Atherosclerotic Plaques Composed of a Large Core of Foam Cells Covered with Thin Fibrous Caps in Twice-injured Carotid Arterial Specimens Obtained From High Cholesterol Diet-Fed Rabbits

Takashi Yano1, Hiroyuki Kawano1, Hidenori Mochizuki1, Osamu Doi2, Takao Nakamura2, and Yasushi Saito3

1Research Center, Mochida Pharmaceutical Co., Ltd., Shizuoka, Japan.
2Department of Animal Science and Technology, Faculty of Agriculture, Gifu University, Gifu, Japan.
3Second Department of Internal Medicine Chiba University, Chiba, Japan.

We attempted to find atherosclerotic plaques including a large lipid core and thin fibrous cap in twice-injured arterial specimens obtained from high cholesterol diet (HCD)-fed rabbits. Rabbits fed a HCD were subjected to carotid artery injury using a balloon catheter. After 2 or 4 weeks of cholesterol feeding, a second mild injury was induced in the same region as the first injury. The rabbits were given a standard diet for 2 weeks after the second injury. Typical atherosclerotic plaques with a fibrous cap formed by smooth muscle cells and extracellular matrix overlying a core formed by macrophage foam cells were observed in the lesion. Gelatin proteolytic activities were found in homogenates containing either media or intima from the injured artery, and activated matrix metalloproteinase-2 (MMP2) was detected. With prolongation of the HCD feeding period (interval between injuries) from 2 weeks to 4 weeks, typical plaque was observed more frequently. Furthermore, the neointimal area and the macrophage foam cells area increased, as did gelatin proteolytic activity. Since the typical atherosclerotic plaques observed in the present study have some histopathological and pathogenic characteristics in common with unstable atherosclerotic plaque, we expect that the typical atherosclerotic plaque found in the present study will be useful for basic studies of plaque stabilization and prevention of acute coronary syndromes. J Atheroscler Thromb, 2000; 7: 83-90.

Key words : Atherosclerosis, Animal model, Fibrous cap, Extracellular matrix Metalloproteinases

Introduction

Recent lipid-lowering trials that used 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors have revealed that lowering plasma cholesterol levels prevented acute ischemic events (1-3). These trials yielded stronger increases in survival rates than amelioration of luminal narrowing of coronary arteries, indicating that lowering the plasma low-density lipoprotein level and stabilization of vulnerable plaques are more important than reducing thickening of the neointima for prevention of acute ischemic events (4). The risk of plaque rupture appeared to be related to the composition of the atherosclerotic plaque. Many researchers (5-7) have suggested that the characteristics of rupture-prone plaques are as follows: 1) a large lipid core underlies a thin and collagen-poor fibrous cap, and 2) macrophage-rich areas are found in the shoulder of the plaque. On the other hand, stable plaques contain mainly smooth muscle cells and collagen fibril (5, 8). Macrophage-rich areas are frequently found in coronary plaques in patients with acute coronary syndromes (9). Macrophages release
proteolytic enzymes that may be responsible for weakening of the fibrous cap and subsequent rupture of the atherosclerotic plaque (8, 10).

For the reasons mentioned above, stabilisation of vulnerable plaques has come to be a new goal in the treatment of atherosclerosis, and an animal model suitable for study of vulnerable plaques is required. Thus far, fibrous and smooth muscle cell-dominated lesions obtained with balloon catheter de-endothelialization alone have mainly been used to investigate smooth muscle cell proliferation and intimal hyperplasia that resemble the histological effects of clinical balloon angioplasty (11, 12). On the other hand, foam cell lesions obtained with high cholesterol diet-fed rabbits may be useful for investigating the relationships between blood lipoproteins and development of atherosclerosis, including the role of inflammatory mechanisms (13-15). Additionally, the combination of arterial balloon injury and subsequent cholesterol diet has been used to induce atherosclerotic lesions in animal models most closely simulating clinically encountered lesions (16, 17). However, animal models of atherosclerosis mimic human atherosclerosis imperfectly, since spontaneous plaque rupture does not occur in such models. Although a few investigators have reported animal models that mimic atherosclerotic plaque rupture (18, 19), their animal models seemed to be commonly used. In the present study, we attempted to conveniently produce atherosclerotic plaques with some characteristics of unstable plaque by hypercholesterolemia induced by cholesterol feeding of rabbits with twice-injured carotid arteries.

