Original Article

Expression of Vasohibin-1 in Human Carotid Atherosclerotic Plaque

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Aim: In patients with carotid plaque, intraplaque hemorrhage arising from ruptured neovascular vessels within the neointima is an important cause of stroke. The expression of Vasohibin-1 (VASH1), a negative feedback regulator of angiogenesis, occurs in the microvessel endothelial cells of various solid tumors and the arterial wall. However, the roles of VASH1 in the pathogenesis of atherosclerotic diseases remain unclear. The present study aimed to clarify the relevance of the VASH1 expression and plaque instability in human carotid plaques.

Methods: We used quantitative real-time PCR and immunostaining to examine 12 atheromatous plaque specimens obtained via carotid endarterectomy. The distal areas of specimens lacking macroscopic atherosclerotic lesions served as controls.

Results: Compared with that observed in the controls, the *VASH1* gene expression increased significantly in the atheromatous plaque ($p = 0.018$). Moreover, the *VASH1* mRNA levels correlated positively with those of *VEGFA*, *CD31* and *VCAM1* ($r = 0.788$, $p = 0.004$; $r = 0.99$, $p < 0.001$; $r = 0.94$, $p < 0.001$, respectively). Finally, the immunohistochemical analyses revealed the VASH1 expression in the neointimal microvessel endothelial cells of carotid plaque.

Conclusions: The VASH1 expression levels in atheroma reflect both enhanced neovascularization and the inflammatory burden. Therefore, the VASH1 level may be a novel biomarker for evaluating plaque instability in patients with carotid arteriosclerosis and predicting ischemic stroke.

Introduction

Carotid atherosclerotic plaque is an important risk factor for stroke, and recent randomized studies indicate that carotid endarterectomy effectively prevents atherothrombotic stroke[1, 2]. Although the degree of stenosis increases the risk of ischemic stroke, this finding lacks high predictive value[3-5]. In contrast, several recent studies have suggested a close association between plaque components and the risk of atherothrombotic cerebrovascular events[6-9]. Histological analyses of human symptomatic carotid plaque demonstrate features characteristic of unstable plaques (i.e., ulceration, disruption, intraplaque hemorrhage, thinning of the fibrous cap and significant accumulation of inflammatory cells, including macrophages and T lymphocytes)[10-13]. Among these characteristics, intraplaque hemorrhage, which results from rupture of the neovascular vessels leading from the adventitial vasa vasorum of the carotid arterial wall[14-17], crucially participates in atherosclerotic plaque progression and

destabilization[8, 18-20]. Therefore, evaluating carotid plaque neovascularization would likely benefit the evaluation of plaque vulnerability and risk of atherothrombotic events.

Recently identified as a negative feedback regulator of angiogenesis[21-26], the vasohibin-1 (VASH1) expression occurs selectively in vascular endothelial cells, induced by vascular endothelial growth factor (VEGF)[21]. Interestingly, VASH1 exists in microvessel endothelial cells in a variety of solid tumors, and the VASH1 expression levels correlate with the prognosis and grade of malignancy[27-34]. Moreover, endogenous VASH1 is expressed in adventitial microvessels of the aortic wall, and exogenous VASH1 prevents neointimal formation by inhibiting adventitial angiogenesis[24]. However, the pathophysiological significance of VASH1 in the development of human atherosclerotic diseases is incompletely understood.

The present study examined the VASH1 expression in human atherosclerotic plaques acquired via carotid endarterectomy and investigated the possible association between the VASH1 expression levels and plaque vulnerability.

Aim

In patients with atherosclerotic plaque, neovascularization is a histological marker of plaque vulnerability and predicts the risk of atherothrombotic diseases, including ischemic stroke. VASH1 functions as an anti-angiogenic factor, and its expression levels may correlate with the degree of intraplaque neovascularization. Therefore, we examined the VASH1 expression in human carotid atherosclerotic plaques in order to clarify the relevance of this parameter for plaque instability.

