High susceptibility of atherosclerotic coronary arteries to the onset of vasospasm and angina pectoris-like symptoms due to coronary spasm in WHHLMI rabbits

Tomonari KOIKE1), Shiori TAMURA2), Ying YU1), Nobue KUNIYOSHI1), and Masashi SHIOMI1,2)

1)Institute for Experimental Animals, Kobe University Graduate School of Medicine, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan
2)Division of Comparative Pathophysiology, Kobe University Graduate School of Medicine, 7-5-1, Kusunoki-cho, Chuo-ku, Kobe, Hyogo 650-0017, Japan

Abstract: We examined the relationship between atherosclerosis and the provocation of coronary spasm as well as the influence of coronary spasm on the onset of acute ischemic myocardial disease. Coronary spasm was provoked in anesthetized normal Japanese white (JW) rabbits and myocardial infarction-prone Watanabe heritable hyperlipidemic (WHHLMI) rabbits, an animal model for coronary atherosclerosis and myocardial infarction, by injecting ergonovine during the infusion of norepinephrine through a marginal ear vein. A decrease in contrast flow in the left circumflex artery was observed on coronary angiograms. Ischemic changes were observed on the electrocardiograms of 29% (2/7) of JW and 79% (27/34, \( P=0.007 \)) of WHHLMI rabbits. The frequency of coronary spasm was significantly high in rabbits with severe coronary plaques showing diffuse lesions. Left ventricle motility in vasospasm-positive rabbits, which was evaluated with echocardiograms, was decreased by 29% following the ergonovine injection (\( P<0.001 \)), and every serum ischemic marker markedly increased 4 h after the provocation of vasospasm. These results demonstrate that atherosclerotic coronary arteries are positively related to the provocation of vasospasm, and vasospasm in severe atherosclerotic coronary segments evokes angina pectoris-like findings and/or non-fatal myocardial infarction. WHHLMI rabbits may be a novel animal model for angina pectoris and acute ischemic heart disease.

Key words: angina pectoris, animal model, coronary atherosclerosis, coronary spasm, WHHLMI rabbit

Introduction

Based on clinical observations, coronary spasm has been implicated as one of the causes of acute ischemic coronary events [5], such as sudden cardiac death [15], lethal arrhythmia [3], variant angina [6], and acute myocardial infarction [17, 20]. Due to its relationship with coronary heart disease (CHD), the gravity of coronary spasm was reconfirmed in the onset of acute ischemic coronary events in Western countries [8]. However, limited evidence is available for the causal link between coronary spasm and CHD. The development of suitable animal models may promote a clearer understanding of the role of coronary spasm in the provocation of acute ischemic heart disease. Several animals have been used as models in the study of coronary spasm. In a swine model fed a cholesterol diet, constriction responses induced by constrictors were significantly augmented [2,
4, 9, 10]. Although these findings have contributed to clarifying the mechanisms involved in the provocation of vasospasm [9], the extensive denudation of arterial endothelial layers does not occur under physiological conditions. Previous studies [4, 7] examined the relationship between coronary spasm and the onset of acute coronary syndromes, but did not evaluate clinical findings.

We developed a myocardial infarction-prone strain of the Watanabe heritable hyperlipidemic (WHHLMI) rabbit [12, 14] by selective breeding [12, 18], and this rabbit develops coronary atherosclerosis and hypercholesterolemia because of a genetic defect in low-density lipoprotein receptors. We previously provoked coronary spasm in WHHLMI rabbits and identified relationships between coronary spasm and plaque disruption as well as ischemic myocardial damage [11]. However, we did not sufficiently examine the relationship between coronary plaques and the provocation of coronary spasm, or the influences of coronary spasm on the development of ischemic heart disease, such as angina pectoris. In the present study, we analyzed stored specimens from our previous study [11] and additional four rabbits in order to examine the suitability of the WHHLMI rabbit as an animal model for coronary spasm and spastic angina.

