Risk Factors of Atherogenecity in Inherently Scorbutic Rats (OD Rats)

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Summary We observed the pathophysiological state of a colony of Wistar rats with a hereditary defect in vitamin C-synthesizing ability (the OD rat) in three kinds of experiments. In experiment 1, OD rats that were not supplemented with L-ascorbic acid (ASA) were killed 36 days after birth. In experiment 2, OD rats that were not supplemented with AsA were killed 63 days after birth. In experiment 3, OD rats that were supplemented with sufficient ASA (100mg/100g body weight/day) for 16 days from 47 days after birth were assayed 63 days after birth. From analyses of serum chemistry, platelet aggregability and counts of blood cells, some atherogenic risk factors were elucidated, that is, there was a decrease of high density lipoprotein in serum and an increase of platelet aggregability. Furthermore, it was observed that OD rats have some characteristics different from latent vitamin C-deficient animals from the viewpoint of malnutrition.

Key Words atherogenecity, vitamin C, OD rat, HDL-cholesterol, platelet aggregability

Several researchers have reported that vitamin C depletion makes atherosclerotic lesions worse in some kinds of animals and humans (1-3). Also, there have been reports indicating there are atherosclerotic intimal thickening and endothelial lesions in guinea pigs with chronic vitamin C deficiency (4, 5). It is worth noticing that vitamin C deficiency is related to some atherogenic risk factors.

Makino and Katagiri (6) established a colony of mutant Wistar rats (OD rats) with a hereditary defect in AsA-synthesizing ability that is controlled by a single autosomal recessive gene. l-Gulonolactone oxidase is lacking in this rat mutant (7). It is enzymatically the same as the state in humans. Because rats can usually synthesize AsA, a dietary supply is not necessary. But if OD rats are not supplied

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with it, they never live longer than approximately 70 days. The growth of OD rats in the homozygotes is promoted by the addition of AsA to a commercial diet and they will grow normally and be fertile. There have been many reports about nutritional states of latent vitamin C deficiency using guinea pigs. But the deficient condition of OD rats is neither latent nor marginal but acute. Therefore Horio et al. (8) suggested a significant difference between the lipid metabolism of OD rats and that of latent vitamin C-deficient animals. In this paper, the authors report on experimental results concerning risk factors in the atherogenesis of this mutant OD rat strain.

MATERIALS AND METHODS

OD rats (osteogenic disorder rats) were generously supplied by Aburahi Laboratories, Shionogi Research Laboratories, Shionogi & Co., Ltd. The homozygotes of the OD 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.

In experiment (Exp.) 1, female OD rats with an average starting weight of 80 g were used. All animals were fed a commercial diet (MM-3, Funahashi Farm Co., Ltd.), the composition of which was percentages: 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 B<sub>1</sub>, 1 mg; vitamin B<sub>2</sub>, 1 mg; vitamin B<sub>6</sub>, 1 mg; vitamin B<sub>12</sub>, 0.5 μg; vitamin D<sub>3</sub>, 200 IU; vitamin E, 5 mg; vitamin K<sub>3</sub>, 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 g of the diet but vitamin C was not. The rats were observed from 34 days after birth in our laboratory and killed 36 days after birth. In this experiment, the observation period in our laboratory was very short, but it was certain that they had received no AsA in the Aburahi laboratories.

In Exp. 2, female rats were used and killed 63 days after birth. All animals were fed the same diet as in Exp. 1. At that time two OD rats were already dead due to scurvy, and thus eight rats were used for experiments.

In Exp. 3, ten male OD rats were fed on basal diets supplemented with 100 mg/100 g body weight/day of AsA from 47 days after birth and the supplementation was stopped 7 days before the day of assay (63 days after birth).

In all experiments, organ weights were measured and blood chemical analysis was performed. Furthermore, pathological changes of aortae in Exp. 1 were observed by light microscopy. Total AsA contents of livers and adrenal glands were measured by the method of Ogawa and Kishigami (9), a modification of the hydrazine method, in Exp. 2. In addition, blood cell counts and platelet aggregability by ADP were observed in Exp. 3.