Methods

Subjects and Tissue Sampling

During the period of June 2012–August 2013, we performed 12 carotid endarterectomies in patients with carotid stenosis (11 men and 1 woman, mean age=75.3±5.0 years) at Kyoto University Hospital. Ten patients (83.3%) presented with clinical symptoms of cerebral ischemic attack related to carotid stenosis. The prevalence of risk factors for atherosclerosis included hypertension in nine patients (75.0%), dyslipidemia in eight patients (66.7%), diabetes mellitus in six patients (50.0%) and ischemic coronary disease in six patients (50.0%).

We obtained samples from the plaque region immediately following endarterectomy. Next, we dissected the stenotic segment and adjacent areas undisrupted as a single specimen, preserving the circumferential integrity as much as possible. All samples were frozen at −80℃ until mRNA extraction. The distal areas of the excision specimens, which lacked macroscopic atherosclerotic lesions, served as normal controls. The institutional review board of Kyoto University Hospital approved the collection of human tissue samples.

Quantitative Real-Time PCR

We extracted total RNA from the specimens using an RNeasy Mini Kit (QIAGEN, Valencia, CA, USA), according to the manufacturer's instructions, and generated first-strand cDNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Foster City, CA, USA). Next, we performed quantitative real-time PCR using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems), according to the manufacturer's instructions. For amplification, we used the following sense (S) and antisense (AS) primer pairs: human *VASH1*; (S) 5'-AGATCC-CCATACCGAGTGTG-3', (AS) 5'-GGGCCTCTT-TGGTCATTTCC-3', β-actin; (S) 5'-ACAATGAG-CTGCGTGTGGCT-3', (AS) 5'-TCTCCTTAATGT-CACGCACGA-3', *CD31*, (S) 5'-GCAGATAATTG-CCATTCCCATG-3', (AS) 5'-CTTCATTCACAGC-AGCACATTGCAG-3', *VEGFA*; (S) 5'-GCAGAAT-CATCACGAAGTGG-3', (AS) 5'-AAGGACTGTTC-TGTCGATGG-3', intracellular adhesion molecule-1 (*ICAM1*); (S) 5'-GGCCTCAGTCAGTGTGA-3', (AS) 5'-AACCCCATTCAGCGTCA-3', vascular cell adhesion molecule-1 (*VCAM1*); (S) 5'-CCGGATT-GCTGCTCAGATTGGA-3', (AS) 5'-AGCGTGGA-ATTGGTCCCCTCA-3'. The data were normalized using β-actin as a reference gene.

Histological Studies

Formalin-fixed, paraffin-embedded samples were cut into 5-μm-thick sections. To assess the immunoreactivity, we deparaffinized and rehydrated the sections and incubated them with boiled citrate buffer (pH 9.0) for 10 minutes. After blocking, we incubated the sections overnight at 4℃ with primary antibodies including anti-human VASH1 mouse monoclonal antibodies (prepared as previously described[21]) diluted at 1:400, anti-human CD31 rabbit polyclonal antibodies (RB-10333-P; Thermo Scientific, Rockford, IL, USA) diluted at 1:50 and anti-human ICAM1 rabbit polyclonal antibodies (sc-8304; Santa Cruz Biotechnology, Dallas, TX, USA) diluted at 1:200. We added horseradish peroxidase-conjugated secondary antibodies (EnVision Labelled Polymer; Dako, Glos-

trup, Denmark) to the sections and incubated them at room temperature for one hour, followed by visualization using 3,3'-diaminobenzidine with hematoxylin and eosin counterstaining.

For the immunofluorescence analysis following primary antibody staining, we incubated the sample sections with Alexa Fluor 488–conjugated donkey anti-mouse antibodies or Alexa Fluor 594–conjugated donkey anti-rabbit antibodies (Molecular Probe, Eugene, Oregon, USA) at room temperature for one hour.

We stained the negative controls with non-immune mouse or rabbit IgG for each experiment.

