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Rapid induction of atherosclerosis in rabbits

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Summary. Japanese white rabbits fed a restricted amount (100 g/head/day) of an atherogenic diet (AD) containing 0.2% cholesterol and 6% peanut oil showed mild and persistent hypercholesterolemia (338 ± 79 mg/dl). They developed atherosclerotic lesions 4 weeks after deendothelialization of aorta carried out at the 4th week of AD-feeding. This rabbit model of atherosclerosis has such advantages as being able to be produced in a short period and having similar biochemical and pathological characteristics with those in human atherosclerosis.

Key words: Atherogenic diet, Atherosclerosis model, Deendothelialization, Rabbit

Introduction

A long-term feeding of rabbits on cholesterol-rich diet has been widely used to produce an animal model of atherosclerosis (Ignatowski, 1909; Anitschkow, 1913; Duff, 1935; Constantinides, 1961; Wissler and Vesselinovitch, 1968). However, rabbits fed such a diet showed an enormously high serum cholesterol level (more than 2000 mg/dl) and developed severe systemic lipidosis (Prior et al., 1961), which is a rare finding in human atherosclerosis. On the other hand, mechanical injury to the aortic endothelium has also been employed to induce intimal thickening without lipid deposition in rabbits (Spaet et al., 1975; Richardson et al., 1980; Faxon et al., 1982; Jackson et al., 1988), rats (Schwartz et al., 1975; Clowes et al., 1983) and monkeys (Stemerman and Ross, 1972). Recently, we were able to produce atherosclerotic lesions in rabbits in a short period by a combination of dietary-induced mild hyperlipidemia and balloon-injury of aortic endothelium. This paper describes the biochemical and pathological characteristics of the aortic lesions.

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Materials and methods

Animals and treatments

A total of 28 male SPF Japanese white rabbits weighing about 2.9 kg (Kitayama Labes Co. Ltd., Kyoto) were housed individually in metal cages in an air-conditioned animal room (temperature; 23 ± 2° C, humidity; 55 ± 5%). Ten animals were fed a standard diet (SD) (GM-1; Funabashi Farms Co. Ltd., Chiba) and the remaining 18 were fed an atherogenic diet (AD) containing 0.2% cholesterol and 6% peanut oil in SD. Throughout the experimental period, 100 g of diet per day and tap water were given to each animal.

At 4 weeks after AD-feeding, deendothelialization was carried out on 10 animals of AD group according to the method of Moore et al. (1982). Briefly, a 4 F embolectomy catheter (model 36-2044; Electro-Catheter Corp. NJ, USA) was inserted into the upper thoracic aorta via the right femoral artery under sodium pentobarbital anesthesia. The balloon was inflated with a constant volume (0.3 ml) of saline and slowly withdrawn to the level of the bifurcation of iliac arteries. This procedure took about 60 seconds and was repeated 3 times. After pulling out the catheter, the incision was sutured. The remaining 8 animals of AD group and all of SD group received sham-operation. After the deendothelialization, AD-feeding was carried out again for additional 4 weeks.

Sample collection for biochemical and pathological analysis

About 3 ml of blood was collected at two-week-intervals from the ear artery of each animal. Thirty minutes before sacrifice under deep pentobarbital anesthesia, 5 ml of 0.45% Evans blue solution was

infused via the ear vein. After animals death, the entire length of the aorta was removed.

For electron microscopic examination, a small segment of about 3 mm length was taken from the distal end of the aorta and fixed in 2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), postfixed in 1.0% osmium tetroxide in the same buffer and embedded in epoxy resin. Semi-thin (1 μm) transverse sections were cut and stained with toluidine blue. Ultra-thin sections were also cut and double stained with uranyl acetate and lead citrate and observed under a JEOL model JEM-100C electron microscope.

The remaining aorta was opened longitudinally and a total of 10 cross segments of about 3 mm length each were obtained; 5 from the thoracic aorta (at the levels of the 1st, 3rd, 5th, 7th and 9th intercostal arterial branches) and 5 from the abdominal aorta (at the levels of celiac trunk, cranial mesenteric artery, left renal artery, and 4th and 5th lumbar arterial branches). Each segment was fixed in 10% neutral-buffered formalin. Six μm frozen sections were made from each segment and stained with oil red O and hematoxylin.

Intima and media of the remaining segments of the aorta were stripped off according to the method of Wolinsky and Daly (1970). Then, aortic lipids were extracted by the modified Folch's method (1957), and the residual defatted tissues were used for the determination of protein and collagen.