Analytical methods. a) Total cholesterol in serum was determined by the enzymatic method using a kit from Daiichi Pure Chemicals Co., Ltd., Tokyo. b) High density lipoprotein (HDL) cholesterol in serum was determined by the heparin

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calcium method using a kit from Daiichi Pure Chemicals Co., Ltd., Tokyo. c) Lipid peroxide in serum was measured by the method of Yagi (10) using a kit from Wako Pure Chemical Industries, Ltd., Osaka in Exps. 2 and 3.

In addition, alkaline phosphatase (ALP), glutamic-oxaloacetic transaminase (GOT) and glutamic-pyruvic transaminase (GPT) in serum were also measured in Exp. 3. These indexes in serum were measured using kits from Daiichi Pure Chemicals Co., Ltd. ALP was determined by the phenyl phosphate method. Activities of GOT and GPT were measured by the enzymatic method (GOT, MDH-UV method; GPT, LDH-UV method).

Statistical analyses. Statistical analyses were carried using Student’s t-test for paired data.

RESULTS

Body weights and scorbutic symptoms in OD rats: On the final day in each experiment, mean body weights of OD rats were (mean ± SD) 91.4 ± 4.0 g in Exp. 1, 65.1 ± 6.0 g in Exp. 2, and 142 ± 24 g in Exp. 3, while those of control rats were 106 ± 4.1 g, 136 ± 16.2 g, and 184 ± 10.2 g, respectively. In all experiments, there were significant differences (p < 0.01) between body weights of OD rats and those of controls (Fig. 1). All the OD rats showed nose bleeding and gait disturbance due to scorbutic state in Exp. 2.

The total AsA contents of animal tissues: It was observed in Exp. 2 that the OD rats had hardly any AsA in any tissues (Table 1).

Weight measurements of each organ: In Exp. 1, weights of the thymus, liver, and adrenal glands were measured. The weights of ten organs were compared between two groups in Exp. 2, and eleven were measured in Exp. 3 (Table 2). The prominently immature organs of OD rats were the thymus, femoral bone, and liver. When AsA was administered to OD rats (Exp. 3), the increase in liver weight was significant.

Table 1. L-Ascorbic acid contents in two organs (Exp. 2).

| Phenotype               | Genotype | L-Ascorbic acid contentsa (µg/g tissue) |
|------------------------|----------|----------------------------------------|
|                        |          | Liver       | Adrenals    |
| Control rats           | +/+      | 556 ± 493   | 329 ± 132   |
| (n = 8)                |          | (n = 6)     |             |
| Osteogenic disorder rats| od/od   | 0           | 31 ± 29     |
| (OD rats)              |          | (n = 3)     | (n = 3)     |
| P value                |          | p < 0.001   | p < 0.001   |

aValues are means ± SD. n, number of animals.
Fig. 1. Body weight changes in OD rats. In Exp. 1, eight female OD rats were used. On the day of assay (36 days after birth), all rats showed scorbutic symptoms. Pathological changes of aortae were observed. In Exp. 2, on the day of assay (63 days after birth), only eight female OD rats were used because two rats had died. The AsA contents of livers and adrenals were measured. In Exp. 3, ten male OD rats were supplemented with AsA from 47 days after birth and the supplementation was stopped 7 days before the day of assay (63 days after birth). Blood cell counts, platelet aggregability, and hydroxyproline contents of aortae were assayed. ○, mean value of body weight in OD rats; ●, mean value of body weight in control rats. *p<0.05, **p<0.01, ***p<0.001.

Blood chemical analysis: As shown in Table 3, we found some differences in blood chemistry between OD rats and control rats. In OD rats, serum total cholesterol (TC) levels were higher than those in control rats only in Exp. 1. Nevertheless, serum high density lipoprotein cholesterol (HDL-C) levels were significantly low. Moreover, levels of HDL-C in OD rats improved considerably when AsA was administered (Exp. 3). In addition, the levels of ALP and transaminase (GOT, GPT) in serum were rather high in OD rats (Exp. 3). Mean values and SD of ALP levels were 22.9±1.6 U in control rats, and 46.4±9.7 U in OD rats (p<0.01). GOT levels in controls and OD rats were 57.8±4.3 U and
### Table 2. Organ weights of OD rats. (g)

