The inhibitory effect of a novel antiatheromatous agent, E5050, on the intimal thickening of aorta in cholesterol-fed rabbit in vivo

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ABSTRACT—The development of atheromatous lesions in the aortic arch of 0.5% cholesterol-fed rabbits was biochemically and morphologically examined. The animals were killed at week twelve (Cont-12W) or sixteen (Cont-16W). Both the micrographic and biochemical studies showed that the main atheromatous lesions in the Cont-12W group were fatty streaks, whereas those in the Cont-16W group were fibrous plaques. In these models, oral ingestion of 0.2% and 0.4% E5050, which has an antiproliferative effect on smooth muscle cells, had no effect on the surface involvement or the lipid content of the aortic arch at the sixteenth week, but reduced the degree of intimal thickening and the DNA content in the aortic arch in a dose-dependent manner. These results strongly suggest that E5050 suppresses the intimal thickening through its inhibitory effect on the proliferation of smooth muscle cells.

The following components have recently been clarified to be the specific cellular constituents in human atherosclerosis (1): The ubiquitous fatty streak is the earliest lesion of atherosclerosis, commonly found in children, and it is a grossly flat, lipid-rich lesion consisting of both macrophages and some smooth muscle (2). The fibrous plaque is representative of increased intimal smooth muscle cells surrounded by a connective-tissue matrix and contains variable amounts of intracellular and extracellular lipid. At the lumen of the artery, this lesion is generally covered by a dense fibrous cap of smooth muscle and connective tissue. Thus, smooth muscle cells can be found in both fatty streaks and fibrous plaques and especially are the predominant type of cell in fibrous plaques (3). Also, the cells can form an enormous amount of connective-tissue matrix (4, 5) and can accumulate lipid. Therefore, it can be expected that prevention of both hypercholesterolemia and abnormal proliferation of vascular smooth muscle cells will lead to suppression of fibrous-fatty plaque formation for the medical treatment of atherosclerosis.

In our recent study (6), it has been demonstrated that a newly synthesized compound, N-[3-[4'-(2''',6'''-dimethylheptyl)phenyl]butanoyl]-ethanolamine (E5050), inhibited the synthesis of DNA as well as the replication of smooth muscle cells stimulated with various mitogens, such as 10% fetal calf serum, platelet extract and purified platelet-derived growth factor (PDGF), with no apparent cytotoxic effect.

The purpose of the present study was to investigate what is the suitable period of cholesterol feeding in rabbits to cause fibrous...
plaque formation in the aortic arch and whether the inhibitory effect of E5050 on smooth muscle cell proliferation can actually contribute to suppressing the development of atherosclerosis in vivo using cholesterol-fed rabbits.

The results in this paper show that the fibrous plaque was formed in the aortic arch of the rabbit fed with cholesterol for 16 weeks and the antiproliferative effect of E5050 suppressed the development of the atherosclerosis.

MATERIALS AND METHODS

Animals and diets
Fifty-four male New Zealand White rabbits weighing approximately 3 kg were randomly separated into 4 groups: Group 1, Rabbits fed an atherogenic diet consisting of 100 g chow/day with 0.5% olive oil and 0.5% cholesterol (by weight) mixed into the chow for 12 weeks (Cont-12W); Group 2, Rabbits fed the same diet described above for 16 weeks (Cont-16W); Group 3, Rabbits given 0.2% (by weight) E5050 (200 mg/head/day) mixed in the diet chow for 16 weeks (0.2% E5050); and Group 4, Rabbits given 0.4% (by weight) E5050 (400 mg/head/day) mixed in the diet chow for 16 weeks (0.4% E5050). The atherogenic diet was prepared to order from the commercial ORC-4 diet (Oriental Yeast Co., Ltd., Tokyo, Japan).

Blood samples
The rabbits were bled from the marginal ear vein after a 24-hr fast, once before the start of the experiment and then at 4, 8, 12 and 16 weeks. Plasma was prepared by centrifugation at 3000 g for 20 min, with EDTA as an anticoagulant. Total cholesterol in plasma was measured by a conventional method, using a commercially available kit (Iatron Lab., Tokyo, Japan). AU-550 (Olympus, Tokyo, Japan) instruments were used to determine blood urea nitrogen (BUN), total protein, albumin, glutamic oxaloacetic transaminase (GOT), glutamic pyruvic transaminase (GPT), phospholipids and triglyceride.

