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Different Role of Macrophages and Vascular Smooth Muscle Cells in Atherosclerotic Lesions of Watanabe Heritable Hyperlipidemic (WHHL) Rabbit between Aorta and Coronary Artery

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ABSTRACT

The WHHL rabbit is often used to investigate the pathogenesis of atherosclerosis. Here we elucidate differences in the pathogenesis of atherosclerosis in coronary arteries (CA) and aorta (AO) by comparing dynamic changes in the distribution of vascular smooth muscle cells (VSMCs), macrophages, collagen and extracellular deposit during atherosclerosis in WHHL rabbits. Sections of CA and AO were obtained at the early, transitional and advanced stages of atherosclerosis and stained with hematoxilin-eosin, elastica van Gieson, antibodies specific to the above components, antibody against proliferated cell nuclear antigen (PCNA) and TUNEL. The relative areas of VSMCs, macrophages, collagen fibers and lipid deposits were calculated. In the early-stage atherosclerosis, VSMCs were predominant in CA lesions, while macrophages were predominant in AO lesions. PCNA-positive VSMCs and macrophages were noted in early-stage atherosclerosis in CA and AO. Collagen type I, III-V fibers were present in early-stage lesions of the AO, while type VI increased in the deep layer during the progression of atherosclerosis. The proportion of apoptotic cells increased in CA and AO lesions with the progression in atherosclerosis. Our results showed differences in the distribution patterns of VSMCs and macrophages at various stages of atherosclerosis in CA and AO of WHHL rabbits.

Key words: *Atherosclerosis, Aorta, Coronary artery*

It is well known that disorders of lipid metabolism are associated with atherosclerosis and resultant coronary artery and cerebrovascular diseases. Since mortality from coronary heart disease has gradually increased worldwide, including in Japan, elucidation of the mechanism of atherosclerosis is important in order to design appropriate therapeutic regimens and programs for prevention of coronary heart disease.

The Watanabe heritable hyperlipidemic (WHHL) rabbit was inbred by Watanabe et al in 1973 and since then has been used to investigate human familial hypercholesterolemia often associated with high atherosclerotic vascular diseases[8,26,27)]. In WHHL rabbits, atherosclerosis is commonly observed in the aorta, where it appears from 2 months of age and is observed in almost all 5-month old rabbits. In contrast to the aorta, atherosclerotic changes in coronary lesions appear from 4 months of age and are present in 70% of 20-month old rabbits[22)]. This difference in the progression of atherosclerosis between the aorta and coronary artery is quite similar to that in human familial hypercholesterolemia.

The present study was designed to elucidate the different mechanism involved in the progression of atherosclerosis affecting coronary arteries and the aorta. For this purpose, we compared the pathological changes in atherosclerosis including vascular smooth muscle cells (VSMCs), macro-

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phages and collagen fibers (type I, III, IV, V and VI collagens), which are considered to be main components of atherosclerotic lesions, between the coronary artery and aorta at the early, transitional and advanced stages by histological and immunohistochemical examinations. We also determined the contribution of different cellular components to cell proliferation or death at each stage of atherosclerosis by proliferated cell nuclear antigen (PCNA) staining and terminal-deoxynucleotidyl transferase medicated d-UTP nick end labeling (TUNEL) at each stage in both coronary and aortic lesions.