| Organ          | Exp. 1 |          | Exp. 2 |          | Exp. 3 |          |
|----------------|--------|----------|--------|----------|--------|----------|
|                | Control | OD       | Control | OD       | Control | OD       |
| Thymus         | 0.37    | ± 0.03   | 0.39    | ± 0.14   | 0.44    | ± 0.06   |
|                | ± 0.32  | ± 0.03   | 0.045   | ± 0.021  | ± 0.07  | ± 0.01   |
|                |         |          |         |          |         |          |
| Heart          | 0.64    | ± 0.06   | 0.41    | ± 0.04   | 0.73    | ± 0.04   |
|                |         |          |         |          |         |          |
| Aorta          | 0.12    | ± 0.05   | 0.11    | ± 0.06   | 0.11    | ± 0.06   |
| Lungs          | 0.89    | ± 0.11   | 0.74    | ± 0.07   | 0.93    | ± 0.04   |
| Kidneys        | 1.66    | ± 0.19   | 1.49    | ± 0.14   | 1.84    | ± 0.10   |
| Liver          | 5.90    | ± 0.42   | 4.94    | ± 0.29   | 8.06    | ± 0.57   |
|                | ± 0.94  | ± 0.29   |         | ± 0.68   | 3.14    | ± 0.29   |
| Adrenal gl.    | 0.042   | ± 0.004  | 0.033   | ± 0.004  | 0.067   | ± 0.015  |
| Pancreas       | 0.44    | ± 0.03   | 0.32    | ± 0.11   | 0.40    | ± 0.06   |
| Spleen         | 0.38    | ± 0.03   | 0.19    | ± 0.06   | 0.37    | ± 0.04   |
| Femoral bone   | 0.41    | ± 0.02   | 0.15    | ± 0.08   | 0.78    | ± 0.04   |
| (one side)     |         |          |         |          |         |          |
| Body weight    | 106     | ± 4.1    | 91.9    | ± 4.1    | 136     | ± 16.2   |
|                |         |          |         |          |         |          |

Values are means ± SD.

87.6 ± 6.0 U (p < 0.001), respectively. GPT levels were 26.6 ± 2.8 U in controls and 65.6 ± 15.0 U in OD rats (p < 0.01), respectively. There was information from the Aburahi Laboratories that some newborn OD strain rats had been suffering from icterus and bile duct dilatation. It was suspected that these pathological conditions were controlled by a gene different from the gene for L-gulonolactone oxidase (personal communication). More detailed analysis of these results is now under
Table 3. Serum chemical analyses of OD and control rats in all experiments.

|                    | Exp. 1 | Exp. 2 | Exp. 3 |
|--------------------|--------|--------|--------|
|                    | Control | OD     | Control | OD     | Control | OD     |
| Total cholesterol  | 100     | 109    | 77.9    | 88.3    | 74.6    | 76.6    |
| (mg/dl)            | ± 7.5   | ± 4.1  | ± 19.7  | ± 11.0  | ± 4.3   | ± 4.7   |
| P value            | p < 0.05|        |         |         |         |         |
| High density lipoprotein cholesterol (mg/dl) | 55.3 | 37.8 | 50.8 | 16.7 | 32.0 | 27.0 |
| P value            | ± 5.1   | ± 9.8  | ± 11.4  | ± 2.5   | ± 1.4   | ± 3.0   |
| Lipid peroxide     | 8.5     | 5.0    | 10.3    | 9.7     | ± 5.2   | ± 1.3   |
| (nmol/ml)          | ± 5.2   | ± 1.3  | ± 1.6   | ± 1.1   |         |         |

Values are means ± SD.

Fig. 2. Platelet aggregation by ADP of OD rats (Exp. 3).

way. Thus we presumed the possibility of disorder in excretion passages of bile juice or liver cell functions in OD rats. The lipid peroxide level in serum indicated no significant difference between the OD and control groups.

Blood cell counts (Exp. 3): The OD rats had relatively low red cell and platelet counts (Table 4). However, an aseptic room was not used in our experiments, so the possible influence on the results cannot be discounted.

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Table 4. Counts of blood cells. (Exp. 3)  

|                | Number of animals | Red blood cell (10^6/mm³) | White blood cell (10^6/mm³) | Platelet (10^3/mm³) |
|----------------|-------------------|---------------------------|-----------------------------|---------------------|
| Control        | 5                 | 695±45                    | 2,920±1,850                 | 50.4±13.8           |
| OD rat         | 5                 | 611±29                    | 4,580±658                   | 29.1±10.1           |
| P value        |                   | p < 0.05                  |                             | p < 0.05            |

Values are means ± SD.

