Cholesterol Metabolism in Inherently Scorbutic Rats (ODS Rats)

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Summary We observed the cholesterol metabolism of a colony of Wistar rats with a hereditary defect in vitamin C synthesizing ability (the ODS (osteogenic disorder-Shionogi) rats) in six kinds of experiments. Female ODS rats aged 36 days had a low HDL (high-density lipoprotein)-cholesterol level in serum as compared with age-matched control rats in spite of the absence of scorbutic symptoms. Female ODS rats aged 63 days which revealed severe scorbutic symptoms had a very low HDL-cholesterol level (mean value: 17 mg/dl). And male ODS rats, whose lives had been prolonged by supplementing with L-ascorbic acid, also had lower serum HDL-cholesterol and had increased total cholesterol in serum and liver when the acid supplement dose was relatively insufficient. On the other hand, we examined HDL\textsubscript{2}- and HDL\textsubscript{3}-cholesterol levels in serum to determine the mechanism of low HDL-cholesterol. As a result, we observed a low HDL\textsubscript{2}-cholesterol level in ODS rats but normal HDL\textsubscript{3}-cholesterol level. But the authors observed no decrease of LCAT (lecithin: cholesterol acyltransferase) activity in serum of ODS rats. These results could be due to disturbance of lipid metabolism in a vitamin C-deficient condition, that is to say, there might be abnormalities of the cholesterol excretion pathway of bile acid from liver, and maturity of the HDL-cholesterol particle due to other factors except that of LCAT activity.

Key Words vitamin C deficiency, ODS rat, total cholesterol, HDL-cholesterol, LCAT

Vitamin C has an important role in a wide number of biochemical reactions. It also appears to play a significant role in cholesterol metabolism. Although ascorbate is reported to have a cholesterol-lowering effect in a number of animal experiments\textsuperscript{(1, 2)} and in some human studies\textsuperscript{(3–5)}, there is disagreement about the effects of vitamin C on cholesterol metabolism\textsuperscript{(6, 7)}. Although some experiments
assessing vitamin C deficiency in animals have been carried out in chronically or marginally deficient states, these conditions produce loss of appetite, resulting in a kind of starvation. But these ODS (osteogenic disorder-Shionogi) rats showed weight loss and symptoms of scurvy three weeks after birth in spite of sufficient intake of a commercial diet without the addition of vitamin C (8). Thus ODS rats represent a pure pathophysiological state of vitamin C deficiency. In this paper, the authors will report significant abnormalities of lipid metabolism as one of the risk factors of atherosclerosis in ODS rats.

EXPERIMENTAL ANIMALS AND METHODS

Makino and Katagiri (9) established a colony of mutant Wistar rats (ODS rats) with a hereditary defect in L-ascorbic acid (AsA)-synthesizing ability that is controlled by a single autosomal recessive gene. In this rat mutant, L-gulonolactone oxidase is lacking (10). It is enzymatically the same as in the human state. Thus ODS rats never live longer than approximately 70 days if they are not supplied with AsA. ODS rats were generously supplied by Aburahi Laboratories, Shionogi Research Laboratories, Shionogi & Co., Ltd. The homozygotes of the ODS rats (strain and gene symbol: ODS-od/od) were used as “abnormal rats,” and the Wistar/Shi rats belonging to this strain with normal gene (ODS-+/+) were used as controls.

