Vasospasm of Atherosclerotic Coronary Arteries Precipitates Acute Ischemic Myocardial Damage in Myocardial Infarction–Prone Strain of the Watanabe Heritable Hyperlipidemic Rabbits

Masashi Shiomi, Tatsuro Ishida, Tsutomu Kobayashi, Norihisa Nitta, Akinaga Sonoda, Satoshi Yamada, Tomonari Koike, Nobue Kuniiyoshi, Kiyoshi Murata, Ken-ichi Hirata, Takashi Ito, Peter Libby

Objective—This study tested the hypothesis that vasospasm can trigger coronary plaque injury and acute ischemic myocardial damage.

Approach and Results—Myocardial infarction–prone strain of the Watanabe heritable hyperlipidemic rabbits received an intravenous bolus of ergonovine maleate (0.45 µmol/kg) during intravenous infusion of norepinephrine (12 nmol/kg per minute) to provoke coronary spasm in vivo. After this treatment, coronary angiography demonstrated vasospasm, and the ECG showed ischemic abnormalities (ST depression/elevation and T-wave inversion) in 77% of animals (23/30). These changes normalized after nitroglycerin injection. In rabbits that demonstrated these ECG findings for >20 minutes, echocardiograms showed left ventricular wall motion abnormality. Serum levels of heart-type fatty acid–binding protein, cardiac troponin-I, and myoglobin increased markedly 4 hours after spasm provocation. In coronary lesions of myocardial infarction–prone strain of the Watanabe heritable hyperlipidemic rabbits with provoked coronary spasm, we observed intimal injury in 60.9% in the form of endothelial cell protrusions (39.1%), denudation (30.4%), and macrophage extravasation (56.5%). Plaque disruption with luminal thrombus, however, was only seen in 2 of 23 animals (8.7%), and mural microthrombus was rarely observed (4.3%).

Conclusions—These observations show that provocation of vasospasm in myocardial infarction–prone strain of the Watanabe heritable hyperlipidemic rabbits associates with subsequent ischemic myocardial damage. Although treatment with spasmogens altered aspects of plaque morphology, for example, endothelial protrusion and macrophage emigration, thrombosis was rare in these animals with chronic atherosclerotic disease. (Arterioscler Thromb Vasc Biol. 2013;33:2518-2523.)

Key Words: cardiovascular diseases ■ coronary atherosclerosis ■ coronary vasospasm ■ ischemia, myocardium ■ models, animal

In humans, disruption of coronary plaques can cause acute coronary syndromes (ACSs). Several factors, such as proinflammatory cytokines, matrix metalloproteinases, oxidative stress, and intraplaque hemorrhage, may increase the fragility of plaques.¹⁻³ Yet, not all thin-capped plaques with macrophage accumulation rupture,¹⁻⁶,⁷ and the trigger(s) of acute plaque disruption events that complicate chronically atherosclerotic coronary arteries remains uncertain. Moreover, frank rupture of the fibrous cap does not cause all fatal myocardial infarctions. A more superficial form of intimal injury or erosion can also provoke fatal coronary thrombosis. The mechanisms that precipitate these forms of coronary plaque disruption remain uncertain.¹⁻⁸

Mechanical factors, such as circumferential stress, may contribute to plaque rupture,³ and coronary vasoconstriction may contribute to the onset of ACSs.¹⁻¹⁰ Yet, no definitive experimental evidence has demonstrated that vasospasm triggers coronary arterial plaque injury—because of a dearth of suitable experimental animal preparations.¹¹⁻¹² Previous investigations have demonstrated disruption of diet-induced brachiocephalic lesions¹³,¹⁴ and coronary lesions¹⁵ in mice although these studies did not identify triggers of plaque disruption. Most studies of plaque injury in mice have focused on non–coronary arteries,¹¹⁻¹⁴ but coronary plaque injury or disruption is particularly important in animal experiments because the composition of atherosclerotic lesions
and arterial structure differs in different beds.16 Moreover, study of coronary arteries is necessary to examine whether vasospasm relates to the development of myocardial damage and decreased ventricular function. Caligiuri et al17 demonstrated that mental stress leads to acute myocardial ischemia in atherosclerotic mice, but they did not observe coronary plaque rupture or document coronary spasm. In pigs with diet-induced atherosclerosis,18,19 repeated coronary spasm evokes intraplaque hemorrhage, luminal occlusion, and endothelial damage without plaque rupture. In rabbits, injection of Russell’s viper venom and angiotensin II induced coronary spasm and the formation of thrombi.20,21 Such treatments that provoke occlusive thrombus formation differ substantially from conditions that likely prevail at the onset of ACSs in humans. In addition, the venom used contains an undefined mixture of biologically active substances. We, therefore, need ways to probe experimentally the pathophysiology of sudden myocardial ischemic events in animals with chronic coronary artery atherosclerosis.