Materials and Methods

Animals and diet

Male Japanese white rabbits (Kitayama Labes Co., Ltd., Nagano, Japan), aged 9 weeks and weighing 2.5-3.0 kg, were used. The rabbits were maintained at a room temperature of 23±2°C, relative humidity of 55±5% and illumination of 12 hours per day from 7:00 a.m. to 7:00 p.m., and were allowed free access to water and food. The animals were given laboratory chow (RC-4 Oriental Yeast Co., Ltd., Tokyo, Japan) as a standard diet and/or 1% cholesterol-containing diet as a high-cholesterol diet (HCD).

Protocol

After one week of HCD feeding, rabbits were anesthetised with 25 mg/kg of sodium pentobarbital and the endothelium of the right carotid artery was denuded by three passages of a Fogarty 3F balloon catheter inserted from the iliac artery. Two and four weeks after the first balloon injury, the same part of the artery was injured again under sodium pentobarbital anesthesia as described above. The second injury was mild, and was made using 3 methods in 6 rabbits each as follows: 1) a Fogarty 2F balloon catheter inserted 10 cm from a branch of the carotid artery was expanded with 40 μl saline, and was passed through the carotid artery 3 times, 2) a Fogarty 2F balloon catheter was not passed through the carotid artery, and was only expanded with 40 μl saline at part of the lesion in the carotid artery, 3) a nylon loop (20) was inserted in the same fashion as the balloon catheter, and was passed through the carotid artery with rotation 3 times. However, since no differences in histopathological or pathogenic characteristics of neo-intima were recognized among these three methods, these methods were handled as the single group of mild second injury. Half of the animals and the remaining animals were given the HCD for 2 weeks (2-week interval group) and 4 weeks (4-week interval group), respectively, continuously between the first and second injuries. The rabbits were sacrificed by exsanguination under sodium pentobarbital anesthesia at 2 weeks after the second injury. After thoracotomy, the heart was exposed and 100 mM phosphate-buffered saline (pH 7.2) was perfused from the left ventricle to wash out residual blood in the carotid artery. The carotid artery region was removed and segments (approximately 5 mm in length each) of both the end and middle portions of each region were fixed in methanol-Carnoy solution for histopathological analysis of carotid arterial specimens. The remaining portions of extracted arteries were used for determination of extra-cellular matrix metalloproteinases. All experimental protocols and procedures were approved by the Institutional Animal Use Committee of our laboratory.

Measurement of serum lipid concentrations

The concentrations of serum cholesterol, triglyceride and phospholipid were measured enzymatically with the following commercially available kits: Cholesterol E Test Wako, Triglyceride E Test Wako and Phospholipid C Test Wako (Wako Pure Chemical Industries, Co., Ltd., Tokyo, Japan).

Histopathological analysis of carotid arterial specimens

After segments of lesions were embedded in paraffin, transverse sections 5 μm in thickness were prepared and stained for elastic fibers and collagen fibers with elastica-Van-Gieson stain and Azan stain, respectively. The smooth muscle cells and macrophages in the segments were also immunostained using monoclonal antibodies to α-smooth muscle actin (HHF-35, Dako Japan, Tokyo, Japan) and rabbit macrophages (RAM-11, Dako Japan, Tokyo, Japan), respectively. Then the components and their configuration in the neo-intima were observed by light microscopy. The intimal area and RAM-11 stained area were measured using an image analyzer (LUZEX-F, NIR-ECO, Tokyo, Japan).

Detection and measurement of gelatinase activities

Gelatinolytic proteinase was detected by substrate-gel
Atherosclerotic Plaque in Rabbit Model

Electrophoresis (21). Extracted arteries except for the adventitia were homogenized. The homogenates were mixed with Tris buffer containing 0.1% SDS and 192 mM glycine (pH 6.8) and applied to 9% acrylamide gels (Tefco, Tokyo, Japan) containing 1 mg/ml of gelatin. The gel was gently shaken in 2.5% Triton X-100 solution for 30 minutes at room temperature and incubated in 50 mM Tris buffer (pH 7.4) containing 5 mM CaCl₂, 200 mM NaCl and 0.02% Brij 35 for 20 hours at 37°C. Then the gel was stained with 0.25% Coomassie brilliant blue in 10% acetic acid – 40% ethanol solution. The MMP activities indicating gelatinolytic activities were detected as unstained bands. The gelatinolytic activities in the homogenate were measured using commercially available kits (Roche Diagnostics, Germany) and indicated as % of degraded gelatin.