Statistical Analysis

The data are presented as the mean ± SEM. The statistical analysis was performed using the paired t-test, and a linear regression analysis was conducted using the R version 3.0.0 software package (Vienna, Austria). A p value of <0.05 was considered to be statistically significant.

Results

Increased Expression of VASH1 mRNA in the Human Carotid Atherosclerotic Lesions

In order to determine whether the VASH1 expression is increased in human atherosclerotic plaques, we isolated total RNA from carotid endarterectomy specimens and evaluated the *VASH1* mRNA levels using quantitative real-time PCR. Compared to that observed in the normal controls, the *VASH1* gene expression was significantly higher in the atheromatous plaque areas ($p = 0.018$) (**Fig. 1**).

According to a univariate analysis, clinical risk factors, such as age ($p = 0.87$), sex ($p = 0.50$), hypertension ($p = 1.00$), dyslipidemia ($p = 0.46$), diabetes mellitus ($p = 0.59$), degree of stenosis ($p = 0.81$), ischemic coronary disease ($p = 0.70$) and the presence of cerebral ischemic symptoms ($p = 0.12$), were not significantly correlated with the VASH1 mRNA levels in the carotid plaque specimens, partially because only a limited number of specimens were examined in this study.

Associations of the VASH1 Expression Levels with Plaque Inflammation and Neovascularization

Next, we investigated whether the VASH1 expression levels reflect plaque vulnerability. Neovascularization and intraplaque inflammation are well-established features of plaque instability. Importantly, VEGFA, which is among the most potent angiogenic factors, targets the VASH1 expression. Accordingly, the intra-
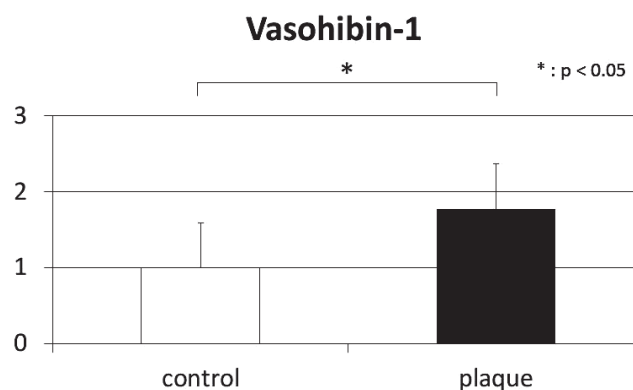

Fig. 1. The VASH1 mRNA expression is increased in human carotid atherosclerotic plaques.

Total RNA was isolated from human carotid endarterectomy specimens ($n = 12$), and the levels of *VASH1* mRNA were measured using quantitative real-time PCR. The distal edge of the harvested samples without macroscopic atherosclerotic plaque served as a control ($n = 12$). The *VASH1* gene expression increased significantly in the atheromatous plaque area vs. controls ($p = 0.018$). The results are presented as the mean ± SEM. *, $p < 0.05$ for the indicated comparison. VASH1, vasohibin-1.

plaque mRNA levels of *VASH1* correlated positively with those of *VEGFA* ($r = 0.788$, $p = 0.004$) (**Fig. 2A**). In addition, the VASH1 mRNA levels showed a direct relationship with the CD31 mRNA levels ($r = 0.99$, $p < 0.001$) (**Fig. 2B**), indicating that the VASH1 expression reflects increased neovascularization in atheromatous plaques.

The increased expression of intercellular adhesion molecule-1 (ICAM1) and vascular cell adhesion molecule-1 (VCAM1) is associated with endothelial activation and dysfunction and leads to intraplaque inflammation. Although not statistically significant, the correlation between the *VASH1* mRNA levels and the *ICAM1* expression in human carotid plaques showed a possible trend toward significance (**Fig. 2C**) ($r = 0.55$, $p = 0.077$). Additionally, the *VASH1* mRNA levels were positively associated with those of *VCAM1* (**Fig. 2D**) ($r = 0.94$, $p < 0.001$).

