labeled to Cy3 (Sigma) was used at a dilution of 1:200.

All antibodies were diluted in PBS containing .3% Triton X-100 and 1% BSA. Nuclear counter staining was performed with DAPI (diamidino-phenylindole) (1 μg/mL; Sigma) for 10 minutes at room temperature. Tissue

sections were analyzed with a confocal laser scanning microscope (LSM-780; Carl Zeiss) using ZEN software.

Statistical Analysis

Continuous variables were presented as mean ± standard deviation (SD), and the Student *t* test was used to detect differences between the 2 groups for the variables that displayed normal distribution. The differences in proportions were compared by using the χ^2 or Fisher test where appropriate, using SPSS 21.0 (SPSS, Chicago, IL). Values of $P \leq .05$ were considered significant.

Results

Clinical Presentation

Demographics and baseline characteristics of the 54 (asymptomatic = 20, symptomatic = 34) patients undergoing CEA for ICAS are presented in **Table 1**. The average age of the study population was 69.4 ± 9.9 years, with no difference between 2 groups. Mean degrees of ipsilateral ICAS stenosis showed no significant difference between asymptomatic and symptomatic patients (79% ± 9% versus 79% ± 11%, $P = .89$). The 2 groups did not differ when it comes to major cardiovascular comorbidities or preoperative medication use. Preoperative aspirin and statin use was similar in both groups [15(75%) versus 30(88.2%), $P = .21$ and 15(75%) versus 20(58.8%), $P = .24$].

Table 1. *Baseline characteristics of patients (n = 54) undergoing carotid surgery for their asymptomatic or symptomatic high-grade internal carotid artery (ICA) stenosis*

Variables	Asymptomatic	Symptomatic	*P* value
	n	n	
Patients (n)	20	34	
Sex (% male)	12 (60)	20 (58.8)	.93
Hypertension (%)	17 (85)	33 (97.1)	.10
Hyperlipidemia (%)	13 (65)	22 (68.8)	.78
Smoking (%)	16 (80)	27 (79.4)	.96
Diabetes mellitus (%)	9 (45)	13 (38.2)	.63
CAD (%)	6 (30)	6 (17.6)	.30
MCI (%)	3 (15)	4 (11.8)	.28
CHF (%)	2 (10)	1 (2.9)	.73
Aspirin (%)	15 (75)	30 (88.2)	.21
Statin (%)	15 (75)	20 (58.8)	.24
ACE-inhibitors (%)	14 (70)	22 (64.7)	.70
Age (years)	70 ± 11	69 ± 10	.90
Body mass index (kg/sqm)	27.6 ± 4.7	27.5 ± 4.79	.91
CRP (mg/dL)	6.4 ± 7.7	7.2 ± 8.9	.79
ICAS ipsilateral (%)	79 ± 9	79 ± 11	.89
ICAS contralateral (%)	48 ± 27	60 ± 23	.40

Expression Pattern of Angiogenic Factors in Human Atherosclerotic Plaques

VEGF, HIF-1α, and Clusterin protein were detected in human carotid atherosclerotic plaques (n = 6) where they colocalized with Von Willebrand factor demonstrating expression of these factors by endothelial cells (Fig 1, a-c).