1) Serum total cholesterol (TC) and high-density lipoprotein-cholesterol (HDL-C) level in rats relatively matured with supply of AsA. In experiment (Exp.) 1, 16 female ODS rats (eight rats of ODS-+/+ and the same number of ODS-od/od) with an average starting weight of 80g were used. They were killed 36 days after birth. They had been given no AsA during their lives. In Exp. 2, 20 female rats (10 rats of the ODS-+/+ and the same number of ODS-od/od rats) were used and killed 63 days after birth. By that time two ODS-od/od rats were already dead due to scurvy, and thus eight rats were used for experiments. In Exp. 3, 14 male ODS-+/+ rats and 14 male ODS-od/od rats were used. All of ODS-od/od rats received 2 mg/day of AsA for 44 days to prolong their lives, and were killed 10 days after the supply of AsA was stopped (103 days after birth). In Exp. 4, 10 male ODS-+/+ rats and 10 male ODS-od/od rats were used. All of the ODS-od/od rats were supplied with 2 mg/day of AsA for only 25 days, and killed 10 days after the supply of AsA was stopped (113 days after birth). In Exps. 1-4, all animals were fed a commercial diet (MM-3, Funahashi Farm Co., Ltd.) containing no AsA. The composition (in percentages) was: water, 7.0; protein, 20.1; lipid, 4.4; fiber, 5.2; ash, 6.4; soluble non-nitrogen compound, 54.5; and digestible protein, 17.7. Vitamin A, 1,000 IU; vitamin B₁, 1 mg; vitamin B₂, 1 mg; vitamin B₆, 1 mg; vitamin B₁₂, 0.5 µg; vitamin D₃, 200 IU; vitamin E, 5 mg; vitamin K₃, 0.5 mg; nicotinamide, 5 mg; pantothenic acid calcium, 1.6 mg; choline, 100 mg; folic acid, 0.1 mg; biotin, 15 µg; and inositol, 10 mg were added to 100 mg of the diet. In the above four kinds of experiments, serum TC was examined by the enzymatic method and HDL-C by the heparin calcium method using kits from Nihon Shoji Co., Ltd., Osaka.

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2) **TC and HDL-C in serum and TC in liver of rats fed with lipid-rich diet.** In Exp. 5, 21 male ODS-+/+ rats and 15 male ODS-od/od rats were divided into three groups, respectively. In the three groups, seven ODS-+/+ and five ODS-od/od rats received 1) commercial diet, 2) diet containing 1% cholesterol, 3) diet containing 1% cholesterol and 0.5% cholic acid, respectively. Rats in groups 2) and 3) received lipid supplementation from 63 days after birth till they were sacrificed. Moreover, all ODS-od/od rats were supplemented with 5 mg/day of AsA from 38 days to 76 days after birth (for 38 days), and killed 84 days after birth together with all ODS-+/+ rats groups which were fed with the cholesterol supplied diet (1% cholesterol and 0.5% cholic acid). Serum TC and HDL-C were measured by the same method, while liver TC was extracted by the Folch method (11) and measured by the Zak method (12).

3) **Serum HDL	extsubscript{2} and HDL	extsubscript{3}-cholesterol level.** In Exp. 5, serum HDL	extsubscript{2}-cholesterol (HDL	extsubscript{2}-C) and HDL	extsubscript{3}-cholesterol (HDL	extsubscript{3}-C) level in three subjects of each group were measured by the method of Kajiyama et al. (13).

4) **Serum lecithin: cholesterol acyltransferase (LCAT) activity.** In Exp. 5, serum LCAT activity of all rats was measured by the enzymatic method using a kit from Daiichi Chemical Pharmacy Co., Ltd., Tokyo.

5) **Effect of the cholesterol supplied diet on vitamin C concentration in liver and body weight gain.** In Exp. 6, 14 male ODS-+/+ rats and the same number of male ODS-od/od rats were divided into two groups respectively. Each group of seven rats received 1) commercial diet, 2) diet containing 1% cholesterol and 0.5% cholic acid from 79 days after birth till they were sacrificed (100 days after birth). In order to prolong the lives of ODS-od/od rats, 2 mg/day of AsA were supplied to all ODS-od/od rats from 42 days after birth to 88 days after birth (for 46 days). Authors observed the changes of body weight gain and scorbutic symptoms during the whole of the experimental period and assayed the AsA concentration in liver, organ weights, and serum lipid level. Vitamin C concentration in liver was determined by the method of Ogawa and Kishigami (14).

**RESULTS**

1) As shown in Table 1, we observed that ODS-od/od rats aged 36 days (Exp. 1) had a low HDL-C level in spite of no sign of weight loss and scorbutic symptoms. In ODS-od/od rats aged 63 days (Exp. 2) that had severe scorbutic symptoms such as nose bleeding and gait disturbance due to bleeding in joints without any loss of appetite, a significant low level of serum HDL-C compared with ODS-+/+ rats was observed. Unexpectedly, it was observed that ODS-od/od rats had a normal level of TC in Exp. 2. Moreover we observed the abnormality of lipid metabolism in mature ODS-od/od rats supplied with AsA (Exps. 3 and 4). Although the TC and HDL-C level in ODS-od/od rats supplemented with 2 mg/day of AsA for 44 days (Exp. 3) indicated no difference when compared with the control, in the case of the same dose supplementation for only 25 days, TC level increased and HDL-C level
Table 1. Serum chemical analyses of ODS rats fed with a commercial diet (Exps. 1–4).