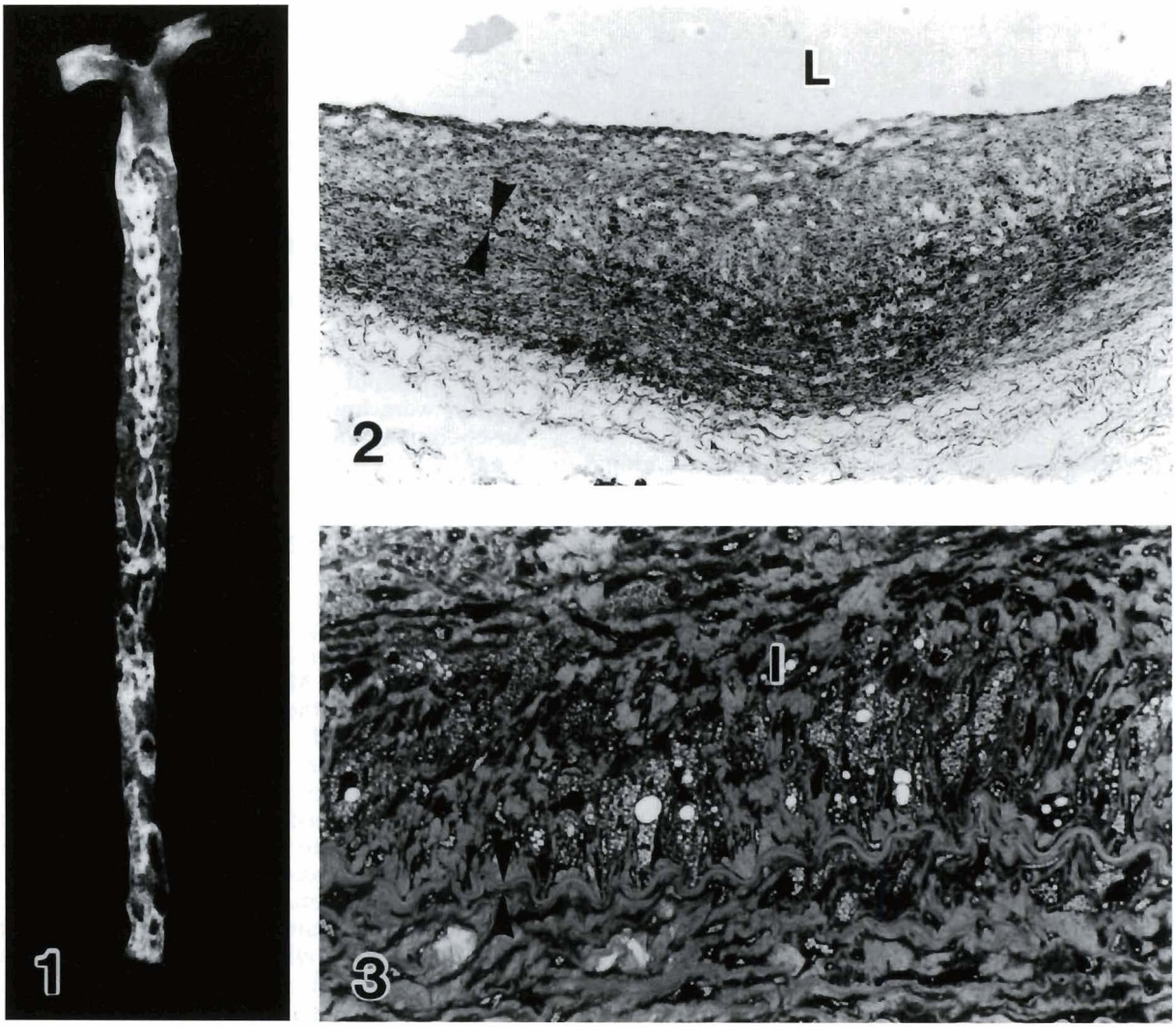

Fig. 1. Macroscopic appearance of luminal surface of a deendothelialized aorta. Reendothelialized regions surrounding ostia of arterial branches look pale while denuded regions dark. Evans blue-stained specimen.

Fig. 2. Abdominal aorta of a deendothelialized and AD-fed animal. The intima is markedly thickened with lipid deposition. L: lumen, arrowheads: internal elastic lamina. Frozen section, oil red 0 stain. × 80

Fig. 3. A deep part of severely thickened intima (I) of abdominal aorta of a deendothelialized and AD-fed animal. Many foam cells are seen above internal elastic lamina (arrowheads). Semi-thin section, toluidine blue stain. × 400