MATERIALS AND METHODS

Animals and sample preparation

Fourteen 4- to 6-week old male WHHL rabbits with an averaged body weight were kindly given by Dr. Shiomi at Kobe University. The rabbits were fed 120 g/day of regular chaw and tap water ad libitum, and bred until 12 to 28 weeks. After midline section of the abdomen under pentobarbiturate anesthesia, the aorta and heart were dissected out, and the heart was cut into 7 pieces and the aorta into 10 pieces[22]. Four of 7 pieces of the heart and 5 of 10 pieces of the aorta were immediately mounted in optimal cutting temperature (OCT) compound (Miles Laboratories, Elkhart) and frozen in liquid nitrogen to prepare sample slices for immunohistochemistry. The other samples were fixed in 4% formalin and mounted in paraffin, and 4-μm thick consecutive slices were prepared for staining by hematoxylin-eosin (HE) and elastica van Gieson. Progression of the atherosclerosis was classified according to the degree of intimal thickening into early, transitional and advanced stages after examination of 40 sequential slices. The early stage was defined as intimal thickening of <10 μm in both coronary arteries and aorta. The transitional stage represented intimal thickening of 40 to 100 μm in coronary arteries and 100 to 250 μm in the aorta. The advanced stage represented intimal thickening of >150 μm in coronary arteries and >300 μm in the aorta. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of Hiroshima University.

Immunohistochemistry

Specific antibodies against rabbit macrophage (RAM-11, Dako Corporation, Carpinteria, CA), alpha-smooth muscle actin (αSMA, IA4, Dako), PCNA (PCNA, PC10, Dako) and collagen fibers (anti-collagen I, II, IV, V and VI, Fuji Pharmaseutical Co., Tokyo, Japan) were purchased from commercial companies. The sample slices fixed in 4% formalin and mounted in paraffin were subjected to immunohistochemical staining with RAM-11, IA4 and PC10 by the ABC method after removal of paraffin according to the protocol provided by the manufacturer. (Histofine SAB-PO(M) kit, Nichirei Co., Tokyo). Briefly, the sample was treated with methanol containing 0.3% H_2O_2 for 30 min at room temperature. After rinsing with tapped water, non-specific blocking was performed with 10% rabbit serum for 30 min at room temperature. Then, the sample was incubated overnight with the primary antibody at 4°C. After rinsing with phosphate-buffered saline (PBS), the sample was treated with biotin-conjugated secondary antibody for 30 min, and then with peroxidase-conjugated streptoavidin for 30 min. After development by reaction with diaminobenzidin, the sample was immersed in hematoxylin for staining the nuclei. For PCNA staining, the sample was immersed in new-fuccin to observe macrophages. For the immunohistochemical staining of collagen fibers, frozen samples were cut at 40-μm thick and fixed in acetone at −20°C before the staining.

TdT-mediated dUTP-biotin nick end labeling (TUNEL)

To evaluate apoptotic cell death in the vessel wall, the TUNEL method was performed using ApopTag® kit (Onco Inc.) and paraffin-fixed sample slices. After deparaffinization, protein-digestion and blocking of endogenous peroxidase activity, the sample was reacted with digoxigenin-conjugated dUTP by TdT enzyme. After termination of the reaction, the sample was treated with antidigoxigenin antibody conjugated with peroxidase, developed by 3,3-diaminobenzidine tetrahydrochloride (DAB), and then stained with hematoxylin.

Quantification of histological and immunohistochemical findings

The stained slices were examined under a light microscope and images were digitized into a personal computer using a 3CCD camera. Stored images were analyzed for the following parameters using the National Institute of Health (NIH) image analysis software: the areas of VSMCs and macrophages within the intima relative to that of α-SMA or RAM 11- positive cells. In a similar manner, we calculated the relative area of collagen and extracellular deposits containing lipid and calcification.

Statistical analysis

Comparison of histological and immunohistochemical changes between the different stages of atherosclerosis was performed by Student t-test. Values were expressed as mean ± SD. A p value less than 0.05 denoted the presence of a statistically significant difference.

RESULTS

Histological changes

In both coronary artery and aortic lesions, the internal lamina was preserved at the early stage of atherosclerosis, but was partially damaged at the transitional stage, and completely destroyed at the advanced stage (Fig. 1). Lipid deposits were observed in the extracellular space from the transitional stage in both coronary artery and aortic lesions, but the size of these deposits was significantly larger and their numbers higher in aortic lesions, especially in the media and adventitia. Scattered areas of calcification were observed in the advanced stage in coronary artery lesions while they were abundant in the aortic lesions at a similar stage.

