Comparison between Dietary Soybean Protein and Casein of the Inhibiting Effect on Atherogenesis in the Thoracic Aorta of Hypercholesterolemic (ExHC) Rats Treated with Experimental Hypervitamin D

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Received September 24, 1996

Atherosclerotic lesions of the thoracic aorta were induced in exogenously hypercholesterolemic (ExHC) rats by treating initially with hypervitamin D₃ and subsequently feeding on hypercholesterolemic diets for 180 days. Dietary soybean protein, in comparison with casein, substantially decreased the degree of atherosclerotic lesions, which was evaluated by intimal thickening, although with a similar topographical distribution. The casein-fed rats tended to maintain a high concentration of serum cholesterol, particularly in triacylglycerol-rich lipoproteins. The concentrations of apo A-I and TBARS in the serum was comparable between the dietary protein groups. The data suggest that dietary soybean protein, compared to casein, produced lipoproteins which were less atherosclerotic by partitioning cholesterol in the triacylglycerol-poor lipoproteins.

Key words: exogenous hypercholesterolemia; cholesterol; atherosclerosis; soybean protein; casein; hypervitaminosis D₃

It is well established that feeding rabbits on a diet containing soybean protein, in comparison with casein, leads to insufficient development of lipid-containing and foam cell-rich atherosclerotic lesions.¹ Diet casein has been repeatedly shown to induce hypercholesterolemia in rabbits fed on cholesterol-containing or -free diets.² Hence, it appears that the concentration of serum cholesterol in rabbits was related to the severity of atherosclerotic lesions. However, several reports have shown that the concentration of serum cholesterol was not always proportional to the severity of lesion in rabbits.³ Alternatively, arterial events such as the metabolic change in the wall components and the interaction of the wall with serum components may be responsible for the differing response of arterial lesions to hypercholesterolemic serum in soybean protein-fed animals.³

Rats also exhibited a lower concentration of serum cholesterol after being fed with soybean protein-containing diets than with casein-containing diets.⁴,⁵ In contrast to rabbits, hypercholesterolemic rats do not generally develop severe artherosclerotic lesions, possibly due to an increased concentration of serum HDL and an arterial wall characteristic that is resistant to the deposition of serum lipids.⁶ Therefore, it is not yet known whether dietary soybean protein is also beneficial for preventing atherosclerotic lesions in rats. If the beneficial effects of dietary soybean protein on the atherosclerotic lesions found in rabbits can be reproduced in rats, this would provide further insight into the role of dietary proteins in human atherogenesis.⁷

ExHC rats, initially isolated from the Sprague-Dawley rat strain by Imai et al.,⁷ are hyper-responsive to exogenous cholesterol without using propylthiouracil, an antithyroid drug which is often used for elevating serum cholesterol in this species.⁸ When ExHC rats were fed on a high-cholesterol diet containing 2% cholesterol, 0.4% sodium cholate and 10% olive oil for 16 weeks,⁹ their aortas had Sudan-Stained deposits and cholesterol accumulation, but developed only weak fatty deposits on the surface of the intima without any accompanying intimal thickening and connective tissue proliferation typical of atherosclerotic lesions.

Bajwa et al. have demonstrated the induction of aortic atherosclerosis in rats fed on a hypervitamin D₃ and cholesterol-containing diets.¹⁰ This animal model has been successfully used for examining a drug’s antiatherogenic effect.¹¹,¹² In the present experiment, we show that dietary soybean protein, compared to casein, prevented lesions from forming in the thoracic aorta of hypercholesterolemic ExHC rats that had been treated with hypervitamin D₃.

Materials and Methods

Animals and diets. Two male ExHC rats (F55-1/2) and six female ExHC rats (F55-2/3/4/5/6) which were originally donated by Takeda Chemical Industries (Osaka, Japan) in 1987, were bred and maintained at Laboratory of Animal Experiments of Kyushu University School of Medicine (Fukuoka, Japan).¹² The rats were maintained in a temperature-controlled room at 22–25°C with a 12-h light/dark cycle and given free access to a nonpurified commercial diet (NMF: Oriental Yeast Co., Tokyo, Japan) and nonionic water until reaching 14 wk old.

The rats were treated with hypervitamin D₃ at 200,000 IU/kg body wt for 4 days according to the schedule originally described by Bajwa et al.¹⁰ The dose of vitamin D₃ was determined by preliminary experiment, because the doses of 350,000 and 250,000 IU/kg body wt, which were originally used on male Long-Evans rats by Bajwa et al.¹⁰ and on male Wistar rats

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Abbreviations: ExHC, exogenously hypercholesterolemic; TBARS, thiobarbituric acid reactive substances.