We developed, as previously described, a myocardial infarction–prone strain of the Watanabe heritable hyperlipidemic (WHHLMI) rabbit22 by selectively breeding the WHHL rabbit that has hypercholesterolemia because of a genetic defect in the low-density lipoprotein receptors.23–25 The WHHLMI rabbit strain has been maintained by selective breeding using the offspring of rabbits with myocardial infarction and severe coronary atherosclerosis. WHHLMI rabbits develop various types of coronary lesions, including thin-capped fibroatheromas with macrophage infiltration, and evidence for old myocardial lesions (fibrosis and myocardial scars) caused by chronic ischemia produced by progression of coronary plaques without acute thrombus formation.22,25,26 We did not detect spontaneous erosive injury or rupture of coronary plaques or the vestiges of coronary plaque rupture in WHHLMI rabbits that died suddenly although macrophages within the thin fibrous cap of a fibroatheroma contain immunoreactive matrix metalloproteinases. These observations suggest that erosion or rupture of coronary plaque involves other precipitants beyond the local expression of proteinases.

The present investigation provoked coronary spasm pharmacologically in WHHLMI rabbits harboring chronically atherosclerotic coronary arteries and tested the hypothesis that plaque disruption with thrombosis, a frequent cause of clinical acute ischemic myocardial injury and decreased left ventricular function, requires 2 hits: a susceptible plaque and a triggering stimulus, one of which may be local arterial spasm.

**Materials and Methods**

Materials and Methods are available in the online-only Supplement.

**Results**

**Characteristics of WHHLMI Rabbits With Provoked Coronary Spasms**

Table I in the online-only Data Supplement shows the characteristics of the WHHLMI rabbits examined. All of these rabbits had hypercholesterolemia and severe atherosclerotic lesions in the coronary arteries.

**Coronary Spasm Provoked in WHHLMI Rabbits**

Figure 1 shows the provocation of coronary spasm in WHHLMI rabbits. After injection of ergonovine during norepinephrine infusion, coronary angiograms showed that contrast flow decreased in the left anterior descending artery and ceased in the left circumflex artery (Figure 1A), and the ECG showed ST-elevation, poor R-wave progression, and ventricular premature depolarization in all leads (Figure 1B and 1C). Nitroglycerin administration reversed the vasoconstriction and ECG changes. Thus, ergonovine injection during norepinephrine infusion provoked coronary spasm in this experimental preparation. Spasm occurred in coronary arteries with

---

**Nonstandard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACS</td>
<td>acute coronary syndrome</td>
</tr>
<tr>
<td>WHHL</td>
<td>Watanabe heritable hyperlipidemic</td>
</tr>
<tr>
<td>WHHLMI rabbit</td>
<td>myocardial infarction–prone Watanabe heritable hyperlipidemic rabbit</td>
</tr>
</tbody>
</table>

---

**Figure 1.** Provocation of coronary spasm in myocardial infarction–prone Watanabe heritable hyperlipidemic (WHHLMI) rabbits. Rabbits received ergonovine (0.45 μmol/kg IV bolus) during norepinephrine infusion (6 mmol/kg per minute). A, Coronary angiogram at baseline, after ergonovine injection, and after nitroglycerin injection. Arrows in A indicate the spastic sites of left anterior descending artery (LAD) and left circumflex artery (LCX). Bars, 100 μm. B, ECG recorded with bipolar limb leads and chest leads at baseline (a), after ergonovine injection (b), and after nitroglycerin injection (c). C, Incidence of ischemic ECG changes in WHHLMI rabbits.
atherosclerotic lesions and caused ECG abnormalities consistent with myocardial ischemia or injury. These ischemic responses occurred in 76.6% of rabbits (23/30) that received the vasoconstrictors (Figure 1C).