Our investigation of the relationship between enhanced neovascularization and the inflammatory burden in atheromatous plaques revealed a significant association between the VEGFA mRNA levels and the *VCAM1* expression (**Fig. 2E**) ($r = 0.927$, $p < 0.001$).

Detection of VASH1 in the Microvessel Endothelium of the Human Carotid Atheroma

Immunofluorescence revealed the VASH1 expression in the microvessels of the human carotid atheromatous plaque. Moreover, VASH1 colocalized with CD31, a marker of the neovascular endothelium
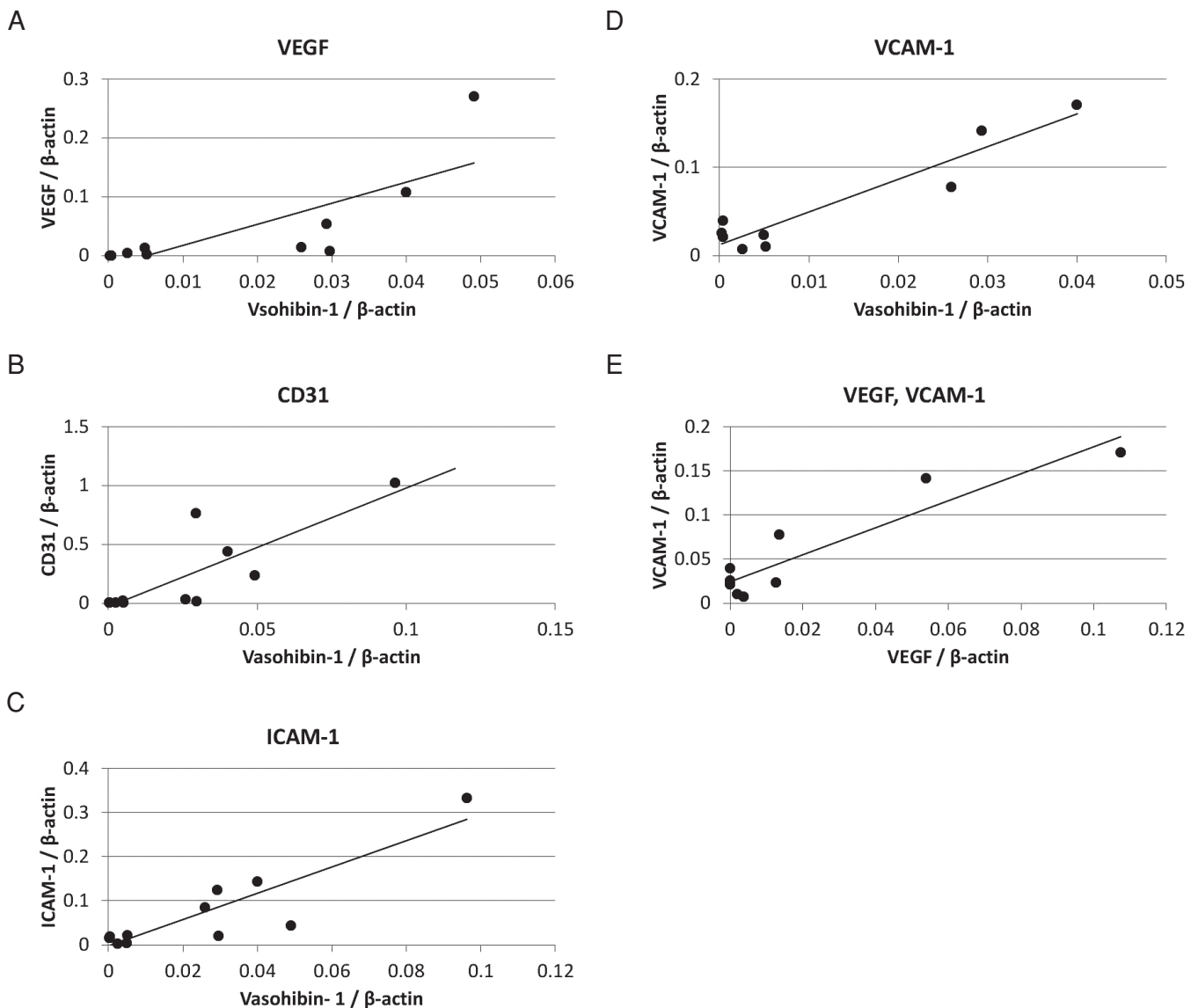
Fig. 2. The VASH1 expression is associated with intraplaque inflammation and neovascularization.