**Materials and Methods**

**Animals**

We used 34 WHHLMI rabbits aged 12–29 months in experiments on the provocation of coronary spasm. WHHLMI rabbits were bred at the Kobe University Graduate School of Medicine. As a control, 7 male Japanese white (JW) rabbits (Kitayama Labes, Co., Ltd., Ina, Japan) aged 8 months were used in the coronary spasm provocation test and examination of left ventricular function. Rabbits resided individually in metal cages (width 550 mm, depth 600 mm, and height 450 mm) with a flat floor, and were given standard rabbit chow (LRC4, Oriental Yeast Co., Ltd., Tokyo, Japan) at 120 g/day and water ad libitum. Animal rooms were maintained under a constant temperature (22 ± 2°C), relative humidity (50–60%), ventilation rate (15 cycles/hour), and lighting cycle (12 h light/dark). This study was approved by the president of Kobe University after being reviewed by the Kobe University Animal Care and Use Committee (approval numbers: P080606 and P091101), and animal experiments were conducted in accordance with the Regulations for Animal Experimentation of Kobe University, and Japanese regulations, such as the Act on the Welfare and Management of Animals (Law No. 105; 1973, revised 2006), Standards Relating to the Care and Management of Laboratory Animals and Relief of Pain (Notification No. 88, 2006), and Fundamental Guidelines for the Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions (Notice No.71, 2006).

**Anesthesia and euthanasia**

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), and anesthesia was continued by the intravenous infusion of ketamine hydrochloride at 60 mg/kg/h. During experiments, oxygen was supplied through a face mask (2.0 l/min for rabbits), and rabbits were warmed with a heating pad. Rabbits were euthanized with exsanguination under the intravenous administration of sodium pentobarbital (30 mg/kg).

**Provocation of coronary spasm**

The provocation and evaluation of coronary spasm were performed as previously described [11], and the study design was shown in Fig. 1. We analyzed the left circumflex artery (LCX) because it is a major coronary artery in rabbits [1, 13], and severe atherosclerotic lesions were observed in LCX; however, the degree of lesions was shown to markedly vary and the frequency of atherosclerotic lesions was low in the anterior descending artery [14].

**Evaluation of ventricular contractile dysfunction and myocardial ischemia**

Echocardiograms were performed on 13 WHHLMI rabbits and 7 JW rabbits using the Philips Envisor C echocardiograph (Philips Inc., Eindhoven, the Netherlands) [11]. Left ventricular function was evaluated by fractional shortening, which was calculated as 1 − [systolic left ventricular diameter (LVDs)] / [diastolic left ventricular diameter (LVDd)]. In our previous study [11], we examined serum markers for ischemic myocardial damage (heart-type fatty acid-binding protein [H-FABP], cardiac troponin-I [cTroponin-I], and myoglobin) in 6 WHHLMI rabbits. In order to ensure the onset of ischemic myocardial damage after the provocation of coronary vasospasm, assays were performed using the sera of 20 WHHLMI rabbits.
rabbits stored at −80°C. These serum biomarkers for ischemic myocardial damage were assayed using ELISA kits (Life Diagnostics Inc., West Chester, PA, USA).

**Preparation of coronary sections**

Rabbits were euthanized after being examined under anesthesia. Hearts were excised, immersion-fixed with 10% neutral buffered formalin solution, and embedded in paraffin. Coronary arterial segments were prepared as reported previously [13]. Sections were stained using Elastic van Gieson staining. Coronary stenosis was evaluated using cross-sectional narrowing (%) in the LCX, which was calculated by dividing the lumen area by the area surrounded by an internal elastic lamina. In the present study, branches of the LCX were not examined.

**Assay of serum lipid levels**

Serum total cholesterol and triglyceride levels were assayed enzymatically with kits when animals were 12 months of age using sera obtained after 15 h of fasting.

**Statistical analyses**

Data are represented as the mean ± standard error of the mean (SEM). Statistical analyses were performed for mean values with the signed Wilcoxon test, Mann-Whitney U-test, or Student’s t-test, and for frequency with the chi-squared test. In order to compare mean values among multiple groups, we performed the Bonferroni test. A value of $P<0.05$ was considered to be significant.

