Atherosclerosis and Lipoprotein

Development of an Animal Model for Spontaneous Myocardial Infarction (WHHLMI Rabbit)

Masashi Shiomi, Takashi Ito, Satoshi Yamada, Seinosuke Kawashima, Jianglin Fan

Objective—Coronary heart disease is the most common cause of death in developed countries. However, there are no suitable animal models that mimic spontaneous myocardial infarction in humans. In this study, we attempted to obtain a rabbit strain with spontaneous myocardial infarction by selective breeding of coronary atherosclerosis–prone Watanabe heritable hyperlipidemic (WHHL) rabbits, designated as WHHLMI rabbits.

Methods and Results—WHHLMI rabbits were characterized by the high incidence of fatal myocardial infarction at ages 11 to 35 months, being increased from 23% to 97% after the selective breeding. The ECG on WHHLMI rabbits showed a typical feature of myocardial infarction. Histological examination of hearts from suddenly deceased WHHLMI rabbits revealed old myocardial infarction accompanied by fresh myocardial lesions. The culprit coronary arteries exhibited severe atheromatous plaques (>90% lumen area stenosis), suggesting that coronary atherosclerosis is responsible for myocardial infarction observed in WHHLMI rabbits. In addition, the coronary plaques showed vulnerable features including macrophage-rich thin cap and large necrotic core.

Conclusions—To the best of our knowledge, this is the first report of spontaneous myocardial infarction in rabbits, and it is suggested that this WHHLMI rabbit strain will be a useful animal model to study human myocardial infarction. (Arterioscler Thromb Vasc Biol. 2003;23:1239-1244.)

Key Words: coronary atherosclerosis ▪ myocardial infarction ▪ vulnerable plaque ▪ WHHLMI rabbit

Coronary heart disease is the major cause of death in developed countries. However, there are no suitable animal models for human myocardial infarction. It is essential to develop animal models for myocardial infarction to reveal its mechanisms and to develop new therapeutic interventions. Although several genetically modified mouse models with hypercholesterolemia and atherosclerosis have been reported,1–3 coronary atherosclerosis and subsequent myocardial lesions resembling those of humans have not been documented, possibly because of the difference in lipoprotein metabolism between humans and mice.4,5 In addition, the mouse is small in size, which may hamper its use in many surgical manipulations and therapeutic interventions. Therefore, there is a need to develop a relatively large animal model for the study of myocardial infarction. Until now, such an ideal model (eg, rabbits) with spontaneous myocardial infarction has not been established.

In 1980, our institute developed Watanabe heritable hyperlipidemic (WHHL) rabbits as a suitable animal model for human familial hypercholesterolemia and atherosclerosis.6 Based on this strain, we additionally developed coronary atherosclerosis–prone WHHL rabbits, a variant strain, which have higher low-density lipoprotein cholesterol and typical coronary atheromatous plaques similar to those of humans.7 However, the incidence of spontaneous myocardial infarction in coronary atherosclerosis–prone WHHL rabbits was extremely low, which limits their use in the study of myocardial infarction. Since 1994, we have attempted to develop an animal model for spontaneous myocardial infarction by serial and selective breeding of the coronary atherosclerosis–prone WHHL rabbits. After 6 years of the selective breeding, we developed a new WHHL strain for spontaneous myocardial infarction and named this myocardial infarction–prone WHHL rabbit strain the WHHLMI rabbit. In this study, we performed a pathological analysis on the hearts of suddenly deceased WHHLMI rabbits and demonstrated that WHHLMI rabbits are potentially useful for the study of myocardial infarction.

Methods

Animals

The rabbits were housed individually in metal cages in a room with constant temperature (22 ± 2°C) and constant lighting cycle (12 hours light/dark) and were fed standard rabbit chow (120 g per day; CR-3, Clea Japan) and water (ad libitum). The serum total cholesterol and triglyceride levels of rabbits were determined by enzymatic methods. This study was approved by the Institutional Animal Care and Use Committee and carried out according to the guidelines of animal experimentation of Kobe University.
Selective Breeding
To develop a rabbit model for spontaneous myocardial infarction, we selected descendants of coronary atherosclerosis-prone WHHL rabbits with the following features: (1) development of myocardial infarction; (2) macrophage-rich coronary plaque; (3) coronary lumen stenosis greater than the values of the mean plus the standard error in their parents’ generation; and (4) plasma total cholesterol levels >21 mmol/L at 12 months old and greater than 18 mmol/L at 24 months old. Using these breeding stocks, we carried out selective breeding and obtained 5 to 7 generations of this colony during 6 years. During the selective breeding, the conditions of housing, feeding, and others were maintained constant and rabbits did not receive any interventions except the selective breeding.

