Cell Compositions of Coronary and Aortic Atherosclerotic Lesions in WHHL Rabbits Differ
An Immunohistochemical Study

Masashi Shiomi, Takashi Ito, Toyohiro Tsukada, Tatsuo Yata, Makiko Ueda

Abstract This study investigated whether coronary atherosclerosis was different from aortic atherosclerosis in Watanabe heritable hyperlipidemic (WHHL) rabbit, which develops hypercholesterolemia and atherosclerosis due to a genetic deficiency of low-density lipoprotein receptor. In contrast, coronary atherosclerosis has shown the opposite features. These results suggested that the role of macrophages and smooth muscle cells in the initiation and/or progression of coronary atherosclerosis differs from the role of these cells in aortic atherosclerosis.

Key Words • immunohistochemical analysis • smooth muscle cell • atherosclerosis • macrophage • WHHL rabbit

Epidemiological and clinical studies suggest that the serum cholesterol level is one of the most important risk factors for coronary heart disease. The Watanabe heritable hyperlipidemic (WHHL) rabbit, which develops hypercholesterolemia and atherosclerosis due to a genetic deficiency of low-density lipoprotein receptors, has long been used in studies on atherosclerosis. However, most of these studies have investigated only aortic atherosclerosis, which has been examined in detail by electron microscopy and immunohistochemical techniques. In contrast, coronary atherosclerosis in WHHL rabbits has not been investigated in detail because both the incidence and severity of coronary atherosclerosis are quite low. Hence, we selectively bred WHHL rabbits to increase the incidence and severity of coronary atherosclerosis.

The risk factors for coronary atherosclerosis apparently differ from those for aortic atherosclerosis; the suppressive or preventive effect of serum cholesterol reduction on coronary atherosclerosis is greater than that on aortic atherosclerosis. These findings suggest that some qualitative differences may exist between coronary and aortic atherosclerosis. However, no studies have examined the differences between these two types of atherosclerosis.

In the present study, we quantitatively examined the qualitative differences between coronary and aortic atherosclerosis. Atherosclerotic lesions consist of several cell fractions and extracellular matrix. We measured areas of macrophages and smooth muscle cells as cell fraction and areas of collagen fibers and extracellular lipid deposits as extracellular matrix in atherosclerotic lesions by using an image analyzer and compared the lesional composition of coronary atherosclerosis with that of aortic atherosclerosis.

Methods

Animals

The WHHL rabbits used in this study were bred at Kobe University. Fifteen rabbits were equally divided into three groups: 3 months old, 11 through 15 months old, and 20 through 24 months old. The rabbits were fed standard rabbit chow (CR-3, Clea Japan Inc) at 120 g/d and were given water ad libitum. According to the method of Rosenfeld et al.,* rabbits were anesthetized with an intravenous injection of sodium pentobarbital (25 mg/kg) and perfused with lactated Ringer's solution and Bouin's fixative using a perfusion apparatus (VPF-1, Nisshin EM). After perfusion fixation, the aorta and heart were excised. All animal experiments were conducted according to the "Guidelines for Animal Experiments at Kobe University School of Medicine."

Preparation of Histological Sections

The hearts and aortas were immersed in Bouin's fixative for at least 48 hours after excision. After immersion fixation, cross sections of the coronary arteries were prepared. In brief, the hearts were divided into six blocks and embedded in paraffin. The blocks containing the origin of the right coronary artery (RCO) and the main trunk of the left coronary artery (LMT) were sectioned at 200-μm intervals, and the other blocks were sectioned at 500-μm intervals. A total of approximately 50 segments from each heart were examined, and 10 4-μm-thick
sections were cut serially from each segment that had an atherosclerotic lesion. Coronary atherosclerosis was then examined in the LMT, RCO, left anterior descending artery (LAD), left septal artery (LSP), left circumflex artery (LCX), and right coronary artery (RCA).

Cross sections of the aorta were prepared by dividing the aortas into five portions (the aortic arch, the proximal and distal thoracic aorta, and the proximal and distal abdominal aorta). The segments that showed the most severe macroscopic lesions in each portion were embedded in paraffin, and 10-μm-thick sections were cut serially from each segment.