| Exp. and sex | Animal       | $n$  | TC (mg/dl) | HDL-C (mg/dl) | Body weight (g) |
|------------|---------------|-----|------------|---------------|-----------------|
| 1 female   | ODS-+/-      | 8   | 100 ± 7.5  | 55.3 ± 5.1    | 106 ± 4.4       |
| P value    |               |     | $p < 0.05$ | $p < 0.01$     |                 |
| 2 female   | ODS-od/od    | 8   | 109 ± 4.1  | 37.8 ± 9.8    | 91.9 ± 4.3      |
| P value    |               |     | $p < 0.01$ | $p < 0.05$     |                 |
| 3 male     | ODS-+/-      | 7   | 79.8 ± 8.9 | 54.0 ± 7.0    | 312 ± 17.7      |
| P value    |               |     | $p < 0.001$| $p < 0.001$    |                 |
| 4 male     | ODS-od/od    | 6   | 82.5 ± 9.0 | 47.9 ± 5.7    | 223 ± 25.7      |
| P value    |               |     | $p < 0.001$| $p < 0.001$    |                 |

Values are means ± SD. $n$, number of animals. The doses of ascorbic acid supplement for ODS-od/od rats are 2 mg/day for 44 days in Exp. 3 and 2 mg/day for 25 days in Exp. 4.

decreased significantly compared with the control in ODS-+/-+ rats. In summary, in every generation of ODS-od/od rats, there was a certain tendency of increase in TC level and decrease in HDL-C level, and a certain relationship between supplement dose of AsA and the severity of disorder of lipid metabolism.

2) As shown in Fig. 1, although serum TC levels in ODS-od/od rats fed with a commercial diet only indicated no significant change, they increased significantly when the diet was supplemented with 1% cholesterol and 0.5% cholic acid. And the increasing rate of HDL-C on a lipid-rich diet was not larger than that of TC, thus the value of atherogenic index, expressed by (TC-HDL-C)/HDL-C, of ODS-od/od rats was significantly high. Moreover, the significant increase of liver TC level in ODS-od/od rats fed with the cholesterol supplied diet is shown in Fig. 2.

3) In Fig. 3, each value of HDL2-C and HDL3-C are plotted on the same column, since there was no overlap of values between HDL2-C and HDL3-C. There was no difference in serum HDL3-C level between ODS-+/-+ rats and ODS-od/od rats on either a commercial diet or the cholesterol supplied diet. However, serum HDL2-C level was to the same extent lower in ODS-od/od rats than in ODS-+/-+ rats in all types of diets. In all rats, a diet containing only 1% cholesterol showed no increase in the serum TC but had lower HDL2-C level compared with that of a commercial diet.

4) There was no difference in serum LCAT activity between ODS-+/-+ rats and ODS-od/od rats (Fig. 4).

5) Figure 5 indicates the liver AsA concentration in rats.

In the group of rats fed with a commercial diet, liver AsA level in all ODS-od/od rats was not measured due to low concentration. However, this parameter
was increased by a cholesterol supplied diet. Indeed, body weights of ODS-od/od rats fed with a cholesterol supplied diet continued to increase but showed weight loss on a commercial diet due to scurvy (Fig. 6). Also in Exp. 5 it is shown that there was an increase of liver AsA concentration in ODS-od/od rats fed with a cholesterol supplied diet but no significant difference in body weight in the two kinds of diet.
Fig. 2. Total cholesterol concentration in livers of ODS rats. Only two groups of each kind were assayed. ●, ODS-+/+ rats; ○, ODS-od/od rats. **p < 0.01.

Fig. 3. HDL2-cholesterol and HDL3-cholesterol in serum of ODS rats. ●, ODS-+/+ rats; ○, ODS-od/od rats. **p < 0.01.
Fig. 4. LCAT activity in serum of ODS rats. White column, mean value of LCAT activity in ODS-+/+ rats; black column, mean value of LCAT activity in ODS-od/od rats.

