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Correlation of Inflammatory Cells in Adventitia and Formation and Extending of Atherosclerotic Lesions in Coronary Artery of Apolipoprotein E Gene Knockout Mice

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Abstract

Accumulating evidence shows that adventitial inflammation contributes to the development of atherosclerotic lesions. The aim of this study was to investigate the relationship between atherosclerotic lesions in coronary artery (CA) and accumulation of inflammatory cells at local adventitia in apolipoprotein E gene knockout (apoE-/-) mice. Modified Movat's pentachrome staining, HE staining, immunohistochemistry and transmission electron microscopy were used to observe and to identify serial paraffin sections of aortic foot and inflammatory cells in CA adventitia of apoE-/- mice of 60 weeks old. There was always accumulation of inflammatory cells in the adventitia of CA with extending lesions from aortic orifice to CA trunks. The CA samples were divided into type I: infiltration of inflammatory cells in CA adventitia without lesions extending in the intima, type II: infiltration of inflammatory cells in CA adventitia with the top of extending lesions in the intima and type III: infiltration of inflammatory cells at CA adventitia with lesions covering all the face of intima. The three types of CA sample represent the different developmental processes of atherosclerotic lesions, respectively. No extending lesions were found in the CA trunks without inflammatory cells in adventitia. In type I samples, 60% of infiltrated inflammatory cells were macrophages 57% of infiltrated cells were neutrophils in type II samples; 67% of infiltrated cells were lymphocytes in type III samples. Our studies revealed that adventitial inflammation may be an early event in the development of atherosclerotic lesions. Different cell types predominate in different stages of CA adventitia. The neutrophils are closely related to the extending of atherosclerotic lesions.

Key Words: apoE knockout mice, artery adventitia, atherosclerosis, coronary artery lesion, inflammatory cell

Introduction

Genetically-engineered mice are useful models for studying atherosclerotic lesions, and one of these is the apolipoprotein E gene knockout (apoE-/-) mouse. All phases of atherosclerosis can be found in the arterial system of these mice (13), especially advanced lesions in the innominate artery of the apoE-/- mice (16, 20). Hu and colleagues reported the distribution of extending and independent athero-

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sclerotic lesions in the coronary arteries of chow-fed, 60-week-old male apoE-/- mice (6). Although we have previously studied the types of atherosclerotic lesions, inflammatory cells were frequently observed in the adventitia. Therefore, we were interested in characterizing these cell types, and had focused on the inflammatory cells in the adventitia, and extended our study to the formation and development of atherosclerotic lesions. In the present study, development of the inflammatory cells in the adventitia shows that accumulation of inflammatory cells in adventitia may be related to the formation and extension of atherosclerotic lesions in coronary arteries (CAs).

Materials and Methods

Preparation of Animals

In this study, 11 60-week-old apoE-/- C57BL/6 mice (Jackson Labs) and 5 wild-type C57BL/6 control mice were used. All mice were fed a rodent chow diet (Harlan Teklad, Hayward, CA, USA) and water *ad libitum*. The animals were perfused first with lactated Ringers solution for 20 sec and then with 10% buffered formaldehyde (cat.#UN-2209 Histology Reagent Co, Lodi, CA, USA) for 4 min. The heart of each mouse was removed and processed and embedded in paraffin for sectioning. Wild-type C57BL/6 mice acted as a control group for the study.

Preparation of Tissue

The hearts were detached from the large vessels at the level of the ascending aorta. The heart was divided at a level 1-2 mm distal from the lower margin of the left and right atrial appendages. The section containing the great vessels was called the base, and the remaining section was called the apex. The two pieces of the heart were processed, and the base was then embedded in paraffin cut-side down whereas the apex was preserved for later investigation. The 16 bases were serially sectioned in 5-μm thick sections. Every fourth slide was stained with modified Movat's pentachrome stain (6). The CAs were mapped in each of the approximately 700 sections per heart to construct the CA tree. To characterize the elastin, collagen and proteoglycan contents of the tissue, paraffin sections were stained with Movat's pentachrome histological stain. Staining was performed according to standard techniques (12) and hematoxylin-eosin (HE) staining was used to identify the type of inflammatory cells in CA adventitia, especially neutrophils. One of the slides was stained with HE and the number of inflammatory cells was counted. Adventitia from outside of the media to 200 μm from the outer edge of the adventitial connective tissue, or adventitia from the outside of media to the neighboring myocardium, was used. Five oil-power fields (×100) for every CA in which inflammatory cells were recruited were counted to determine the number of inflammatory cells.