Serial sections from each segment were immunohistochemically or conventionally stained. Immunohistochemical staining with a muscle actin–specific monoclonal antibody (HHF35) and a rabbit macrophage–specific monoclonal antibody (RAM-11) was performed. The sections were also stained with Azan-Mallory, Kossa stain as calcium deposits. In Azan-Mallory-stained sections, fibers with three prime color elements (red, green, and blue) in the color image analyzer were detected as elastin, collagen, and blue) in the color image analyzer were detected as elastin, collagen, and extracellular areas showing black staining with muscle cells, cells with black reaction products after RAM-11 and Azan-Mallory staining as extracellular lipid deposits, and extracellular areas showing black staining with Kossa stain as calcium deposits. In Azan-Mallory–stained sections, fibers with three prime color elements (red, green, and blue) in the color image analyzer were detected as elastin, while fibers having only the blue element were considered as collagen. In addition, the average degree of aortic intimal thickening and coronary narrowing was calculated.

Statistical Analysis
Data were represented as mean±SEM. Statistical analysis was performed by using the Mann-Whitney U test for comparing mean values, and the χ² test or Fisher’s exact probability test was used for comparing frequency of histological findings.

Results
Progression of Atherosclerosis With Age
Table 1 shows the coronary artery narrowing in each age group. Coronary atherosclerosis had developed in all arteries except the LSP by 3 months of age, and coronary narrowing was markedly advanced with aging in the LMT, RCO, and LCX. Atherosclerosis had also developed in the aorta by 3 months of age, and the lesions progressed with aging at every site examined (data not shown). The most severe aortic luminal narrowing was 38.4±0.1% in the distal thoracic aorta at 20 through 24 months of age. However, aortic luminal narrowing was generally mild compared with the coronary lesions.

Since the severity of atherosclerosis varied widely in every arterial portion, we defined the stages of atherosclerosis as follows. Early lesions had an average intimal thickening of less than 10 μm in both the coronary arteries and aorta and were observed in 3-month-old rabbits. Transitional lesions had an average intimal thickening of 40 to 100 μm in the coronary arteries and 100 to 250 μm in the aorta and were observed at 11 through 15 months of age. Advanced lesions had an average intimal thickening of over 150 μm in the coronary arteries and over 300 μm in the aorta and were observed at 20 through 24 months of age.

Histological Findings
Early Lesions
Fig 1 shows an example of early coronary atherosclerosis. Some macrophages were scattered in the superficial portion of the intimal lesion (top), and numerous smooth muscle cells were observed throughout the intimal lesion (bottom). Fiberization of the intimal lesion was already in progress (data not shown).

Fig 2 shows an example of early aortic atherosclerosis. Macrophages formed a single layer on the internal elastic lamina (top, left), and numerous macrophages were observed throughout the intimal lesion (top, right). Macrophages were occasionally identified within the media. However, no smooth muscle cells were observed in the intimal lesion (bottom).

Transitional Lesions
Fig 3 shows an example of transitional coronary atherosclerosis. Macrophages were observed in the superficial portion, and the deep part of the lesion contained extracellular lipid deposits (top). Some of the macrophages had become small foam cells. Smooth muscle cells were widely observed underneath the macrophage layer, and HHF35 staining was decreased in the medial smooth muscle cells underlying the intimal lesion (bottom). Numerous collagen fibers were also observed throughout the intimal lesion (data not shown).

Fig 4 shows an example of transitional aortic atherosclerosis. Macrophages and foam cells were observed throughout the intimal lesion, and the deeper portions contained large extracellular lipid deposits (top). Large foam cells filled with cholesteryl ester were observed in the deep parts of the intimal lesion. Macrophages were also present in the media. Smooth muscle cells were scattered among the macrophages in the superficial part of the lesion, and HHF35 staining was decreased in these cells around the macrophages in the media (bottom). Collagen fibers were observed throughout the