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by Tsutsumi et al., but were fatal to ExHC rats. Normal rats received olive oil alone through a stomach tube. The vitamin D₃-treated rats were subsequently, maintained on a purified diet containing cholesterol, and the normal rats, on the nonpurified diet for 180 days.

The purified diet was formulated according to the AIN76 formula (in weight %) as 10 olive oil (Nacalai Tesque, Kyoto, Japan), 20 casein (Wako Pure Chemicals, Osaka, Japan) or isolated soybean protein (Fuji Oil, Osaka, Japan), 15 corn starch (Mitsubishi Shoji, Tokyo, Japan), 5 cellulose (Nihon Noso Kogyo, Tokyo, Japan), 3.5 mineral mixture (AIN76 from Nihon Noso Kogyo), 1 vitamin mixture (AIN76 from Nihon Noso Kogyo), 0.3 dl-methionine (Nacalai Tesque), 0.2 choline bitartrate (Wako Pure Chemicals), and sucrose (Dainippon Seitou, Tokyo, Japan) to 100. To this diet were supplemented 1% cholesterol (Nacalai Tesque) and 0.25% sodium cholate (Nacalai Tesque). All aspects of the experiment were approved by Kyushu University Animal Policy and Welfare Committee.

Tissue preparation for histologically determining atherosclerosis. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital, and sacrificed by withdrawing blood from the heart. Each aorta was perfused with 50 ml of physiological saline via a needle inserted into the right ventricle, unrestricted efflux being provided from an incision in the vena cava. Perfusion was continued with 50 ml of a 10% neutral formalin buffer solution at pH 7.4 (Wako Pure Chemicals). The heart and entire aorta with its main branches were dissected from each animal as a unit, the bulk of the fat and tissue adhering to the adventitia being dissected from the aorta as much as possible in situ. The aorta was sectioned from the aortic valve to the ascending aorta parallel to a line drawn from the aortic root, and then the thoracic aorta was divided into seven sections as shown in Fig. 1. The tissues were preserved in a 20% neutral formalin buffer solution at pH 7.4 (Wako Pure Chemicals), before paraffin embedding in the routine manner to prepare 4 µm-thick serial sections. The cut sections were stained with hematoxylin and eosin, Von Kossa stain and Van Gieson elastic fiber stain. Intimal thickness was measured on the Von Kossa- and hematoxylin and eosin-stained slides by using a Nikon microscope fitted with a video camera, X 10 objective, digitizing pad and PC-9821X2 with a Nexus Qube image analysis processor (Nexus, Tokyo, Japan). The thickness of 20-40 consecutive cross sections from each of the 7 parts was measured, and the mean of 6 sections with the greatest intimal height in each part was determined.

Analytical methods. Blood samples obtained from the tail vein at 30, 60, 90, 120, and 150 days were subjected to a determination of cholesterol. Serum lipids were determined by commercially available kits (Cholesterol C Test, Triglyceride G Test, and Phospholipid B Test, each product from Wako Pure Chemicals). Serum thiobarbituric acid reactive substances (TBARS) were measured by a commercially available kit (LPO-Test, Wako Pure Chemicals). Serum apolipoprotein (apo) A-I was measured by rocket immunoelectrophoresis as described elsewhere. The concentration of apo A-I in the serum was determined by comparing its rocket heights and is expressed as a relative percentage to normal groups.

Statistical analyses. Data were analyzed by Student's t-test and Duncan's new multiple-range test, differences being considered significant at p<0.05.

Results

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The growth of the rats fed on casein was no different from that when fed on soybean protein (the final body weights of the casein- and soy protein-fed rats were 541 ± 25 g and 531 ± 33 g, respectively). As shown in Fig. 2, in accordance with the results of Imai et al., the high-cholesterol diet resulted in a concentration of serum cholesterol in the ExHC rats greater than 1,000 mg/dl during the feeding period. The serum cholesterol levels at 90 and 150 days tended to be lower in the rats fed on soybean protein than in those fed on casein, cholesterol concentration at 180 days was significantly higher in the casein-fed rats than in the soybean protein-fed rats (Table). The Table also shows the serum lipid, apo A-I and TBARS levels at 180 days. The concentration of triacylglycerols and phospholipid was higher in the casein-fed rats than in the soybean protein-fed rats. The ratio of triacylglycerols to cholesterol was significantly higher in the casein-fed rats than in the soybean protein-fed rats, suggesting that the former animals predominantly carried the cholesterol in triacylglycerol-rich lipoproteins, and the latter animals, in triacylglycerol-poor lipoproteins. The concentration of serum apo A-I, measured as an index for evaluating the level of serum HDL, was lower in the soybean protein-fed