A mouse has three main branches of CAs from the aorta, which are named as left coronary artery (LCA), right coronary artery (RCA) and septal artery (SA), which goes through the interventricular septum. The primary segment of the CA is called the CA trunk (6). Inflammatory cells were observed in the adventitia surrounding the CA. Three types of CA tissue were defined which represent the different developmental processes of atherosclerotic lesions: the initial stage, the advanced stage, and the mature stage, respectively. In type I tissues of the initial stage, there was infiltration of inflammatory cells in CA adventitia without lesions extended to the intima; type II were characterized by infiltration of inflammatory cells in CA adventitia with the top of extending lesions in the intima; type III tissues were characterized by infiltration of inflammatory cells at CA adventitia with lesions covering the entire intima surface.

Immunohistochemical Analyses

Immunohistochemical (IHC) analyses were used to assess the cellular components of the paraffin-embedded sections involved in adventitial inflammation such as macrophages and lymphocytes. Serial sections from formalin-fixed, paraffin-embedded tissue blocks were cut into 5-μm sections, deparaffinized and dehydrated. Sections were pretreated in a microwave. Immunohistochemical staining with mouse monoclonal anti-$CD79_\alpha$ at 1/200 dilution (Neomarkers, Fremont, CA, USA) was used to identify B lymphocytes (2), and was then stained with the vector M.O.M. immunodetection Kit (PK-2200, Vector Laboratories, Burlingame, CA, USA). For a negative control, the primary antibody was omitted from the stain. The infiltrated lymphocytes ($CD79_\alpha$+) were classified as B-lymphocytes. The lymphocytes that were negative for $CD79_\alpha$ were thought to be T-lymphocytes. To stain macrophages, the mouse monoclonal anti-CD68 (ab955, 1/100 dilution, purity: tissue culture supernatant) (Abcam, Cambridge, UK) was used.

Transmission Electron Microscopy (TEM)

Three paraffin-embedded heart bases of apoE-/- mice were serially sectioned in 5-μm thick sections. Every slide was stained with HE before the next section was cut. No further sections were cut when the tissues showed the connection between the coronary arteries and the aortic root. The paraffin-

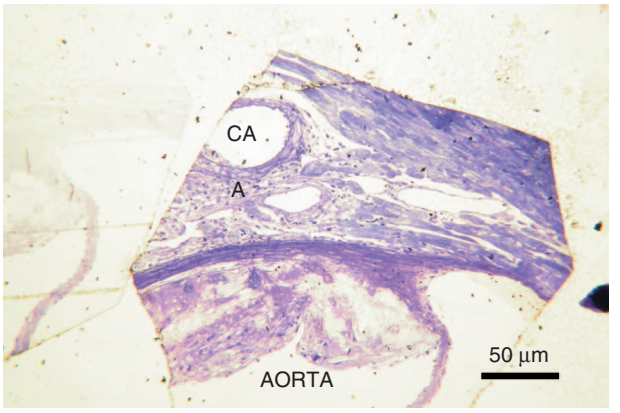

Fig. 1. TEM examination of a 1-µm thick section stained with toluidine blue dye showing inflammatory cells. Inflammation cells scattered in adventitia. CA: coronary artery; A: adventitia.

