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Augmented angiogenesis in adventitia promotes growth of atherosclerotic plaque in apolipoprotein E-deficient mice

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ABSTRACT

Objective: Accumulating evidence suggests that exaggerated formation of vasa vasorum (VV) plays an important role in the pathogenesis of atherosclerosis. However, it remains unclear whether augmented angiogenesis in the adventitia could promote hyperlipidemia-induced atherosclerotic lesion formation.
Methods and results: First, we analyzed the time course of VV development in apolipoprotein E-deficient (ApoE−/−) mice. VV proliferation was observed only after atherosclerotic lesion formation. Next, we investigated whether forced perivascular angiogenesis could promote plaque progression. Basic fibroblast growth factor (bFGF) (100 μg/body) incorporated in acid gelatin hydrogel microspheres (AGHM) (bFGF + AGHM group, $n = 10$), AGHM alone (AGHM group, $n = 7$), or PBS (control group, $n = 8$) was administered into the periaortic area of the retroperitoneal space in 10- to 11-week-old male ApoE−/− mice. At 13 weeks after the operation, lesions were significantly larger in the bFGF + AGHM group than in others (bFGF + AGHM: $3.4 \pm 0.7 \times 10^4$ μm^2; AGHM: $0.1 \pm 0.1 \times 10^4$ μm^2; control: 0 μm^2; $p < 0.0001$), which was associated with increased neovascularization in the adventitia. The number of adventitial capillaries correlated with plaque size ($r = 0.69$, $p < 0.0001$). In the bFGF + AGHM group, an increase in the number of VV and accumulation of Mac3-positive macrophages were observed prior to atherosclerotic lesion formation.
Conclusions: Our findings demonstrated that local administration of bFGF in the adventitia induced development of VV and accelerated plaque progression in ApoE−/− mice, supporting the notion that VV formation plays a crucial role in the pathogenesis of atherosclerosis.

1. Introduction

The vasa vasorum (VV) are the microvasculature present in the adventitial layer of the vessel wall, presumably supplying nutrients to the vessel wall of large arteries [1]. VV play a significant role in maintaining vessel integrity. Previous postmortem studies showed that adventitial VV developed in human atherosclerotic coronary arteries [2,3]. Chronic inflammation of large arteries is associated with proliferation of VV which function to perfuse the vessel wall beyond the limit of diffusion from the luminal side [2]. It has been proposed that VV could function as a conduit supplying inflammatory cells and lipid into atherosclerotic plaques [4]. VV may also cause intraplaque hemorrhage, which contributes to lesion progression and destabilization [3,4]. Neovascularization was reported to increase in ruptured plaques in the human aorta [5].

Recent advances in imaging techniques, particularly micro CT technique, have enabled us to visualize VV [6]. Micro CT has made it possible to perform quantitative analysis of the number of VV, spatial density, vascular area fraction, and endothelial surface fraction [7]. Recently, Kampschulte et al. investigated the spatio-temporal distribution of vasa vasorum (VV) relative to advanced atherosclerotic lesions of mice using high-resolution nano-CT [7]. The authors convincingly demonstrated that atherosclerotic lesion type is correlated to the number and cross-sectional area of VV. Although these studies suggested that VV formation plays a crucial role in the pathogenesis of atherosclerosis, it has been unclear whether proliferation of adventitial microvessels is a cause or a result of atherosclerotic lesion progression. Precise role of VV in progression and destabilization of atherosclerosis remains to be clarified.

Histological analysis can provide high resolution images of capillary plexuses in close proximity to the vascular wall, particularly at sites where atherosclerosis develops, with information on inflammatory cells, cytokine expression, connective tissues and surrounding tissues [8]. Here, we carefully analyzed the time course of atherosclerotic lesion formation and VV progression in ApoE−/− mice. We also tested the hypothesis that forced angiogenesis in the adventitia could promote atherosclerotic lesion formation in ApoE−/− mice.

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2. Materials and methods

2.1. Animals

ApoE−/− mice were originally purchased from Jackson Laboratory [9]. In protocol 1, ApoE−/− mice were fed a regular diet. At the indicated time points, the abdominal aorta and perivascular soft tissue were harvested to evaluate the relationship between atherosclerotic lesion progression and adventitial VV number in the natural time course (Online Supplement Fig. 1). In protocol 2, ApoE−/− mice received slow-release hydrogel in the para-aortic area and were fed a Western type diet (protein 17.3%, carbohydrate 48.5%, fat 21.2% per g diet). All procedures involving experimental animals were performed in accordance with protocols approved by the institutional committee for animal research of The University of Tokyo and complied with the *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 86-23, revised 1985).

2.2. Slow-release form of basic fibroblast growth factor

Recombinant human bFGF was supplied by Kaken Pharmaceutical Co., Ltd., Tokyo, Japan. AGHM with a diameter of 30–75 μm and 90–92 vol% water content were prepared from gelatin with an isoelectric point of 4.9 (Nitta Gelatin, Osaka, Japan) as described previously [10]. Degradation time of AGHM was approximately 30 days [10]. Then, 100 μg bFGF was added to 3 mg AGHM and incubated at 37 °C for 1 h to obtain bFGF in AGHM [11]. bFGF in AGHM solution was suspended in 100 μl PBS. AGHM without bFGF were also made as a control.