Fig. 5. Ascorbic acid concentration in livers of ODS rats in the two cases of commercial diets and lipid-rich diets. Lipid-rich diet: 1% cholesterol and 0.5% cholic acid. ●, ODS-+/+ rats; ○, ODS-od/od rats. ***p<0.001.
DISCUSSION

We previously reported the increased platelet aggregability of ADP in ODS-od/od rats, and also emphasized that the abnormality of lipid metabolism was atherogenic, that is, increased TC level and significantly decreased HDL-C in serum of ODS-od/od rats (8). Although the accumulation of TC in serum of ODS-od/od rats fed with a commercial diet was not so much higher than the results of experiments using guinea pigs chronically fed with a vitamin C-deficient diet (15, 16), ODS-od/od rats fed with a cholesterol supplied diet resulted in a significant increase of TC in serum and liver. Our result is the same as that of Horio et al. using ODS rats fed with a diet supplied 0.5% cholesterol and 0.25% cholic acid (17). We assume the possibility that the difference in species between rats and guinea pigs explains the difference of accumulation of cholesterol. From the above results, exogenous loading of an inadequately large dose of lipid, we also can assume that ODS-od/od rats have a disorder in the excretion pathway of cholesterol. Ginter (16) reported that vitamin C is a cofactor of a key enzyme, 7α-hydroxylase, in this pathway to bile acid (18). There have been only a few reports proving this hypothesis because the method of measurement of this enzyme is not easy. But the results of our experiments using ODS rats neither deny nor support his hypothesis.

Secondly, plasma HDL-C has attracted the interest of many investigators because of the putative antiatherogenic role in man (19), and we could assume the possibility of disturbance in maturity of HDL-C in humans with a vitamin C-deficient state because of the results from low HDL₂-C in ODS-od/od rats. There
Table 2. Change of organ weights in ODS rats fed with cholesterol supplied diet (Exp. 6). (g)

| Animal    | Diet       | n  | Thymus       | Heart      | Aorta      | Lung       | Kidney     |
|-----------|------------|----|--------------|------------|------------|------------|------------|
| ODS-+/+   | Commercial | 7  | 0.39 ± 0.09  | 1.26 ± 0.18| 0.08 ± 0.03| 2.28 ± 0.35| 3.16 ± 0.24|
|           | Lipid rich | 7  | 0.39 ± 0.07  | 1.31 ± 0.18| 0.08 ± 0.02| 2.04 ± 0.27| 3.33 ± 0.40|
|           | P value    |    |              |            |            |            |            |
| ODS-od/od | Commercial | 7  | 0.29 ± 0.08  | 1.05 ± 0.16| 0.07 ± 0.02| 1.70 ± 0.27| 2.86 ± 0.26|
|           | Lipid rich | 7  | 0.36 ± 0.006 | 1.14 ± 0.11| 0.07 ± 0.01| 1.71 ± 0.18| 2.98 ± 0.28|

Values are means ± SD. n, number of animals. *p = 0.08. Cholesterol supplied diet: 1% cholesterol and 0.5% cholic acid.
are two hypotheses to explain this disturbance—a decrease of LCAT activity (19, 20), and a decrease of lipoprotein lipase (LPL) activity (21–23)—the results of our experiments do not support the former. It is suggested that different kinds of experiments using a large number of animals are necessary for resolving these problems.

Finally, many reports using guinea pigs chronically fed with a vitamin C-deficient diet suggest that a cholesterol supplied diet results in a decrease of vitamin C concentration in blood and liver, while our experiments suggested that a cholesterol supplied diet gave the effect of "vitamin C preservation" in liver. As an explanation of this mechanism, we supposed that the weight gain in ODS-od/od rats fed with a lipid-rich diet in Exp. 6 was due to the effect of vitamin C on the increasing rate of weight of each organ compared with those of od/od rats fed with a commercial diet (Table 2). Indeed, these organs had a certain correlation with vitamin C supplement as compared with other organs (8). We stress that the relationship between vitamin C and lipid metabolism from our results and those of numerous other investigators cannot be ignored. Clinically, there are at least over ten previous experiments involving gram dose AsA (mainly 1 or 2 g/day taken orally) administration for healthy subjects or patients of hyperlipidemia and atherosclerosis. But the results of these experiments produced two opposing opinions: one recognized the cholesterol-lowering effect of vitamin C, the other did not. Some investigators who observed the result that supplementation of vitamin C increased TC in serum, assumed the possibility that vitamin C transferred cholesterol from liver and/or aorta to blood (24).

If so, an adequate dose of vitamin C administration may be a good therapy for patients with atherosclerosis on fatty liver, etc., in spite of increased cholesterol level in serum. Also from this point of view, the relationship between vitamin C and the etiology of diseases in the aged and the effect of gram dose of vitamin C on diseases require further investigation.

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