Statistical analysis

Values represent the mean ± standard error of rabbits in each group for measurements of serum cholesterol levels, neointimal area and rate of occupancy by macrophages, and gelatinolytic activities in the lesions of carotid arteries. The statistical significance of differences between experimental groups was evaluated by the Student’s t-test, and findings of p < 0.05 were considered significant.

Fig. 1. Changes in serum cholesterol levels in rabbit atherosclerotic plaque models. After one week of a high cholesterol diet, the endothelium of the right carotid artery was denuded by a balloon catheter inserted from the iliac artery. Two or four weeks after the first balloon injury, the same part of the artery was mildly injured again. The animals were given high cholesterol diet for 2 weeks (2-week interval group) or 4 weeks (4-week interval group) continuously between the first and second injuries, and then diet was changed from high cholesterol diet to standard diet for 2 weeks. Each point represents the mean ± S.E. for 9 animals in each group.

Fig. 2. Photomicrographs of type 1 plaque in histopathological analysis of twice-injured carotid arterial specimens obtained from high cholesterol diet-fed rabbits. The section was subjected to elastica-Van-Gieson staining (EVG) and Azan staining (AZ). Smooth muscle cells and macrophages were immunostained using HHF-35 (HHF-35) and RAM-11 (RAM-11), respectively. Original magnification ×140.
Results

Serum cholesterol levels

Changes in serum total cholesterol levels are indicated in Fig. 1. At the beginning of the experiment, the serum total cholesterol levels in the 2-week interval group and 4-week interval group were 31 ± 3 mg/dl and 35 ± 5 mg/dl, respectively. When the animals were first injured, the serum total cholesterol levels were already increased to 678 ± 55 mg/dl and 688 ± 43 mg/dl, respectively, after a high-cholesterol diet for one week. Serum cholesterol levels continuously increased until the animals were subjected to the second injury, and reached 1,446 ± 112 mg/dl and 1,917 ± 150 mg/dl in the 2-week interval group and 4-week interval group, respectively. At 2 weeks after the second injury, serum cholesterol levels were decreased to 445 ± 69 mg/dl and 614 ± 81 mg/dl by change to the standard diet.

Histopathological analysis of carotid arterial specimens

The plaques in the lesions observed were classified by histopathological properties. Atherosclerotic plaques composed of a core of foam cells covered with fibrous cap were selected and they were sorted into two typical types, those having a fibrous cap thicker or thinner than one half of the thickening of the neointima were defined

Fig. 3. Photomicrographs of type 2 plaque in histopathological analysis of twice-injured carotid arterial specimens obtained from high cholesterol diet-fed rabbits. The section was subjected to elastica-Van-Gieson staining (EVG) and Azan staining (AZ). Smooth muscle cells and macrophages were immunostained using HHF-35 (HHF-35) and RAM-11 (RAM-11), respectively. Original magnification ×140.

Fig. 4. Changes in the incidence of typical atherosclerotic plaques in twice-injured carotid arterial specimens obtained from high cholesterol diet-fed rabbits. Type 1 plaques had a thin layer formed by smooth muscle cells and extracellular matrix overlying a lipid core formed by macrophage foam cells. Type 2 plaques had less foam cell area than type 1 plaques and were observed in only the core of the neointima. Each value represents % of incidence of each type of the atherosclerotic plaques in all specimens obtained from each group of animals.
as type 1 or type 2, respectively. Photomicrographs of transverse sections of type 1 and type 2 plaques and incidences of these plaques are indicated in Figs. 2-4, respectively. Type 1 plaques had a thin layer of smooth muscle cells weakly stained by HHF-35 and collagen fibers also stained weakly by Azan stain, while many foam cells were present in the core part of the neointima and were RAM-11-positive macrophages. Type 1 plaques were recognized more frequently in the 4-week interval group than in the 2-week interval group. On the other hand, type 2 plaques had less foam cell area than type 1 plaques and were observed only in the core of the neointima. The superficial smooth muscle cells in type 2 plaques stained well with HHF-35, and collagen fibers in the same layer were also stained well by Azan stain.

Type 2 plaques were observed more frequently in the 2-week interval group than in the 4-week interval group.

**Neointimal area and rate of occupancy by macrophages**

Figure 5 shows the neointimal area and the rate of occupation by macrophages on histopathological analysis of carotid arterial specimens in rabbit models. The neointimal area in the 4-week interval group was larger than that in the 2-week interval group, and the area occupied by macrophages stained by RAM-11 was also higher in the 4-week interval group.