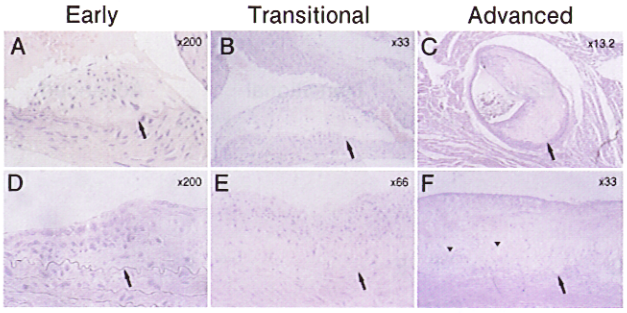

Fig. 1. Hematoxylin-eosin staining of atherosclerotic lesion in coronary artery (A, B, C) and aorta (D, E, F) at each pathological stage.
Arrow and arrowhead show internal elastic lamina and lipid core, respectively. Numbers in the right upper corner represent magnifications.

Changes in vascular smooth muscle cells

At the early stage of atherosclerosis, a few VSMCs were observed in atherosclerotic lesions of the coronary arteries, but few spindle-shaped VSMCs were found in aortic lesions. At the transitional stage, although several VSMCs were observed in both coronary artery and aortic lesions, the distribution of these cells was different between the vessels (Fig. 2). In coronary artery lesions, VSMCs were found in the superficial to middle layer of the intimal wall, while most of those cells were localized in the superficial layer of intimal lesions in the aorta. At the advanced stage, VSMCs were present in the superficial

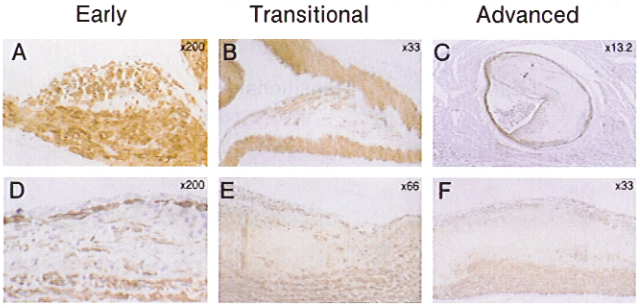

Fig. 2. Immunohistochemical staining of atherosclerotic lesion in coronary artery (A, B, C) and aorta (D, E, F) with anti-α-SMA at each pathological stage.
VSMCs are stained brown. Numbers in the right upper corner represent magnifications.

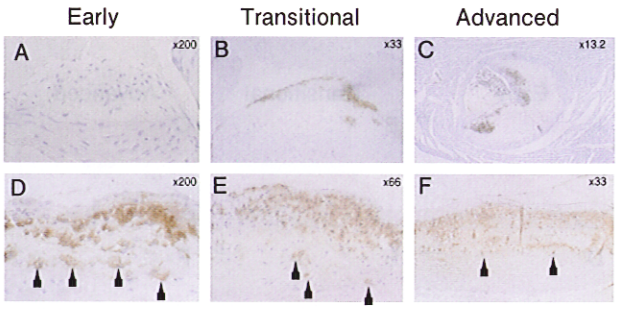

Fig. 3. Immunohistochemical staining of atherosclerotic lesion in coronary artery (A, B, C) and aorta (D, E, F) with RAM 11 at each pathological stage.
Macrophages are stained brown. Arrowhead shows stained macrophages located in media. Numbers in the right upper corner represent magnifications.