**Ventricular Dysfunction and Myocardial Ischemia After Provocation of Coronary Spasm**

Figure 2 shows the influence of coronary spasm on left ventricular function and on myocardial ischemia in WHHLMI rabbits. The echocardiogram (Figure 2A) shows markedly reduced motion of the anterior wall and increased end-diastolic left ventricular size after administration of spasmodic agents. Left ventricular fractional shortening calculated from the echocardiogram (Figure 2B) decreased by 30% during the administration of spasmodic agents. Nitroglycerin injection mitigated these changes in echocardiograms. In addition, serum levels of myocardial injury markers (heart-type fatty acid–binding protein, cardiac troponin-I, and myoglobin) increased markedly 4 hours after the provocation of coronary spasm although those levels were normal at baseline (Table 1). Taken together, these results indicate that coronary spasm induced substantial myocardial ischemia and injury and impaired left ventricular function.

**Influence of Coronary Spasm on Coronary Lesions**

Administration of spasmodic agents altered the morphology of coronary arterial lesions (Table 2). WHHLMI rabbits that showed provoked coronary spasm had a similar burden of coronary plaques because WHHLMI rabbits did not respond to spasmodic agents. Nontreated WHHLMI rabbits or WHHLMI rabbits that did not respond to the spasmodic agents did not have damage to coronary plaques. These observations suggest that neither plaque burden nor morphology affected the susceptibility to provoked coronary vasospasm, and that thin-capped atheromata did not readily disrupt in WHHLMI rabbits without provocation. We observed damage to coronary plaque in 19 WHHLMI rabbits with provoked coronary vasospasm (82.6%; 19/23). In these rabbits, endothelial injury (60.9%; 14/23), and apparent macrophage efflux indicating injury of coronary plaques (56.5%; 13/23) occurred frequently, but only 2 of 23 animals (8.7%) had plaque disruption with luminal thrombus, and only 1 animal (4.3%) displayed mural microthrombus. We did not observe these changes in WHHLMI rabbits without vasospasm and nontreated WHHLMI rabbits. The present results suggest that provocation of coronary spasm causes morphologic changes in coronary plaques (Figures 3 and 4).

Figure 3A to 3D shows a typical thin-capped atheroma with macrophage accumulation from a WHHLMI rabbit without coronary spasm. This representative plaque has a large necrotic core, containing foam cell debris, lipid, cholesterol crystals, and erythrocytes. A thin fibrous cap covered this large necrotic core (black arrows). Although matrix metalloproteinase–positive macrophages localized in the thin fibrous cap, one feature mechanistically linked with plaque disruption, the plaque was not disrupted and had a morphologically intact endothelial layer. Figure 3E to 3V shows superficial injury of collagen and smooth muscle cell–rich plaques and an advanced lesion from WHHLMI rabbits with provoked coronary spasm. Figure 3F to 3H shows lifting of CD41-positive endothelial cells and subendothelial edema in the lesion-free coronary wall (black arrows) and the lesion surface (blue arrows). These CD31-positive cells did not stain for macrophages (RAM-11; Figure 3H) or smooth muscle α-actin (1A4; data not shown). At the lesion surface, RAM-11–positive macrophages localized under the region of subendothelial edema. These findings occurred in 39% of rabbits (9/23; Table 2). Figure 3I to 3L shows micrographs from specimens from rabbits that underwent fixation by immersion rather than by perfusion fixation. In contrast to a rabbit without spasm (Figure 3A–3D), the coronary arterial lumen contained numerous cells (Figure 3I) positive for CD31 (Figure 3K) but negative for 1A4 (Figure 3L)—consistent with endothelial cell denudation. Subtotal occlusion in distal coronary artery segments sections (500–1000 μm downstream, data not shown) may account for accumulation of denuded endothelial cells in the lumen. Thirty percent of rabbits showed denudation of endothelial cells (7/23; Table 2). Figure 3M to 3P shows an advanced plaque. Macrophages situated in the subendothelial region and extended into the coronary arterial lumen (Figure 3N–3P, black arrows), where endothelial cells seemed to protrude to the lumen and form an interendothelial gap (Figure 3O). Because we did not observe monocytes

