RELATION BETWEEN MONOCYTES IN PERIPHERAL BLOOD AND DEVELOPMENT OF ATHEROSCLEROSIS IN HYPERCHOLESTEROLEMIC RABBITS

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The relation among circulating monocytes, serum cholesterol and LDL cholesterol in manifestation of atherosclerosis was investigated in hypercholesterolemic rabbits. Serum cholesterol increased sharply and reached a plateau at 12 weeks after the start of cholesterol diet feeding; LDL cholesterol gradually increased until 24 weeks, and the number of monocytes in blood started to decrease abruptly around 12 weeks and resulted in less than 1% total white blood cells at 24 weeks, as reflected by a severe progression of atheroma formation. This result indicated that the decrement of monocyte number in blood was predictive of the presence of severe atherosclerotic plaques.

KEYWORDS atherosclerosis; macrophage; foam cell; serum cholesterol; LDL cholesterol

Hypercholesterolemia is a major risk factor for development of atherosclerosis and coronary heart diseases.[1,2] Histochemical and immunochemical studies showed that smooth muscle cells and macrophages played important roles in the pathogenesis of atherosclerosis and were identified in atheroma.[3-5] Recent studies indicated that an earliest characteristic of atherosclerosis was the adherence of monocytes to the arterial endothelium.[6] When rabbits are fed a high -cholesterol diet, monocytes but not platelets and lymphocytes are immunohistochemically detected on the arterial wall at the outset[7]. These monocytes migrate into the subendothelial space and take up large amounts of modified LDL that is not catabolized by itself, resulting in foam cell formation.[8] Macrophage-derived foam cells were detected in fatty streak and fibrous plaques using antibody raised against monocyte/macrophage surface antigen.[9] Furthermore, macrophages differentiated from monocytes in the subendothelium secreted growth factors for mesenchymal cells and T cells, which could play an important role in hypertrophy of the intima[10-12] and facilitate atherosclerosis. In spite of a great deal of energetic work in this field, there is no way to predict the existence of atherosclerotic lesion in the human aorta. We therefore tried to clarify the relationship between the events evoked in blood and the progression of atherosclerosis in order to find an indicator showing the existence of atherosclerotic lesion.

MATERIALS AND METHODS

Animals and diet: Sixteen New Zealand White rabbits weighing 1.5-2.0 kg were ramdomly separated into 2 groups: Group 1, rabbits fed a commercial diet (CR-3, Nippon Crea Co. Ltd.) containing 1.0% cholesterol in 30 g chow/kg of body weigh/day; Group 2, rabbits fed a commercial diet. Every four weeks each rabbit was weighed and, following an overnight fast, blood was drawn from auricular veins to obtain serum. Low density lipoprotein(LDL) was prepared from the serum of rabbits, following sequential ultracentrifugal flotation. Serum and LDL cholesterol were determined enzymatically.[13]

Preparation of monoclonal antibody against monocyte: Monoclonal antibody was prepared by injection into BALB/c mice, with the rabbit monocyte partially purified by means of Ficoll-paque density gradient. Isolation and screening of hybridoma were carried out by the standard procedure.[14] Monocytes were analyzed with a flow cytometer (FACScan, Becton Dickinson Co. Ltd.) after immunostaining.

RESULTS

Rabbits were fed a normal diet or a 1% cholesterol fortified diet restricted to 30g per kg of body weight per day for 24 weeks. During the period of the study, their body weights increased smoothly, and significant differences were not seen between the body weight of the two groups (data not shown). The serum cholesterol and LDL cholesterol in rabbits fed a high cholesterol diet increased as represented in Fig.1. Serum cholesterol reached a plateau at 12 weeks after the start of cholesterol diet feeding. On the other hand, LDL cholesterol gradually increased until 24 weeks. However, no increases in serum cholesterol and LDL cholesterol were observed in the control group. We therefore prepared antibody reacted with rabbit monocytes to measure monocyte number using a flow cytometer. Only one clone that we obtained produced antibody reacted with not only rabbit monocytes but also neutrophils(data not shown). As we made use of a flow cytometer to measure monocyte number, the specificity of the antibody caused no problem in this experiment. The antibody was