<table>
<thead>
<tr>
<th>Age, mo</th>
<th>LMT</th>
<th>LAD</th>
<th>LCX</th>
<th>LSP</th>
<th>RCO</th>
<th>RCA</th>
</tr>
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<tbody>
<tr>
<td>3</td>
<td>12.5±5.6</td>
<td>6.0±4.2</td>
<td>5.1±2.3</td>
<td>0</td>
<td>44.6±6.7</td>
<td>11.1±1.0</td>
</tr>
<tr>
<td>11-15</td>
<td>61.2±4.0</td>
<td>23.4±13.7</td>
<td>34.8±5.6</td>
<td>10.8±3.5</td>
<td>88.0±3.2</td>
<td>10.0±1.0</td>
</tr>
<tr>
<td>20-24</td>
<td>70.2±1.8</td>
<td>16.8±8.8</td>
<td>63.2±5.3</td>
<td>10.4±4.6</td>
<td>81.2±2.2</td>
<td>12.4±8.6</td>
</tr>
</tbody>
</table>

WHHL indicates Watanabe heritable hyperlipidemic; LMT, main trunk of the left coronary artery; LAD, left anterior descending artery; LSP, left septal artery; RCO, origin of the right coronary artery; and RCA, right coronary artery. Luminal narrowing was calculated as follows: (lesional area/lesional area+luminal area)×100. Data are mean±SEM.

Quantitative Analysis of Atherosclerotic Lesions
Each section was observed under a color image analyzer (SP-500, Olympus Co) at magnifications of 56× through 280×, and various parameters were measured. We defined cells with black reaction products after HHF35 staining as smooth muscle cells, cells with black reaction products after RAM-11 staining as macrophages, extracellular vacuoles and lacunae with RAM-11 and Azan-Mallory staining as extracellular lipid deposits, and extracellular areas showing black staining with Kossa stain as calcium deposits. In Azan-Mallory–stained sections, fibers with three prime color elements (red, green, and blue) in the color image analyzer were detected as elastin, while fibers having only the blue element were considered as collagen. In addition, the average degree of aortic intimal thickening and coronary narrowing was calculated.

Statistical Analysis
Data were represented as mean±SEM. Statistical analysis was performed by using the Mann-Whitney U test for comparing mean values, and the χ² test or Fisher’s exact probability test was used for comparing frequency of histological findings.

Table 1. Luminal Narrowing of Coronary Arteries in WHHL Rabbits

<table>
<thead>
<tr>
<th>Coronary Narrowing, %</th>
<th>Age, mo</th>
<th>LMT</th>
<th>LAD</th>
<th>LCX</th>
<th>LSP</th>
<th>RCO</th>
<th>RCA</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>12.5±5.6</td>
<td>6.0±4.2</td>
<td>5.1±2.3</td>
<td>0</td>
<td>44.6±6.7</td>
<td>11.1±1.0</td>
<td></td>
</tr>
<tr>
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<td>61.2±4.0</td>
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<td>10.0±1.0</td>
<td></td>
</tr>
<tr>
<td>20-24</td>
<td>70.2±1.8</td>
<td>16.8±8.8</td>
<td>63.2±5.3</td>
<td>10.4±4.6</td>
<td>81.2±2.2</td>
<td>12.4±8.6</td>
<td></td>
</tr>
</tbody>
</table>

Luminal narrowing was calculated as follows: (lesional area/lesional area+luminal area)×100. Data are mean±SEM.
intimal lesion (data not shown). The internal elastic lamina was partly fragmented or had disappeared.

**Advanced Lesions**

Fig 5 shows an example of advanced coronary atherosclerosis. Macrophages were observed only in the superficial part of the intimal lesion, and many small extracellular lipid deposits were scattered throughout the intimal lesion (top). Granules considered to be the necrotic debris of foam cells were observed in the area of extracellular lipid deposits. Smooth muscle cells were observed under the macrophage layer, and HHF35 staining was decreased in the medial smooth muscle cells beneath the intimal lesion (bottom). Collagen fibers were observed throughout the intimal lesion (data not shown).

Fig 6 shows an example of advanced aortic atherosclerosis. Large foam cells filled with lipid, large extracellular lipid deposits, and numerous cholesterol crystals were observed between the middle and deep parts of the intimal lesion (top), and smooth muscle cells were scattered in the superficial portion (bottom). Collagen fibers were distributed throughout the lesion, the internal elastic lamina was fragmented or absent, and the media was thinned (data not shown).