Postmortem studies

Morphological studies: At the termination of the experiment, all the rabbits were anesthetized with pentobarbital and exsanguinated from the femoral artery. Immediately after laparotomy, the aorta was excised, and the grossly adherent adventitial tissues were removed. The aortic arch and thoracic aorta were separated at 5-mm proximal from the outlet of the first intercostal artery. These tissues were then longitudinally cut into two halves of about equal size. One half was fixed in buffered formalin and stained with Sudan IV. The positively stained areas were measured by an image analyzer (Nachet, France). Three representative cross-sections (ascending, middle and descending aortic arch) were taken from the aortic arch for histochemical studies. The cross-sections were stained with H.E. and Azan. The degree of intimal thickening was expressed as the intima-media volume ratio, using the image analyzer.

Biochemical studies: Intimal tissue of the other unfixed half was homogenized by the method of Morisaki et al. (7). An aliquot of the homogenate was used for protein and DNA assays, and the remainder was used for lipid analysis. Protein concentration was estimated by the method of Lowry et al. (8). DNA and lipids were assayed by the methods of Kissane and Robbins (9) and Morisaki et al. (10), respectively.

Statistical analysis
Significance of differences was evaluated by Student's t-test.

RESULTS

In vivo studies
Body weights were comparable in all groups throughout the study, irrespective of drug treatment. No animal showed evidence of anorexia. There were no significant differences in the plasma levels of blood urea nitrogen.
(BUN), total protein, albumin, glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) among the groups.

Table 1 shows plasma cholesterol levels in rabbits on the atherogenic diet during the course of the experiment, with or without drugs. The plasma cholesterol levels in all groups were increased from approximately 40–70 mg/dl to approximately 2000–2500 mg/dl after 12 weeks and remained at that level throughout the experiment. The plasma cholesterol level of the Cont-12W group showed the same pattern as that of the Cont-16W group for 12 weeks. There was no cholesterol-lowering effect in the two E5050 groups.

The plasma phospholipids and triglyceride levels in all groups were increased from approximately 70 mg/dl and 45 mg/dl to approximately 750 mg/dl and 150 mg/dl after 16 weeks, respectively. E5050 treatment had no effect on plasma phospholipid or triglyceride level (data not shown).

### Postmortem studies

**Morphological results:** The surface involvement of positively stained areas of the aortic arch and thoracic aorta was measured using an image analyzer and the results are shown in Table 2. In both the Cont-12W group and Cont-16W group, there was a greater degree of atherosclerosis in the aortic arch (66.8 ± 6.8% and 71.2 ± 5.7%, respectively) than in the thoracic aorta (15.8 ± 3.8 and 28.6 ± 4.8, respectively) or in the abdominal aorta (14.1 ± 2.2 and 21.0 ± 4.0%, respectively). In the aortic arch, the surface involvement in the Cont-12W group was similar in degree to that seen in the Cont-16W group (94% of Cont-16W). On the other hand, in the thoracic and abdominal aorta, the degree of atherosclerosis in the Cont-12W group was lower than that in the Cont-16W group (55% and 67% of Cont-16W, respectively), although the differences were not statistically significant. E5050 had no significant effect on the surface involvement in terms of the sudanophilic area in any portion of the aorta.

| Group   | Pre (mg/dl) | 8W (mg/dl) | 12W (mg/dl) | 16W (mg/dl) |
|---------|-------------|------------|-------------|-------------|
| Cont-12W | 71.8 ± 10.4 | 1819.5 ± 226.7 | 2447.0 ± 249.5 |             |
| Cont-16W | 58.3 ± 6.0  | 1874.7 ± 132.0 | 2434.4 ± 164.7 | 2199.1 ± 175.5 |
| 0.2% E5050 | 64.2 ± 10.1 | 1942.9 ± 193.6 | 2373.3 ± 178.6 | 2294.6 ± 145.5 |
| 0.4% E5050 | 42.0 ± 4.1  | 1568.3 ± 196.9 | 1917.9 ± 203.0 | 1944.5 ± 156.7 |

Values are means ± S.E. Pre, before feeding; W, weeks after feeding; Cont-12W, rabbits fed cholesterol for 12 weeks (n = 13); Cont-16W, rabbits fed cholesterol for 16 weeks (n = 15); 0.2% E5050, rabbits fed cholesterol for 16 weeks with 0.2% E5050 administration (n = 13); 0.4% E5050, rabbits fed cholesterol for 16 weeks with 0.4% E5050 administration (n = 13).