IgG_{2a} and recognized two proteins, whose molecular sizes were 76kD and 81kD in isolated monocyte membrane(data not shown). Fig.2 represents the FACS pattern staining with anti-monocyte/neutrophil antibody, indicating that the forward scatter representing cell volume is shown on the ordinate and the intensity of fluorescence on the abscissa. The antibody stained monocytes in peripheral blood, as indicated with an arrow. In peripheral blood from a rabbit fed a cholesterol diet , the monocyte number obviously decreased as compared with a control rabbit (Fig.2c). We therefore examined the monocyte number in blood from rabbits fed a cholesterol diet throughout the experiment of 24 weeks. As shown in Fig.3, although constant untill 8 weeks, the monocyte number sharply decreased at 12 weeks, resulting in less than 1% at 24 weeks. With feeding of a cholesterol diet, variation of monocytes in the forward scatter and in the side scatter indicating the density of intracellular granules were observed on flow cytometer (data not shown). Atheromatous plaque in the thoracic aorta, particulaly in aortic arch, began to be observed sparsely at 4-8 weeks (less than 5% of the aorta surface) after the start of cholesterol diet feeding, and small plaques were formed in the descending aorta at 8-12 weeks (about 20% of aorta surface) in another experiment (data not shown). Finally, after 24 weeks on a cholesterol diet, atheromatous plaques were detected in almost all inner surface of the aortic arch and descending aorta in the cholesterol group.

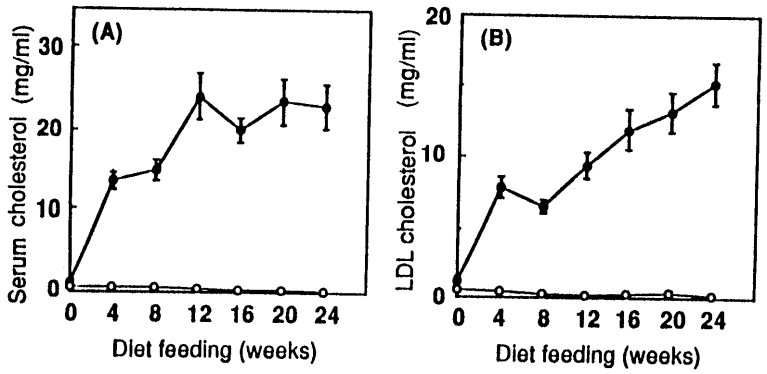

Fig.1. (A) Serum Cholesterol and (B) LDL Cholesterol in Rabbits Fed a High Cholesterol Diet or Normal Diet
Every four weeks blood was drawn from auricular veins to obtain serum. LDL was prepared from the serum by sequential ultracentrifugal flotation. ○, control diet; ●, high cholesterol diet. Values represent means ± S.E. of eight rabbits.

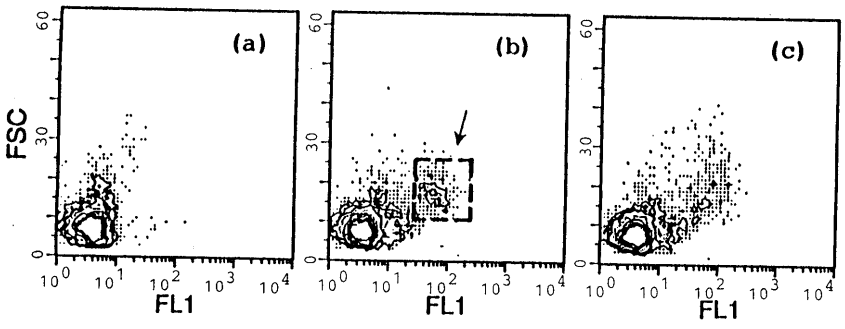

Fig.2. FACS Analysis of Peripheral Rabbit Blood
Blood collected in tubes containing 10mM EDTA in PBS was stained with anti-monocyte/neutrophil antibody, followed by fluoresceinisothiocyanate-conjugated goat F(ab)2 anti-mouse IgG (Caltag Laboratories Inc.) after erythocytes were lyzed with lysing solution (Becton Dickinson Co. Ltd.). Each figure shows forward scatter on the ordinate vs green fluorescence intensity on the abscissa. Data analyzed by setting a gate to lymphocytes and monocytes were shown above. (a) control rabbit without 1st antibody, (b) control rabbit (16 weeks of diet feeding) (c) rabbit fed a cholesterol diet (16 weeks). The arrow in Fig.2b indicates the monocyte area stainted with antibody.