**Results**

**Baseline data of WHHLMI rabbits**

Table 1 shows the baseline data of WHHLMI rabbits used in this study. We added data on blood pressure, heart rate, and animals with more than 75% coronary stenosis to our previous findings [11], and updated data on 30 rabbits from our previous study [11] for 34 rabbits in the present study. As shown in Table 1, all WHHLMI rabbits showed hypercholesterolemia and had atherosclerotic lesions in the coronary arteries. Maximum stenosis was
78.2 ± 3.3%. Coronary stenosis (evaluated as cross-sectional narrowing) of more than 75% was observed in 67.6% of rabbits (23/34).

Occurrence of coronary spasm

Figure 2 shows the ECG results of rabbits. In analyses, we added data obtained from JW rabbits (Fig. 2B) to our previous findings [11], and updated the ECG results of 30 WHHLMI rabbits in our previous study [11] for 34 WHHLMI rabbits in the present study. Furthermore, a 12-lead ECG (Fig. 2A) was presented instead of the 9-lead eCG in the previous study [11]. During the vasospasm provocation test, ECG showed ischemic changes in WHHLMI rabbits (Fig. 2), e.g. ST depression/elevation, T-wave elevation/inversion, poor R-wave progression, a deep Q-wave, and the ventricular premature complex. The frequency of these abnormalities was markedly higher in WHHLMI rabbits (27/34, 79.4%, \(P=0.007\)) than in normal JW rabbits (2/7, 28.6%).

Figure 3 shows the provocation of angiographical coronary spasm in WHHLMI rabbits in vivo. We did not analyze the relationship between coronary stenosis and the provocation of coronary spasm in our previous study [11]. Contrast flow was markedly decreased after the injection of ergonovine (Fig. 3A), and the area with decreased perfusion in the LCX corresponded to segments with plaques (sections 7–16) (Fig. 3B). Regarding the relationship between coronary plaques and the development of coronary spasm (Fig. 3C), maximum coronary stenosis was significantly larger in vasospasm-positive rabbits (76 ± 4%, \(P=0.034\)) than in vasospasm-negative rabbits (57 ± 10%). The average of coronary stenosis in each atherosclerotic lesion was significantly larger in vasospasm-positive rabbits (65 ± 4%, \(P=0.023\)) than in vasospasm-negative rabbits (33 ± 6%). In addition, the frequency of coronary segments with more than 75% stenosis was significantly higher in vasospasm-positive WHHLMI rabbits (32 ± 6%, \(P=0.036\)) than in vasospasm-negative WHHLMI rabbits (8 ± 6%). These results demonstrated that atherosclerotic coronary arteries were highly susceptible to the provocation of vasospasm.

Changes in the motility of the left ventricular wall

Table 2 shows the results of echocardiograms during the vasospasm provocation test. In this examination, we added data from JW rabbits to our previous findings [11], and updated data on 10 WHHLMI rabbits in the previous study [11] for 13 WHHLMI rabbits. Although fractional shortening was already significantly lower at the baseline in WHHLMI rabbits (32.5 ± 1.2, \(P<0.004\)) than in normal JW rabbits (41.5 ± 3.1), it decreased further after the spasmoden treatment (23.0 ± 0.6, \(P<0.001\)). The reduction observed in fractional shortening was mainly due to the depression of left ventricular contractions (11.6 ± 0.5 vs. 9.8 ± 0.6, \(P=0.009\)). These results indicate that coronary spasm leads to cardiac dysfunction in WHHLMI rabbits.

Table 1. Baseline data of WHHLMI rabbits provoked with coronary spasm

| Parameter                                      | Value        |
|------------------------------------------------|--------------|
| Examined rabbits                               | 34           |
| Gender (female : male)                         | 11:23        |
| Age (months)                                   | 18.0 ± 0.8   |
| Body weight (kg)                               | 3.32 ± 0.07  |
| Serum lipid levels at 12 months old (mg/dl)    |              |
| Total cholesterol                              | 845 ± 31     |
| Triglyceride                                   | 364 ± 34     |
| Coronary plaques                               |              |
| Examined segments                              | 21.6 ± 1.0   |
| Segments with lesions                          | 14.4 ± 1.4   |
| Animals with more than 75% coronary stenosis   | 67.6% (23/34) |
| Segments with more than 75% stenosis           | 9.7 ± 1.6    |
| Maximum stenosis (%)                           | 78.2 ± 3.3   |
| Blood pressure at the femoral artery (n=13, mmHg) |            |
| Systolic                                       | 131 ± 4.6    |
| Diastolic                                      | 68 ± 3.5     |
| Heart rate (beats/min)                         | 243 ± 5.4    |

Data are represented as the mean ± SEM.

