Ascorbic Acid Deficiency Stimulates Hepatic Expression of Inflammatory Chemokine, Cytokine-Induced Neutrophil Chemoattractant-1, in Scurvy-Prone ODS Rats

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Summary  ODS rat has a hereditary defect in ascorbic acid biosynthesis and is a useful animal model for elucidating the physiological role of ascorbic acid. We previously demonstrated by using ODS rats that ascorbic acid deficiency changes the hepatic gene expression of acute phase proteins, as seen in acute inflammation. In this study, we investigated the effects of ascorbic acid deficiency on the production of inflammatory chemokine, cytokine-induced neutrophil chemoattractant-1 (CINC-1), in ODS rats. Male ODS rats (6 wk of age) were fed a basal diet containing ascorbic acid (300 mg/kg diet) or a diet without ascorbic acid for 14 d. Obvious symptoms of scurvy were not observed in the ascorbic acid-deficient rats. Ascorbic acid deficiency significantly elevated the serum concentration of CINC-1 on d 14. The liver and spleen CINC-1 concentrations in the ascorbic acid-deficient rats were significantly elevated to 600% and 180% of the respective values in the control rats. However, the lung concentration of CINC-1 was not affected by ascorbic acid deficiency. Ascorbic acid deficiency significantly elevated the hepatic mRNA level of CINC-1 (to 480% of the value in the control rats), but not the lung mRNA level. These results demonstrate that ascorbic acid deficiency elevates the serum, liver and spleen concentrations of CINC-1 as seen in acute inflammation, and suggest that ascorbic acid deficiency stimulate the hepatic CINC-1 gene expression.

Key Words  ascorbic acid, chemokine, CINC-1, inflammation, ODS rat

The ODS rat (genotype od/od) with a hereditary defect in ascorbic acid biosynthesis (1) is a useful model for investigating the physiologic role of ascorbic acid. This rat cannot synthesize ascorbic acid because of the lack of L-gulono-γ-lactone oxidase (EC 1.1.3.8), which catalyzes the terminal step of ascorbic acid biosynthesis (2). Previously, we (3) suggested the dietary requirement of ascorbic acid in ODS rats, and examined the physiological significance of ascorbic acid using ODS rats (4–8).

In ODS rats, we (9) found that ascorbic acid deficiency changes the hepatic gene expression of positive and negative acute phase proteins, as seen in acute inflammation. That is, ascorbic acid deficiency elevates the hepatic mRNA levels and serum concentrations of negative acute phase proteins, such as apolipoprotein A-I, albumin and α2u-globulin. Moreover, the serum concentration of interleukin-6 (IL-6) was significantly elevated in ascorbic acid-deficient ODS rats (3). These results suggest that ascorbic acid deficiency causes physiological changes similar to those that occur in acute inflammation.

Acute inflammation involves the secretion of inflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin-1 (IL-1), IL-6, and IL-8. TNFα and IL-1 are the primary inflammatory cytokines and mediate other inflammatory cytokines such as IL-6 and IL-8. These cytokines cause the accumulation of neutrophils in local tissues, induction of adhesion molecules, and positive acute phase protein expression (10–12). Cytokine-induced neutrophil chemoattractant-1 (CINC-1) is a member of the IL-8 family in rats. CINC-1 has a potent chemotactic activity mediating the accumulation of neutrophils in rat tissues, and is called a chemokine. Therefore, CINC-1 is one of the inflammatory chemokine members, and its production is stimulated in acute inflammation. CINC-1 is also involved in process of tissue damage provoked by inflammation.
The aim of this study was to investigate, for the first time, the effect of ascorbic acid deficiency on CINC-1 production in ODS rats, in order to more precisely understand the physiological changes observed in ascorbic acid deficiency.

**MATERIALS AND METHODS**

*Animals and diets.* Male ODS (od/od) rats, 5 wk of age, were purchased from CLEA Japan, Inc. (Tokyo, Japan). They were housed in individual wire screen-bottomed cages in the animal colony of Nagoya University and maintained at 24°C with a 12-h light cycle (lights on from 08:00 to 20:00 h). The rats were allowed free access to water and a purified diet. The composition of the basal diet is shown in Table 1. The dietary addition of 300 mg of ascorbic acid/kg diet is sufficient for maximum growth and prevents the development of scurvy in ODS rats (3). All rats were fed the basal diet for 7 d before the start of the experiment, and then the experimental diet from day 1 through 14. In this experiment, the rats were killed by decapitation between 10:00 and 11:00 h, and all procedures were performed in accordance with the Animal Experimentation Guides of Nagoya University.

