Induction of Atherosclerosis in Brown Norway Rats by Immunization with Ovalbumin

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A study was carried out to establish an animal model that would be suitable for evaluating the role of the diet in immune cell-mediated atherogenesis. Brown Norway rats were initially treated with hypervitamin D, for 4 days and then fed on an atherogenic diet for 3 months, during which period the rats were either immunized with ovalbumin plus Al(OH)₃ (OVA group) or with Al(OH)₃ alone (control group) every 3 weeks. Aortic lesions were mainly composed of foam cells, the lesions evaluated by the intimal thickness of the ascending aorta being more severe in the OVA group than in the control group. The OVA group, in comparison with the control group, showed prominently increased serum levels of OVA-specific IgG and rat chymase, an indicator of mast cell degranulation. The intimal thickness was positively correlated with the level of chymase. Immunization had no effect on the serum lipid levels. These results support the hypothesis that mast cells play a role in the early stage of atherosclerosis and suggest that this animal model could be useful for evaluating the role of the diet in immune-related atherogenesis.

Key words: atherosclerosis; Brown Norway rat; immunization; mast cell; rat chymase

Introduction

T lymphocytes and macrophages are present in significant numbers at all stages of the development of human atherosclerotic lesions. In an experimental model of this disease such as the cholesterol-fed rabbit, T lymphocytes and monocytes adhere to the endothelium of the artery within 7 days of commencing consumption of the experimental diet, and by 2 months, a lesion was evident that resembled a fatty streak consisting mainly of macrophages and T lymphocytes. Hence, it has been suggested that there was an association between T cell infiltration and macrophage proliferation. Recent immunohistochemical observations on atherosclerotic lesions in human aorta and coronary arteries have revealed that these lesions contained mast cells. Mast cells, better known for their action in the allergic reactions of skin and mucosal surfaces, are filled with cytoplasmic secretory granules that contain histamine, heparin and neutral proteases, notably chymase. When activated, these cells degranulate, and in this way may influence the lipoprotein metabolism in their immediate environment. Indeed, tissue-culture studies have suggested that exocytosed mast cell granules may carry LDL into peritoneal macrophages, and so induce their conversion into foam cells. Furthermore, recent findings suggest that the chymase-dependent angiotensin-II-forming pathway may be related to the pathogenesis of atherosclerosis, because angiotensin II plays a crucial role in the promotion of vascular tissue remodeling. In fact, chymase from the human and monkey, but not from the rabbit and rat, efficiently generated angiotensin II, and the mRNA level of chymase in the monkey aorta was significantly higher when the monkey had been fed with an atherogenic diet. However, these possible pathophysiological hypothesis cannot be evaluated in vivo in experimental animals. Schwartz et al. have reported a high level of tryptase, another type of mast cell-derived protease, in one patient with acute myocardial infarction.

In order to clarify the combined actions of immunological injury and diet that have just been described, it is preferable to use a small animal such as the rat or mouse, although they are less susceptible to atherosclerosis than the rabbit. We have previously reported experiments in which a combination of hypervitaminosis and an atherogenic diet caused atherosclerosis in rats. In a separate experiment, we also found that repeated immunization of Brown Norway (BN) rats with a food antigen resulted in a serum elevation of chymase, which is one of the most reliable markers for the degranulation of mast cells in vivo. Accordingly, it appeared reasonable to hypothesize that immunological injury-mediated atherosclerosis could be induced in BN rats by the combined action of hypervitaminosis and feeding an atherogenic diet, and by repeatedly injecting a foreign protein. We show here that the level of serum chymase was positively correlated to aortic lesion development in BN rats.

Materials and Methods

Animals and diets. BN rats were obtained from Seac Yoshitomi Co. (Fukuoka, Japan) and maintained in a temperature-controlled room at 22–25°C with a 12-h
light/dark cycle with free access to a nonpurified diet (NMF; Oriental Yeast Co., Tokyo, Japan) and nonionic water until reaching 8 weeks old. The rats were treated with hypervitamin D$_2$ (Nacalai Tesque, Kyoto, Japan) at 200,000 IU/kg of body weight for 4 days as described previously. The vitamin D$_2$-treated rats were subsequently maintained on a purified diet containing cholesterol and cholate (the atherogenic diet) for 3 months. The purified diet was formulated according to the AIN-93G formula (in weight%) as 9.6 coconut oil, 0.4 safflower oil, 20 casein, 13.2 α-corn starch (Nihon Shokuhin Kakou Co., Aichi, Japan), 10 sucrose (Nishinohon Sugar Manufacturing Co., Fukuoka, Japan), 5 cellulose (Nihon Nosan Kogyo Co., Osaka, Japan), 3.5 mineral mixture (Nihon Nosan Kogyo Co., Osaka, Japan), 1 vitamin mixture (Nihon Nosan Kogyo Co., Osaka, Japan), 0.3 l-cystine (Nacalai Tesque, Kyoto, Japan), 0.25 choline bitartrate (Wako Pure Chemicals Co., Osaka, Japan), and corn starch (Nihon Shokuhin Kakou Co., Aichi, Japan) to 100. To this diet were supplemented 1% cholesterol (Nacalai Tesque, Kyoto, Japan) and 0.25% sodium cholate (Nacalai Tesque, Kyoto, Japan). The rats were peritoneally injected with 500 µg/ml of ovalbumin (OVA; Sigma Chemicals Co., St. Louis, MO, U.S.A.) containing 3% Al(OH)$_3$ (Sigma Chemicals Co., St. Louis, MO, U.S.A.) as an adjuvant at 1, 4, 7 and 10 weeks. This amount of OVA resulted in a significant elevation of serum chymase in BN rats. All aspects of the experiment were approved by Kyushu University Animal Policy and Welfare Committee.