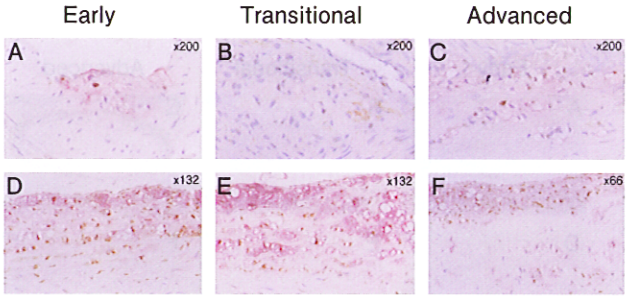

Fig. 4. Immunohistochemical double staining of atherosclerotic lesion in coronary artery (A, B, C) and aorta (D, E, F) with anti- PCNA and RAM 11 at each pathological stage.
PCNA is stained brown and macrophages are stained red. Numbers in the right upper corner represent magnifications.

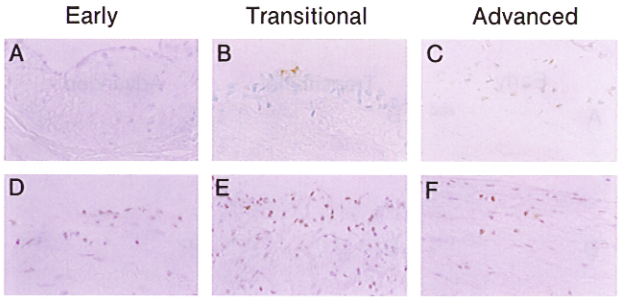

Fig. 5. TUNEL staining of atherosclerotic lesion in coronary artery (A, B, C) and aorta (D, E, F).
TUNEL positive cells are stained red-brown. Magnification of all panels is × 132.

layer of both coronary artery and aortic lesions. Interestingly, thinning of the media and reduction of VSMCs stained with anti-smooth muscle actin in that area were observed in some parts of the vessels, suggesting that change in medial VSMCs is characteristic of the progression of atherosclerosis.

Changes in macrophages

In coronary artery lesions, few macrophages were found at the early stage of atherosclerosis, and such cells were localized in the superficial layer of intima at the transitional stage (Fig. 3). At the advanced stage of atherosclerosis, foamy cells were observed in the middle layer of the intima. In aortic lesions, the whole intima was occupied by foamy cells during the progression of atherosclerosis. At the advanced stage of aortic lesions, foamy cells were observed even beyond the lamina interna, suggesting that the macrophages had

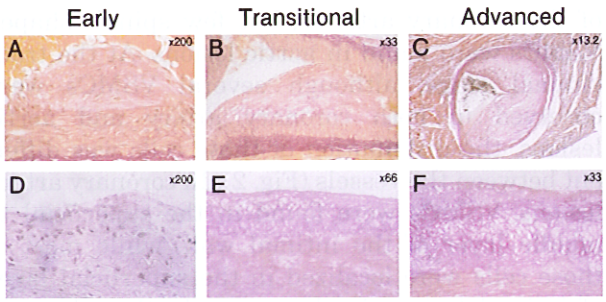

Fig. 6. Elastica van Gieson staining of atherosclerotic lesion in coronary artery (A, B, C) and aorta (D, E, F) at each pathological stage.
Numbers in the right upper corner represent magnifications.

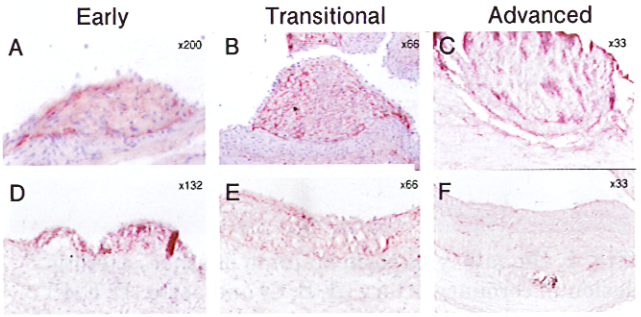

Fig. 7. Immunohistochemical staining of type I collagen in atherosclerotic lesion of coronary artery (A, B, C) and aorta (D, E, F) at each pathological stage.
Collagen fibers are stained red. Numbers in the right upper corner represent magnifications.