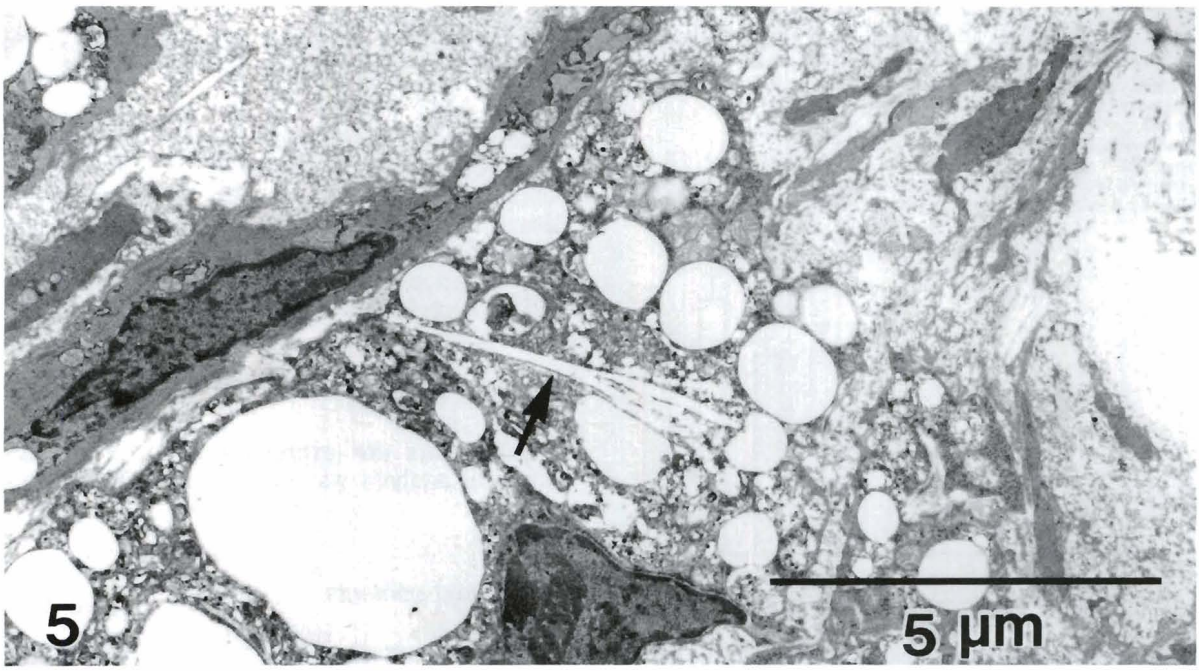

Fig. 4. Lipid droplets are mainly seen in the cytoplasm of both SMC-like (arrows) and foam cells (arrowheads) in aortic intima of a deendothelialized and AD-fed animal. L: lumen. Bar = 10 μm.

Fig. 5. A cholesterol crystal (arrow) is seen in a foam cell in aortic intima of a deendothelialized and an AD-fed animal. Bar = 5 μm.

Morphometry

Stained sections of the aorta on a slide glass were put directly in a photographic enlarger and were printed under the same magnification. Areas of the aortic intima and media on the prints were measured by a computer-assisted image analysing system (YHP 9845B and 9111A; Yokogawa Hewlet Packard Co. Ltd., Tokyo). Percentage of the intimal area to the medial one was provided as intimal thickening index (ITI).

Table 1. Body weight and serum cholesterol, triglyceride and phospholipid levels at sacrifice

Group	n	BW[a] (g)	TC[b] (mg/dl)	TG[c]	PL[d]
SD[e] sham-operated	10	[e]3403±42[f]	21±3	40±4	4±4
AD[g] sham-operated	8	[e]3511±69	322±48**	46±7	168±22**
AD deendothelialized	10	[e]3407±64	338±79**	48±12	171±30**

a) Body weight.
b) Total cholesterol.
c) Triglycerides.
d) Phospholipis.
e) Standard diet.
f) Valnes are means ± SEM.
g) Atherogenic diet.
** : $p < 0.01$, vs SD sham-operated group.

Table 2. Aortic total cholesterol, triglyceride, phospholipid and collagen contents

Group	n	Total cholesterol		Triglycerides		Phospholipids		Collagen (% of total protein)	
		(μg/mg of dry weight tissues)							
		T[a]	A[b]	T	A	T	A	T	A
SD[c] sham-operated	10	5.3±0.1[d]	5.8±0.1	10.9±2.0	16.5±2.3	17.5±0.8	17.8±1.5	61.1±1	68±3
AD[e] sham-operated	8	5.4±0.2	7.5±0.5*	10.4±2.2	16.4±4.6	16.8±1.5	17.4±2.0	63±2	66±3
AD deendothelialized	10	20.2±4.9 *# *#	28.6±6.3**	13.5±1.9	20.0±3.2	19.0±2.2	21.5±3.3	71±3**#	76±2

a) Thoracic aorta.
b) Abdominal aorta.
c) Standard diet.
d) Values are means ± SEM.
e) Atherogenic diet.
** : $p < 0.01$, * : $p < 0.05$, vs SD sham-operated group.
# : $p < 0.01$, # : $p < 0.05$, vs AD sham-operated group.