embedded bases were immersed three times in xylene for 3 h, and incubated twice in pure ethanol for 15 min. The tissues were dehydrated with gradient ethanol from 95% to 70%. The position at which the CAs connected with the aortic root was confirmed using an anatomical microscope. Several parts were divided into samples for transmission electron microscopy (TEM) to confirm the different kinds of inflammatory cells in the adventitia around the CA. The samples for electron microscopy were immersed in 2.5% glutaraldehyde overnight and post-fixed with 1% O_sO_4 for 2 h, followed by dehydration through graded ethanols. They were embedded in the Epon mixture. Ultra-thin sections were doubly stained with uranyl acetate and lead citrate and were examined with an JEM-1200EX electron microscope. Toluidine blue dye was applied to 1-µm-thick sections that were preliminarily examined by light microscopy to determine the location of the opening of the aorta into the coronary and to identify the cell-type (Fig. 1). The blocks were trimmed to centre on the aorta and CA under electron microscopy analysis. The blocks were sectioned and viewed with a transmission electron microscope (JEM-1200EX, Tokyo, Japan).

Results

All mouse CA branches were classified as one of three types: type I, type II and type III (Fig. 2, A, B and C, respectively). In type I tissues (Fig. 3, A and B), the initial stage, adventitial inflammatory cells were seen in the coronary of the specimen. Sixty percent of the infiltrated inflammatory cells were macrophages positive for CD68 (Fig. 3, A and Table 1, $P < 0.0001$), which was significantly more than in type II (27%) or type III (15%) tissues. The typical morphology of single macrophages was also examined

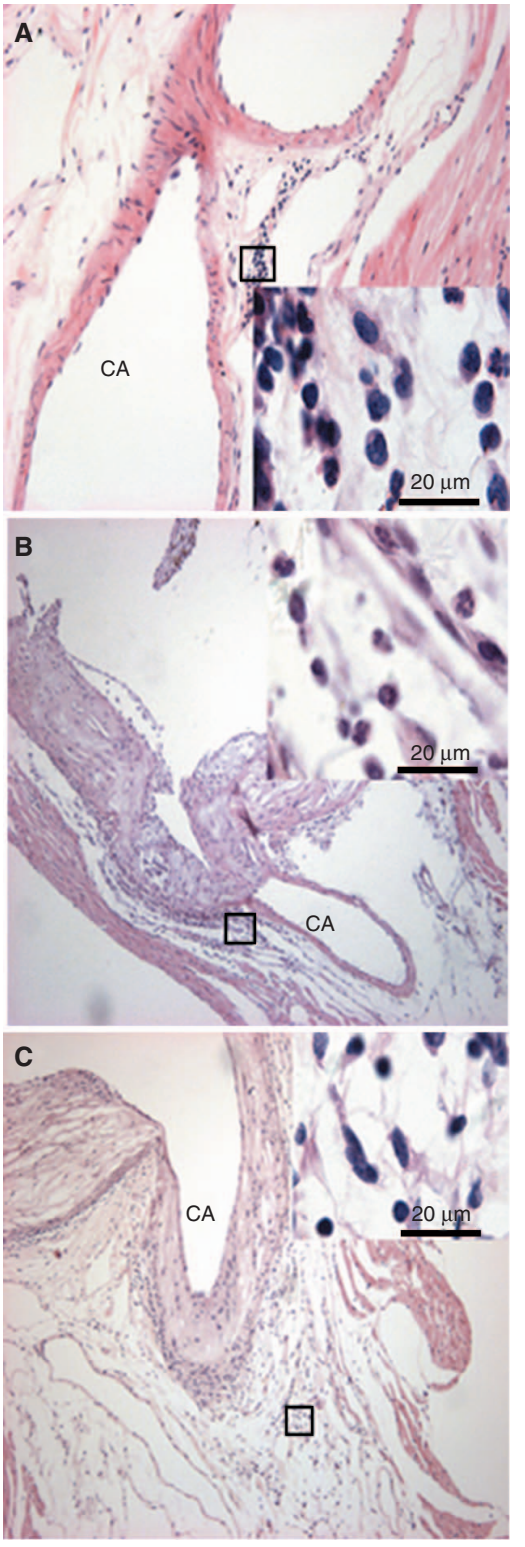

Fig. 2. Three types of CA tissue. A. Type I shows infiltration of inflammatory cells in the CA adventitia but no lesions in the intima (HE ×20). B. Type II shows infiltration of inflammatory cells in the CA adventitia with the front part of extending lesions in the intima (HE ×10). C. Type III shows infiltration of inflammatory cells at the CA adventitia with lesions covering the intima surface (HE ×10).