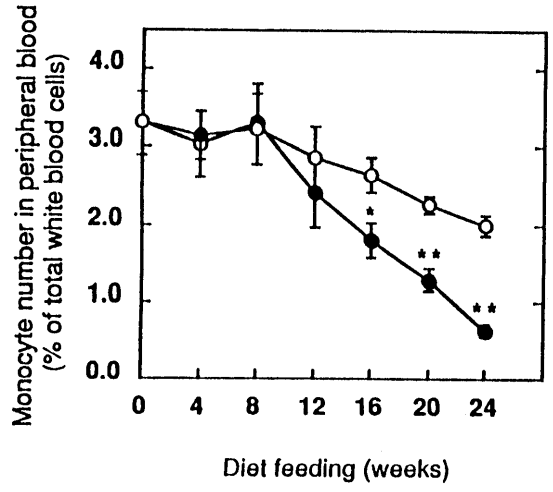

Fig.3. The Effect of Cholesterol Feeding on Monocyte Number in Peripheral Blood
○ , control diet; ● , high cholesterol diet. Values represent means ± S.E. of eight rabbits. * $p<0.05$, ** $p<0.01$ vs control group (Student's t test).

DISCUSSION

It is evident that hypercholesterolemia can be a necessary and sufficient cause of premature atherosclerosis. Although hypercholesterolemia is not the only cause of atherosclerosis, a number of studies have shown that it is a determining cause in many cases. The work of Goldstein and Brown concerning scavenger receptor[15] sheds light on the mechanism by which hypercholesterolemia accelerates atherogenesis. Thereafter, several groups reported that Probucol, an antioxidant, prevented the progression of atherosclerosis[16]; cDNA sequences of scavenger receptors were determined,[17] and modified LDLs (desialylated and oxidized LDL) were detected in atherosclerotic patients[18,19] and WHHL rabbits.[20] Furthermore, it became evident that monocytes played important roles in manifestation of atherosclerosis. Although macrophages accumulated in atherosclerotic lesion were confirmed by histochemical and immunohistochemical methods,[3-5] there is no way to detect the existence of atherosclerotic lesion in the aorta in human beings. We therefore investigated the manifestation and the progress of atherosclerosis by examining a change in monocyte number in peripheral blood, serum cholesterol and LDL cholesterol in order to search for an indicator showing the existence of atherosclerotic lesion and examined the progress of atherosclerosis in detail. A high cholesterol diet rapidly increased serum and LDL cholesterol in NZW rabbits. Although it was reported that monocytes attached to the endothelium of the artery were detected immunohistochemically even 1-4 weeks after the start of the diet,[3,4] monocyte number in peripheral blood was not changed until 8 weeks despite a high cholesterol level in serum compared with the control group. This result indicates that monocyte number in peripheral blood cannot reflect the early progress of atheromatous plaque formation. However, monocytes started to decrease sharply at 12 weeks when atherosclerotic plaques started to progress rapidly, and resulted in less than 1% of total white blood cells at 24 weeks when the surface of thoracic aorta were all covered with atheromatous plaques in rabbits fed a high cholesterol diet. On the other hand, no fatty streak or atheromatous plaque was detected on thoracic aortas from control rabbits. These data support the idea that circulating monocytes are linked to progression of atheromatous lesion and start to decrease when atheromatous plaque formation is developed beyond a certain severity. The reason monocytes decreased after 12 weeks is not yet understood . However, changes sufficient to recruit large numbers of monocytes to the artery could be related to monocyte function, the arterial wall or the nature of LDL around 12 weeks. Although high serum and LDL cholesterol can become a cause of atherosclerosis, they are not predictive of the degree of severity of atheroma formation. Finally, this study may suggest the possibility that we can diagnose or predict the existence of slightly developed atherosclerosis in the aorta by determining the number of moncytes in peripheral blood.

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