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Original Article

Aortic adventitial angiogenesis and lymphangiogenesis promote intimal inflammation and hyperplasia

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Abstract

Introduction: Adventitial inflammation is known to influence neointimal formation and vascular remodeling. The present study was aimed to clarify the relationship between neointima hyperplasia and adventitial angiogenesis and lymphangiogenesis after balloon-induced aortic endothelial injury. **Methods:** Seventy male Wistar rats were randomly divided into six interventional groups and one control group. The intimal area/medial area ratio (I/M ratio), the adventitial macrophage index, and the number of adventitial microvessels (Ad-MV) and lymphatic vessels (Ad-LV) in the aorta were measured, and the mRNA expressions of VEGF-A, VEGFR-1, VEGF-C, VEGFR-3, PDGF-B, and PDGFR-β in the aortic wall were quantified by real-time RT-PCR. **Results:** Compared with the control group, the I/M ratio, macrophage index, Ad-MV, Ad-LV, and the mRNA expressions of VEGF-A, VEGFR-1, VEGF-C, VEGFR-3, PDGF-B, and PDGFR-β in interventional groups increased significantly after balloon-induced injury. I/M ratio showed significant correlations with Ad-MV and Ad-LV after balloon intervention. Multiple linear regression analysis indicated that Ad-MV and Ad-LV were independent factors of intimal hyperplasia. **Conclusion:** Adventitial angiogenesis and lymphangiogenesis are induced by intimal inflammation after balloon injury, and these neogenetic vessels in turn promote intimal inflammation and hyperplasia probably via delivery and activation of inflammatory cells. © 2009 Elsevier Inc. All rights reserved.

Keywords: Angiogenesis; Lymphangiogenesis; Intimal injury; Inflammation

1. Introduction

It is well known that lymphatic vessels, which commonly accompany blood vessels in tissues, drain extravasated bloodless fluid, protein, and inflammatory cells from the tissues and take an active part in inflammatory diseases [1,2]. Recent studies have reported that lymphatic vessels were enlarged in chronic inflammatory skin diseases and that rejected kidney transplants contained abundant lymphatic vessels [3,4]. Lately, it was found that lymphangiogenesis prevented mucosal edema and these neogenetic lymphatic vessels persisted in chronic airway inflammation [5]. Although the presence of adventitial inflammatory infiltration in close proximity to intimal atherosclerotic plaques has been recognized for more than four decades [6], the clinical relevance of this finding was not clear until recently when inflammatory infiltration was proved to be present in adventitia after coronary balloon injury [7]. Inflammatory

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cells like macrophages can secret cytokines and growth factors to promote angiogenesis and lymphangiogenesis [8,9]. However, the relationship between adventitial lymphangiogenesis and intimal hyperplasia is still unknown. In the present study, we tested the hypothesis that adventitial angiogenesis and lymphangiogenesis are initially induced by intimal inflammation after endothelial injury and these neogenetic vessels in turn promote intimal inflammation and neointimal hyperplasia by transportation and activation of inflammatory cells.

2. Materials and methods

2.1. Animal model

Seventy male Wistar rats weighing 450 to 500 g were obtained from the Animal Center of Shandong University and divided randomly into seven groups with 10 rats in each group. Group 1 to Group 6 rats underwent balloon-induced aortic endothelial injury, while Group 7 rats received only sham operation and served as a control group. Rats were housed under conditions of constant room temperature (22°C) and a 12-h dark/12-h light cycle, and fed a normal diet. All rats underwent anesthesia with intraperitoneal injection of pentobarbital (30 mg kg^{-1}) and the left external carotid artery was exposed to introduce a 2-F embolectomy catheter (Baxter Healthcare Corp., Irvine, CA, USA) into the distal abdominal aorta. The balloon catheter was then inflated with 50 μl of saline and went back and forth in the abdominal aorta three times to induce endothelial injury in Group 1 to Group 6 rats [10–13]. Sham operation was performed in Group 7 rats that underwent the same catheterization procedure but without balloon inflation. Group 1 to Group 6 rats were euthanized by intraperitoneal injection of a lethal dose of phenobarbitone on Days 1, 3, 7, 14, 28, and 90 after the procedure, respectively, and Group 7 rats were euthanized on Day 1 after sham operation. After the abdominal aorta was harvested, aortic segments were snap frozen in liquid nitrogen and stored at −80°C for quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) analysis, or fixed with 4% phosphate-buffered formaldehyde for 24 h for immunohistochemical and morphometric analysis. All animal procedures in the present study conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the Guide for the Care and Use of Laboratory Animals published by the Chinese National Institutes of Health.

2.2. Morphometric analysis

Aortic segments were embedded in paraffin and cut into 6-μm-thick sections which were stained with hematoxylin and eosin, and analyzed using a computer-assisted morphometric analysis system (Image-Pro Plus 5) with particular attention to the intimal and the medial thickness. Vascular area within the external elastic lamina (EELA) and the internal elastic lamina (IELA) as well as the lumen area (LA) was measured. The I/M ratio was calculated as: I/M=(IELA−LA)/(EELA −IELA). All parameters were measured from three sections selected from the proximal, the middle, and the distal portion of the aortic segment, and the values averaged.

2.3. Antibodies

The primary antibodies used were as follows: goat polyclonal antibody against human CD34 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA) to identify the blood endothelial cells; rabbit polyclonal antibody against human LYVE-1 (1:100; Santa Cruz Biotechnology) to tag lymphatic endothelial cells; rabbit polyclonal antibody against human CD68 (1:400; Santa Cruz Biotechnology) to mark macrophages; mouse monoclonal anti-α-SMA antibody (1:2000; ab7817, Santa Cruz Biotechnolgy) to label vascular smooth muscle cells (VSMCs); rabbit polyclonal antibody against mouse VEGF-A (1:50; Santa Cruz Biotechnology); rabbit polyclonal antibody against human VEGF-C (1:50; Santa Cruz Biotechnology), and rabbit polyclonal antibody against PDGF-BB (1:100; ab15499, Abcam, Cambridge, UK). Second antibodies against IgG in rabbits, mice, and goats were obtained from Beijing Zhongshan Biotechnology Co., Ltd. (ZSBIO, Beijing Zhongshan Golden Bridge Technology, China).

2.4. Immunohistochemistry

Sections were deparaffinized and incubated with 5% goat serum or 5% BSA for 20 min to minimize the nonspecific binding to the primary antibody and incubated with the primary antibodies overnight at 4°C in a moisture chamber. The sections were then incubated with the appropriate secondary antibody for 30 min at room temperature. To inhibit any endogenous peroxidase activity, the sections were incubated with 0.3% H_2O_2 in absolute methanol for 30 min. A peroxidase substrate solution containing 0.02% H_2O_2 and 0.1% 3,3′-diaminobenzidine tetrahydrochloride (ZSBIO) in PBS was applied to display the reaction product with a brown color, and the sections were then counterstained with hematoxylin. Incubation with PBS instead of the primary antibody was used as a negative control.

2.5. Quantification of adventitial angiogenesis and lymphangiogenesis

The total number of microvessels (CD34+) (Fig. 1A) or lymph vessels (LYVE-1+) (Fig. 1B) in the whole adventitia was counted under a light microscope at a high power magnification (×400) [14–17]. Vascular and lymph vessels were identified as morphologically circumferential brown products formed by one or more stained endothelial cells with at least one counterstained nucleus. In this case, a single brown dot was not counted. The number of adventitial microvessels (Ad-MV) and adventitial lymphatic vessels

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