Table 3 shows changes in serum markers for ischemic myocardial injury in WHHLMI rabbits. In this examination, we updated data on 6 WHHLMI rabbits in our
previous findings [11] for 20 WHHLMI rabbits in order to ensure the development of ischemic myocardial injury after the provocation of coronary spasm. These serum markers were within normal human ranges at baseline. However, they markedly increased after the provocation of vasospasm, which confirmed that coronary spasm induced ischemic injury in the myocardium.

**Discussion**

The present study demonstrated that vasospasm was frequently induced in coronary arteries with diffuse atherosclerotic plaques showing more than 75% coronary stenosis, and cardiac dysfunction and ischemic myocardial injury developed in these rabbits. These results suggest that WHHLMI rabbits are a suitable animal model for coronary spasm, angina pectoris-like findings, and/or non-fatal myocardial infarction.

In the present study, the frequency of coronary spasm in WHHLMI rabbits with severe coronary stenosis and diffuse atherosclerotic plaques was significantly higher than that in WHHLMI rabbits with less stenosis and focal plaques in spite of similar serum cholesterol levels (851 ± 37 mg/dl vs. 824 ± 58 mg/dl, *P*=0.735). Previous *ex vivo* studies using endothelial-denuded coronary strips demonstrated that atherosclerotic coronary strips

![Fig. 2. Electrocardiograms (ECG) of rabbits treated with an ergonovine bolus injection under the norepinephrine infusion. (A) Representative ECG changes in a WHHLMI rabbit during the experiments. (B) Frequency of ischemic patterns on ECG in normal rabbits (n=7) and WHHLMI rabbits (n=34). Statistical analyses were performed with the chi-squared test.](image-url)
showed markedly greater sensitivity and reactivity against vasoconstrictors than normal coronary strips [21]. The present results are consistent with these findings. Since the 1990s, atherosclerotic lesions have been detected at the site of focal vasospasm in the coronary arteries of patients [19], and the existence of atherosclerotic lesions is considered to be related to the onset of vasospasm [15]. Therefore, we considered coronary arteries with severe atherosclerotic plaques to be related to the onset of coronary spasm. Regarding gender differences in coronary spasm, all females (11/11) developed coronary spasm, whereas its incidence was 70% (16/23) in males. One of the causes for this gender difference may be differences in the degree of coronary stenosis. Coronary atherosclerosis was more severe in females than in males (data not shown).

After the provocation of coronary spasm, left ventricle motility in rabbits with coronary spasm evaluated with echocardiograms was decreased, and serum markers for ischemic myocardial injury were markedly increased, which is consistent with our previous findings [11]. Wang et al. also observed elevations in serum cardiac troponin-I levels in patients after the provocation of coronary spasm [17]. The present results suggest the onset of angina pectoris-like symptoms and/or non-fatal myocardial infarction; however, the frequency of occlusive

Fig. 3. Relationship between coronary plaques and coronary spasm in WHHLMI rabbits. (A) Coronary angiograms after the ergonovine injection during the norepinephrine infusion. (B) Coronary stenosis evaluated as cross-sectional narrowing of the left circumflex artery as indicated on the angiogram. (C) Relationship between coronary plaques and the provocation of coronary spasm. Data are represented as the mean ± SEM. Statistical tests were performed with the Mann-Whitney U-test. LAD, left anterior descending artery, LCX, left circumflex artery.
thrombi after the disruption of coronary plaques was very low [11]. Previous studies reported that coronary spasm plays an important role in the pathogenesis of not only variant angina, but also ischemic heart disease, including other forms of angina, acute myocardial infarction, arrhythmias, and ischemic sudden death [3, 5, 6, 16, 17, 20]. These findings are consistent with the results of the present study; therefore, we speculated that the WhhLmi rabbit is useful as an animal model of experimentally provoked coronary spasm and subsequent myocardial ischemia.