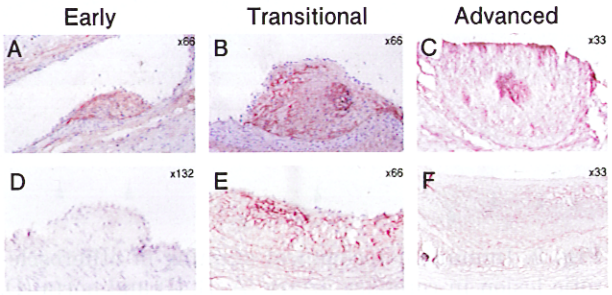

Fig. 8. Immunohistochemical staining of type III collagen in atherosclerotic lesion of coronary artery (A, B, C) and aorta (D, E, F) at each pathological stage.
Collagen fibers are stained red. Numbers in the right upper corner represent magnifications.

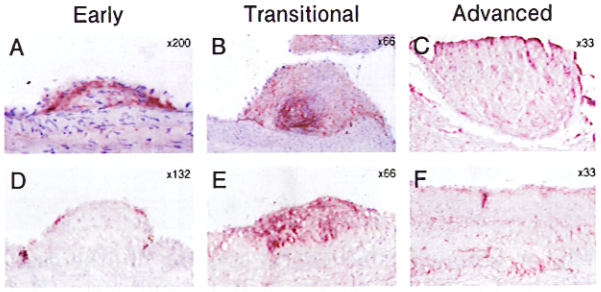

Fig. 9. Immunohistochemical staining of type IV collagen in atherosclerotic lesion of coronary artery (A, B, C) and aorta (D, E, F) at each pathological stage.
Collagen fibers are stained red. Numbers in the right upper corner represent magnifications.

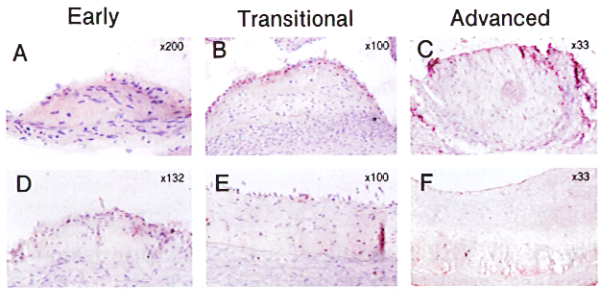

Fig. 10. Immunohistochemical staining of type V collagen in atherosclerotic lesion of coronary artery (A, B, C) and aorta (D, E, F) at each pathological stage.
Collagen fibers are stained red. Numbers in the right upper corner represent magnifications.

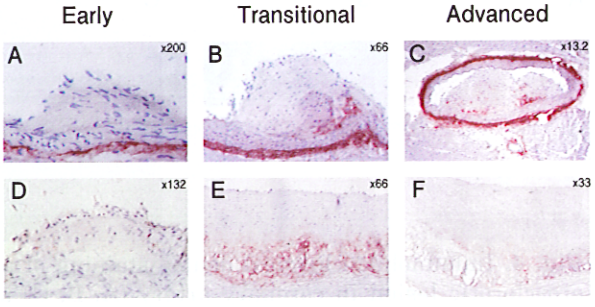

Fig. 11. Immunohistochemical staining of type VI collagen in atherosclerotic lesion of coronary artery (A, B, C) and aorta (D, E, F) at each pathological stage.
Collagen fibers are stained red. Numbers in the right upper corner represent magnifications.

invaded the microvessels generated by neovascularization.

