Papain-Hydrolyzed Pork Meat Reduces Serum Cholesterol Level and Premature Atherosclerosis in Dietary-Induced Hypercholesterolemic Rabbits

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Summary The effects of the low-molecular-weight fraction of papain-hydrolyzed pork meat (LMF) on the plasma cholesterol level and the generation of atherosclerosis were studied in rabbits fed a cholesterol-enriched diet. In LMF-fed rabbits, the plasma and liver cholesterol concentrations were both significantly lower (p<0.01) than in rabbits fed untreated pork meat (PM). Similarly, the cholesterol concentrations of the chylomicron and VLDL fractions were significantly lower in LMF-fed rabbits than in rabbits fed PM. Deposition of lipid in transverse sections of the aortic arch was significantly less in rabbits fed LMF than in those fed PM. Electron microscopic studies revealed preventive effects against premature atherosclerotic lesions in the aorta of rabbits fed LMF. These results indicate that LMF has a hypocholesterolemic action and preventive effects against premature atherosclerosis.

Key Words New Zealand white rabbits, papain-hydrolyzed pork meat, plasma cholesterol, liver cholesterol, premature atherosclerosis

Many epidemiological, biochemical, and clinical studies have demonstrated that hypercholesterolemia is one of the most important risk factors for atherosclerosis, which is responsible for coronary heart disease. It is important to improve dietary conditions and/or to develop new dietary materials if we are to help prevent the high level of plasma cholesterol seen in many persons among the general population, then to keep it within the desirable range. Dietary proteins and fat, are important factors in determining the plasma cholesterol level. In animals and humans, soy protein has been shown to be more effective than casein at reducing the serum cholesterol level (1–5). After Sugano et al. (5, 6) had found that a protease hydrolyzate of isolated soy protein had a more marked hypocholesterolemic effect than undigested soy protein, Morimatsu et al. (7, 8) reported that a papain hydrolyzate of pork meat produced a greater suppression of the plasma cholesterol level than soy protein did in rats with dietary-induced hypercholesterolemia. The rat, however, is less sensitive to a cholesterol-rich diet than the rabbit is, and it is difficult to induce a distinct atherosclerosis in the former species (9, 10). Consequently, in the present study we chose the rabbit for our investigation of the effects of a papain hydrolyzate of pork meat on the plasma and liver cholesterol levels and the formation of atherosclerotic lesions in animals fed a cholesterol-rich diet for 4 wk.

MATERIALS AND METHODS

Preparation of papain-hydrolyzed pork meat. Pork meat protein was prepared by cutting swine dorsal longissimus muscles into pieces, defatting them with hexane, and lyophilizing. This freeze-dried pork meat (PM) was used as an experimental source of protein for the control group. After adding water to adjust the protein concentration to 5%, PM was hydrolyzed by using papain (Wako Pure Chemical Industries Ltd., Osaka, Japan) by the method of Morimatsu et al. (8). The papain-hydrolyzate thus obtained was passed through a gauze filter and centrifuged at 5,000×g for 20 min. The filtrate was then subjected to ultrafiltration through a membrane that separated molecular weights below and above 150,000 (Carbosep, Sumitomo Heavy Industries Ltd., Tokyo, Japan). The permeable solution (filtrate) was freeze-dried as a low-molecular-weight fraction (LMF). The molecular weight of LMF is below 10,000, and its amino acid composition is almost the same as that of PM (8).

Animals, diet, and experimental design. The experi-
ments were carried out on 3-mo-old male New Zealand white rabbits (Kitayama Laves Inc., Kyoto, Japan). All procedures were carried out in accordance with the appropriate committee in line with the Guide for the Care and Use of Laboratory Animals. After the rabbits had been fed a commercial diet (RC-4, Oriental Yeast Co. Ltd.) with 2% PM and 0.5% cholesterol added for 3 d, they were divided into 3 groups. The division was made on the basis of the animals' serum cholesterol levels (PM group, 9.37±0.68 mm; LMF group, 9.54±0.71 mm; NC group, 8.29±1.89 mm). The PM group subsequently received 2% PM and 0.5% cholesterol added to the commercial diet (n=6), and the LMF group received 2% LMF and 0.5% cholesterol added to the commercial diet (n=6). The NC group (as the histomorphological control) received the commercial diet in an unmodified form (n=4). The rabbits were housed individually in wire cages in an air-conditioned room (22–25°C; lighting on from 8:00 to 20:00). The rabbits were given experimental diets at 120 g/d/animal for 4 wk, and their blood was sampled every week from the central auricular vein after overnight fasting. For the final experiment, after overnight fasting the rabbits were anesthetized with pentobarbital sodium (30 mg/kg body weight, i.v.), then sacrificed by bleeding from the femoral vein. The liver was excised immediately for the determination of its lipid content. The aorta was excised from the origin of the ascending aorta to the bifurcation of the common iliac artery for pathological observations.