Histological Analysis
We examined 201 WHHL rabbits during the selective breeding. We estimated elapsing time up to excision of hearts after decease based on daily observation of rabbits’ health at 9:00 AM, 1:00 PM, and 5:00 PM in addition to regular care. After the sudden death of rabbits, the hearts were excised within 30 minutes to 16 hours. According to the method reported previously,7 the immersion-fixed hearts with 10% formaldehyde fixative, or periodate-lysine-paraformaldehyde fixative were cut into 6 blocks and were embedded in paraffin. To examine atherosclerotic plaques of the upstream coronary arteries associated with the myocardial lesions, we prepared 4 paraffin blocks of the upper quarter of the heart (from the base of the heart) containing the main stem of the left coronary artery, the origin portion of the right coronary artery, or the left circumflex artery (LCX) in addition to the left anterior descending artery (LAD), left septal artery (LSP), and right coronary arteries, respectively. Using these blocks, coronary arteries were sectioned at 500-μm intervals transversely. The remainder (the 3 quarters of the apex site) was cut into 2 blocks. These 2 blocks were sectioned at 1000-μm intervals transversely for the observation of myocardial lesions. Sections were stained with H&E staining, elastic van Gieson staining, Azan-Mallory staining, or immunohistochemically using mouse monoclonal antibodies for smooth muscle actins (1A4; Dako A/S) and for rabbit macrophages (RAM-11; Dako A/S). Immunohistochemical staining was carried out using the Vectastain ABC kit (Vector Laboratories Inc) with hematoxylin counterstaining. The degree of coronary stenosis was expressed as the percent lumen area stenosis evaluated by 2 independent researchers. The lesion stage of myocardial infarction in rabbits was evaluated by the method of Schoen.8

Monitoring of the ECG
To examine whether the ECG showed representative changes of myocardial infarction, we monitored each WHHLMI rabbit (>12 months old) with the ECG monthly under anesthesia by intravenous injection of ketamine hydrochloride (10 mg/kg) and xylazine hydrochloride (3 mg/kg). ECG was monitored using unipolar leads and extremity leads using a Polygraph System RM-6000 equipped with a bioelectric amplifier AB-621G (Nihon-Kohden Co), and measurements were performed with PowerLab/8SP (AD Instruments Japan Inc).

Statistical Analyses
Values were expressed as mean±SEM. Statistical analyses were carried out using Fisher’s exact probability test for incidence, using the Mann-Whitney U test for coronary stenosis, and using Student’s t test or Aspin-Welch’s t test for serum lipid levels. Statistical significance was set at P<0.05.

Results
Serum Lipids, Coronary Stenosis, and Incidence of Myocardial Infarction
The Table shows the serum lipid levels, the degree of coronary stenosis, and the incidence of myocardial infarction.

Comparison of Serum Lipid Levels, Coronary Stenosis, and Incidence of Myocardial Infarction Between WHHL (Before Selective Breeding) and WHHLMI (After Selective Breeding) Rabbits

<table>
<thead>
<tr>
<th></th>
<th>WHHL Rabbits</th>
<th>WHHLMI Rabbits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum lipid levels at 12 months, mmol/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>15.7±0.4 (n=106)</td>
<td>22.5±0.7 (n=51)*</td>
</tr>
<tr>
<td>Triglyceride</td>
<td>2.9±0.1 (n=106)</td>
<td>3.1±0.1 (n=51)</td>
</tr>
<tr>
<td>Average coronary stenosis (11 to 35 months old), %</td>
<td>60±4 (n=57)</td>
<td>93±1 (n=34)*</td>
</tr>
<tr>
<td>Incidence of myocardial infarction (11 to 35 months old), %</td>
<td>23 (8/35)</td>
<td>97 (33/34)*</td>
</tr>
</tbody>
</table>

Values are represented as mean±SEM. *P<0.001 compared with control WHHL rabbits.