Proliferation and apoptosis of cells during progression of atherosclerosis

Figure 4 shows immunohistochemical staining of cells stained with anti-PCNA antibody. In early stage lesions, few PCNA-positive cells were found only in the superficial layer of the thickened intima layer of coronary arteries. However, these cells were observed throughout the entire intima layer of early-stage aortic lesions. In transitional to advanced stage atherosclerosis, PCNA-positive cells were also detected in the inner layer that contained foamy cells in coronary artery lesions, while large numbers of VSMCs and macrophages present throughout the whole layer of aortic lesions were stained with anti-PCNA antibody.

Figure 5 shows apoptotic cells stained by the TUNEL method at each stage in aortic lesions. TUNEL-positive cells gradually increased during the progression of atherosclerotic aortic lesions and were identified as both VSMCs and macrophages. In contrast, few TUNEL-positive cells were identified in coronary artery lesions at all stages of atherosclerosis.

Change in collagen fibers

The number of collagen fibers stained by elastica van Gieson increased with the progression of atherosclerosis in both coronary artery and aortic lesions. Comparison between the coronary artery and aortic lesions shows that number of collagen fibers was higher in coronary artery than in aortic lesions at early and transitional stages of atherosclerosis (Fig. 6).

The distribution of collagen isoforms type I and type III was similar (Fig. 7, Fig. 8). Both types were observed in early stage lesions in both the coronary arteries and aorta, and were distributed throughout the proliferated intima. In advanced stage lesions, they were mainly located in the superficial to intermediate layers of the proliferated intima, and there was no difference in their distribution pattern in the coronary artery and aortic lesions.

Type IV collagen was generally observed in the extracellular space of the media in intact vessels. In atherosclerotic lesions, type IV collagen was also distributed widely in the proliferated intima throughout the different stages of atherosclerosis in both coronary artery and aortic lesions (Fig. 9). The amount of collagen fiber was larger in the intima than in the media and the distribution was not different between coronary artery and aortic lesions irrespective of the stage of atherosclerosis.

Type V collagen was expressed a little later than other collagen fibers (Fig. 10). In early-stage atherosclerosis, a few type V collagen fibers were identified in the superficial layer only of proliferated intima. With the progression of atherosclerosis, the expression of this collagen type increased to the whole layer of the intima, but the expression was weaker than that of other collagens. The distribution was not different between coronary artery and aortic lesions.

Expression of type VI collagen also appeared also later than type I, III and IV collagen fibers (Fig. 11). No expression was observed at the early stage, whereas, at the transitional to advanced stage, the expression was limited to the internal lamina of the proliferated intima. The distribution of type VI collagen was similar in both coronary artery and aortic lesions.

Quantitation of macrophages, VSMCs, collagen fibers and extracellular deposits during different stages of atherosclerosis

Figure 12 shows quantitative analysis of the area of macrophages, VSMCs, collagen and extracellular deposits relative to intimal thickening during different stages in coronary artery and aortic atherosclerotic lesions. In coronary lesions, VSMCs and collagen formed significant areas of the intima in early-stage atherosclerosis. However, progression of atherosclerosis from the transitional to advanced stages was associated with a reduction of VSMCs while the relative area of collagen fibers remained stable.

Macrophages were not prevalent during the different stages of atherosclerosis in coronary arteries. In contrast to coronary artery lesions, macrophages occupied most of the intima in aortic lesions, but became fewer with the progression of atherosclerosis. In contrast, the relative area of collagen fibers gradually increased with the progression of atherosclerosis, whereas the content of VSMCs remained similar at different stages of atherosclerosis. The proportional area of extracellular deposits, including lipid and calcification, increased with the progression of atherosclerosis in both coronary artery and aortic lesions. At the advanced stage of atherosclerosis, the relative areas of macrophages, VSMCs, collagen fibers and extracellular deposits were identical in coronary artery and aortic lesions.