![Fig. 1. Line Graphs Showing the Arteries in ExHC Rats.](image_url)

Sites subjected to microscopic analyses are indicated by the numbers.
rats than in normal rats. However, no significant difference was observed in the apo A-I level between the soybean protein-fed and casein-fed rats, suggesting no significant difference in the concentration of HDL-cholesterol between either dietary protein group. The concentration of TBARS was higher in the cholesterol-fed rats than in normal rats, but the dietary proteins did not influence this concentration.

Histological findings

In accordance with observations by Bajwa et al.,\textsuperscript{10} the rats fed on the hypercholesterolemic diets with vitamin D\textsubscript{2} exhibited a number of pathological changes: intimal thickening and calcification, degeneration, metachromasia, lysis and fragmentation of the elastic lamina in the media as shown in Fig. 3. These changes occurred principally in the ascending thoracic aorta and in the arch of the rats fed on both protein diets. Intimal thickening was exclusively marked in the areas of calcification, but calcification was rare in the intima. Mesenchymal cell proliferation was also observed in the area of thickened intima. As shown in Figs. 4 and 5, the extent of intimal thickening was markedly greater in all sections of the casein-fed rats than of the soybean protein-fed rats. In addition, judging from the microscopic observations, the lesion area was spread more in the casein-fed rats than in the soybean protein-fed rats. However, the topological distribution of calcification in the media and intimal thickening was apparently similar between the rats fed on casein and soybean protein. In the sections from 4 to 7, intimal thickening in the soybean protein-fed was statistically similar to that in normal rats.

Table  Concentrations of Serum Lipids, Apo A-I and TBARS in Casein-fed, Soybean Protein-fed, and Normal Rats

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Casein</th>
<th>Soybean protein</th>
<th>Normal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triacylglycerols (mg/dl)</td>
<td>1010 ± 180\textsuperscript{a}</td>
<td>472 ± 61\textsuperscript{c}</td>
<td>68.8 ± 18.1\textsuperscript{a}</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>1450 ± 150\textsuperscript{a}</td>
<td>1060 ± 90\textsuperscript{b}</td>
<td>89.1 ± 6.2\textsuperscript{c}</td>
</tr>
<tr>
<td>Phospholipids (mg/dl)</td>
<td>1550 ± 200\textsuperscript{a}</td>
<td>1010 ± 110\textsuperscript{b}</td>
<td>124 ± 10\textsuperscript{c}</td>
</tr>
<tr>
<td>Triacylglycerols/cholesterol</td>
<td>0.678 ± 0.058\textsuperscript{a}</td>
<td>0.440 ± 0.029\textsuperscript{b}</td>
<td>0.768 ± 0.064\textsuperscript{a}</td>
</tr>
<tr>
<td>Apo A-I (relative percentage to control)</td>
<td>93.5 ± 6.2\textsuperscript{b}</td>
<td>70.1 ± 5.6\textsuperscript{a}</td>
<td>110 ± 12\textsuperscript{b}</td>
</tr>
<tr>
<td>TBARS (nmol/dl)</td>
<td>531 ± 27\textsuperscript{a}</td>
<td>495 ± 47\textsuperscript{b}</td>
<td>306 ± 22\textsuperscript{b}</td>
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</tbody>
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Each values is the mean ± SEM for 7 rats per group.

\textsuperscript{a,b} Different letters show significant difference at \( p < 0.05 \).

Discussion

The present study shows that ExHC rats fed on casein, in comparison to soybean protein, developed more severe atherosclerotic lesions in the thoracic aorta when fed on a high-cholesterol diet after the administration of hyper-vitamin D\textsubscript{2}. The lesions evaluated by intimal thickening were strongest in the aortic root and weakest in the arch and descending aorta. This is the first report showing that a soybean protein diet as effective to prevent atherosclerotic lesions in rats, and this observation is in accordance with previous results from rabbits fed on cholesterol-free or -containing diets.\textsuperscript{11}