The serum total cholesterol levels of WHHLMI rabbits were significantly increased by 1.4-fold after the selective breeding compared with control WHHL rabbits, although the serum triglyceride levels were unchanged. The coronary stenosis of WHHLMI rabbits 11 to 35 months of age was significantly increased by 1.6-fold. Although the incidence of coronary plaques with a lumen area stenosis >90% was only 21% in the control WHHL rabbits, the incidence in WHHLMI rabbits was >90%. WHHLMI rabbits started to die suddenly from 11 months of age without apparent symptoms. Histological analysis revealed that myocardial infarction was observed in 97% of rabbits that died at up to 35 months of age, and the incidence was increased by 4.1-fold compared with the control WHHL rabbits before the selective breeding. There was no sex difference in myocardial infarction in terms of the incidence, distribution, and lesion size.

Myocardial Lesions of WHHLMI Rabbits
The myocardial lesions were widely distributed from the left ventricle, right ventricle, and ventricular septum, where coronary arteries showed severe coronary stenosis (see below). Based on the anatomical location, myocardial infarction is classified into subendocardial infarction, intramural infarction, transmural infarction, and subepicardial infarction. In many WHHLMI rabbits, old myocardial infarction with pathological features equivalent to chronic myocardial infarction was often observed, which was accompanied by fresh myocardial lesions (combined myocardial infarction) (Figure 1). Fresh myocardial lesions consisted of hyperemia (panel A), eosinophilic myocardial cells (panel B), and infiltration of inflammatory cells (panel C). These findings were observed in the vicinity of myocardial fibrosis at the subendocardial zone. Figure 2 shows old myocardial infarction. In the old myocardial lesions, there was a prominent calcification (panel A) and subepicardial infarction (panel B), which is frequently found in old myocardial infarction in humans. In addition, there was dissolution of myocardial cells and replacement of fibrosis at the posterior right region (panel A) and ventricular septum (panel C). The left ventricle wall was thinned (panels A, C, and D), and a transmural myocardial lesion was observed at the lateral wall (panel D). In classical WHHL rabbits before selective breeding, the myocardial lesions were...
small (focal or patchy) and the lesions were limited at the subendocardial region or periarterial region. We did not detect any transmural lesions or subepicardial infarction in addition to myocardial calcification. Therefore, after selective breeding, myocardial lesions became more extensive and advanced.

Proximal Atheromatous Plaque of the Culprit Coronary Arteries

Because myocardial infarction was present in all WHHLMI rabbits, we next examined the coronary atherosclerotic lesions in terms of distribution, stenosis, severity, and stability of the lesions. As summarized in Figure 3, atherosclerotic lesions were found in all upstream coronary arteries associated with the myocardial lesions; the most severe coronary branch was the LCX followed by LSP, LAD, and RCA. In the LCX, 73% of sections showed atheromatous plaques having >90% lumen area stenosis. In addition, the frequency of coronary lesions with >70% area stenosis was 97.2% in the LCX, 54.7% in the LAD, and 59.2% in the LSP, respectively. These findings suggest that the serial advanced atheromatous plaques observed in the upstream culprit coronary arteries were related to myocardial lesions as described above. The culprit plaques of the LCX are either foam cell–rich or matrix-rich plaques that produce a marked lumen stenosis or concentric occlusion (Figure I, available online at http://atvb.ahajournals.org). The typical plaques were frequently found in large branches and were associated with complicated changes, including calcification (Figure I, panel B) and intraplaque hemorrhage (Figure I, panel C). In some lesions, the plaque was extremely fragile (Figure 4); they had a thin cap (panel A and B), beneath which there were accumulating macrophages, lipid cores, and calcium deposits (panel D) but few smooth muscle cells (panel C). Despite such vulnerable plaques, we were unable to detect the real rupture or thrombus formation in any coronary plaques of WHHLMI rabbits.