### Table 2. The surface area with atherosclerotic involvement in each group

| Group   | No of rabbits | Arch (%) | Thoracic (%) | Abdominal (%) |
|---------|---------------|----------|--------------|---------------|
| Cont-12W | 13            | 66.8 ± 6.8 | 15.8 ± 3.8    | 14.1 ± 2.2    |
| Cont-16W | 15            | 71.2 ± 5.7 | 28.6 ± 4.8    | 21.0 ± 4.0    |
| 0.2% E5050 | 13          | 64.8 ± 6.5 | 29.7 ± 8.6    | 26.1 ± 5.6    |
| 0.4% E5050 | 13          | 65.9 ± 5.0 | 22.8 ± 4.5    | 20.0 ± 3.1    |

Cont-12W, Cont-16W, 0.2% E5050 and 0.4% E5050: See the footnote to Table 1. All results are expressed as the mean ± S.E.
The degree of intimal thickening in the ascending, middle and descending portions of the aortic arch was measured by an image analyzer, and the results are summarized in Table 3. In the Cont-12W group, there was a lower intima-media volume ratio present in all sections compared to the Cont-16W group, and significant differences were observed in both sections 1 and 2. E5050 treatment lowered the mean intima-media volume ratio only in section 2, in which the intima-media volume ratio was the largest. E5050 dose-dependently lowered the intima-media volume ratio; and in particular, the 0.4% E5050 treatment significantly reduced it in section 2.

Figure 1 (a, b and c) shows micrographs of a cross-section of the aortic arch from a rabbit in the Cont-12W group, the Cont-16W group and 0.4% E5050 group, respectively. The atheromatous lesions in the middle portion of the aortic arch in the Cont-12W group mainly consisted of foam cells, and those in the middle portion of the Cont-16W group consisted of foam cells with proliferation of smooth muscle cells and increased fibrous components. On the other hand, the atheromatous lesions of the aortic arch in the 0.4% E5050 group mainly showed fatty streaks like those in the Cont-12W group.

Biochemical results: Table 4 shows the contents of protein and lipids extracted from the intima-media of the aortic arch. There were no significant differences in the contents of protein, free cholesterol, phospholipids or triglyceride of the aortic arch among all experimental groups. The cholesterol ester content in the Cont-12W group was slightly lower, but not significantly so, as compared to the Cont-16W group. Table 5 shows the DNA content in the aortic arch of rabbits on the atherogenic diet, with or without the drug. The DNA content in the Cont-12W group was

| Table 3. The intima-media volume ratio of aortic arch in each group |
|---------------------------------------------------------------|
| Group              | No. of rabbits | Intima-media volume ratio (%) |       |       |       |
|                   |                | section 1          | section 2          | section 3          |
| Cont-12W          | 13             | 41.9 ± 11.5*      | 51.1 ± 11.9**      | 48.7 ± 17.4        |
| Cont-16W          | 15             | 81.8 ± 10.4       | 103.6 ± 12.1       | 65.6 ± 19.3        |
| 0.2% E5050        | 13             | 82.7 ± 17.3       | 79.1 ± 12.7        | 49.5 ± 14.7        |
| 0.4% E5050        | 13             | 68.9 ± 13.9       | 62.0 ± 10.6*       | 50.7 ± 15.8        |

Three representative cross-sections were taken from the aortic arch as follows: section 1, ascending; section 2, middle; section 3, descending arch. Cont-12W, Cont-16W, 0.2% E5050 and 0.4% E5050: See the footnote to Table 1. All results are expressed as the mean ± S.E. *: P < 0.05, **: P < 0.01, compared to Cont-16W.

| Table 4. Lipid contents in the aortic arch in each group |
|--------------------------------------------------------|
| Group                  | Protein (mg/g wet weight) | CE (μg/g wet weight) | FC (μg/g wet weight) | PL (μg/g wet weight) | TG |
| Cont-12W               | 70.0 ± 1.2                | 46.1 ± 10.9          | 7.4 ± 1.5            | 15.0 ± 2.3           | 7.0 ± 1.5 |
| Cont-16W               | 67.8 ± 1.8                | 53.2 ± 8.3           | 10.8 ± 1.7           | 14.4 ± 1.7           | 5.6 ± 1.1 |
| 0.2% E5050            | 63.5 ± 3.5                | 48.0 ± 7.2           | 11.7 ± 2.2           | 15.4 ± 1.6           | 4.8 ± 0.9 |
| 0.4% E5050            | 68.1 ± 1.2                | 40.7 ± 7.9           | 11.0 ± 2.0           | 18.0 ± 2.4           | 4.4 ± 0.6 |

CE: cholesterol ester, FC: free cholesterol, PL: phospholipids, TG: triglyceride. Cont-12W, Cont-16W, 0.2% E5050 and 0.4% E5050: See the footnote to Table 1. All results are expressed as the mean ± S.E.
significantly lower than that in the Cont-16W group. E5050 treatment significantly decreased the DNA content per both wet weight and mg protein, in a dose-dependent manner, except for the DNA content per mg protein in the 0.2% E5050 group.