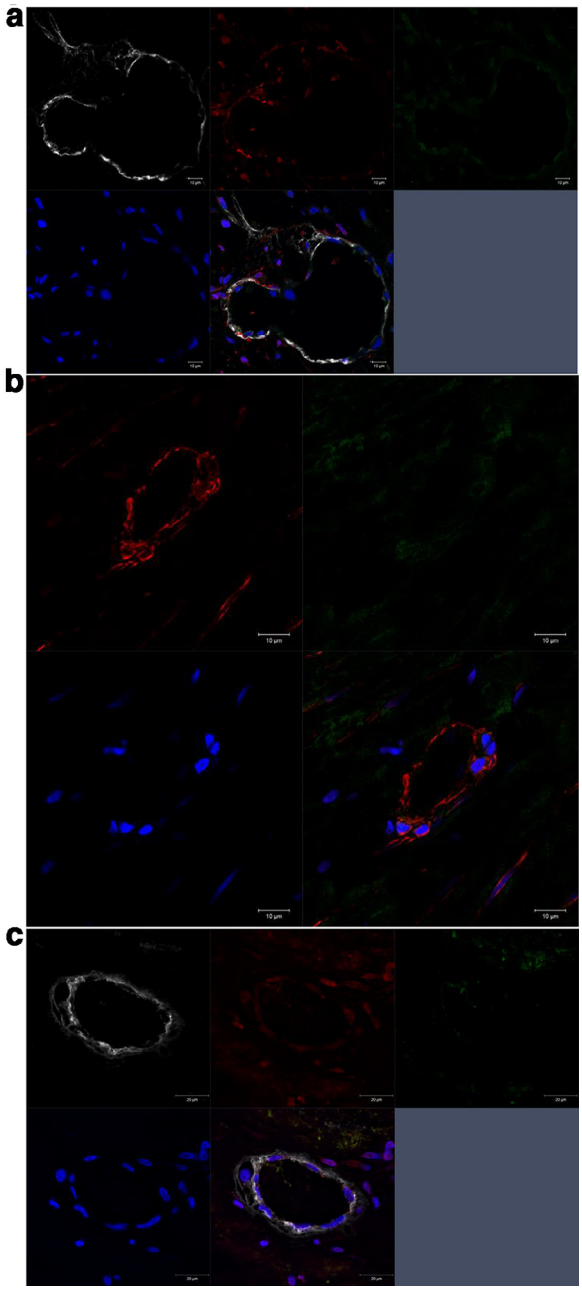

Figure 1. *VEGF, HIF-1α, and Clusterin are colocalized in microvessels of human atherosclerotic plaques (a-c). Staining for SMA (red), vWF (gray), and cell nucleus (blue). VEGF (green in a), HIF-1α (green in b), and Clusterin (green in c). Staining was performed with atherosclerotic samples from 6 different donors. Representative pictures are shown. (Color version of figure is available online.) Abbreviations: HIF-1α, hypoxia inducible factor-1 alpha; VEGF, vascular endothelial growth factor; Vwf, Von Willebrand factor.*

These factors were also colocalized with α-smooth muscle actin in the vessel wall (Fig 1, a-c), demonstrating expression of the factors by SMCs. In accordance with the results of rt-PCR analysis, VEGF was rather detectable in carotid plaque of symptomatic patients, whereas HIF- 1α and Clusterin in those of asymptomatic patients.

Quantitative Estimation of Expression of Angiogenic Factors

In symptomatic patients, the expression of VEGF was increased .7-fold (P = .017), whereas HIF-1α and Clusterin mRNA levels were reduced (1.5-fold, P = .054 and 1.8-fold, P = .021, respectively; Fig 2, a-c).

Discussion

Our study revealed several findings: (1) VEGF was rather detectable in plaques of symptomatic patients, (2) Clusterin mRNA levels were significantly higher in plaques of asymptomatic patients, (3) there was no significant difference in the expression level of HIF1-α between 2 groups.

Growth of atherosclerotic plaques leads to intraplaque hypoxia which triggers neoangiogenesis.[3,4,19] These neovessels in human atherosclerosis have an important role in the atherosclerotic plaque progression and contribute to the rupture of atheroma with subsequent thromboembolic carotid-related ischemic stroke.[20,21]

Under hypoxic conditions within the plaque, HIF-1 initiates VEGF-driven angiogenesis.[7] As mentioned above, serum levels of VEGF have already been associated with carotid stenosis,[9] neovascularization, and carotid plaque vulnerability.[21,22] VEGF also increases the permeability of blood vessels leading to the accumulation of inflammatory cells and cytokines.[23] In our study, in symptomatic patients VEGF was rather expressed in the endothelium of microvessels of the carotid plaques. This is an important finding as in experimental studies a bevacizumab-eluting stent (monoclonal antibody specific for VEGF-eluting stent) was