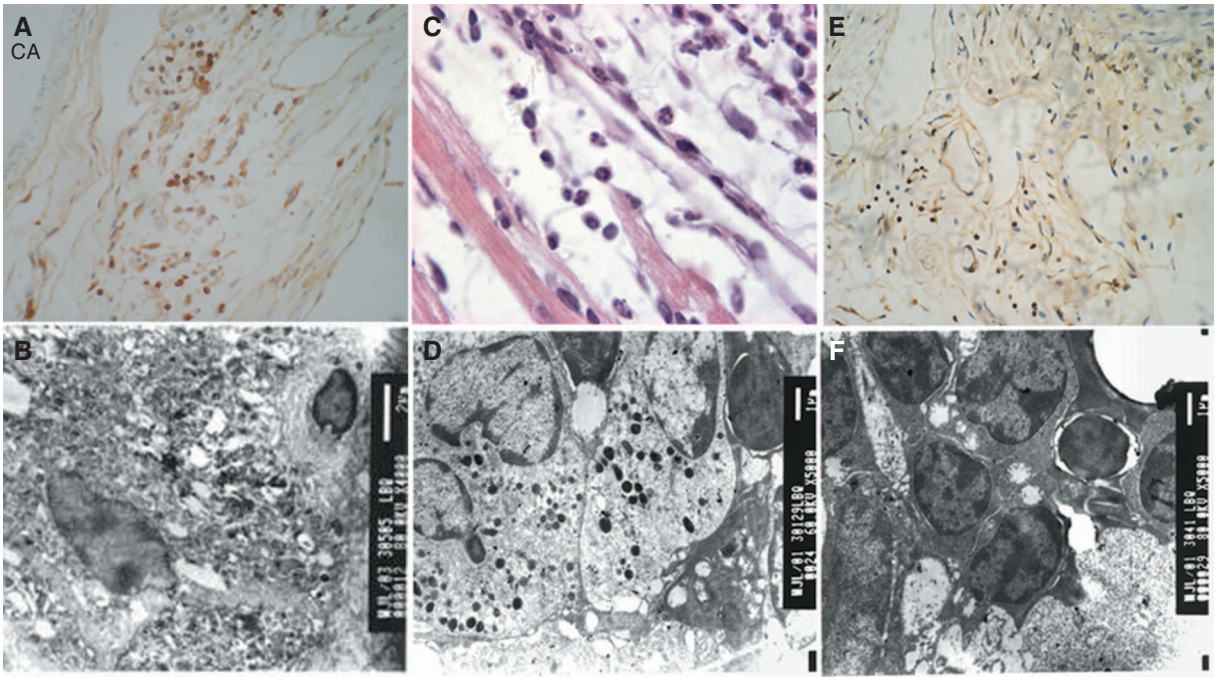

Fig. 3. A section of CA adventitia without extending lesions (type I). The section was taken from the same series as the section in Fig. 3A. A. A macrophage stained by immunohistochemical staining (IHC ×40). B. Electron microscopic image of a macrophage in the CA adventitia showing an anomalous external shape the bulk of which is enlarged. The image shows some nucleoplasm attached to the interior of the nuclear membrane, the size of phagosomes and residual bodies collected in cytoplasm (80.0 kV ×4000). C. Neutrophils stained by HE in type II tissues (nucleus generally has 3-6 segments and chromatin appears granular; HE ×100). D. Two adjacent neutrophils are in the CA adventitia with highly electron-dense granules in the cytoplasm (60.0 kV ×5000). E. Infiltration of inflammatory cells at the CA adventitia with lesions covering the intima surface. IHC staining shows that most of these cells are lymphocytes in type III tissues. $CD79_\alpha$ positive lymphocytes were shown to be B lymphocytes, whereas the other lymphocytes ($CD79_\alpha$ negative) were usually T lymphocytes (IHC ×40). F. Several lymphocytes in the CA adventitia had highly electron-dense nuclei (80.0 kV ×5000).