**Changes of Lesion Composition With Disease Progression**

Changes of the lesion composition with the progression of coronary atherosclerosis were analyzed in the LMT, LAD, LCX, and RCA because the atherosclerotic lesions that developed at the RCO and LSP differed from those that developed in the other coronary arteries. RCO lesions were relatively rich in macrophages, and some of the developing LSP lesions showed fibrous thickening without macrophages and extracellular lipid deposits (data not shown).

Table 2 shows the areas of each lesional component of atherosclerosis. Early lesions were observed in 12 coronary artery segments and 11 aortic segments from five rabbits; the average intimal thickening was 2.7±0.6 μm and 3.2±0.6 μm, respectively. Transitional lesions were observed in 11 coronary artery segments and 13 aortic segments from five rabbits; the average intimal thickening was 70±7 μm and 173±14 μm, respectively. Advanced lesions were observed in 10 coronary artery segments and 13 aortic segments from five rabbits; the average intimal thickening was 238±19 μm and 481±33 μm, respectively. In coronary lesions, the area of each component increased with lesion progression. In aortic lesions, however, the smooth muscle cell area did not increase, and the macrophage area showed a downward trend with progression from transitional to advanced lesions.

Fig 7 compares each lesional component area by percent in the three lesion stages. The percent macrophage area in the coronary lesions was significantly
lower than in the aortic lesions, especially in the early (9.2±5.3% versus 43.5±6.8%, P<.005) and transitional (4.5±1.4% versus 28.2±3.6%, P<.001) stages. In contrast, the percent smooth muscle cell area in coronary lesions was significantly greater than that in aortic lesions (25.7±6.1% versus 3.3±1.2%, P<.005 [early] and 4.7±1.2% versus 1.7±0.5%, P<.05 [advanced]). In early and transitional lesions, the percent collagen fiber area in the coronary lesions was significantly greater than in aortic lesions (P<.05). Although the cell composition of atherosclerotic lesions showed large variation in the early stage, variation decreased as the lesions progressed. Cell composition in advanced lesions was similar in both aortic and coronary lesions. The ratio of macrophage area to smooth muscle cell area was markedly lower in coronary lesions compared with aortic lesions (P<.001; Table 3). The apparent contradiction in the data in Tables 2 and 3 is due to the data that showed very small values in the areas of smooth muscle cells from aortic lesions and macrophages from coronary lesions.

**Other Histological Findings**

Table 4 compares other histopathologic findings in coronary and aortic lesions. In transitional and advanced lesions, cholesterol crystal deposits were significantly less common in the coronary lesions than in the aortic lesions. In addition, fragmentation or disappearance of the internal elastic lamina in transitional coronary lesions was significantly less common than in aortic lesions.

**Discussion**

We performed immunohistochemical and conventional staining of the major components of atherosclerotic lesions and quantitatively compared the lesional composition between the coronary arteries and the aorta. Coronary atherosclerotic lesions were rich in smooth muscle cells and contained a small number of macrophages, whereas aortic atherosclerotic lesions had the opposite features. In early lesions, the macrophage area was notably small and the smooth muscle cell area large in coronary lesions (Figs 1 and 7 and Table 2), whereas most of the cells observed in the intimal lesions were macrophages in the aorta (Figs 2 and 7 and Table 2). These results suggested that the role of macrophages and smooth muscle cells in the initiation and/or progression of coronary atherosclerosis differs from that in aortic atherosclerosis.

The differences in the cell composition of the two types of atherosclerotic lesion may be explained by structural differences between the coronary arteries and the aorta. The coronary arteries are muscular and have a single layer of elastin fibers (internal elastic lamina) surrounded by several layers of smooth muscle cells and collagen fibers; the aorta is an elastic artery and has several layers of elastin fibers along with smooth muscle cells and collagen fibers. In the coronary arteries, therefore, plasma components may more readily enter the media, and smooth muscle cells can more easily
migrate from the media to the intima compared with the aorta.