Relationship between the *VASH1* gene expression in carotid atherosclerotic plaques and that of *VEGFA* (A), *CD31* (B), *ICAM1* (C) and *VCAM1* (D), respectively. Statistically significant correlations existed between the *VASH1* mRNA levels and those of *VEGFA*, CD31 and *VCAM1*. The *VEGFA* mRNA levels also correlated positively with those of *VCAM1* (E). The gene expression levels were evaluated using quantitative real-time PCR, and the mRNA levels of β-actin served as an internal control for normalization between samples ($n=11-12$). The results are presented as the mean ± SEM. VEGFA, vascular endothelial growth factor A; ICAM1, intercellular adhesion molecule-1; VCAM1, vascular cell adhesion molecule-1

(**Fig. 3A**), and the immunohistochemical analysis detected the VASH1 expression in ICAM1-positive endothelial cells (**Fig. 3B**).

Discussion

This study examined the VASH1 expression in microvascular endothelial cells from human carotid atherosclerotic plaques and explored the positive relationship between the *VASH1* gene expression and that of *VEGFA*, *CD31* and *VCAM1*.

Recent reports have identified VASH1 to be a soluble and potent negative feedback regulator of angiogenesis[21-26]. Although the VASH1 expression is restricted to vascular endothelial cells, its function in the arterial wall remains unclear. Yamashita *et al.*
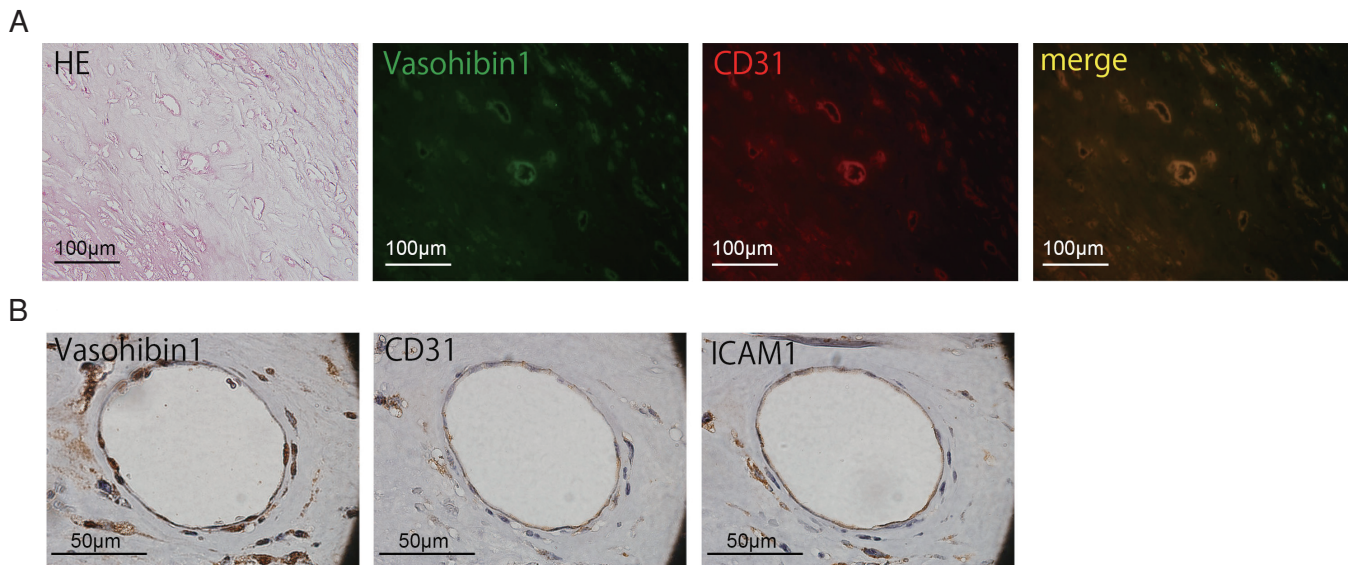
Fig. 3. VASH1 exists in the microvessel endothelium of human carotid atheroma.