### Table 1. Serum Markers of Myocardial Injury in WHHLMI Rabbits With Coronary Spasm

<table>
<thead>
<tr>
<th></th>
<th>H-FABP, ng/mL</th>
<th>cTroponin-I, ng/mL</th>
<th>Myoglobin, ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human normal ranges</td>
<td>&lt;6.2</td>
<td>&lt;0.04</td>
<td>20–80</td>
</tr>
<tr>
<td>WHHLMI rabbits (n=6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>1.48±0.32</td>
<td>0.00±0.00</td>
<td>23.8±7.4</td>
</tr>
<tr>
<td>4 hours after onset</td>
<td>19.0±6.66</td>
<td>1.67±0.58</td>
<td>676±292</td>
</tr>
<tr>
<td>P value</td>
<td>0.036</td>
<td>0.036</td>
<td>0.036</td>
</tr>
</tbody>
</table>

Data are represented as the mean±SEM. Statistical analyses were performed with the signed Wilcoxon test. A value of *P<0.05* was considered statistically significant. cTroponin-I indicates cardiac troponin-I; H-FABP, heart-type fatty acid–binding protein; and WHHLMI rabbits, myocardial infarction–prone strain of the Watanabe heritable hyperlipidemic rabbits.

---

**Figure 2. Ventricular dysfunction after provocation of coronary spasm in myocardial infarction–prone Watanabe heritable hyperlipidemic (WHHLMI) rabbits.** Rabbits were injected intravenously with ergonovine (0.45 μmol/kg) during norepinephrine infusion (6 nmol/kg per minute). **A,** Echocardiograms (M-mode images) at baseline and after ergonovine injection. **B,** Motility of the left ventricle of 10 WHHLMI rabbits with coronary spasm evaluated by echocardiography. Data are represented as the mean±SEM. Statistical analyses were performed with the Dunnet test between each group. □, Baseline; ■, norepinephrine; ●, norepinephrine+ergonovine; and ▼, nitroglycerin.
adherent to the plaque surface, these findings suggest, but do not prove, that spasm can provoke extrusion of macrophages from plaques into the lumen. Examination of coronary sections prepared with or without perfusion fixation showed similar findings in 56.5% of rabbits (13/23; Table 2). Figure 3Q to 3V shows specimens in which macrophages localized on the plaque surface in association with microthrombus (Figure 3Q), and the endothelial monolayer seemed distorted (Figure 3S). We observed many eosin-positive structures at the plaque surface (Figure 3T–3V). By the Fraser–Lendrum method (Figure 3U) and the Martius scarlet-blue staining (Figure 3V), red staining identifies fibrin and stains erythrocytes yellow. Although these findings suggest that microthrombi can form at the plaque surface showing endothelial abnormality, this finding occurred in only 1 case.

Figure 4A to 4D shows serial sections of a coronary plaque observed in a rabbit euthanized >20 hours after spasm provocation. This plaque has a less fibrous character and only a rudimentary fibrous cap (Figure 4A–4C) but has abundant foam cell debris (Figure 4D) and lipid accumulation (Figure 4A–4C). In this plaque, we observed plaque fragments (white arrows) in the lumen and blood components (yellow arrows) in the plaque (Figure 4B). The Martius scarlet-blue stain indicates fibrin in red and erythrocytes in yellow,27 demonstrating fibrin thrombus in the lumen (Figure 4C). These results suggest that coronary spasm can provoke morphologic changes in plaques and local thrombus formation.

**Discussion**

Using pharmacologic provocation of coronary vasospasm in WHHLMI rabbits, this study demonstrates that exaggerated vasoconstriction can trigger morphologic abnormalities of coronary artery atheromata and acute ischemic myocardial damage in WHHLMI rabbits. Photomicrographs presented in Figure 4 show spasmogen-related plaque disruption and subsequent thrombus formation although the morphological features did not resemble human thin-capped plaques, and the plaque disruption occurred in only 2 animals. The present results demonstrated, however, that coronary spasm evoked injury or disruption of coronary plaques, and that injury of coronary plaques correlated with acute myocardial ischemic injury—as determined electrocardiographically, biochemically, histologically, and by impaired left ventricular function. Previous studies using animals did not document influences of coronary plaque injury because of vasospasm on ischemic myocardial damage, including impaired left ventricular function.1,1,11–18 Therefore, the present study provides novel mechanistic insight into the pathogenesis of acute ischemic myocardial damage, including abnormal left ventricular function.