Fig. 1. Histological appearance of the middle portions of the aortic arch in the Cont-12W group (a), the Cont-16W group (b) and the 0.4% E5050 group (c). Azan stain (×162), Bar, 100 μm.
DISCUSSION

In the cholesterol-fed experimental model (11–17), the formation of atheromatous lesions consists of the following two steps: 1) Adhesion of monocytes to endothelial cells, invasion of monocytes into the subendothelial space and intracellular lipid accumulation by monocyte-derived macrophages (foam cell formation); and 2) Migration of smooth muscle cells from the media into the intima, proliferation of intimal smooth muscle cells and secretion of excessive amounts of collagen, elastin and glycosaminoglycan by the increased number of intimal smooth muscle cells.

We confirmed both morphologically and biochemically that the atheromatous lesions of the aortic arch in rabbits fed the atherogenic diet for 12 weeks were fatty streaks that corresponded to step 1 described above and that the lesions in rabbits fed the atherogenic diet for 16 weeks were fatty-fibrous plaques that corresponded to step 2 described above. The surface involvement of the aortic arch in the Cont-16W group was almost the same as that in the Cont-12W group (Table 2). On the other hand, the surface involvement of the thoracic aorta in both the Cont-12W and Cont-16W groups (15.8 ± 3.8% and 28.6 ± 4.8%, respectively) was lower than that of the aortic arch in the Cont-16W group (71.2 ± 5.7%), and that in the Cont-12W group was 55% of that in Cont-16W group. Thus, after cholesterol-feeding, the accumulation of lipids into the aortic arch occurs first, followed by accumulation of lipids into the thoracic aorta. After 12 weeks of cholesterol-feeding, the development of surface involvement in the aortic arch is saturated, while the surface involvement of the thoracic aorta is progressing even at 16 weeks of cholesterol-feeding. In the Cont-16W group, the atheromatous lesions in the aortic arch were large and confluent plaques, while those of the thoracic aorta appeared only at the sites adjacent to the ostia of the intercostal arteries. Also, the atheromatous lesions in the ascending and middle portions of the aortic arch were fibrous plaques, whereas those in the descending portions of the aortic arch and in the thoracic aorta were fatty streaks. Thus, after cholesterol-feeding for 16 weeks, the fibrous plaques pervaded the ascending and middle portions of the aortic arch, whereas the lesions in the thoracic aorta were still restricted. Therefore, we examined atheromatous lesions in the aortic arch in order to investigate biochemically the conversion of fatty streaks to fibrous plaques.

The protein and lipid contents of the aortic arch in the Cont-16W group were almost the same as in the Cont-12W group (Table 4), but the intima-media volume ratio (Table 3) and DNA content (Table 5) of the aortic arch in the Cont-16W group were significantly higher than those in the Cont-12W group. These results were consistent with the microscopic changes (Fig. 1, a and b). Gaton and Wolman, using histochemical techniques, have observed a stratification of two cell types in atheroma (18) and Watanabe et al., using immunoperox-
idase techniques with monoclonal antimacrophage antibody, have shown that lipid-filled macrophages are the cells that predominate in the fatty streak, whereas in the fibrous plaque, smooth muscle cells are increased in number (19). Therefore, our present results indicate that after the saturation of lipid accumulation into the aortic arch, smooth muscle cell proliferation and synthesis of fibrous components occurred; and 16 weeks after the start of cholesterol-feeding, the advance of the lesions was continuing. These findings are consistent with data in reports on human coronary arteries (20).

Thus, the examination of the atheromatous lesions in the aortic arch of rabbits on the atherogenic diet for 16 weeks is a suitable approach for investigating whether the prevention of smooth muscle cell proliferation suppresses the conversion of fatty streaks to fibrous plaques. In this model, treatment with E5050 had no effect on the surface involvement or lipid content of the aortic arch. However, E5050 treatment did dose-dependently reduce the DNA content in the aortic arch and significantly decreased the degree of intimal thickening in the middle portion, which was the most advanced plaque lesion in the three portions of the aortic arch, as shown in Fig. 1c. We have demonstrated that E5050 inhibited smooth muscle cell proliferation stimulated by various mitogenic factors, and this inhibitory effect was positively correlated with the E5050 uptake into smooth muscle cells, in vitro (6). Furthermore, in our preliminary study, we have confirmed the uptake of unchanged [14C]-E5050 into the aorta by oral administration to beagles (data not shown). These results suggest that the suppressive effect of E5050 on intimal thickening in this model results from the inhibitory effect on smooth muscle cell proliferation in atherosclerotic lesions and that E5050 should be useful for preventing and treating atherosclerosis, although the clinical relevance of our finding must await completion of appropriate human studies.

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