Another possible explanation is associated with the role of endothelial cells in atherogenesis. The secretion of prostaglandin $I_2$ from coronary endothelial cells is increased compared with that from aortic endothelial cells.$^{19}$ Cybulsky and Gimbrone$^{20}$ have shown the expression of monocyte adhesion molecules on aortic endothelial cells during atherogenesis. If the functioning of endothelial cells in the coronary arteries differs from that of aortic endothelial cells, the macrophage content in atherosclerotic lesions from different vessels

| TABLE 2. Area of Atherosclerotic Lesion Components In WHHL Rabbits |

<table>
<thead>
<tr>
<th>Lesion Type</th>
<th>Early</th>
<th>Transitional</th>
<th>Advanced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of segments analyzed</td>
<td>12</td>
<td>11</td>
<td>10</td>
</tr>
<tr>
<td>Macrophages</td>
<td>0.03±0.01</td>
<td>0.75±0.22*</td>
<td>3.42±2.09*</td>
</tr>
<tr>
<td>Smooth muscle cells</td>
<td>0.08±0.03</td>
<td>1.02±0.27*</td>
<td>4.41±1.03*‡</td>
</tr>
<tr>
<td>Collagen</td>
<td>0.13±0.05</td>
<td>5.95±1.71*</td>
<td>30.0±5.79*‡</td>
</tr>
<tr>
<td>Extracellular lipid deposits</td>
<td>0.003±0.003</td>
<td>0.61±0.23*</td>
<td>5.18±1.21*†</td>
</tr>
<tr>
<td>Aortic</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of segments analyzed</td>
<td>11</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Macrophages</td>
<td>1.13±0.34</td>
<td>62.7±8.71*</td>
<td>43.4±13.7*</td>
</tr>
<tr>
<td>Smooth muscle cells</td>
<td>0.07±0.02</td>
<td>8.1±1.98*</td>
<td>9.3±1.88*</td>
</tr>
<tr>
<td>Collagen</td>
<td>0.33±0.29</td>
<td>26.4±5.18*</td>
<td>173.5±24.9*‡</td>
</tr>
<tr>
<td>Extracellular lipid deposits</td>
<td>0</td>
<td>11.7±2.30*</td>
<td>59.9±12.6*‡</td>
</tr>
</tbody>
</table>

WHHL indicates Watanabe heritable hyperlipidemic. Data are mean±SEM. The unit of area is $10^{-2}$ mm$^2$. For definitions of lesion stage, see "Methods."

*P<.005 vs the early lesion group by Mann-Whitney $U$ test.

†P<.05, ‡P<.005 vs the transitional lesion group by Mann-Whitney $U$ test.
will probably vary. Thus, the various functions of the endothelial cells in coronary arteries probably differ from those of aortic endothelial cells.

The cells that stained positive with HHF35 were decreased in the media beneath intimal lesions in both the coronary arteries and aorta despite normal staining of cells in the media at sites without intimal lesions in the same sections. Although normal smooth muscle cells are stained equally by various monoclonal antibodies, ie, muscle actin-specific HHF35, smooth muscle cell actin-specific CGA7, and vimentin-specific 4Z3E8, Ueda et al report that the reactivity of smooth muscle cells in atherosclerotic lesions is variable. We examined the HHF35-negative portion in the media using HHF35 and V9, a monoclonal antibody for vimentin (Dako Laboratories); most cells in the media stained positive with both HHF35 and V9 (T.I., H.S., September 1993, unpublished data). Moreover, cell number increased in the HHF35-negative portion in the media compared with the HHF35-positive portion, which was close to the HHF35-negative portion. Therefore, the number of smooth muscle cells is apparently decreased in the HHF35-negative portion in the media.

Klurfeld, who immunohistochemically stained macrophages in human coronary and aortic atherosclerotic lesions, observed foam cells in all aortic and half of the coronary lesions. His observations suggest that macrophages are a major component of aortic but not coronary atherosclerotic lesions. In our study, aortic atherosclerotic lesions in WHHL rabbits were relatively rich in macrophages and had a small number of smooth muscle cells, whereas coronary atherosclerotic lesions showed opposite features (Table 3 and Fig 7). Although various conditions (eg, classification of lesion, difference between hypercholesterolemia or normal cholesterol levels, age of specimen, and difference between quantitative analysis and observation of findings) varied between our rabbit study and the human study, the cellular composition of aortic atherosclerotic lesions in WHHL rabbits appears similar to those in humans. In addition, our immunohistochemical findings in aortic atherosclerosis were identical to observations previously reported in WHHL rabbits with aortic atherosclerosis.