In this study, injury of the coronary plaque after spasm provocation occurred in 82.6% of rabbits, as observed previously,14 but no damage occurred in coronary plaques of WHHLMI rabbits without vasospasm or those of nontreated WHHLMI rabbits. The length of coronary artery sampled (an ≈10-mm long segment of the left circumflex artery from its origin), therefore, lay in the zone affected by vasospasm as determined angiographically. These observations suggest that provoked vasospasm contributed to coronary plaque injury or disruption. In addition, increased serum markers of myocardial injury and impaired left ventricular function after vasospasm provocation demonstrate acute ischemic myocardial damage. In a rabbit with evoked plaque disruption and thrombus formation in the lumen after vasospasm provocation (Figure 4), the serum markers for ischemic myocardial injury—at near normal levels before spasm provocation—increased markedly 4 hours after spasm provocation (from 2.5 to 34.0 ng/mL in heart-type fatty acid–binding protein; from 0.0 to 0.7 ng/mL in cardiac troponin-I; and from 47 to 283 ng/mL in myoglobin).

### Table 2. Influence of Vasospasm on Coronary Lesions From WHHLMI Rabbits

<table>
<thead>
<tr>
<th>Rabbits Treated With Vasoconstrictors</th>
<th>With Spasm</th>
<th>Without Spasm</th>
<th>P Value</th>
<th>Nontreated</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animals examined</td>
<td>23</td>
<td>7</td>
<td>100</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex ratio (male:female)</td>
<td>12:11</td>
<td>4:3</td>
<td>59:41</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Frequency of rabbits with coronary lesion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibrous lesion</td>
<td>21 (91.3%)</td>
<td>7 (100%)</td>
<td>0.954</td>
<td>99 (99%)</td>
<td>0.158</td>
</tr>
<tr>
<td>Macrophage-filled lesion</td>
<td>18 (78.3%)</td>
<td>2 (28.6%)</td>
<td>0.047</td>
<td>41 (41%)</td>
<td>0.003</td>
</tr>
<tr>
<td>Fibroatheroma</td>
<td>16 (69.6%)</td>
<td>6 (85.7%)</td>
<td>0.720</td>
<td>67 (67%)</td>
<td>0.084</td>
</tr>
<tr>
<td>Thin-capped fibroatheroma with macrophage infiltration</td>
<td>9 (39.1%)</td>
<td>2 (28.6%)</td>
<td>0.952</td>
<td>46 (46%)</td>
<td>0.715</td>
</tr>
<tr>
<td>Frequency of rabbits showing</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Injury to coronary lesions</td>
<td>19 (82.6%)</td>
<td>0 (0.0%)</td>
<td>&lt;0.001</td>
<td>0 (0.0%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Injury to endothelium</td>
<td>14 (60.9%)</td>
<td>0 (0.0%)</td>
<td>0.017</td>
<td>0 (0.0%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Protruded endothelium</td>
<td>9 (39.1%)</td>
<td>0 (0.0%)</td>
<td>0.132</td>
<td>0 (0.0%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Denudation of endothelial cells</td>
<td>7 (30.4%)</td>
<td>0 (0.0%)</td>
<td>0.247</td>
<td>0 (0.0%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Microthrombus at lesion surface</td>
<td>1 (4.3%)</td>
<td>0 (0.0%)</td>
<td>0.521</td>
<td>0 (0.0%)</td>
<td>0.420</td>
</tr>
<tr>
<td>Macrophage extravasation accompanying with endothelial damage</td>
<td>13 (56.5%)</td>
<td>0 (0.0%)</td>
<td>0.027</td>
<td>0 (0.0%)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Disruption of plaque</td>
<td>2 (8.7%)</td>
<td>0 (0.0%)</td>
<td>0.954</td>
<td>0 (0.0%)</td>
<td>0.040</td>
</tr>
</tbody>
</table>

Values in parentheses indicate frequency. Statistical analyses were performed between the vasospasm-positive rabbit group and other groups with the χ² test. WHHLMI rabbit indicates myocardial infarction–prone strain of the Watanabe heritable hyperlipidemic rabbit.
fibroatheroma with macrophage infiltration, similar to the
plaques remaining intact. Only WHHLMI rabbits
subjected to provoked coronary spasm displayed plaque dis-
ruption and acute ischemic myocardial damage, observations
that support coronary artery spasm as 1 possible triggering
mechanism for endothelial injury and macrophage emigra-
tion from the plaque in association with acute ischemic myo-
cardial damage.1