Limitations of the study

In patients, angina pectoris is defined as unstable chest pain and ischemic ECG changes. Since rabbits do not have the ability to express chest pain similar to humans, it is difficult to diagnose the present results obtained after a spasmogen injection as the provocation of angina pectoris.

The Japanese Circulation Society reported that it is possible to establish a diagnosis even in patients without a chest pain by recording a 12-lead ECG, and more than half of patients had asymptomatic attacks [15]. In the present study, coronary spasm caused ischemic changes in ECG, cardiac dysfunction, and ischemic injury to the myocardium. These results suggest that angina pectoris-like findings and/or non-fatal myocardial infarction developed due to the provocation of coronary spasm.

In conclusion, the present results suggest that atherosclerosis in coronary arteries contributes to the development of coronary spasm, and coronary spasm in severe atherosclerotic lesions results in acute ischemic myocardial damage, which resembles angina pectoris in humans. The WHHLMI rabbit is a good animal model for coronary spasm and the related ischemic heart disease accompanying cardiac dysfunctions.

Conflict of Interests

The authors have declared that no competing interests exist.

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, and by Grants-in-Aid for scientific research from the Ministry of Education, Culture, Sports, and Technology, Japan 23300157, and 24591813).

Acknowledgments

We would like to thank Dr. Norihisa Nitta and Dr. Akinaga Sonoda, Shiga University of Medical Science, Japan, for coronary angiography on rabbits.