**Lipid analysis.** Plasma total cholesterol and triacylglycerol were analyzed by enzymatic assay, using kits purchased from Wako Pure Chemical Industries Ltd. Liver lipids were extracted by the methods of Folch et al. (11) and cholesterol concentration was measured (12).

Plasma lipoproteins were separated sequentially (13) by ultracentrifugation (TL-100: Beckman Instruments Inc., California, USA). The plasma density was adjusted to 1.006 g/mL with NaCl and NaBr, and the plasma was then centrifuged for 20 min at 20,000 rpm in a TLA-100.2 rotor. The chylomicrons fraction was removed, and the density of the plasma was readjusted to 1.006 g/mL with NaCl and NaBr and centrifuged in a TLA-100.2 rotor at 100,000 rpm for 4 h 10 min. The very-low-density lipoproteins (VLDL: d<1.006) fraction was removed. For the low-density lipoproteins (LDL: 1.006<d<1.063) and high-density lipoproteins (HDL: 1.063<d<1.210), the plasma density was adjusted with NaBr to 1.063 and 1.210, respectively. It was then centrifuged as mentioned for VLDL. The cholesterol concentration of each lipoprotein fraction was measured by an enzymatic colorimetric method similar to that used for the plasma cholesterol assay.

**Determination of the lipid deposition area in photomicrographs of histological sections.** Representative aortic segments were sampled from the aortic arch around the orifice of the intercostal arteries and from the abdominal aorta around the orifice of the mesenteric artery, where early atherosclerotic lesions are selectively generated. The segments were fixed in a 10% buffer-formalin solution, embedded in paraffin, and stained with Sudan III. Images of the deposited lipid, colored orange, in the histological sections which was fed into an image-analyzing system (LUZEX IID: Nireco Corporation, Japan) through a microscope (Nikon Eclipse E-600; Nikon Corporation, Japan) and a CCD-video camera operated by a camera control unit. The images were converted to sliced video images before processing by the main processor. The images of the lipid deposits were discriminated in outline by the intensity, hue, and purity of their color, then extracted. The area of the lipid deposit and the total sectional area were binarized, and the intensity and tint were adjusted with respect to the background, the lipid-deposition area being red, the rest of the area light green. The area of the lipid deposit and the total area examined were measured with the aid of the main processor. The lipid deposition area was then expressed as a percentage of the total area examined in each histological section.

**Scanning electron microscope preparations.** Representative pieces of the aortic segments about 2×2 mm square were sampled from the aortic arch and a distal region at the orifice of the mesenteric artery. The samples were fixed in 3% glutaraldehyde with 0.1 M cacodylate buffer for 1 h at 4°C, rinsed several times with the same buffer solution, postfixed in 1% osmium tetroxide for 30 min, then washed with distilled water. Initially they were dehydrated through a graded ethanol series (70–99%) and isoamyl acetate, then carefully critical point dried. They were mounted on metal stubs, sputter-coated with silver, and examined under a scanning electron microscope (S-4000; Hitachi Inc., Japan) at 20 kV.