This study revealed damage to the plaque surface in fibrous
plaques (60.9% in rabbits with provoked vasospasm). Plaque
injury with apparent extrusion of intimal macrophages also
occurred in fibroatheroma with macrophage infiltration in the
subendothelial region (56.5% of rabbits with provoked
vasospasm; Figure 3M; Table 2). Thus, vasospasm seems to
counter both superficial plaque injury (in more fibrous
lesions) and actual plaque disruption (in lesions of a more ath-
emomatous morphology). Yet, we observed luminal thrombus
rarely in injured coronary plaques. These observations suggest
that contact of blood components with macrophages in situ
may not suffice to trigger thrombus formation.

Limitations
Coronary plaque disruption with thrombus formation in
WHHLMI rabbits after spasm provocation does not rep-
licate precisely coronary plaque rupture in humans. The
triggers of coronary plaque rupture and subsequent ACSs
remain uncertain,1,8 despite much study.1,11,12 Although
these animal experiments have many obvious differences
from the clinical situation, the present study does provide
experimental validation of the concept that coronary artery
vasospasm might be 1 mechanism of triggering coronary
plaque injury and subsequent acute ischemic myocardial
damage, including impaired left ventricular function. The
present study was performed to examine the hypothesis that
the onset of acute ischemic myocardial damage requires 2
hits: a susceptible plaque and a triggering stimulus. Despite
these limitations, the present experimental study does iden-
tify vasospasm as 1 possible second hit in the precipitation

Figure 3.

Figure 4.
of acute ischemic myocardial damage. Extrapolation of the present experimental results to human ACSs will require further study.

Acknowledgements
We thank Dr Kazuya Miyagawa and Dr Seimi Kobayashi, Cardiovascular Medicine, Kobe University Graduate School of Medicine, for imaging echocardiograms. We also thank Sara Karwacki, Brigham and Women’s Hospital, for editing the article.

Sources of Funding
This work was supported, in part, by a grant for Research on Biological Resources and Animal Models for Drug Development from the Ministry of Health, Labor, and Welfare of Japan, by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, and Technology, Japan (23300157), by a research grant from Daiichi-Sankyo Co Ltd, Tokyo, Japan, and by a grant from the US National Institutes of Health (R01-HL080472) to Dr Libby.

Disclosures
None.

References

Significance
This study indicates several novel and provocative observations that add to the understanding of the relation of vasospasm of atherosclerotic coronary artery lesions to the onset of acute ischemic myocardial damage. The present study was performed to examine the hypothesis that the onset of acute ischemic myocardial damage requires 2 hits: a susceptible plaque and a triggering stimulus. Using pharmacologic provocation of coronary arterial spasm in myocardial infarction–prone strain of the Watanabe heritable hyperlipidemic rabbits, which developed coronary atherosclerosis spontaneously, the present study does provide experimental validation of the concept that coronary artery vasospasm might be 1 mechanism of triggering coronary plaque injury and subsequent acute ischemic myocardial damage, including impaired left ventricular function. Despite the limitations of animal studies, the present experimental study does identify vasospasm as 1 possible second hit in the precipitation of acute ischemic myocardial damage.
Vasospasm of Atherosclerotic Coronary Arteries Precipitates Acute Ischemic Myocardial Damage in Myocardial Infarction–Prone Strain of the Watanabe Heritable Hyperlipidemic Rabbits
Masashi Shiomi, Tatsuro Ishida, Tsutomu Kobayashi, Norihisa Nitta, Akinaga Sonoda, Satoshi Yamada, Tomonari Koike, Nobue Kuniyoshi, Kiyoshi Murata, Ken-ichi Hirata, Takashi Ito and Peter Libby

Arterioscler Thromb Vasc Biol. 2013;33:2518-2523; originally published online August 29, 2013;
doi: 10.1161/ATVBAHA.113.301303
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2013 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/33/11/2518