DISCUSSION

The WHHL rabbit is generally used as a model of familial hypercholesterolemia and for the investigation of atherosclerosis[8,26,27]. It is also suitable as an experimental model to investigate the atherosclerotic process in the aorta because almost all rabbits develop atherosclerosis of the aorta at 3 months after birth[22,27]. Although several investigators have described the atherosclerotic changes in the aorta of the WHHL rabbit, only a few reports have investigated atherosclerosis in the coronary artery of these rabbits[22]. This is proba-

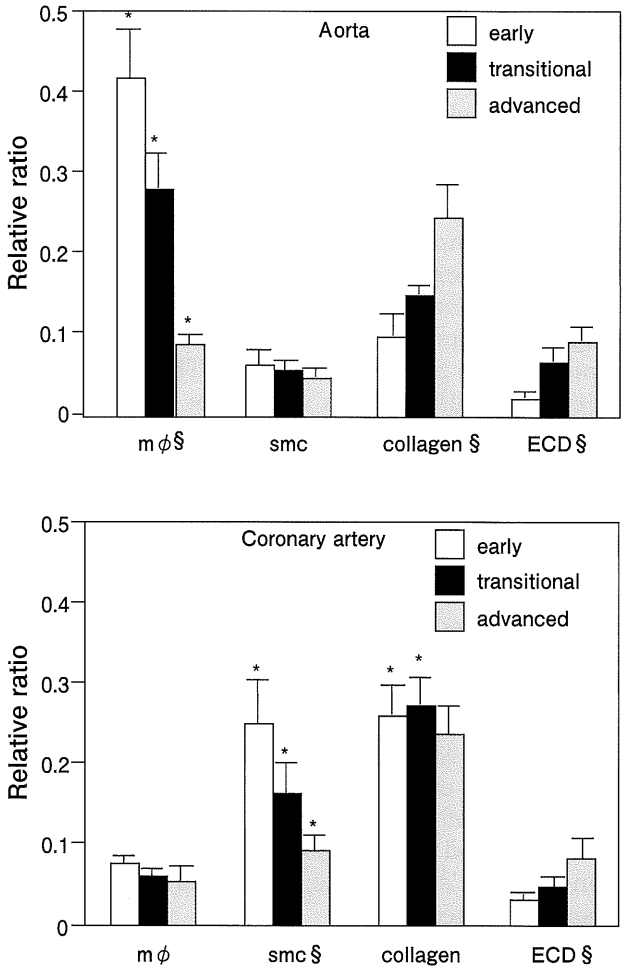

Fig. 12. Relative content of macrophages (mø), VSMCs (smc), collagen fibers (collagen) and extracellular matrix deposit (ECD) containing calcification and lipid core in intimal thickness.
Calculation of the ratio is described in Materials and Methods. * indicates a significant difference ($p<0.05$) between aorta and coronary artery, and § between the stages ($p<0.05$).

bly due to the belief that coronary heart disease is rare in WHHL rabbits. However, it is important to investigate the pathological changes in coronary arteries of WHHL rabbits to determine whether the pathogenesis of atherosclerosis in coronary arteries is different from that in the aorta. In the present study, we examined simultaneous pathological changes, including changes in collagen fiber composition, in atherosclerotic lesions in both the aorta and coronary arteries at the early, transitional and advanced stages.

Our results showed that the process of atherosclerosis is quite different between the aorta and coronary arteries of WHHL rabbits. In the coronary artery, proliferation of VSMCs and accumulation of collagen fibers (which might be generated from differentiated and migrated VSMCs) were predominant events from the early stage of atherosclerosis. In comparison, only few macrophages were observed at the early stage of atherosclerosis in the coronary artery lesions. With the progression of atherosclerotic lesions, the number of VSMCs gradually decreased, while apoptotic cells were widely observed, suggesting that the decrease in VSMCs in the transitional and advanced stages was due to apoptosis of these cells. The content of collagen fibers in the coronary artery lesions did not change during the different stages of atherosclerosis despite the apoptosis of VSMCs. These results suggest that the collagen fibers produced by VSMCs at the early stage are retained without any degradation in the late stages of atherosclerosis.