We showed qualitative differences between coronary and aortic atherosclerotic lesions in WHHL rabbits. In addition, we observed that the aortic valve cusps in aged WHHL rabbits were diffusely thickened by fibrous tissue containing foam cells (data not shown). Atherosclerosis of the aortic valve cusps is frequently observed in patients with familial hypercholesterolemia.

Gown et al report an immunohistochemical analysis of the cellular components of atherosclerotic lesions in humans from 34 to 58 years of age. Fibro-fatty lesions in their studies were composed almost exclusively of macrophages and were almost devoid of smooth muscle cells, whereas advanced plaques were characterized by complex layers of macrophages and smooth muscle cells. They also report that although variable numbers of smooth muscle cells and macrophages were present in the fibrous plaque, the predominant cell type was smooth muscle cells. Aqel et al report similar results in human aortic atherosclerotic lesions. In our study, macrophages predominated in early lesions, whereas in advanced lesions macrophages decreased and the lesions became more complex in their composition (Fig 7). Although various conditions (eg, classification of lesion, difference between hypercholesterolemia or normal cholesterol levels, age of specimen, and difference between quantitative analysis and observation of findings) varied between our rabbit study and the human study, the cellular composition of aortic atherosclerotic lesions in WHHL rabbits appears similar to those in humans. In addition, our immunohistochemical findings in aortic atherosclerosis were identical to observations previously reported in WHHL rabbits with aortic atherosclerosis.

We showed qualitative differences between coronary and aortic atherosclerotic lesions in WHHL rabbits. In addition, our findings suggested that the role of macrophages and smooth muscle cells in the initiation and/or progression of coronary atherosclerosis differs from that in aortic atherosclerosis.

### Table 3. Ratio of Macrophage Area to Smooth Muscle Cell Area in Atherosclerotic WHHL Rabbits

<table>
<thead>
<tr>
<th>Lesion Type</th>
<th>Lesion Stage</th>
<th>Early</th>
<th>Transitional</th>
<th>Advanced</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coronary</td>
<td></td>
<td>1.0±0.5 (n=12)</td>
<td>1.1±0.4 (n=11)</td>
<td>0.9±0.5 (n=10)</td>
</tr>
<tr>
<td>Aortic</td>
<td></td>
<td>150±88 (n=11)</td>
<td>20.4±9.9 (n=13)</td>
<td>10.2±3.8 (n=13)</td>
</tr>
</tbody>
</table>

WHHL indicates Watanabe heritable hyperlipidemic. Values were calculated using the data in Table 2 and are mean±SEM.

P<.001 coronary vs aortic lesions at all lesion stages by Mann-Whitney U test.
We wish to thank Toshiaki Tamura, Ken-ichi Yoneda, Shinti Saruki, and Seiji Katayama for their excellent technical assistance.

References


Acknowledgments

We wish to thank Toshiaki Tamura, Ken-ichi Yoneda, Shinti Saruki, and Seiji Katayama for their excellent technical assistance.

TABLE 4. Frequency of Other Features of Atherosclerosis in WHHL Rabbits

<table>
<thead>
<tr>
<th>Lesion Stage</th>
<th>Early</th>
<th>Transitional</th>
<th>Advanced</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CA</td>
<td>Aorta</td>
<td>CA</td>
</tr>
<tr>
<td>No. of sections examined</td>
<td>12</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Cholesterol crystals</td>
<td>0</td>
<td>0</td>
<td>9</td>
</tr>
<tr>
<td>Calcification</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Fragmentation or loss of internal elastic lamina</td>
<td>0</td>
<td>0</td>
<td>64</td>
</tr>
<tr>
<td>Medial thinning</td>
<td>0</td>
<td>0</td>
<td>36</td>
</tr>
</tbody>
</table>

*P<0.05 by χ² test or Fisher's exact probability test.

WHHL indicates Watanabe heritable hyperlipidemic; CA, coronary artery. All values are percent except number of sections examined.

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