**Gelatinolytic activities**

Results of zymographic analysis of gelatinolytic activities are shown in Fig. 6. Active MMP2 was recognized in the lesions of carotid arteries in both the 2-week interval group and 4-week interval group, but not in the arteries of normal animals. On the other hand, pro-MMP2, which is non-active, was recognized in carotid arteries of animals in all groups.

The gelatinolytic activities in the lesions of carotid arteries are indicated in Fig. 7. Although gelatinolytic

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**Fig. 5.** Changes in neointimal area and rate of occupancy by macrophages in twice-injured carotid arterial specimens obtained from high cholesterol diet-fed rabbits. The neointimal area (A) in elastica-Van-Gieson-stained specimens and macrophage area in RAM-11-stained specimens were measured using an image analyzer, and the ratio of occupancy by macrophages (B) to neointimal area was determined. Each column represents the mean ± S.E. for 9 animals in each group. Significant differences between the 2-week interval group and 4-week interval group by Student's t-test are marked **: p < 0.01 and ***: p < 0.001, respectively.

**Fig. 6.** Detection of gelatinolytic activity in homogenate from twice-injured carotid arterial lesions obtained from high cholesterol diet-fed rabbits. Each column represents the mean ± S.E. for 9 animals in each group. Significant difference between 2-week interval group and 4-week interval group by Student's t-test is marked *: p < 0.05

**Fig. 7.** Changes in gelatinolytic activities in homogenate from twice-injured carotid arterial lesions obtained from high cholesterol diet-fed rabbits. Each column represents the mean ± S.E. for 9 animals in each group. Significant difference between 2-week interval group and 4-week interval group by Student's t-test is marked *: p < 0.05

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**Fig. 6.** Detection of gelatinolytic activity in homogenate from twice-injured carotid arterial lesions obtained from high cholesterol diet-fed rabbits.
activities were not detected in normal arteries, they were 28.3±7.4% in the 2-week interval group and markedly increased to 54.3±7.9% in the 4-week interval group.

Discussion

The risk of plaque rupture appears to be related to the composition of atherosclerotic plaque. The cellular composition of advanced atherosclerotic plaques is known to be heterogeneous (22, 23). The purpose of the present study was to conveniently produce an atherosclerotic plaque with some characteristics of unstable plaque by hypercholesterolemia induced by cholesterol feeding of rabbits with twice-injured carotid arteries. Although several types of plaques were observed on histopathological analysis of carotid arterial specimens in twice-injured carotid arteries in the present study, two types of atherosclerotic plaques were noted. Type 1 plaques had a thin-layer fibrous cap composed of HHF-35-negative synthetic smooth muscle cells and extracellular matrix overlying a large core composed of macrophage foam cells, while type 2 plaques had a superficial thick layer of HHF-35-positive smooth muscle cells as well as a small area of macrophage foam cells observed only in the core of the neointima. It is generally believed that atherosclerotic plaques with a thin fibrous cap overlying a large lipid core present a high risk of rupture (5, 6). Monocytes adhering to the endothelial surface enter the intima at sites of lesion predilection and then accumulate lipid and transform into macrophage foam cells. Activated macrophages in the plaques are capable of releasing various extracellular-matrix metalloproteinases responsible for weakening of the fibrous cap and subsequent rupture of the atherosclerotic plaque (6). Actually, rupture-prone lesions usually have prominent macrophage accumulation (24) and macrophage-rich areas are frequently found in coronary plaques of patients with acute coronary syndromes (3). Comparison of these pathological findings and the features of the type 1 plaques found in the present study suggests that type 1 plaques have similarities to unstable atherosclerotic plaques, at least morphologically. On the other hand, smooth muscle cells may counteract some of the adverse effects of macrophages by producing extracellular matrix, collagen, and inhibitors of extracellular-matrix metalloproteinases (8). Since the type 2 plaques observed in the present study had a thick smooth muscle cell layer and dense collagen fibrils, they may have a more stable cellular composition than type 1 plaques.

Although two types of plaques were recognized in both the 2-week interval group and the 4-week interval group, the type 1 plaques were recognized more frequently in the 4-week interval group than in the 2-week interval group. In addition, with prolongation of the cholesterol diet from 2 weeks to 4 weeks, the neointimal area of lesions increased. The areas occupied by RAM-11-positive macrophage foam cells were also higher in the 4-week interval group than in the 2-week interval group. These results suggested that enhancement of the neointimal hyperplasia by prolongation of the cholesterol-fed period in the present rabbit atherosclerotic plaque model was principally due to the increase in macrophage foam cell-rich areas. The combination of atherosclerotic plaque denudation and subsequent high-cholesterol diet induces foam cell-rich atherosclerotic plaques in animal arteries, and has thus been used to produce experimental atherosclerotic plaques (25-27). In the present study, since serum cholesterol levels were already increased enough to form foam cell-rich plaques at the time of first injury, development of cores which contained mainly macrophage foam cells might have depended on the length of the cholesterol-fed period.

