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Original Article

Laser microdissection-based analysis of hypoxia- and thioredoxin-related genes in human stable carotid plaques

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Abstract

Background: Neovascularization in the carotid atherosclerotic plaque is a common pathogenetic feature in carotid artery stenosis. To investigate whether the neovascular region of the stable plaque differentially expresses specific genes, we analyzed the patterns of angiogenesis-related gene expression in regions of the plaque isolated by laser microdissection and examined by immunohistochemistry and real-time reverse transcription (RT)–polymerase chain reaction (PCR). **Methods:** Carotid plaque samples were obtained by carotid endarterectomy in 27 clinically asymptomatic patients with high-grade internal carotid artery stenosis. Among these 27 plaque samples, 23 plaques were confirmed to be stable pathologically, and 14 stable plaques had neovascularization. The medial, shoulder, and neovascular regions of the 14 carotid plaques were determined by immunohistochemical staining. These 3 regions were microdissected, and total RNA was extracted for real-time RT-PCR analysis. The expressions of hypoxia inducible factor 1α, vascular endothelial growth factor-A, thioredoxin, and thioredoxin interacting protein were analyzed at mRNA level. **Conclusions:** Real-time RT-PCR was performed on 42 laser microdissected regions of 14 plaques. The expressions of all four genes examined were significantly lower in the medial region at mRNA level. High expressions were noted in both shoulder and neovascular regions, with no significant difference between the two. Furthermore, these expression patterns were related significantly to macrophage infiltration. In conclusion, hypoxia- and thioredoxin-related genes are significantly overexpressed in human stable carotid atherosclerotic plaques and strongly correlate with macrophage infiltration rather than neovascularization. Macrophage infiltration may lead to overexpression of these genes and promote angiogenesis in stable carotid plaques.

Keywords: Carotid atherosclerotic plaque; Neovascularization; Macrophage infiltration; Laser microdissection; Hypoxia inducible factor 1; Thioredoxin

1. Introduction

Atherosclerosis is a multifactorial disease characterized by smooth muscle cell migration and proliferation, cholesterol accumulation, connective tissue formation, inflammatory cell infiltration, thrombus formation, and calcification [1,2].

Pathological neovascularization is a consistent feature of atherosclerotic plaque development and disease progression [3]. Microvessels may play a key role in progression of unstable plaques [4]. However, neovascularization is observed even in stable plaques. In addition, the pathophysiological roles of these new vessels in stable plaques remain unclear.

Hypoxia and the redox state have great impact on neovascularization. To investigate whether there is differential gene expression that stimulates neovascularization in the neovascular region of human stable carotid atherosclerotic plaques, we analyzed the expression of hypoxia- and thioredoxin (TRX)-related genes at mRNA level. We used

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laser microdissection (LMD) to isolate the neovascular and shoulder regions from human stable carotid plaques and compared the gene expression patterns of these two regions to macroscopically normal medial smooth muscle cell layer.

Hypoxia-inducible factor 1 (HIF-1) is a heterodimeric transcription factor composed of α and β subunits. Although HIF-1β is constitutively expressed in many cell types, stability of the α subunit and its transcriptional activity is precisely controlled by the intracellular oxygen concentration [5]. Hypoxia results in rapid accumulation of HIF-1α in the nucleus, leading to the transcriptional activation of vascular endothelial growth factor (VEGF) and several dozens of other known target genes [5].

TRX is a 12-kDa redox protein [6]. Previous report demonstrated that TRX may play an antioxidative role in atherosclerotic lesions, where increased oxidative stress is speculated [7]. Thioredoxin interacting protein (TXNIP) is a negative regulator of TRX [8]. Reactive oxygen species (ROS) have been shown to play a crucial role in vascular angiogenesis [9].

The aims of the present study were to determine whether the neovascular region of stable atherosclerotic plaque exhibits specific expression patterns of hypoxia- and thioredoxin-related genes, which may potentially change the stable plaque to vulnerable plaque through promoting neovascularization.