References

1. Clozel, J.P., Lengsfeld, H., Kuhn, H., and Baumgartner, H.R. 1988. Decreased coronary vascular reserve in Watanabe heritable hyperlipidemic rabbits. *Arteriosclerosis* 8: 310–320. [Medline] [CrossRef]
2. Egashira, K., Tomoike, H., Yamamoto, Y., Yamada, A., Hayashi, Y., and Nakamura, M. 1986. Histamine-induced coronary spasm in regions of intimal thickening in miniature pigs: roles of serum cholesterol and spontaneous or induced intimal thickening. *Circulation* 74: 826–837. [Medline] [CrossRef]
3. Krahn, A.D., Healey, J.S., Chauhan, V., Birnie, D.H., Simpson, C.S., Champagne, J., Gardner, M., Sanatani, S., Exner, D.V., Klein, G.J., Yee, R., Skanes, A.C., Gula, L.J., and Gollob, M.H. 2009. Systematic assessment of patients with unexplained cardiac arrest: Cardiac Arrest Survivors With Preserved Ejection Fraction Registry (CASPER). *Circulation* 120: 278–285. [Medline] [CrossRef]
4. Maseri, A., Beltrame, J.F., and Shimokawa, H. 2009. Role of coronary vasoconstriction in ischemic heart disease and search for novel therapeutic targets. *Circ. J.* 73: 394–403. [Medline] [CrossRef]
5. MacAlpin, R.N. 1993. Cardiac arrest and sudden unexpected death in variant angina: complications of coronary spasm that can occur in the absence of severe organic coronary stenosis. *Am. Heart J.* 125: 1011–1017. [Medline] [CrossRef]
6. Nakamura, M. and Abe, S. 1997. An experimental induction of acute myocardial infarction and arterial thrombosis in rabbits. *Ann. N. Y. Acad. Sci.* 811: 424–428. [Medline] [CrossRef]
7. Ong, P., Athanasiadis, A., Borgulya, G., Voehringer, M., and Sechtem, U. 2011. 3-year follow-up of patients with coronary artery spasm as cause of acute coronary syndrome: the CASPAR (coronary artery spasm in patients with acute coronary syndrome) study follow-up. *J. Am. Coll. Cardiol.* 57: 147–152. [Medline] [CrossRef]
8. Shimokawa, H. 2000. Cellular and molecular mechanisms of coronary artery spasm: lessons from animal models. *Jpn. Circ. J.* 64: 1–12. [Medline] [CrossRef]
9. Shimokawa, H., Tomoike, H., Nabeyama, S., Yamamoto, H., Ishii, Y., Tanaka, K., and Nakamura, M. 1985. Coronary artery spasm induced in miniature swine: angiographic evidence and relation to coronary atherosclerosis. *Am. Heart J.* 110: 300–310. [Medline] [CrossRef]
10. Shiono, K., Tanaka, T., and Nakamura, T. 1988. The Watanabe heritable hyperlipidemic rabbit. *Arterioscler. Thromb. Vasc. Biol.* 8: 2518–2523. [Medline] [CrossRef]
11. Shiomi, M., Ishida, T., Kobayashi, T., Nitta, N., Sonoda, A., Yamada, S., Koike, T., Kuniyoshi, N., Murata, K., Hirata, K., Ito, T., and Libby, P. 2013. Vasospasm of atherosclerotic coronary arteries precipitates acute ischemic myocardial damage in myocardial infarction-prone strain of the Watanabe heritable hyperlipidemic rabbits. *Arterioscler. Thromb. Vasc. Biol.* 33: 2518–2523. [Medline] [CrossRef]
12. Shiomi, M. and Ito, T. 2009. The Watanabe heritable hyperlipidemic (WHHL) rabbit, its characteristics and history of development: a tribute to the late Dr. Yoshio Watanabe. *Atherosclerosis* 207: 1–7. [Medline] [CrossRef]
13. Shiomi, M., Ito, T., Shiraisi, M., and Watanabe, Y. 1992. Inheritability of atherosclerosis and the role of lipoproteins as risk factors in the development of atherosclerosis in WHHL rabbits: risk factors related to coronary atherosclerosis are different from those related to aortic atherosclerosis. *Atherosclerosis* 96: 43–52. [Medline] [CrossRef]
14. Shiomi, M., Ito, T., Yamada, S., Kawashima, S., and Fan, J. 2003. Development of an animal model for spontaneous myocardial infarction (WHHLMI rabbit). *Arterioscler. Thromb. Vasc. Biol.* 23: 1239–1244. [Medline] [CrossRef]
15. The Japanese Circulation Society Joint Working Groups. 2008. Guidelines for Diagnosis and Treatment of Patients with vasospastic angina (coronary spastic angina) (JCS 2008). *Circ. J.* 72 (Suppl. IV): 1239–1252.
16. Koga, T., Tagawa, H., Tomoike, H., Mitsuoka, W., Egashira, S., Ohara, Y., Takeshita, A., and Nakamura, M. 1993. Role of coronary artery spasm in progression of organic coronary stenosis and acute myocardial infarction in a swine model. Importance of mode of onset and duration of coronary artery spasm. *Circulation* 87: 573–582. [Medline] [CrossRef]
17. Wang, C.H., Kuo, L.T., Hung, M.J., and Cherng, W.J. 2002. Coronary vasospasm as a possible cause of elevated cardiac troponin I in patients with acute coronary syndrome and insignificant coronary artery disease. *Am. Heart J.* 144: 275–281. [Medline] [CrossRef]
18. Watanabe, Y. 1980. Serial inbreeding of rabbits with hereditary hyperlipidemia (WHHL-rabbit). *Atherosclerosis* 36: 261–268. [Medline] [CrossRef]
19. Yamagishi, M., Miyatake, K., Tamai, J., Nakatani, S., Koyama, J., and Nissen, S.E. 1994. Intravascular ultrasound detection of atherosclerosis at the site of focal vasospasm in angiographically normal or minimally narrowed coronary segments. *J. Am. Coll. Cardiol.* 23: 352–357. [Medline] [CrossRef]
20. Yasue, H., Takizawa, A., Nagao, M., Nishida, S., Horie, M., Kubota, J., Omote, S., Takaoka, K., and Okumura, K. 1988. Long-term prognosis for patients with variant angina and inflammatory factors. *Circulation* 78: 1–9. [Medline] [CrossRef]
21. Yokoyama, M., Akita, H., Mizutani, T., Fukuzaki, H., and Watanabe, Y. 1983. Hyperreactivity of coronary arterial smooth muscles in response to ergonovine from rabbits with hereditary hyperlipidemia. *Circ. Res.* 53: 63–71. [Medline] [CrossRef]