Fig. 3. Pathological findings of thoracic aorta (Exp. 2). × 200. HE, hematoxylin-eosin stain; EvG, elastica van Gieson stain; TB, toluidine blue stain.

**Platelet aggregability** (Exp. 3): Platelet aggregation induced by ADP in OD rats was accelerated (Fig. 2).

**Pathological findings of aorta** (Exp. 2): Although the media of the thoracic aorta was slightly thinner in OD rats as compared with control rats, the difference was not significant (Fig. 3). Moreover there was no endothelial cell injury or thrombosis formation in the coronary arteries, renal arteries and another main arteries.

**DISCUSSION**

In OD rats, we found abnormalities in two factors related to atherosclerosis:
lipid metabolism and platelet aggregability: 1) hypo-HDL-cholesterolemia and 2) accelerated platelet aggregability. It is well known that the administration of a large dose of AsA causes an increase of serum HDL-C. There is a report that acutely scorbutic guinea pigs have lower HDL-C levels than pair-fed controls(11), and similar observations have been reported in the acutely scorbutic state(12) and the chronically marginal scorbutic state in guinea pigs(13) and rhesus monkeys(14). It is necessary to note that we used female rats in Exps. 1 and 2, whereas we used male rats in Exp. 3. But we considered that serum HDL-C levels of OD rats were definitely lower than those of controls because these relationships were observed in all experiments. It is also well known that there is cholesterol accumulation in the blood and liver of scorbutic animals(15, 16), because AsA is understood to work as a cofactor of cholesterol 7a-hydroxylase(17), a rate limiting enzyme in the metabolic process from cholesterol to bile acids. In our results, the degree of hypercholesterolemia of OD rats was not so significant. Moreover the reason why serum TC levels of OD rats were not so high as compared with previous reports of latent vitamin C-deficient animals is unknown. The solution to this problem awaits further studies regarding OD rats.

Platelet aggregation induced by ADP in OD rats was accelerated. This result is very different from the experimental results, reported by Ikezawa et al.(18). Moreover it was found that the mean value of platelet cell count in OD rats was less than that of controls in our experiments. We know of no reports stating that vitamin C is necessary for platelet production; thus it is assumed that platelet aggregation increased after platelet consumption for bleeding due to vitamin C deficiency. If so, platelet aggregation of OD rats was exceedingly strong in this state (Exp. 3: a moderately scorbutic state). Therefore it is necessary for investigators to study mutant animals in mild deficiency, moderate deficiency, and severe deficiency.

Ikezawa et al. reported that platelet aggregability in guinea pigs fed a vitamin C-deficient diet for 30 days decreased. We postulate that the phenomenon of "pseudohypoaggregability" of platelets in previous reports was produced by an increase of nonfunctioning platelets in latent, long-term scorbutic animals.

In addition, there were significant differences in the organ weights reported by Ginter et al.(15) and those by the present authors. In a comparative study of scorbutic guinea pigs fed 0.5 mg/day of AsA and another group fed 10 mg/day, Ginter et al. reported that the average ratio of liver to body weight of the deficient group was larger than that of controls (4.1%: 3.3%), and that the relative weights of adrenals were on the same order. However, our data showed that the ratio of the liver to body weight of OD rats was slightly smaller than that of control rats (5.0%: 5.3%) and that of the adrenals was larger than in control rats (0.035%: 0.022%) in Exp. 2. The results of Ginter et al. were the same as our results in Exp. 3. In Exp. 3, OD rats received a sufficient dose of AsA to gain weight when young, and the same dose of AsA especially promoted liver weight increase. Thus, the ratios of organ weights to body weights in latent vitamin C-deficient guinea pigs and OD rats were not the same.

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Altho the some experiments to assess vitamin C deficiency in animals have been done in late or marginally deficient states, these conditions make the animals lose appetite, and resulting in a kind of starvation. Therefore, it is impossible in long-term experiment to analyze a purely vitamin C-deficient state without deficiency of other dietary components as well. We found differences between our results and some previous reports related to latent vitamin C deficiency in terms of organ weights, platelet aggregation, and so on. The OD rats showed weight loss and symptoms of scurvy three weeks after birth in spite of sufficient intake of a commercial diet without vitamin C added. Thus, OD rats represent a pure pathophysiological state of vitamin C deficiency. Differences between OD rats and scorbutic guinea pigs exist, of course, due to fundamental species differences and experimental methods may differ as well.