It is not clear how fibrous caps were formed by sequential injury in the present study. In sequential injury animal models, the second balloon injury is typically followed by cell proliferation and neointimal formation (28), and these animal models have been used to study the mechanisms of restenosis after percutaneous transluminal angioplasty (26, 27, 29, 30). A disadvantage of sequential injury animal models is the heterogeneous nature of the lesions induced. Our preliminary examination also showed that the second injury with severe damage to aortic lesion by similar severity with inflation and passages of first balloon injury resulted in amplification of hyperplasia and foam cell formation, but disorder of the structure of neointima and failure to form the fibrous cap (data not shown). In contrast, the mild second injuries induced using the three methods described in “Materials and Methods” were equally successful in inducing formation of thin fibrous caps overlying a large core of macrophage foam cells. These results suggest that it is important that the second injury be mild, to induce fibrous cap formation and retain the lipid core induced by the first injury. van Erven et al. suggested that even mild injury produces maximal release of mitogenic factors from vascular cells (31). Additionally, Stadius et al. (32) reported in a study of sequential balloon injury in an experimental angioplasty rabbit model that the second of sequential injuries initiated a cell proliferation response in the arterial wall but did not alter the neointimal area or affects the lumen caliber of the primary injury. The same phenomenon might be apparent in our study. Namely, severe sequential injury resulted in a variety of neointimal structures, because the features of the lesion destroyed by the second severe injury might be complicated. On the other hand, the second mild injury, which might have injured only the surface of the lesion formed by the first injury, was successful in inducing formation of thin fibrous caps composed of HHF-35-negative synthetic smooth muscle cells and poor collagen fibrils. Second mild injury on the surface of the lipid plaque induced by first injury might thus result in fibrous cap formation.
Macrophage-rich areas are frequently found in coronary plaques of patients with acute coronary syndromes (9). Macrophages are the principal inflammatory cells in atherosclerotic plaques. It appears that macrophages are capable of releasing lytic enzymes that may be responsible for the weakening of the fibrous cap and subsequent rupture of atherosclerotic plaques (10). Various types of MMPs have been detected in atherosclerotic plaques in humans and rabbits (33, 34). Aikawa et al. (35) demonstrated constitutive expression of MMPs by macrophage foam cells within atheroma in hypercholesterolemic rabbits. In the present study, latent MMP2 (pro-MMP2) and activated MMP2 were observed in homogenates prepared from atherosclerotic lesions of rabbit carotid arteries using gelatinolytic zymography. On the other hand, only pro MMP2 was detected in normal arteries. We also detected activated MMP2 and MMP9 in the conditioning medium from the tissue culture of the atherosclerotic artery using the same model as tested in the present study (data not shown). Since homogenates include many proteins, MMPs other than MMP2 might have been masked by other proteins in the present study. The total quantities of gelatinolytic activity were measured in the homogenate prepared from the atherosclerotic lesions, and the activities in the 4-week interval group were stronger than those in the 2-week interval group. On the other hand, gelatinolytic activities were not detected in the homogenate prepared from normal arteries. The increase in MMP activities with prolongation of the cholesterol diet feeding period was accompanied by an increase in neointimal area and particularly by an increase in the area occupied by macrophage foam cells.

These changes were accompanied by an increase in plaque with a thin fibrous cap overlying a large core of macrophage foam cells as well as a decrease in plaque with a solid cap of smooth muscle cells. Libby et al. (36) also suggested that macrophage–related proteolysis within atheroma may contribute to weakness of the protective fibrous cap of plaque. It is suggested that in the present study the MMPs produced by macrophages might have contributed to formation of the thin fibrous cap of type 1 atherosclerotic plaques.

It appears that the atherosclerotic plaque in hypercholesterolemic rabbits induced by cholesterol feeding with twice-injured carotid artery has some characteristics similar to those of vulnerable plaques, and is therefore useful for basic study of plaque stabilization and prevention of acute coronary syndromes. We expect that the typical atherosclerotic plaque found in present study will be useful for the basic study of plaque stabilization and prevention of acute coronary syndromes.

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