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/
METHODS and MATERIALS

Animals

We used 30 myocardial infarction-prone Watanabe heritable hyperlipidemic (WHHLMI) rabbits aged 13–29 months in the experiments involving provocation of coronary spasm. For comparison with general features of coronary lesions, we analyzed coronary sections from 100 WHHLMI rabbits that did not receive spasmogens, aged 10-24 months old, that had been sacrificed previously. WHHLMI rabbits were bred at the Kobe University Graduate School of Medicine. Rabbits resided individually in metal cages (550 mm wide, 600 mm deep, and 450 mm high) with a flat metal floor, and consumed standard rabbit chow (LRC4, Oriental Yeast Co., Ltd., Tokyo, Japan) at 120 g/day and water ad libitum. The animal rooms were maintained under a constant temperature (22 ± 2°C), relative humidity (50–60%), ventilation rate (15 cycles/hour), and lighting cycle (12 hours light/dark). This study was approved by the Kobe University Animal Care and Use Committee (approval numbers: P080606, P091101), and animal experiments were conducted in accordance with the Regulations for Animal Experimentation of Kobe University, Act on Welfare and Management of Animals (Law No. 105; 1973, revised 2006), the Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notification No. 88, 2006), and the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions (Notice No.71, 2006).

Provocation of Coronary Spasm

Coronary spasm was provoked under anesthesia. Based on the results obtained in preliminary experiments that monitored isometric tension in WHHLMI coronary helical strips with atherosclerotic plaques, and demonstrated marked hypercontraction to the combination treatment with norepinephrine and ergonovine (data not shown), WHHLMI rabbits received a bolus of ergonovine maleate (0.45 µmol/kg i.v.) during infusion of norepinephrine (12 nmol/kg/min) through a marginal ear vein. To reverse the coronary spasm, nitroglycerin (Hikari Pharmaceutical Co., Ltd., Tokyo, Japan) was injected intravenously (10 µg/kg) 20–30 minutes after electrocardiogram (ECG) changes began. During the spasm provocation, rabbits were anesthetized with an intravenous injection of ketamine hydrochloride (15 mg/kg, Daiichi-Sankyo Co. Ltd., Tokyo, Japan) plus midazolam (1 mg/kg, Dormicum, Astellas Pharma Inc., Tokyo, Japan) via a marginal ear vein, and anesthesia was continued by infusion of ketamine hydrochloride at 60 mg/kg/h. In addition, oxygen was supplied through a face mask (2.0 L/min), and rabbits were warmed with a heating pad. Rabbits were euthanized by intravenous injection of sodium pentobarbital (150 mg/kg) or exsanguination from the carotid artery under anesthesia with intravenous administration of sodium pentobarbital (30 mg/kg), in combination with local administration of lidocaine hydrochloride (1.0 mg/kg) for histopathological examination.

Evaluation of Coronary Spasm

Coronary angiograms and ECGs monitored the occurrence of coronary spasm. Electrodes were positioned to mimic the sites used in humans. Coronary angiography was performed using an X-ray apparatus (OPESCOPE PLENO; Shimadzu Corporation, Kyoto, Japan). The resolution of this X-ray apparatus is 3 line pairs/mm (166.6 μm). Contrast medium (Omnipaque 350; Daiichi-Sankyo Co. Ltd., Tokyo, Japan) was injected at the coronary artery ostia through a sheath catheter (4Fr, Terumo Clinical Supply Co. Ltd., Tokyo, Japan) inserted from the carotid artery. ECGs were monitored with bipolar limb leads (leads I, II, and III) and chest leads (leads V1, V2, V3, V4, V5, and V6) using an amplifier (AB-621G; Nihon Kohden, Tokyo, Japan), and were recorded with a PowerLab/8SP (ADInstruments Pty
Evaluation of Ventricular Contractile Dysfunction and Myocardial Ischemia

Signs of ventricular contractile dysfunction or myocardial ischemia/injury were monitored with echocardiograms and with serum biomarkers. Echocardiographic imaging was performed using a Philips Envisor C echocardiograph (Philips Inc., Eindhoven, the Netherlands). Left ventricular internal diastolic diameter (LVDd) and systolic diameter (LVDs) were measured from M-mode images. Left ventricular function was evaluated by fractional shortening of the left ventricle’s diameter. Fractional shortening (%) was calculated as 1 - LVDs/LVDd. Serum ischemic markers (heart-type fatty acid-binding protein [H-FABP], cardiac troponin-I [cTroponin-I], and myoglobin) were assayed with ELISA kits (Life Diagnostics Inc., West Chester, PA, USA) before the injection of ergonovine plus norepinephrine, and 4 hours after ischemic changes occurred in the ECG.