Dietary soybean protein, compared to casein, is generally hypocholesterolemic in various animal species including rats.\textsuperscript{5,6} In the present study, with the high-cholesterol diet, the ExHC rats had a serum cholesterol concentration exceeding 1000 mg/dl, although dietary soybean protein tended to lower this concentration. The susceptibility of ExHC rats to dietary cholesterol might have obscured the hypocholesterolemic effect during this period of dietary soybean protein feeding. Serum triacylglycerols are mainly carried in the fractions with a density of less than 1.006 g/ml (very-low-density lipoproteins) while cholesterol is predominantly carried in the fractions with a density greater than 1.063 g/ml (low- and high-density lipoproteins); thus the increased ratio of triacylglycerols to cholesterol means an increased mass of cholesterol in the lower-density lipoprotein fractions when the concentration of serum

**Fig. 2.** Changes in the Serum Cholesterol Concentration in Rats Fed on Casein and Soybean Protein

Each point and bar shows the mean ± SEM for 7 rats per group.
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cholesterol was not markedly different between the two groups. The dietary protein effect was significant only at 180 days. Hence, the serum cholesterol concentration per se was not the major cause for the differing response of arterial lesions to dietary proteins.

The results of the present study suggest that dietary protein altered the distribution of serum cholesterol in the lipoprotein fractions, because the ratio of serum triacylglycerols to cholesterol was higher in the rats fed on casein than in those fed on soybean protein.\(^{13}\) This finding may be relevant to explain the different responses of arterial lesions to hypercholesterolemia, because the cholesterol distribution in different lipoprotein classes is involved in the severity of atherosclerotic lesions in hypercholesterolemic animals.\(^{17}\) In fact, Paley et al. have demonstrated that a different partitioning of cholesterol into the lipoprotein fractions influenced the lesion area, volume and thickness, but not the topographic distribution, in rabbits, although the animals exhibited a comparable concentration of serum cholesterol.\(^{2}\) Furthermore, \(\beta\)-VLDL and VLDL remnants are directly recognizable by intimal macrophages,\(^{18,19}\) although LDL must be modified before uptake by these cells.\(^{20}\) Thus, relatively triacylglycerol-poor lipoproteins in the rats fed on soybean protein may result in reducing the potential for serum cholesterol that leads to severe atherosclerotic lesion formation. Conversely, development of the severe atherosclerotic lesions in the casein-fed rats may be related to the preferential distribution of cholesterol into such triacylglycerol-rich lipoproteins as \(\beta\)-VLDL and IDL.\(^{21}\) The serum HDL concentration appeared not to be related to the different development of arterial lesions, as the concentration of serum apo A-I, one of the major protein components of serum HDL, was not significantly different between the soybean protein-fed and casein-fed rats. It is also unlikely that dietary proteins differently affected the oxidative degeneration of serum lipoproteins\(^{22}\) as the concentration of TBARS, one of the markers of lipoperoxidation, was comparable between the rats fed on either dietary protein.

In accordance with the experiments on rats treated with hypervitamin D\(_2\),\(^{10}\) intimal thickening exclusively occurred at the site where calcium was deposited, suggesting that calcium deposition in the artery is a prerequisite for the infiltration of serum components into intima under the state of lipemic serum.\(^{23}\) Although the mechanism for calcium-dependent arterial injury is not precisely understood, Ca\(^{2+}\) channel blocker nifedipine is known to be effective in reducing atherosclerotic lesions in rabbits.\(^{24}\) Hence, in addition to the distribution of cholesterol in the serum lipoproteins, it remains a possibility that dietary proteins may directly affect calcium-dependent arterial injury.

Imai et al. have shown that ExHC rats developed hypercholesterolemia to exogenous cholesterol without an antithyroid drug and exhibited fatty streak-like lesion in the abdominal and descending aorta.\(^{9}\) They did not however, observe intimal thickening in these lesions, as was observed in the present study with hypervitamin D\(_2\). Hence, the present animal model is useful for evaluating the preventive role of dietary soybean protein and its constituents (isoflavons)\(^3,25\) in atherosclerotic lesion formation.
Fig. 4. Cross Sections in the Ascending Aorta Stained with Van Gieson Elastica Stain.

(a), (b), and (c), arteries from normal rats; (d), (e), and (f), arteries from casein-fed rats; (g), (h), and (i), arteries from soybean protein-fed rats; (a), (d), and (g) correspond to part 1 in Fig. 1; (b), (c), and (h) to part 4; (c), (f), and (i) to part 6 in Fig. 1. The internal elastic lamina is indicated by arrows. Magnification is 300.
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Fig. 5. Intimal Thickness in the Thoracic Aorta of Casein-fed, Soybean Protein-fed, and Normal Rats.
The numbers from 1 to 7 correspond to the parts shown in Fig. 1. Each bar shows the mean ± SEM for 7 rats per group.
different letters show significant difference at p<0.05.

References