The pathological data of the aorta in the present study is consistent with the mechanism of atherosclerosis as a response to injury urgued by Ross[20], but those of coronary artery do not accord with the hypothesis. Siomi et al pointed out in their report that the pathogenesis of atherosclerosis in WHHL rabbits was quite different between the aorta and coronary artery[22,23]. This might be the result of the different histological construction of the vessels. It is also reported that the pathology of atherosclerosis in human coronary artery is characterized by intimal thickening rather than the lipid deposits which are usually observed in the aortic lesion, and that the arteriosclerotic lesion is mostly occupied by VSMCs in coronary artery and by macrophages in aorta[13].

Six types of collagen fibers, type I, III, IV, V, VI, and VIII, are distributed in the human arterial wall, and constitute 20% of the dried weight of large arteries and in 40% of middle to small arteries[2]. Morton and Burleigh et al reported that the ratio of type I to type III collagen fibers was about 3 to 1 in both media of normal aorta and intima of atherosclerotic lesions[4,6,18]. In the present study, more type I and III collagen fibers were distributed in the thickening intima than in the media of the coronary artery lesion, and the expressions were maintained during the stages. Furthermore, it was observed that expressions of type IV, V and VI collagen gradually increased with the progression in arteriosclerosis, and that type V collagen was expressed in the superficial layer of the lesion and type VI in the profound. These data suggested that the expression pattern of collagen fibers might be related to the pathogenesis of progression in atherosclerosis[11,15,16,19]. Ross et al demonstrated that type VI collagen was distributed in plunged lesions, suggesting that type VI collagen might serve as a protector against blood flow pressure[21]. In the present study, the expression of type VI collagen was observed in the profound lesion of thickening intima of coronary artery. Type VI collagen might be generated to maintain the profound structure which becomes fragile with the progression in atherosclerosis[10,12,24]. In contrast, type V and VI collagens were observed less in aor-

tic lesions, although type I and III collagens were expressed at similar stages to coronary artery. Although the functions of collagen fibers are unclear in each lesion, the different distribution in collagen fibers may reflect the different pathogenesis of progression in atherosclerosis between coronary artery and aorta.

It is generally demonstrated that apoptotic cells are more common in atherosclerotic lesions than in normal vessels during all stages of the progression of atherosclerosis[7,9], and that the expression of apoptosis-related signals such as Fas, Bcl-2, Bax and p53 is more elevated in atherosclerotic lesions in comparison with normal vessels[3,14,25]. It is also reported that VSMCs in coronary atherosclerotic lesions are more sensitive to the induction of apoptosis than those in normal arterial wall[7]. In the present study, TUNEL positive cells were found in the lesions of both coronary artery and aorta together with a proliferation of VSMCs and macrophages during the study period. These data suggest that the apoptosis of VSMCs and macrophages may be a protective response to cell migration and proliferation during progression in atherosclerosis. It has been demonstrated that unstable plaque in which the fibrous cap constructed by proliferated VSMCs and collagen fibers is fragile and may give rise to an acute coronary event[5]. Therefore, excretion of matrix metalloproteinase from foam cells and apoptosis of VSMCs might negatively contribute to the stabilization of atherosclerotic plaque, leading to plaque rupture[1,17,25]. Although the relationship between apoptotic cells and stabilization of atherosclerotic plaque was unclear in the present study, apoptosis of the VSMCs and macrophages found during the study period may be an inhibitory response to proliferation of the cells.

In summary, we demonstrated the different distribution of macrophages, VSMCs, apoptotic cells and collagen fibers in atherosclerotic lesions of the aorta and coronary artery in WHHL rabbits. This difference in distribution is attributed to functional disparities of endothelial cells and VSMCs between the aorta and coronary artery. Although rabbits and humans are different species, the present data may be useful for understanding the atherosclerotic lesions of the aorta and coronary artery in patients with familial hypercholesterolemia.

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