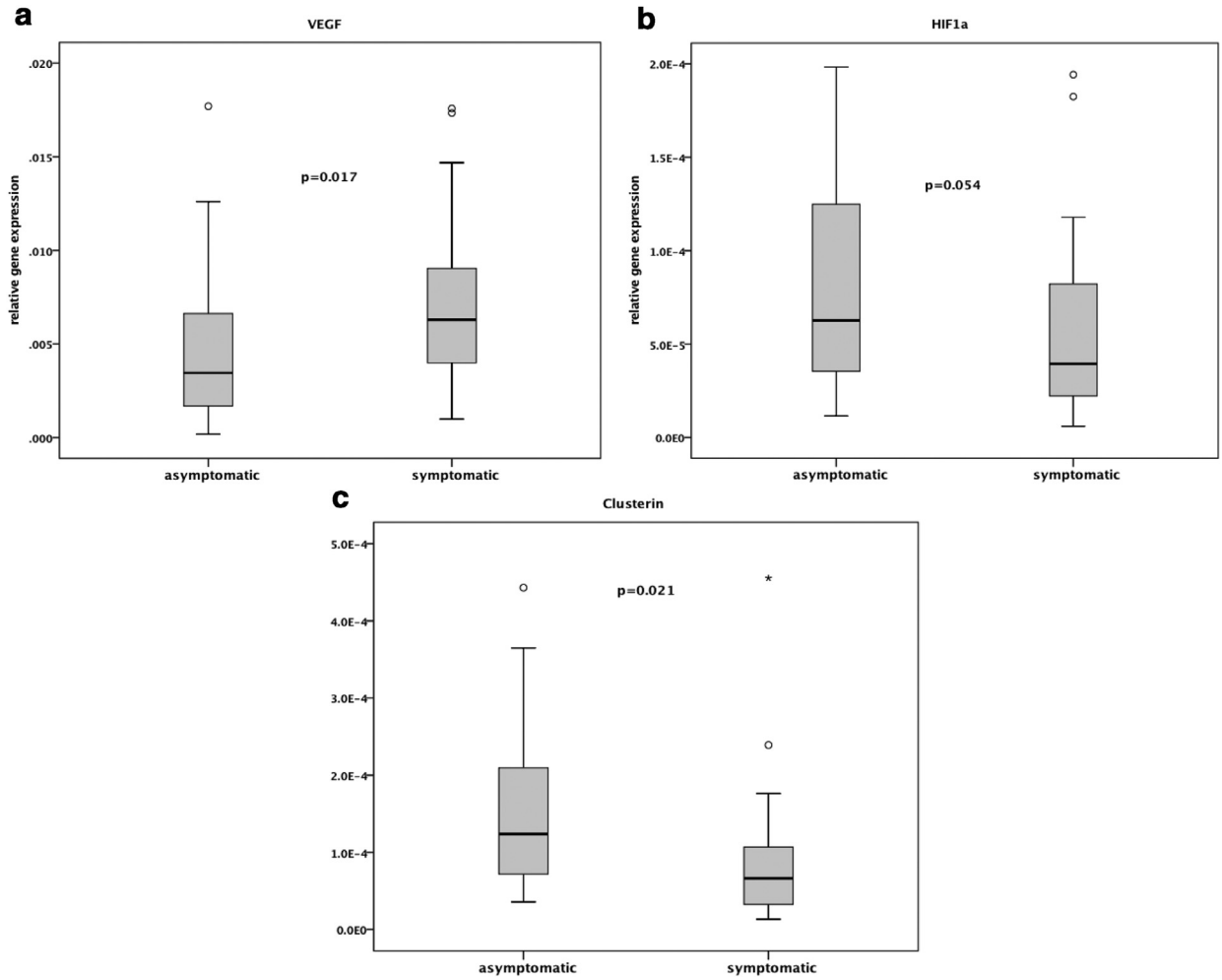

Figure 2. *Quantification of HIF-1α, Clusterin, and VEGF mRNA levels in human atherosclerotic plaques. RNA was isolated from human carotid atherosclerotic plaques (n = 54). VEGF (a), HIF-1α (b), and Clusterin (c) mRNA were determined by real-time PCR. mRNA levels were correlated with patients' symptomatology. Abbreviations: HIF-1α, hypoxia inducible factor-1 alpha; PCR, polymerase chain reaction; VEGF, vascular endothelial growth factor.*

responsible for high-risk plaque stabilization through inhibition of neovascularization in coronary and iliac arteries in humans and rabbits.[24,25] Consequently our findings support further investigations of VEGF as a potential treatment target in patients with unstable, that is, symptomatic carotid artery stenosis.

HIF-1s are heterodimers consisting of 2 subunits, named alpha and beta. Alpha (α) is oxygen-regulated and beta (β) is constitutively expressed in the nucleus. Previous studies have associated HIF-1α with plaques angiogenesis, leading to plaque progression, hemorrhage, and ulceration.[26,27] In our study, rt-PCR analysis of HIF1-α showed no significant difference in the expression between asymptomatic and symptomatic patients. Our findings agree with the observation of Chen et al that the expression of HIF-1α does not vary between asymptomatic and symptomatic coronary atherosclerosis patients.[28] This is possibly the consequence of alternative regulatory pathways, such as nuclear factor kappa B, mutation of p53, and activation of the Ras/mitogen-activated protein kinase pathway that might play a role in the regulation of the expression of VEGF in our samples,[29] which has to be addressed in future studies.

Clusterin (Clu) showed a significant higher expression in the asymptomatic patients of this study. Clu, also known as Apolipoprotein J, is a subset of high-density lipoprotein[30] which has important antiatherosclerotic properties. Interestingly, several studies have shown a correlation of the progression of aortic atherosclerosis with the expression of clusterin.[31,32] Previously, the distribution of Clu in the aortic wall has been analyzed: it increased with the degree of atherosclerosis.[33] The authors of this study hypothesized Clu might have a protective role by its involvement with cholesterol transport. Another study showed that Clu upregulation during vascular injury may have a protective role in the development of neointimal hyperplasia, an acknowledged precursor of atherosclerosis.[34] These findings agree with our study showing significantly higher levels of Clu mRNA in asymptomatic patients. Consequently, our findings might indicate to Clusterin as a potential new agent for atherosclerotic plaque stabilization and this may pose a reasonable hypothesis that deserves further investigation.

Study Limitations

Our study is limited by relatively small sample size. Furthermore, this study design did not allow for establishing a causal relationship between the assessed markers and neurological adverse events in the patients with carotid disease could not be definitely established.

Conclusions

In the present study, we could show a significant association of VEGF and Clusterin with the neurological presentation of patients with high-grade internal carotid artery stenosis. Although further work is required, the findings of the present study may have clinical relevance. After the 2 studies, which clearly linked peripheral blood levels of VEGF with carotid stenosis and endarterectomy, our work definitely confirms the expression of VEGF in the symptomatic plaque.[9,35] Therefore, VEGF serum levels might directly serve as risk stratification tool in asymptomatic patients. Even more importantly, as the growth of atherosclerotic carotid plaque leads to its instability, new therapeutic agents regulating neoangiogenesis, by targeting VEGF or using Clusterin, might serve as an innovative approach to carotid disease.

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