**Transmission electron microscope preparations.** Representative samples of the aortic wall were taken from the aortic arch and a distal aortic region just around the orifice of the mesenteric artery. The specimens were fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer (pH=7.2) for 2 h at 4°C, rinsed several times with 0.1 M cacodylate buffer, then postfixed with 2% osmium tetroxide for 2 h at 4°C. They were washed with 0.1% sodium acetate, stained on block with 2% uranyl acetate, washed again with 0.1% sodium acetate, dehydrated through a graded ethanol series (50 to 100%), and embedded in Spurr’s low-viscosity resin as the final step. After the embedded specimens had been sectioned at approximately 0.5 μm thick and stained with toluidine blue solution, areas selected for observation were further sectioned at 0.1 μm thick by using an ultramicrotome, then stained with 3% uranyl acetate in 30% ethanol followed by treatment with Raynold’s lead citrate. The ultrathin sections were examined under a transmission electron microscope (Phillips CM 120) operating at 20 kV.

**Statistical analysis.** Values are given as mean±SE. Data were analyzed by an analysis of variance (ANOVA) with Scheffe’s multiple comparison test.

**RESULTS**

In the three groups, there were no changes in body
weight during the experimental period for the mild restricted feeding. In the PM group, liver weight was slightly greater, but there is no significant difference compared with the LMF group (PM: 34.0±1.6 g, LMF: 32.6±2.7 g). Table 1 shows the plasma cholesterol and triacylglycerol levels in the experimental animals. After 4 wk of feeding on various diets, the total cholesterol concentration in rabbits fed LMF was about 10 times higher than in the NC group, but it was significantly lower than in rabbits fed PM over the same period. In contrast, little difference was noted in plasma triacylglycerol concentration among these three groups. The cholesterol concentration in each plasma lipoprotein fraction at the end of the 4 wk experimental period is shown in Table 2. The cholesterol concentration in chylomicrons and VLDL was significantly lower in rabbits fed LMF than in those fed PM. The NC group showed normal to low levels of cholesterol in each lipoprotein fraction. Table 3 shows the lipid concentrations in the livers of the experimental animals. The increased levels of cholesterol and triacylglycerol because of feeding on a cholesterol-enriched diet were significantly suppressed in the LMF group by comparison with the PM group. No significant differences were noted in phospholipid content among the three groups. Table 4 compares the lipid deposits in circumferential histological sections of the aortic arch and the abdominal aorta taken from just above the orifice of the mesenteric artery. The lipid deposition area in the aortic arch as a percentage of the total area examined was 0.57% in the LMF group and 5.28% in the PM group, these values indicating a significant suppression of lipid deposition in this region by the inclusion of LMF in the diet (p<0.01). The area of lipid deposition in the section just above the orifice of the mesenteric artery could not be compared between the PM and LMF groups because the deposition was very slight in this region in both groups.

Electron micrographs of the intima in the aortic wall facing, but just above, the orifice of the mesenteric artery in the PM, LMF, and NC groups are illustrated in Fig. 1. In the NC group, the intimal surface is intact, and the endothelial cells are regularly arranged along the direction of blood flow. In the PM group, the endothelial cells are rounded, irregular, and partially degenerated, and the intercellular junctions are dilated. It is interesting that some monocytes are adhered to the injured endothelial cells (Fig. 2A). Many platelets are aggregated and adhered to the surface of the endothelial cells. Monocytes are considered to migrate to the subendothelial space to become macrophages and to accumulate cholesterol. Fibrin fibers clung to monocytes, platelets, and erythrocytes. In the LMF group, the endothelial cells are well
Fig. 1. Scanning electron micrographs of the intima in the aortic wall facing, surrounding the orifice of the mesenteric artery. ×2,000. NC group: the endothelial cells are intact. PM group: the endothelial cells are rounded, irregularly arranged, and partly degenerated. Cell debris is attached to the injured portion. Intercellular junctions are dilated. LMF group: the regularity of the endothelial cells is better preserved than in the PM group. Intercellular junctions are partly dilated.