**Histological observation.** 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 Co., Kyoto, Japan). 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 tissues were preserved in a 20% neutral formalin buffer solution at pH 7.4 (Wako Pure Chemicals Co., Kyoto, Japan), before embedding in paraffin in the routine manner to prepare cross sections. Two hundred consecutive cross sections of 4 µm in thickness were made from the end of the aortic root to the aortic arch. The cut sections were stained with hematoxilin and orcein. Intimal thickness was measured by using a Nikon microscope and NIH Image software as described. The thickness with the greatest intimal height from each aorta was recorded.

**Analytical methods.** Reaginic antibodies, OVA-specific IgG and rat chymase were measured as described previously. Briefly, the reaginic antibodies were measured by a passive cutaneous anaphylaxis assay on Sprague-Dawley rats. The reaction was estimated by measuring the diameter of the blue spot on the inner surface of the skin. The concentration of the serum IgG antibody specific to OVA was measured by an enzyme-linked immunosorbent assay (ELISA) kit (Moredun Institute, Edinburgh, U.K.). IgG obtained from Cappel (West Chester, PA, U.S.A.) was used as a standard. Goat anti-rat IgG antibodies conjugated with peroxidase were obtained from Zymed (CA, U.S.A.). Serum rat chymase was measured by an ELISA kit (Moredun Institute, Edinburgh, U.K.). Lipoprotein fractionation from the serum was performed by ultracentrifugation as described by Havel et al. The lipoproteins were separated at a density of 1.006 g/ml and 1.063 g/ml. Serum total cholesterol, high-density lipoprotein (HDL)-cholesterol, triglycerides, and phospholipids were measured by using commercially available kits.

**Statistical analysis.** All data were analyzed by Student’s t-test, differences being considered significant at $P<0.05$.

**Results**

There were no significant differences in the growth or food intake of the rats between the control group and OVA group (the mean final body weight of the control group was 283±4 g and of the OVA group was 268±8 g, and the average food intake of the control group was 11.6±0.3 g/day and of the OVA group was 10.8±0.3 g/day).

Figure 1 shows representative lesions of the thoracic aorta in the one third closest to the heart in the OVA group. These lesions mainly comprised diffused intimal thickening with degenerated elastic fiber in the media (Fig. 1A) and multilayered foam cells in the intima accompanied by severely calcified regions (Fig. 1B). In our previous experiments, treating exogenously hypercholesterolemic (ExHc) rats with hypervitamin D$_2$ and feeding them subsequently on an atherogenic diet for 6 months resulted in more severe advanced lesions co-

**Fig. 1. Cross Sections of the Ascending Aorta Prepared from Brown Norway Rats Fed on a High-cholesterol Diet for 3 Months.** The internal elastic lamina is indicated by arrows. Magnification is 300.
covered with fibrous caps. In the present experiment, although the BN rats received hypervitamin D$_2$ and an atherogenic diet, they had no such advanced lesions.

Figure 2 shows the thickness with the greatest intimal height from each aorta. The intimal thickness in the OVA group was significantly higher than that in the control group.

As shown in Fig. 3A, the OVA group, in comparison with the control group, had a markedly increased serum concentration of IgG specific to OVA at 3 months. In contrast, the OVA group had no detectable reaginic antibodies (IgE) specific to OVA at 3 months. In the early period when the rats were immunized with OVA, reaginic antibodies were detected in 3 out of 7 rats at 2 weeks and in 1 out of 7 rats at 8 weeks. As shown in Fig. 3B, chymase was detected in the serum from both groups. The concentration of chymase in the OVA group was more than 2-fold higher than that in the control group. More than 92% of the serum chymase was included in the serum with a density greater than 1.063 g/ml of the lipoprotein fraction (HDL and lipoprotein-free fraction), and the distribution pattern was similar between the groups. As shown in Fig. 4, the concentration of serum chymase was positively correlated with the lesion thickness.

Table 1 shows the concentrations of cholesterol, tryglycerides and phospholipids in the serum and lipoprotein fractions in the OVA group and control group. These BN rats exhibited mild hypercholesterolemia, but OVA-immunization exerted no significant influence on the concentrations of serum and lipoprotein lipids.