by electron microscopy (Fig. 3, B). The other infiltrated cells were lymphocytes and neutrophils. The histological construction of the coronary wall appeared normal under electron microscopic observation. In type II tissues (Fig. 3, C and D), the advanced stage, 57% of the inflammatory cells at the CA adventitia were neutrophils (Table 1, $P < 0.0001$) which was significantly more than in type I (7%) or type III (5%) tissues. Some other inflammatory cells, including macrophages, lymphocytes, a few plasma cells, mast cells and eosinophils, were also found in the CA adventitia (data not shown). In type II tissues, the adventitia became thicker than the normal adventitial construction. Accompanying thicker adventitia, fiber structure proliferation and richer vasa vasorum (VV) in the CA adventitia with the front part of extending atherosclerostic lesions in the intima were seen. The more aggregated inflammatory cells were seen in coronary adventitia. In type III tissues (Fig. 3, E and F), the mature stage, 67% of the infiltrated inflammatory cells in the CA adventitia were lymphocytes (Table 1, $P < 0.0001$), which was significantly more than in type I and type II tissues. Most of the lymphocytes were B lymphocytes positive for $CD79_\alpha$. Few coronary contained dense nodular or lymphonodulus inflammatory infiltrates in the adventitia adjacent to intimal plaques. The adventitia became thicker than that of the normal coronary. In these specimens, the pre-existing media were markedly attenuated.

Table 1. The constituent ratio of inflammatory cells in three types of coronary arteries adventitia of apoE-/-mice (%)

Cell Type	Type I	Type II	Type III
Macrophage	60	27	15
Neutrophil	7	57	5
Lymphocyte	33	12	67
Other cells	0	4	13

$\chi^2 = 164.334$, $P < 0.0001$.

Five male C57BL/6 mice also aged 60 weeks formed the control group. Five CA samples of the C57BL/6 mice were tested and there were no athero-

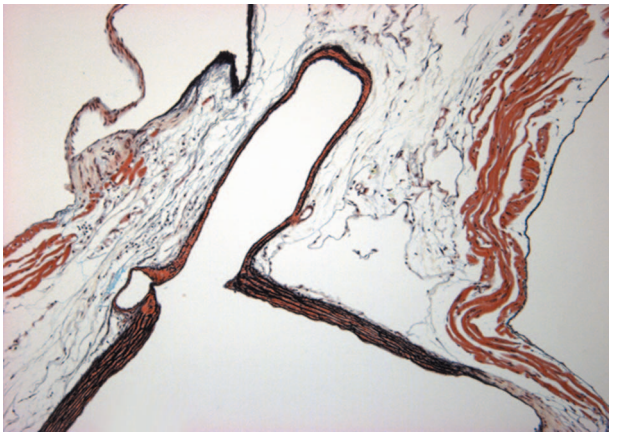

Fig. 4. In C57BL/6 mice aged 60 weeks, no CA was found extending from the aorta and no inflammatory cells were seen in the adventitia. (Movate ×20).

sclerotic lesions in the intima and no infiltration of inflammatory cells within all CA adventitia (Fig. 4).

Discussion

Inflammatory cells were reported in the adventitia surrounding atherosclerotic arteries by Albutt in 1915 (1). In 1962, Schwartz and Mitchell studied more than 440 tissue blocks from 111 randomly selected men and women aged more than 35 years, and found that there was a positive relationship between the degree of adventitial inflammation and the severity of atherosclerotic disease in the overlying arterial wall, which is often found associated with necrosis or thinning of the medial wall (18). Seo *et al*. reported that adventitial inflammation occurs in innominate arteries with advanced atherosclerotic lesions in apoE-/- mice (20). Most researchers have proposed that adventitial inflammation is the result of atherosclerosis and is a response to atherosclerotic lesions.

