Atherosclerosis is a chronic disease that progresses with age and leads to serious complications, including heart attack, stroke, or even death. Although it remains asymptomatic for decades, the atherogenic process is reported to begin in fetal arteries.\(^1\) Moreover, the extent of atherosclerotic changes in children is correlated with the presence of the same risk factors as identified in adults.\(^2\)

A high plasma cholesterol level is one of the most important risk factors for atherosclerosis. The current primary strategy for treating atherosclerosis involves lowering plasma concentrations of low-density lipoprotein cholesterol (LDL-C) by following an appropriate diet or administering drugs such as statins. There is a notion that lifestyle changes have in -

**METHODS**

**Ethics Statement** This study was performed in strict accordance with the Guidelines for the Animal Care and Use of Otsuka Pharmaceutical Co., Ltd., which conforms to the international norms stipulated by the Ministry of Health, Labour and Welfare of Japan. The study protocol was approved by the Institutional Animal Care and Use Committee of Otsuka Pharmaceutical Co., Ltd. (permit number: 11–0060). All efforts were made to maximize the welfare of the animals and minimize their suffering.

**Animals** Male New Zealand White rabbits (specific pathogen free) were purchased from Kitayama Labes Co., Ltd. (Nagano, Japan) and were acclimated and housed in individual cages in animal rooms maintained at 23 ± 2°C and 60 ± 10% humidity. The rabbits were fed a 0.5% cholesterol (w/w)-containing diet (80 g/d) from 8 (younger group, n = 6) or 12 (older group, n = 5) weeks of age for 8 weeks (Fig. 1). The cholesterol-containing diet was prepared by Oriental Yeast Co., Ltd. (Osaka, Japan). Body weights and plasma concentrations of lipids were measured at 1, 2, 4, 6, and 8 weeks after the initiation of cholesterol feeding (Fig. 1).

**Measurement of Plasma Lipid Concentrations** After overnight fasting, blood was drawn from the ear vein of the rabbits without anesthesia and collected in heparinized syringes. Plasma was obtained by centrifugation of blood samples at 3000 rpm for 10 min at 4°C. Plasma concentrations of lipids [total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), phospholipid (PL), and triglyceride (TG)] were measured in an autoanalyzer (BioLis24i; Tokyo Boeki Medisys Inc., Tokyo, Japan) using the reagents L-type CHO M, L-type HDL-C M2, L-type PL, and L-type TG M, respectively (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

**Anatomical Analysis and Analysis of Atherosclerotic Lesion Areas** Anatomical analysis and analysis of the areas of atherosclerotic lesions were performed based on our previous study.\(^3\) At the end of cholesterol feeding, the animals were
euthanized by exsanguination under pentobarbital anesthesia, and their aortas were isolated. The ascending and thoracic aortic regions were dissected partly for histological analysis. The remaining aorta was opened longitudinally after carefully removing the surrounding connective tissues, and luminal atherosclerotic lesions were stained with Sudan IV. The areas of atherosclerotic lesion and entire luminal surface were measured using WinROOF software (version 5.8.0; Mitani Corporation, Tokyo, Japan) in a blind manner. Ratios of the area of atherosclerotic lesions relative to the area of the entire luminal surface (relative atherosclerotic lesion area) were calculated for the entire aorta. For histological analysis, Elastica van Gieson staining and immunohistochemical staining of macrophages using RAM11 (Dako Japan, Inc., Tokyo, Japan) were performed in paraffin sections.

**Statistical Analysis** Data are presented as mean±S.D. Differences in the areas of atherosclerotic lesions and plasma lipid concentrations between the younger and older groups were analyzed using the unpaired t-test and two-way repeated-measure ANOVA, respectively. Differences were considered significant at 5% in the two-tailed test. SAS software (Release 9.1; SAS Institute Inc., Tokyo, Japan) was used for all statistical analyses.

**RESULTS**

Plasma concentrations of TC increased and reached >1000 mg/dL at 4 weeks after the initiation of cholesterol feeding (Fig. 2A). Plasma concentrations of TC in control rabbits aged 12 weeks (n=4) fed normal chow were 42±5 mg/dL (data not shown). Analysis of lipoprotein profiles using HPLC showed that the increase in plasma concentrations of TC was essentially because of elevated plasma concentrations of very-low-density lipoprotein and LDL cholesterol (data not shown). Plasma concentrations of HDL-C increased moderately during cholesterol feeding. After 8 weeks of cholesterol feeding, plasma concentrations of HDL-C in the younger group were 23% lower than those in the older group (Fig. 2B). Plasma concentrations of PL (Fig. 2C) and TG (Fig. 2D) also increased during cholesterol feeding; however, the differences between the two groups were not statistically significant. After 8 weeks of cholesterol feeding, the body weights of rabbits in the younger and older groups were 2.38±0.13 and 2.85±0.06 kg, respectively. The difference in body weights between the two groups indicated that average cholesterol intake per body weight in the younger group was 1.3-fold higher than that in the older group during the test period (193±15 µg/kg/d in the younger group and 148±5 µg/kg/d in the older group).