2.3. Retroperitoneal periaortic injection of slow-release bFGF in ApoE−/− mice and wild-type mice

In protocol 2, 10- to 11-week-old ApoE−/− male mice were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital. A midline incision was made in the abdomen. The intestine, testes and bladder were pushed away to expose the inferior vena cava (IVC) and abdominal aorta. Then, 100 μl solution containing bFGF in AGHM (bFGF + AGHM group), AGHM alone (AGHM group) or PBS (control group) mixed with Evans blue was injected into the retroperitoneal perivascular space just beside the IVC and aorta through a 25G needle. The injected gelatin solution stayed in the perivascular area because it was slightly sticky. Similarly, 100 μg of bFGF in AGHM was injected into the retroperitoneal perivascular space of 10- to 11-week-old C57BL/6 mice. Mice were sacrificed and abdominal aortas and perivascular tissues were harvested at 4 weeks ($n = 4$), 9 weeks ($n = 3$), 13 weeks ($n = 4$).

2.4. Adventitial microvessel staining

To visualize the microvessels in the adventitia, endothelial cells were stained by perfusion of biotinylated *Lycopersicon Esculentum* (Tomato) lectin as described by Thurston et al. with some modifications [12] (Online Supplement Fig. 1).

2.5. Immunohistochemical staining

Macrophages were detected by immunohistochemical staining using an anti-mouse Mac3 antibody (M3/84, BD Pharmingen,) and HRP-conjugated anti-rat IgG (MAX-PO, Nichirei, Tokyo) and a DAB substrate kit (Vector). Von Kossa staining was performed as previously described [13].

2.6. Morphometric analysis

Morphometric analysis was performed on digitalized images using image analysis software (Image J, National Institute of Health).

2.7. Immunofluorescence-double stating

Immunofluorescence double staining was performed on thick frozen sections (40 μm) of a 104-week-old male ApoE−/− mouse fed regular diet using antibodies against FITC conjugated α-SMA antibody (clone 1A4, Sigma) and rat anti-mouse CD31 antibody (clone MEC 13.3, BD Bioscience), followed by Cy3 conjugated anti-rat IgG (Jackson). The sections were observed under a confocal microscope (FLUOVIEW FV1000, Olympus, Tokyo). Images were evaluated by Z plane analysis.

2.8. Observation of vascular cast with plastic resin under scanning electron microscope

After fixation with 2.5% glutaraldehyde, a red colored synthetic resin (Mercox®, DIC, Tokyo) was injected through a 22 G plastic cannula inserted into the left ventricle of an 82-week-old ApoE−/− female mice fed regular diet. The sample was kept at room temperature for 1 h until the liquid resin became completely polymerized. Then, the heart, the aorta and the perivascular soft tissues were harvested en bloc. The soft tissues were solved by incubation in 20% potassium hydroxide solution at 50 °C for 3 days. To dissolve the fat tissues completely, the samples were incubated with proteinase K in buffer ATL (QIAGEN) for a day. Next the sample was washed in 0.5% nonidant P 40. The microvascular casts were mounted on stubs, coated with platinum/palladium, and examined under a scanning electron microscope (S-3500N, Hitachi, Tokyo).

2.9. Statistics

All data are expressed as mean ± SEM. Comparisons among the three groups were made by one-way ANOVA followed by Bonferroni test. Comparison of regression lines was performed by standard method [14]. A p value <0.05 was considered statistically significant.

3. Results

3.1. Time course of atherosclerotic plaque progression and increase in VV number in abdominal aorta

In protocol 1, at earlier time points (7, 17, 24 weeks, $n = 4$ respectively), no atherosclerotic lesion was detected in the abdominal aorta (Fig. 1A and B). At 34 weeks, an atherosclerotic lesion was detected in one of the three mice. At 49 weeks, all three mice had lesions; however, the plaque size was moderate, without increase in the number of microvessels in the adventitia (Fig. 1C). At 67–94 weeks ($n = 9$), all mice had large plaques with a lipid core. The number of VV increased in the adventitia surrounding the atherosclerotic plaque (Fig. 1D–I). These results indicate that the number of VV increases following atherosclerotic plaque progression in the abdominal aorta of ApoE−/− mice. 3D observation of the microvasculature by scanning electron microscopy (Fig. 2A–C) revealed a network of microvessels in perivascular area of abdominal aorta of 82-week-old female ApoE−/− mice fed on regular diet. Capillaries communicate with the atherosclerotic lesion. We also detect communication between aortic lumen and atherosclerotic lesions. Double immunofluoresce imaging against α-smooth muscle actin and CD31 of abdominal aorta of 104-week-old ApoE−/−