Rayner and colleagues studied sections of aorta in apoE-/- mice by *in-situ* hybridization and found that expression of monocyte chemotactic protein-1 (MCP-1/JE) and chemotactic cytokines receptor-2 (CCR-2, MCP-1 receptor) mRNA occurs earlier in the adventitia than in the intima (15), indicating that adventitial inflammation may be involved in the early stages of atherosclerosis development. Isik used a combination of *in situ* hybridization and immunocytochemistry to localize JE gene expression in specific cell types using a rat aortic transplant model of acute arterial graft rejection. Within 3 days of transplantation, JE mRNA expression was detected in adventitial mesenchymal cells, probably fibroblasts. Intimal JE mRNA expression was detected 7-20 days after transplantation (7). The apoE-/- mice were fed a hyperlipidic diet with 5-bromo-2-deoxyuridine (BrdU). The early proliferative changes occurred in the adventitia and BrdU-labeled cells first emerged in the adventitia, and were then observed in the intima (22). In addition, there is an association between VV and atherosclerotic plaque formation. Ritman's group hypothesized that low densities of vasa vasorum may play a role in the localization of early atherogenesis (3). Their researches showed that adventitial inflammation and atherosclerosis were related, because in normal arteries adventitial inflammation was absent; however, once atherogenesis occurred, adventitial inflammation increased with the extent and severity of atherosclerotic plaque formation (9, 10, 17). The inflammatory reaction within the adventitia is associated with increased VV, indicating that inflammatory cells might use VV as their own means of transportation into the aortic wall. These data indicated that the relationship with adventitial inflammation may be an early event in the development of atherosclerotic lesions. And during the development of intimal lesions, most accumulated cells were neutrophils; when the lesions extended and covered the surface of the intima, most accumulated cells were lymphocytes (8). The cell populations worked in concert to evoke an inflammatory response that propagated inward toward the intima (11). A more significant finding was the occurrence of perivascular inflammation without intimal lesions. These findings support the hypothesis that adventitial inflammation may be an early event in the development of atherosclerotic lesions. The work of Houtkamp *et al*. (5) has also shown that most infiltrated cells at mature lesions in CA adventitia are B lymphocytes rather than T lymphocytes. In addition, Schwartz and Johnson (19) have reported that lymphocytes and macrophages are recruited at the abdominal aorta and femoral artery of the adventitia in apoE-/- mice aged 9 months, in agreement with our findings that most infiltrated cells at the CA adventitia were lymphocytes and that most were B lymphocyte during the later stages of atherosclerotic lesion development.

Up to now, the mechanism by which inflammatory cells develop at the CA adventitia has been unclear. It is possible that hypercholesterolemia stimulates the mRNA expression and secretion of MCP-1 protein in adventitial fibroblasts (21). Increased MCP-1 levels attract monocytes from the vasa vasorum, which can then differentiate into macrophages. Interleukin-8 (IL-8) and granulocyte-macrophage colony-stimulating factor (GM-CSF) can lead to recruitment of neutrophils to the adventitia. MCP-1, IL-8 and other cytokines could be transferred to the intima from the vasa vasorum to accelerate the spread of lesions to the CA trunks (4, 14).

The inflammation in CA adventitia plays an

important role in the formation of atherosclerotic lesions of apoE-/- mice. Atherosclerosis is now regarded as a chronic inflammatory disease. These cell-type changes in CA adventitia, however, indicate that adventitial cells change in component with the development of atherosclerotic lesions. Different cell types predominate in different stages of CA adventitia. The neutrophils are closely related to the extending of atherosclerotic lesions in apoE-/- mice.

Acknowledgments

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