**Statistical Analysis** Data are presented as mean±S.D. Differences in the areas of atherosclerotic lesions and plasma lipid concentrations between the younger and older groups were analyzed using the unpaired t-test and two-way repeated-measure ANOVA, respectively. Differences were considered significant at 5% in the two-tailed test. SAS software (Release 9.1; SAS Institute Inc., Tokyo, Japan) was used for all statistical analyses.

En-face analysis showed that the areas of atherosclerotic lesions differed significantly between the two groups. The areas of atherosclerotic lesions in the younger group (32±21%) were significantly larger than those in the older group (3.3±0.3%; Figs. 3A, B). Lipid-rich plaques formed throughout the aortic area in the younger group, whereas these plaques were seen in limited regions such as the aortic arch and some branches of the thoracic aorta in the older group (Fig. 3A). Immunohistological analysis of the ascending and thoracic aortic
regions showed that atherosclerotic lesions in the intima were essentially composed of RAM11-positive foamy macrophages, which were more prominent in the ascending aortic region than in the thoracic aortic region. The extent of intimal thickening was also obviously larger in the younger group than in the older group (Fig. 3C). Neointimal areas in the ascending aortic regions in the two groups were $1.34 \pm 0.75 \text{mm}^2$ ($n=3$) and $0.05 \pm 0.08 \text{mm}^2$ ($n=3$), respectively. En-face analysis confirmed that control rabbits fed normal chow showed no atherosclerotic lesions in any aortic region (data not shown).

**DISCUSSION**

This study clearly showed that in juvenile rabbits, dietary cholesterol-induced atherosclerotic lesion formation changed markedly by only a 4-week time lag in the initiation of cholesterol feeding. Rabbits have been used for studying the development of atherosclerotic lesions for many decades \(^5\); however, changes in atherogenesis during the juvenile period have not been studied previously. To the best of our knowledge, this is the first study to show that atherogenic susceptibility to cholesterol feeding changed with a 4-week difference in rabbit age.

Several studies reported that atherosclerotic lesions form even during fetal development in humans \(^1\) and rabbits. \(^5\) In addition, fetal fatty streaks are enhanced by maternal hypercholesterolemia and regress temporarily after birth. \(^9\) These results suggest that the immature aorta could be susceptible to
high concentrations of cholesterol, although this susceptibility may change to become increasingly resistant to high concentrations of cholesterol during the growth process. A recent study has found nearly complete regression of early atherosclerotic lesions by lowering concentrations of cholesterol and regression resistance of mature and advanced lesions, suggesting that early-stage atherosclerosis has higher reactivity to plasma concentrations of cholesterol than mature-stage atherosclerosis. Thus, it can be assumed that changes in arterial properties during the growth period result in less atherogenic susceptibility.

However, the major cause of changes in atherogenic susceptibility during the growth period remains unknown. No significant differences were observed in the lipid profiles of the two rabbit groups in this study, except for the difference in plasma concentrations of HDL-C by 8–11 mg/dL (Fig. 2B), which would have affected the atherogenic susceptibility of rabbits in both groups. However, the difference in the plasma concentration of HDL-C was relatively less than that in plasma concentrations of TC (>800 mg/dL). In addition, no correlation was observed between plasma concentrations of HDL-C and the area of atherosclerotic lesions (data not shown), suggesting that these were not the major causes of the difference in atherosclerotic lesion formation between the two groups.

Our previous study on rabbits fed cholesterol under the same conditions as younger rabbits in the present study showed a significant increase in plasma oxidized LDL levels and excessive accumulation of oxidized LDL in atherosclerotic plaques. Those results suggested that oxidative stress was associated with atherogenesis in these rabbits and that the extent of LDL oxidation in blood or accumulation of oxidized LDL in vascular tissue could change during a short juvenile period. Another possible reason may be a change in the inflammatory response because atherosclerotic lesions in this model essentially contained accumulated macrophages. Testosterone reduces the expression of vascular cell adhesion molecule 1, which plays an important role in the recruitment of monocytes into arterial walls and atherosclerotic lesion formation in male rabbits. Plasma concentrations of testosterone increase markedly during the juvenile period. Therefore, a low plasma concentration of testosterone in the early juvenile period and in senescence could be a risk factor for atherosclerosis in rabbits.

In conclusion, atherogenic susceptibility to cholesterol feeding changed unexpectedly during a short period in juvenile rabbits. This finding should be noted for research on atherosclerosis in juvenile rabbits and may be important for comprehensive pediatric care to prevent the emergence of atherosclerotic disorders in adults.

Conflict of Interest Yuka Keyamura, Chifumi Nagano, Masayuki Kohashi, Manabu Niimi, Masanori Nozako, Takashi Koyama and Tomohiro Yoshikawa are employees of Otsuka Pharmaceutical Co., Ltd. Hiroyuki Itabe has no conflict of interest.

REFERENCES