(A) Human carotid endarterectomy specimens were analyzed using HE staining (left) and immunostaining for VASH1 (green, left middle) and CD31 (red, right middle; right, merged picture). Scale bars: 100 mm. (B) The samples were immunostained with anti-VASH1 (left), anti-CD31 (middle) and anti-ICAM1 antibodies. Scale bars: 50 μm. HE, hematoxylin and eosin.

observed VASH1 in the adventitial microvessels of the human atherosclerotic aorta and determined that adenoviral *VASH1* gene transfer inhibits neointimal formation in mouse femoral artery cuff placement by preventing adventitial angiogenesis and macrophage infiltration[24]. Because neovascular vessel formation in the arterial wall plays a pivotal role in the accumulation of inflammatory cells and subsequent plaque vulnerability[12, 35, 36], the exogenous administration of VASH1 may contribute to the prevention of atheromatous plaque development and the onset of atherothrombogenic diseases. However, the impact of endogenous VASH1 remains undetermined, especially regarding its clinical relevance to human atherosclerotic diseases. Our study demonstrates that the VASH1 expression in human carotid arteries is more prominent in atheromatous plaque than in normal arterial regions and is probably associated with the amount of neovascularization. The present data showed that the VASH1 level may be a novel marker for evaluating the severity of human carotid atherosclerosis.

Vascular endothelial cells play central roles in the development of atherosclerosis. Various stimuli (e.g., mechanical and metabolic stress) deteriorate the endothelium that surrounds the vessel wall and induce its dysfunction[37-39]. The defective and activated endothelium secretes adhesion molecules of inflammatory cells, such as ICAM1 and VCAM1, key steps in the initiation and formation of a vicious cycle of vascular

inflammation and plaque instability[40]. Unsurprisingly, the soluble ICAM1 and VCAM1 levels are associated with carotid artery inflammation[41] and increased in patients with acute ischemic stroke[42, 43]. However, controversy remains regarding the ability of the circulating levels of cell adhesion molecules to predict the onset or prognosis of ischemic stroke[44], probably because endothelial activation occurs systemically and may not always reflect the vulnerability of culprit lesions in carotid atheroma. In the current study, the VASH1 expression levels in the plaque correlated positively with the levels of endothelial adhesion molecules. Therefore, the amount of VASH1 may be associated with the amount of neovascularization as well as the degree of vascular inflammation in carotid plaques. Taken together, VASH1 may be a potent biomarker for evaluating the clinical severity of human carotid arteriosclerosis.

Study Limitations

This study is associated with several limitations. First, the present study was conducted retrospectively and in a limited number of patients. Explaining the clinical role of VASH1 in human carotid atheroma will require a prospective study in a large population. Second, because ethical limitations prohibited us from harvesting samples from normal carotid arteries, we used samples of the distal media and endothelium

from carotid endarterectomy specimens as control lesions.

Conclusion

This study identified the VASH1 expression in human carotid atherosclerosis. We also determined that VASH1 exists in intraplaque microvessel endothelial cells and that the VASH1 expression levels are positively associated with the VEGFA and VCAM1 expression levels in carotid plaques. Our data suggest that the VASH1 expression in atheroma reflects both enhanced neovascularization and the inflammatory burden. Therefore, VASH1 may be a novel biomarker for evaluating the clinical severity of carotid arteriosclerosis and predicting ischemic stroke.

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Conflicts of Interest

The authors declare that no competing interests exist.

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