Finally, light microscopic examinations of the aortae in OD rats showed no significant alterations in the intimal walls.

However, we observed only one slice per rat. Most other investigators who have examined the pathology of the aorta in vitamin C-deficient animals have also failed to find lesions. Rat aortae have been known to be resistant to atherosclerosis, and therefore there may be a relationship between vitamin C deficiency and aortic lesions. Because aortic lesions in OD rats may appear if their lifespan can be elongated, further studies are necessary on these mutant animals.

REFERENCES

1) Yavorsky, M., Almaden, P., and King, C. G. (1934): Vitamin C content of human tissue. J. Biol. Chem., 106, 525–529.
2) Becker, R. R., Burch, H. B., Salomon, L. L., Venkilasubramanian, T. A., and King, C. G. (1953): Accumulation of cholesterol in arteries in ascorbic acid deficiency. J. Am. Chem. Soc., 75, 2020–2022.
3) Spittle, C. R. (1971): Atherosclerosis and vitamin C. Lancet, ii, 1280–1281.
4) Willis, G. C. (1953): An experimental study of the intimal ground substance in atherosclerosis. Can. M. A. J., 69, 17–22.
5) Sulkin, N. M., and Sulkin, D. F. (1975): Tissue changes induced by marginal vitamin C deficiency. Ann. N. Y. Acad. Sci., 258, 317–328.
6) Makino, S., and Katagiri, K. (1980): Osteogenic disorder rat. Exp. Anim. (Japan), 29, 374–375.
7) Mizushima, Y., Harauchi, T., Yoshizaki, T., and Makino, S. (1984): A rat mutant unable to synthesize vitamin C. Experimentia, 40, 359–361.
8) Horio, F., Ozaki, K., Yoshida, A., Makino, S., and Hayashi, Y. (1985): Requirement for ascorbic acid in a rat mutant unable to synthesize ascorbic acid. J. Nutr., 115, 1630–1640.
9) Ogawa, Y., and Kishigami, M. (1957): Study regarding to the measurement method for vitamin C in tissues. Annu. Rep. Shionogi Res. Lab. (in Japanese), 7, 635–647.
10) Yagi, K. (1976): A simple fluorometric assay for lipoperoxide in blood plasma. Biochem. Med., 15, 212–216.
11) Bates, C. J., Mandal, A. R., and Cole, J. J. (1977): H.D.L. cholesterol and vitamin-C status. Lancet, ii, 611.

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12) Banerjee, S., and Bandyopadhyay, A. (1963): Plasma lipids in scurvy: effect of ascorbic acid supplement and insulin treatment. *Proc. Soc. Exp. Biol. Med.*, 112, 372–374.

13) Ginter, E., Bobek, P., and Gerbolova. M. (1965): The influence of scorbut and prolonged low intake of vitamin C on serum lipoproteins in guinea-pigs. *Nutr. Dieta*, 7, 103–107.

14) Banerjee, S., and Bandyopadhyay, A. (1965): Plasma lipids in ascorbic acid-deficient rhesus monkeys. *Am. J. Physiol.*, 208, 329–333.

15) Ginter, E., Ondreicka, R., Bobek, P., and Šimko, V. (1969): The influence of chronic vitamin C deficiency on fatty acid composition of blood serum, liver, triglycerides and cholesterol esters in guinea pigs. *J. Nutr.*, 99, 261–266.

16) Banerjee, S., and Singh, H. D. (1958): Cholesterol metabolism in scorbutic guinea pigs. *J. Biol. Chem.*, 233, 336–339.

17) Bjorkhem, I., and Kallner, A. (1976): Hepatic 7α-hydroxylation of cholesterol in ascorbate-deficient and ascorbate-supplemented guinea pigs. *J. Lipid Res.*, 17, 360–365.

18) Ikezawa, K., Matsuoka, A., and Okamoto, S. (1983): Vitamin C in platelets as a activator of platelets. *Bitamin (Vitamins)*, 57, 39.