Fig. 2. Scanning electron micrographs of the monocytes and other corpuscles adhered to the endothelium of the aortic wall facing, surrounding the orifice of the mesenteric artery in the PM (A) and LMF (B) groups. ×2,000. In the PM group, some monocytes are adhered to the injured endothelial cells. Many platelets have adhered and aggregated, and some erythrocytes are on the surface of the endothelial cells. The platelet contacts with the monocyte. Fibrin fibers clung to these corpuscles. In the LMF group, only a few small monocytes and some cell debris are observed on the endothelial surface.

preserved in their regularity by comparison with those in the PM group, although the intercellular junctions are slightly dilated and partly unclear. A few small-sized monocytes and some cell debris are found adhered to the endothelial surface, which is only slightly injured (Fig. 2B). The adhesion and aggregation of platelets and interaction between monocytes and platelets are little observed. Similar changes were in the intima at the aortic arch in these groups. Figure 3 shows photomicrographs of thick sections of that part of the aortic wall facing, surrounding the orifice of the mesenteric artery. The intima consists of a single layer of endothelial cells, and the media is composed of multilayered smooth muscle cells bordering the elastic lamina in the PM, LMF, and NC groups. A slight thickening of the intima is observed in the PM group. The NC and LMF groups show no histopathological changes.

Figure 4 shows transmission electron micrographs of the intima and media in aortic regions similar to those shown in Fig. 3. In the NC group, the intima and media are both intact. In the PM group, the endothelial cell is partly injured and isolated. Macrophage laden abundant of cholesterol is observed (Fig. 5). Lipid deposition is advanced in the media, which crashes the nucleus, degenerates the smooth muscle cells, and finally causes necrosis in serious cases. In the LMF group, lipids are not deposited in the smooth muscle, only in the intima. Smaller numbers of foam cells were observed in com-
Fig. 3. Photomicrographs of thick sections from the wall of the aorta facing, surrounding the orifice of the mesenteric artery. Toluidine blue. ×200. The intima is slightly thickened in the PM group. Noteworthy histopathological changes are not observed in the LMF or NC groups.

Fig. 4. Transmission electron micrographs of the intima and media from the aortic wall facing, surrounding the orifice of the mesenteric artery. ×5,000. EC: endothelial cell; SMC: smooth muscle cell. NC group: the endothelial and smooth muscle cells are both intact. PM group: the deposition of lipid is advanced in smooth muscle cells. LMF group: the deposition of lipid is restricted to the intima. The smooth muscle cells are less injured than in the PM group.

Comparison with those in the PM group. Injury to the smooth muscle cell is much less severe in the LMF group than in the PM group.