**Discussion**

The results of the present study show that immunization with OVA plus Al(OH)$_3$ as an adjuvant resulted in increased arterial intimal thickening in BN rats that had initially been treated with vitamin D$_2$ and subsequently fed with an atherogenic diet. The lesions in the OVA group were at an early stage of atherosclerosis since they predominantly comprised diffused intimal thickening and foam cell layers. There were no advanced lesions such as fibrous plaques, although ExHC rats initially subjected to hypervitaminosis and subsequent feeding on an atherogenic diet had more advanced lesions. These differences might have been due to the level of hypercholesterolemia, since the BN rats in the present study exhibited only mild hypercholesterolemia.

This is not the first report to show that immunizing
cell activation may be involved in arterial lesion development in rats. The important role of mast cells in early aortic lesion formation has been reported by Kovanen.\(^3\) He has presented a mechanism by which lipoproteins, both LDL and HDL, can be first modified for uptake by macrophages and then interact with activated mast cells. He also showed that the ratio of mast cells to T lymphocytes in normal human aortic intima was 2:1, and that mast cells, some largely degranulated, were about as frequent in atherosclerotic plaque in humans as T-cells. Thus, among the blood-born cells in aortic intima in humans and animals, mast cells comprise a significant cell population.

It is known that rats have two types of chymase, chymase I and chymase II. Chymase I and chymase II are derived from connective tissue-type mast cells and mucosal-type mast cells, respectively, in rats.\(^6\) The elevation of serum chymase II in rats indicates activation and degranulation of mucosal mast cells in the intestines. It is also known that connective tissue-type mast cells respond to immunization with foreign proteins and are then activated to release the chymase.\(^9\) Therefore, it is reasonable to assume that the mast cells present in the arterial walls might be locally activated by repeated immunization with OVA and modify LDL so as to be easily recognized by macrophages present in the arterial wall. This point, however, should be clarified in a future experiment.

It deserves comment on how chymase in the serum was elevated in the OVA group, because there was no appreciable elevation of reaginic antibodies (IgE) specific to OVA at 3 months, in spite of a prominent elevation of serum IgG specific to OVA. The classic example of mast cell stimulation is their activation by IgE.\(^19\) In IgE-mediated degranulation, the relevant antigen is bound by two or more of the IgE molecules bound to receptors with high affinity for IgE (FcεRI) on the mast cell surface. Therefore, it remains a possibility that our method for detecting OVA specific IgE was not sensitive enough. Alternatively, it has been reported that cultured mast cells could be triggered to degranulate by a number of non-IgE factors, including IgG, complement component C3a and C5a and histamine-releasing factors.\(^21\)

It has been established that atherosclerotic lesions in humans and rabbits contained a large number of T lymphocytes.\(^1\) The presence of T cells in atherosclerotic lesions could be important, since these cells can secrete factors chemotactic for monocytes/macrophages and smooth muscle cells.\(^22\) It has also been reported that rabbits repeatedly immunized with foreign protein antigens had peripheral blood lymphocytes with a higher degree of proliferation to the antigens and increased aortic lesion areas than non-immunized rabbits.\(^6\) Therefore, it remains a possibility that activated T lymphocytes as well as mast cells would contribute to arterial lesion formation in BN rats.

In summary, the present study has demonstrated that an immunologically mediated inflammatory process can induce atherosclerotic lesions in rats without influencing the serum cholesterol concentration. Therefore, this animal model could be useful for evaluating the role of

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**Fig. 4.** Correlation between the Serum Chymase Concentration and Aortic Intimal Thickness.

**Table 1.** Concentrations of Serum Total Cholesterol, Triglycerides and Phospholipids in Brown Norway Rats Fed on a High-cholesterol Diet for 3 Months

<table>
<thead>
<tr>
<th></th>
<th>Control (mg/dl)</th>
<th>OVA (mg/dl)</th>
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</thead>
<tbody>
<tr>
<td><strong>Total cholesterol</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>162±9</td>
<td>170±7</td>
</tr>
<tr>
<td>VLDL</td>
<td>37.7±3.0</td>
<td>39.0±1.4</td>
</tr>
<tr>
<td>LDL</td>
<td>60.5±6.2</td>
<td>69.7±4.1</td>
</tr>
<tr>
<td>d≥1.063</td>
<td>64.5±4.4</td>
<td>61.2±2.4</td>
</tr>
<tr>
<td><strong>Triglycerides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>51.7±7.6</td>
<td>46.8±4.3</td>
</tr>
<tr>
<td>VLDL</td>
<td>44.5±5.1</td>
<td>38.3±3.9</td>
</tr>
<tr>
<td><strong>Phospholipids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serum</td>
<td>127±3</td>
<td>124±7</td>
</tr>
<tr>
<td>VLDL</td>
<td>21.5±1.5</td>
<td>20.5±1.3</td>
</tr>
<tr>
<td>LDL</td>
<td>30.0±2.4</td>
<td>34.7±1.6</td>
</tr>
<tr>
<td>d≥1.063</td>
<td>76.2±2.1</td>
<td>73.2±1.9</td>
</tr>
</tbody>
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Each values is the mean±S.E. for 6 rats in the control group and 7 rats in the ovalbumin (OVA) group.
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the diet in immunologically mediated atherosclerosis.

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