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 Ltd., Sydney, Australia).
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. Sections were sliced serially at 5 µm thick, and were stained with elastic van Gieson, Azan, hematoxylin and eosin (HE), Martius scarlet-blue, and Fraser-Lendrum methods. Sections also underwent immunohistochemical evaluation with monoclonal antibodies to RAM-11 (Dako A/S, Glostrup, Denmark) specific for rabbit macrophages, 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.