**DISCUSSION**

This study demonstrates that in rabbits fed a cholesterol-enriched diet, the inclusion of LMF in the diet has a suppressing effect on plasma and liver cholesterol concentrations in comparison with the levels seen in rabbits with PM included in the diet. Similar effects on the plasma cholesterol and liver cholesterol levels in rats fed a cholesterol-enriched diet were reported by Morimatsu et al. (8). They also noted an increase in the fecal excretion of neutral and acid steroids and proposed that the major hypocholesterolemic action of LMF is exerted via intradigestive tract effects. Furthermore, they suggested the possibility that the indigestibility of LMF may lead to an increased degradation of cholesterol. Although we cannot be sure, the effects of LMF on the fecal excretion of steroids was not examined in the present study; the hypocholesterolemic effect of LMF seen here may have partly resulted from a suppression of cholesterol absorption and/or from an acceleration of the destructive metabolism of cholesterol. Nevertheless, in our histological study we observed that LMF has a definite repressive effect on the deposition of lipid in the aorta and a preventive effect on premature atherosclerosis. We also observed a decreased concentration of cholesterol in the chylomicron and VLDL fractions in the LMF group of cholesterol-fed
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rabbis by comparison with the PM group, even after fasting for over 16 h. It has been reported that the increased total plasma cholesterol level seen in cholesterol-fed rabbits is for the most part attributable to an increase in chylomicron remnant and/or β-VLDL cholesterol, which does not exhibit typical VLDL or LDL patterns (14-16). Chylomicron remnants, including β-VLDL, are taken up by the liver partly through the LDL receptor as well as by the ordinary pathway through the chylomicron remnant receptor (17). The removal of these lipoproteins from the plasma has been shown to be impaired in cholesterol-fed rabbits (18-20), an effect that is responsible for the accumulation of cholesterol and triacylglycerol in the liver and for their increase in the plasma VLDL. This would contribute to a delay in the catabolism of VLDL to LDL. This hyperlipidemic pattern shows a similarity to human type III hypercholesterolemia (21), a condition that contributes to the generation and development of atherosclerosis (14-16, 21-23). In this study, the increased lipoproteins in the fraction of density less than 1.006 is thought to represent mainly cholesterol-rich chylomicron remnants or β-VLDL instead of triacylglycerol-rich chylomicrons or VLDL. In rabbits of the LMF group, the cholesterol concentration in the lipoprotein fraction of <1.006, which is implicated in the generation and development of atherosclerosis, was about half that found in the rabbits of the PM group. Despite the markedly higher plasma cholesterol level in the LMF group than in the NC group, the histological appearance of the aorta in the LMF group fed a cholesterol-enriched diet was much the same as in the NC group. Thus LMF seems partly to suppress the increase in chylomicron remnants and/or β-VLDL induced by cholesterol feeding, resulting in a prevention first of cholesterol deposition in the intima, and second of the generation of atherosclerosis. It is suggested that the functional mechanism by which LMF prevents the generation of atherosclerosis is related not only to a suppression of dietary cholesterol absorption, but also to an effect on lipoprotein metabolism. Atherosclerotic lesion is generally generated in the ascending aorta and around the orifice of the branch arteries, such as the brachiocephic, intercostal, celiac, mesenteric, and renal arteries, and progresses toward the peripheral aortic region in heritable hyperlipidemic (24, 25) and cholesterol-fed rabbits (26). Atherosclerotic lesion is generally considered to be primarily initiated in endothelial injury or activation because of various risk factors, e.g., hypercholesterolemia, hypertension, oxidative stress, diabetics, and smoking (27). In the primary stage of atherogenesis, monocytes adhesion to endothelium has been demonstrated to be followed by an expression of adhesion mononuclear leukocyte adhesion molecules in hypercholesterolemic rabbits (27-29). Truskey et al. (30) reported that an expression of vascular cell adhesion molecules were increased at lesion-prone sites in correlation with the integration of plasma cholesterol in cholesterol-fed rabbits, and that macrophage densities at these sites were also positively correlated with integrated plasma cholesterol. In the secondary stage, a proliferation of macrophages (31, 32), peroxidation of LDL or β-VLDL, and intake of it by macrophages (33-35) play important roles in the formation of fatty streaks in the subendothelial space. A decrease in serum cholesterol level in hyperlipidemic patients by an improvement of dietary conditions greatly contributes to a decrease in atherosclerotic disease, such as coronary heart disease (36-38). A lowering of serum lipid level by diet is also demonstrated to progressively reduce macrophage content in atheromatous plaque in rabbits (39). In the present study, the adhesion of the monocyte and platelet to the endothelium cell was decreased by the diet, including LMF. On the other hand, a phenotype transition of the smooth muscle cell (SMC) from contractile to synthetic state is also an early event of atherogenesis (27, 40, 41). Cytokines produced by endothelial cell, platelet, and macrophage play a major role in the phenotype modulation of SMC (40, 41). Platelet-derived growth factor (PDGF), interleukin-1, transforming growth factor-β, angiotensin II, and so forth are known to promote the phenotype transition of SMC, whereas nitric oxide (NO) is revealed to have relaxing and protecting actions on SMC. The synthetic SMC produces cytokines, proliferates, migrates from the media to the intima, and intake cholesterol like a macrophage does. Macrophages modulate the phenotype of SMC, stimulate the proliferation of SMC, and enhance the binding of β-VLDL and cholesterol ester accumulation in SMC (42, 43).

In the present study, SMC was relatively well preserved in the LMF-fed group in comparison with that in PM-fed groups. Although the detailed mechanism of the prevention of atherosclerosis because of LMF should be clarified in future studies, LMF might exert on suppressing actions in the early stages of atherogenesis.
mainly associated with monocyte/macrophage functions in cholesterol-fed rabbits. Thus LMF can be expected to be one of the antihypercholesterolemic and antiatherosclerotic materials derived from animal protein.

The effects exerted by LMF on the other mechanisms involved in atherogenesis (e.g., oxidation of LDL, monocyte/macrophage functions, platelet adhesion and aggregation, and the cytokine network) cannot be deduced from the present study. These mechanisms should be investigated in future studies.

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