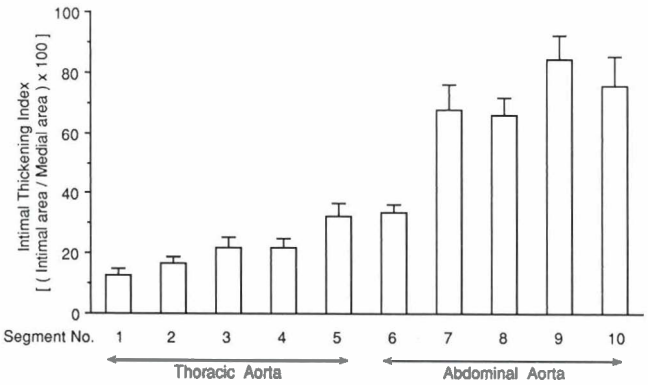

Fig. 6. Intimal thickening indices of thoracic and abdominal aortas of rabbits 4 weeks after deendothelialization. Data are shown as mean ± SEM (n = 10).

Measurement of lipids and collagen

Serum lipids, and aortic cholesterol and triglycerides were enzymatically measured in an automatic analyzer (Hitachi 705; Hitachi Co. Ltd., Tokyo) using enzymatic kits (Cholestezyme-V and Triglyzyme-V; Eiken Chemical Co. Ltd., Tokyo). Serum high density lipoprotein (HDL) cholesterol was measured on ultracentrifugally-separated fractions.

Inorganic phosphorus of aortic phospholipids was measured according to Allen's method modified by Nakamura and Mori (1958). The defatted aortic tissues were hydrolyzed with 6 N HC1 and then neutralized with a NaOH for hydroxyproline determination by the method of Neuman and Logan (1950). Protein was measured by the method of Lowry et al. (1951).

Statistical analysis

All values are expressed as mean ± SEM, and statistical analysis was done using Scheffe's test.

Results

Serum lipid contents

Within 4 weeks after the start of AD-feeding, the level of total cholesterol content showed moderate elevation while those of HDL cholesterol and phospholipid contents exhibited mild elevation. These levels were maintained during the additional feeding period of 4 weeks (Table 1). On the other hand, the level of triglyceride content showed no significant change throughout the experimental period.

Aortic lipid and collagen contents

The contents of aortic lipid and collagen showed no significant changes by AD-feeding alone. However,

following catherization, the content of total cholesterol increased markedly while those of triglycerides, phospholipids and collagen increased slightly (Table 2).

Pathological findings of aorta

Macroscopic findings: Luminal surface of the deendothelialized aorta was strongly stained with Evans blue dye while that of the sham-operated control was not (Fig. 1). Moreover, aortic wall of the deendothelialized animal was thicker and harder than that of the sham-operated animal.

Light and electron microscopic findings: In the aorta of AD-fed and deendothelialized animals, there were a large number of smooth muscle cell (SMC)-like cells and focal aggregation of foam cells, resulting in intimal thickening (Fig. 2). In these thickened intima, collagen fibres increased in the intercellular space (Fig. 3). Lipid droplets were found in the cytoplasm of SMC-like cells and foam cells in the thickened intima (Fig. 4), and some of the foam cells also contained cholesterol crystals in their cytoplasm (Fig. 5). In addition, a few lipid droplets in SMCs and focal calcification were observed in the media of about 60% of aortic segments. Aortic sections from the sham-operated animals exhibited no significant light and electron microscopic changes.

Morphometric findings

The ITI value of the deendothelialized aorta was generally greater in the abdominal portion than in the thoracic one (Fig. 6).

Discussion

In the present study, rabbits fed a restricted amount (100 g/head/day) of AD containing 0.2% cholesterol exhibited mild and persistent hyperlipidemia and they developed atherosclerotic lesions 4 weeks after deendothelialization (i.e. 8 weeks after the start of AD-feeding). They accompanied no or slight lipid deposition in the organs other than the aorta. The induction period was about half of that reported in the similar experiment by Minick et al. (1979).

The thickened aortic intima was composed of a large number of lipid-laden SMC-like cells, focal aggregation of foam cells and increased interstitial collagen fibres, and such a pathological picture is similar to that of human atherosclerosis (World Health Organization, 1958).

The thoracic aorta were more severely affected than the abdominal aorta when rabbits were fed a lipid-rich diet for a long period (Duff, 1935). On the other hand, intimal thickening was severer in the abdominal aorta than in the thoracic one when normolipidemic rabbits received deendothelialization (Moore et al., 1982). In the present study, intimal thickening with atherosclerotic lesion was more prominent in the abdominal aorta than in the thoracic one, and such a tendency is similar to that in human atherosclerosis (Wissler et al., 1985).

Judging from the above-mentioned findings, our rabbit model seems to be a useful tool for the investigation of atherosclerosis as well as for the efficacy-estimation of anti-atherogenic drugs.

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