Preparation of Coronary Sections
Rabbits with provoked vasospasm were euthanized 4–20 hours after the development of coronary vasospasm. Hearts were excised and immersion-fixed with a 10% neutral buffered formalin solution, with or without prior perfusion fixation (15 rabbits each) with the same fixative, and embedded in paraffin. To seek emigration of macrophages from lesions and accumulation of sloughed endothelial cells in the arterial lumen, we did not perform perfusion fixation on 15 rabbits. For perfusion fixation, a needle was inserted into the left ventricle from the apex; the aortic root was clamped; and the heart was removed. Approximately 300 mL of saline was perfused from the needle with a perfusion apparatus to wash the blood in the coronary arteries. A neutral buffered formalin solution was then perfused through the needle. To minimize disturbance of newly formed and potentially friable thrombus and preserve the intra vitam morphological state of the intimal surface, we set the perfusion pressure at 60 mmHg for rabbits treated with spasmogens. Coronary arterial segments were prepared as reported previously at 250-µm or 500-µm intervals.4Sections were sliced serially at 5 µm thick, and were stained with elastic van Gieson, Azan, hematoxylin and eosin (HE), Martius scarlet-blue 5, and Fraser-Lendrum methods 5. Sections also underwent immunohistochemical evaluation with monoclonal antibodies to RAM-11 (Dako A/S, Glostrup, Denmark) specific for rabbit macrophages,6 1A4 (Dako A/S) specific for smooth-muscle cell α-actin, MMP-9 (Daiichi Fine Chemical Co., Ltd. Takaoka, Japan), or CD31 (Dako A/S). CD31 is strongly expressed in endothelial cells and weakly expressed in megakaryocytes, platelets, occasional plasma cells, lymphocytes, and neutrophils. We observed coronary lesions with an optical microscope equipped with 40x or 100x magnification objectives.

Assay of Serum Lipid Levels
Serum total cholesterol and triglyceride levels were measured at 12 months of age. Blood samples were taken after 15 hours of fasting. Serum lipid levels were assayed enzymatically with kits.

Statistical Analyses
Data are presented as the mean ± standard error of the mean. Statistical analyses were carried out for mean values with the signed Wilcoxon test; and for frequency with the chi-square test. For the comparison of mean values among multiple groups, we performed the Dunnet test. A value of P<0.05 was considered statistically significant.
References
SUPPLEMENTAL MATERIALS

Vasospasm of Atherosclerotic Coronary Arteries Precipitates Acute Ischemic Myocardial Damage in WHHLMI Rabbits

Masashi Shiomi, Tatsuro Ishida, Tsutomu Kobayashi, Norihisa Nitta, Akinaga Sonoda, Satoshi Yamada, Tomonari Koike, Nobue Kuniyoshi, Kiyoshi Murata, Ken-ichi Hirata, Takashi Ito, Peter Libby

Adresses for Corresponding:
Masashi Shiomi, Ph.D.
Institute for Experimental Animals, and Division of Comparative Pathophysiology
Kobe University Graduate School of Medicine
7-5-1, Kusunoki-cho, Chuo-ku, Kobe 650-0017, Japan
Phone: (+81) 78 382 6900; Fax: (+81)78 382 6904; Email:ieakusm@med.kobe-u.ac.jp
Supplemental Table I. Characteristics of WHHLMI rabbits with coronary spasms.

1. Animals examined 30
2. Age at experiment (months) 18.1 ± 0.8
3. Serum lipid levels at 12 months of age (mmol/L)
4. Total cholesterol 21.9 ± 0.8
5. Triglyceride 3.9 ± 0.4
6. Examination of coronary lesions
7. Segments examined 78.9 ± 5.4
8. Number of segments with lesions 61.0 ± 6.9
9. Maximum coronary stenosis (%) 79.7 ± 2.8
10. Frequency of >70% stenosis 0.37 ± 0.05

Coronary stenosis was evaluated grossly with every 10% stenosis.

Data are represented as the mean ± SEM.