ECG of a WHHLMI Rabbit

The electrocardiograms from a WHHLMI rabbit monitored immediately before sudden decease (Figure II, available online at http://atvb.ahajournals.org) showed the typical change of acute myocardial infarction in humans. These features on the ECG were never recorded when rabbits were injected an overdose anesthetic for euthanasia. Moreover, we also recorded the ECG of classical WHHL rabbits but did not find the pattern of myocardial infarction (data not shown).

Discussion

In this study, we successfully developed a rabbit model for spontaneous myocardial infarction (WHHLMI rabbits) by selective breeding of coronary atherosclerosis-prone WHHL rabbits without any interventions such as ligation. By the selective breeding, coronary lumen area stenosis was advanced from 60% to 93%, and the cumulative incidence of myocardial infarction was increased from 23% to 97%. This suggests that progression of coronary plaques with >90% stenosis led to myocardial infarction in this model. Because the stressless environmental conditions including feeding were consistent during the selective breeding period, the high
incidence of myocardial infarction in WHHLMI rabbits suggests that some genetic factors may be involved in severe coronary atherosclerosis and the subsequent onset of myocardial infarction. Although serum cholesterol levels in WHHLMI rabbits were increased by selective breeding, high cholesterol levels may not be the major determinant for high incidence of myocardial infarction, because nonselective WHH rabbits with equally high serum cholesterol levels did not suffer from myocardial infarction or severe coronary plaques. This suggests that factors other than serum cholesterol levels may play an important role in the development of severe coronary plaques and subsequent myocardial infarction.

Almost all myocardial lesions of WHHLMI rabbits were old myocardial infarctions accompanied by fresh myocardial lesions (Figure 1). The myocardial lesions were mainly observed at the subendocardial region circumferentially (Figure 2), and the related culprit coronary arteries located at the upper stream showed advanced atheromatous plaques with >90% stenosis in the serial sections (Figure 3). This suggests that serial nearly occluded coronary lesions related to the development of myocardial infarctions. In autopsied human hearts, multivessel disease was frequently encountered in the subendocardial infarction because of chronic insufficient blood supply.9,10 Therefore, the features of myocardial lesions of WHHLMI rabbits may resemble those of patients with multivessel coronary disease.

In several plaques of the culprit coronary arteries, the fibrous cap was thin and macrophage accumulation was prominent at the plaque surface (Figure 4 and Figure 1). Macrophage-rich coronary plaque was a feature of WHHLMI
rabbits and was distinct from the coronary plaques of WHHL rabbits before the selective breeding, in which the lesions were macrophage-poor and fibromuscular component-rich.\textsuperscript{11} Several coronary lesions showed nearly rupture features: thin fibrous cap below which a large lipid core and calcium accumulation were mixed with a large number of macrophages (Figure 4). In addition, we observed intraplaque hemorrhage, calcium accumulation, and occlusion of the coronary lumen with accumulation of macrophages (Figure I). These features are consistent with vulnerable plaques,\textsuperscript{12–14} which may trigger plaque rupture and possibly potentiate acute coronary syndromes. Despite these observations, we did not detect any rupture or luminal thrombosis in the coronary plaques of WHHLMI rabbits. Previous studies reported by Nakamura et al\textsuperscript{15} demonstrated that coronary thrombosis was observed in WHHL rabbits (19-month-old average) treated with Russell’s viper venom in combination with angiotensin II but not in age-matched WHHL rabbits treated with saline or angiotensin II alone. Therefore, we considered that coronary thrombosis was not induced spontaneously in WHHL rabbits if coronary plaques did not rupture.