2. Materials and methods

2.1. Carotid endarterectomy specimens

Human carotid endarterectomy (CEA) specimens (n=27) were obtained from 27 patients (23 males; average age ±SD, 69.4±5.95 years) with asymptomatic high-grade extracranial internal carotid artery stenosis (>60% luminal narrowing). CEA was performed as described in our previous reports [10,11]. An arterial stenosis was defined as clinically asymptomatic if there were no ipsilateral symptoms or signs of cerebral or retinal ischemia. The plaques were cut into <1-cm blocks, embedded in TissueTeck OCT medium (Sakura Finetechnical Co. Ltd., Tokyo, Japan) and then snap-frozen in liquid nitrogen-cooled isopentane within 30 min after resection. All studies were approved by the Ethics Committee of the Tokyo Women's Medical University Hospital.

2.2. Immunohistochemistry

For immunohistochemistry, antibodies to anti-CD34 and anti-CD68 (Dako Denmark A/S, Glostrup, Denmark), anti-HIF-1α (sc-13515) and anti-VEGF (sc-7269) (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), and to anti-smooth muscle actin antibodies (Nichirei, Tokyo, Japan) were used as primary antibodies. The frozen tissues were sliced by a cryomicrotome at a thickness of 6 μm. The sections were air-dried for 10 min and fixed with 4% paraformaldehyde for 30 min (for CD34 and CD68) or 1% Triton X-100 in 10% formaldehyde for 20 min (for HIF-1α, VEGF, and smooth muscle actin). Endogenous peroxidase activity was blocked with 3% H_2O_2 in methanol for 10 min. Samples were preincubated with a blocking solution (Dako North America Inc., Carpinteria, CA, USA) for 5 min to block unspecific binding and then incubated with a primary antibody (CD34: 1:50, overnight at 4°C; CD68: 1:50, 60 min at room temperature; HIF-1α and VEGF: 1:200, 60 min at 37°C; smooth muscle actin: 1:1, overnight at 4°C). Then, the polymer reagent of the DakoCytomation LSAB+ kit (Dako North America Inc., Carpinteria, CA, USA) was applied for 30 min. Samples were incubated with DAB substrate according to the manufacture's instruction. The sections were rinsed in water, counterstained with hematoxylin, again rinsed in water, and mounted. Negative control was included by replacing antibody with Tris-buffered saline.

2.3. Target determination and LMD

2.3.1. Target determination

For LMD, 3 regions were determined from the result of immunohistochemistry as shown in Fig. 1A. (1) *Medial region* was defined as the medial muscle layer containing internal elastic lamia. (2) *Shoulder region* was defined as the triangular shape area where the bulking of the plaque was initiated. (3) *Neovascular region* was defined as the area where neointimal migration of smooth muscle cells and CD34-positive microvessels were noted.

2.3.2. Preparation of frozen samples and LMD

The tissues were sliced by a cryomicrotome at a thickness of 8 μm, and each tissue section was affixed to a slide to which an original thin film (provided by Meiwa Shoji Co., Ltd., Osaka, Japan) had been attached by silicone adhesive (GE Toshiba Silicone, Tokyo, Japan). The glass slides were pretreated at 200°C for 8 h to inactivate ribonuclease (RNase). The frozen sections were placed at room temperature for 5 min and fixed in 100% methanol for 5 min, and then washed with RNase-free water. The target regions in the sections were microdissected with a Laser Microbeam System (P.A.L.M, Munich, Germany).

2.3.3. RNA extraction from microdissected samples and cDNA synthesis

Total RNA was extracted independently from each laser-microdissected region. Briefly, the microdissected cells within the cap were covered with 200 μl buffer solution, 4 M guanidinium thiocyanate, 25 mM sodium citrate, and 0.5% sarcosyl, and the cap was placed in a tube and vortexed. After adding 20 μl of 2 M sodium acetate, 220 μl of water-saturated phenol, and 60 μl of chloroform-isoamyl alcohol, the tube was centrifuged at 15,000×g at 4°C for 30 min to separate the aqueous and organic phases. The aqueous layer was transferred to a