There are several possibilities for this outcome. First, the rabbit coronary lesions (macrophage number or functions and matrix components) may be insufficient to trigger the plaque rupture and subsequently lead to thrombus formation. Several factors such as matrix metalloproteinases, tissue factors, and lipoprotein (a) are considered to play major roles in triggering the plaque rupture and thrombus formation.\textsuperscript{12–14} In future studies, we will crossbreed our WHHLMI rabbits with lipoprotein (a) transgenic rabbits, which have advanced atherosclerosis and calcification,\textsuperscript{16} or with macrophage-specific expression of metalloproteinase-12 transgenic rabbits (Fan J, et al, unpublished data, 2003). Therefore, it will be interesting to test these hypotheses by introducing these risk factors into WHHLMI rabbits. Second, the general stressless environment for WHHLMI rabbits, which is in contrast to humans exposed to multiple environmental conditions and risk factors (social stress, hypertension, diabetes, and smoking), may also protect the plaque from rupture. To test this hypothesis, we will expose WHHLMI rabbits to stressful environments in future studies. Having vulnerable plaques without detectable rupture or luminal thrombi suggests that old myocardial infarctions in WHHLMI rabbits were caused by progressing coronary occlusions; thus, acute coronary syndromes may be not manifested in these rabbits. Consistent with this notion, clinical studies showed that coronary thrombosis

\textbf{Figure 4.} Representative micrographs of a vulnerable plaque of the circumflex arteries of WHHLMI rabbits. The sections were stained with elastic van Gieson staining (A) or Azan-Mallory staining (B). To define the cellular components in the lesions, the sections were also stained immunohistochemically using monoclonal antibodies against smooth muscle cells (C) and macrophages (D) with hematoxylin counterstaining. Arrowheads indicate the calcium accumulation. Vulnerable plaque contains a thin cap beneath which there is a lipid core with calcification (arrowheads) and macrophages (cells stained dark brown, D). A few smooth muscle cells (cells stained dark brown, C) are sparsely around the lipid core.
bosis was not detected in patients with subendocardial infarction.9,10,17–19

The areas of fresh myocardial lesions were small despite showing typical ST elevation in ECG (Figure II). WHHLMI rabbits, which we could monitor, deceased within 15 minute after a heart attack. Therefore, fresh myocardial infarction can hardly be observed under a light microscopy within such a short time. We observed many nuclei of myocardial cells with hollow, myolysis of myocardial cells, and intermyocardial edema in the hearts of WHHLMI rabbits excised within 30 minute after death (data not shown), although it is difficult to distinguish these findings from findings by artifacts. In addition, WHHLMI rabbits may decease from myocardial infarction directly or complications of the myocardial ischemia such as fatal arrhythmia or cardiogenic shock attributable to the coronary occlusion.

In conclusion, we successfully developed WHHLMI rabbits as a potential human myocardial infarction model. Coronary plaques with complicated lesions are the major determinant for the myocardial infarction found in these rabbits. It is suggested that WHHLMI rabbits will be a useful model for the study of human myocardial infarction and be particularly valuable to study the mechanism of chronic ischemia and plaque formation of coronary arteries. It is also possible that these WHHLMI rabbits can be used as a tool for the development of new therapeutic drugs and interventions. Efforts are being made toward investigating the mechanisms of plaque stability, and, hopefully, plaque rupture or acute coronary syndromes can be produced in WHHLMI rabbits through the introduction of other risk factors.

Limitation of this Study
Coronary plaque rupture and subsequent intravascular thrombosis are the major cause for acute coronary syndromes in humans.12–14 whereas WHHLMI rabbits did not show these findings despite the presence of vulnerable coronary plaques. These observations indicate that the mechanisms for myocardial infarction in WHHLMI rabbits are different from those in humans. In addition, the features of WHHLMI rabbits suggest that additional risk factors other than vulnerable plaque may be required to trigger the rupture of coronary plaques. Therefore, we consider that WHHLMI rabbits will be a suitable model to elucidate risk factors for plaque rupture and the possible mechanisms.

Acknowledgments
This work was supported in part by unrestricted research grants from Sankyo Co, Ltd (M.S.) and a grant of the Center for Tsukuba Advanced Research Alliance at the University of Tsukuba (J.F.). The authors are grateful to T. Tamura for help with rabbit breeding and maintenance.

References
Development of an Animal Model for Spontaneous Myocardial Infarction (WHHLMI Rabbit)
Masashi Shiomi, Takashi Ito, Satoshi Yamada, Seinosuke Kawashima and Jianglin Fan

Arterioscler Thromb Vasc Biol. 2003;23:1239-1244; originally published online May 8, 2003; doi: 10.1161/01.ATV.0000075947.28567.50
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/23/7/1239

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2003/07/08/23.7.1239.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/