*Experimental procedures.* During the experimental period, the rats were fed the basal diet containing 300 mg of ascorbic acid/kg diet (control group) or the diet without ascorbic acid (ascorbic acid-deficient group). Five rats in each group were killed on the morning of day 14 after the start of the experiment. The food intake of the ascorbic-acid deficient rats began to decrease slightly on day 12. Therefore, the rats in the control group were pair-fed the mean amount consumed by the rats in the ascorbic-acid-deficient group from day 12 to 14. On day 12, blood was collected from tail vein of each rat. On day 14, rats were killed by decapitation and blood was collected simultaneously. Serum was prepared from whole blood by centrifugation at 1,600 g for 10 min. The serum was immediately used for the measurement of the CINC-1 concentration. The liver, spleen and lungs were removed, frozen immediately in liquid nitrogen, and stored at −80°C until use for the measurement of CINC-1, extraction of total RNA and determination of ascorbic acid.

**Assay of serum alkaline phosphatase activity.** The serum alkaline phosphatase activity was assayed using a commercial assay kit (Alkaline phosphor B Test, Wako Pure Chemical Industries, Ltd., Osaka, Japan).

**Determination of tissue and serum ascorbic acid concentration.** The liver, spleen and lung were homogenized in ice-cold 5% (w/v) metaphosphoric acid and centrifuged for 10 min at 1,600×g. In the case of serum, 10% metaphosphoric acid was added to the serum (1:1) and kept in ice for 1 min. This mixture was centrifuged for 10 min at 1,600×g. The resulting supernatant was used for the measurement of ascorbic acid concentration by the dinitrophenylhydrazine method (14), with a modification in which the oxidation of ascorbic acid was accomplished with 2,6-dichlorophenol-indophenol.

**Determination of serum and tissue CINC-1 concentrations.** The serum CINC-1 concentration on day 12 or 14 was measured by enzyme-linked immunosorbent assay (ELISA) with a commercial assay kit according to the manufacturer’s directions (Wako Pure Chemical Industries, Ltd.).

The liver, spleen and lung were homogenized in 0.1 M phosphate buffer (pH 7.4) and centrifuged for 15 min at 3,000×g. CINC-1 in the supernatant was determined by the commercial ELISA kit described above.

**Northern blot analysis and cDNA clones.** The total RNA was extracted from the liver, spleen and lungs by the method of Chomczynski and Sacchi (15). Twenty micrograms of the extracted RNA from each tissue was used for Northern blot analysis. Northern blot analysis was performed according to the method described previously (9).

The cDNA clones for rat CINC-1 (16), rat Apo E (17) and rat glyceraldehyde 3-dehydrogenase (GAPDH) (18) were kindly provided by K. Konishi of The Nippon Dental University (Tokyo, Japan), J. M. Taylor of Gladstone Foundation Laboratories (San Francisco, CA) and K. Hitomi of Nagoya University (Nagoya, Japan), respectively.

**Statistical analysis.** The values in the text are the mean±SE. The mean values obtained for the control and ascorbic acid-deficient groups were compared using Student’s t test (Statview, version 4). Differences with a p-value <0.05 were regarded as significant.

**RESULTS**

**Body weight, tissue weight, and serum and tissue ascorbic acid concentration**

The final body weight, relative liver weight, relative spleen weight and relative lung weight did not differ in the control and ascorbic acid-deficient groups (Table 2). No signs of scurvy were observed in any rats of the ascorbic acid-deficient group during the course of the experiment. The serum activities of alkaline phos-
Haptase, whose activity is known to be lowered in the ascorbic acid deficiency of ODS rats (1), were not different between the control and ascorbic acid-deficient groups. The serum concentration of haptase in the ascorbic acid-deficient group was significantly lower than that in the control group. Moreover, the liver, spleen and lung concentrations of this vitamin in the ascorbic acid-deficient group were also remarkably lower than the respective values in the control group.

**CINC-1 concentration in serum, liver, spleen and lungs**

On day 12, there was a tendency for the ascorbic acid deficiency to elevate the serum concentration of CINC-1, but not significantly. However, on day 14, the serum concentration of CINC-1 was significantly elevated to 160% of that in the control group (Fig. 1). On day 14, the hepatic concentration of CINC-1 in the ascorbic acid-deficient group was significantly higher than that in the control group, reaching 600% of the control value (Fig. 2). The spleen concentration of CINC-1 in the ascorbic acid-deficient group was also significantly elevated to 180% of the control value. However, the lung concentrations of CINC-1 in the control and ascorbic acid-deficient groups were not different.

**Hepatic mRNA levels of CINC-1, haptoglobin, apolipoprotein A-I, apolipoprotein E, and lung mRNA level of CINC-1**

The hepatic mRNA level of CINC-1 in the ascorbic acid-deficient group was significantly greater than that in the control group, reaching 480% of the control value (Fig. 3). The haptoglobin mRNA level was significantly higher (170% of the control value) in the ascorbic acid-deficient group than in the control group. In contrast, the Apo A-I mRNA level in the ascorbic acid-deficient group was significantly lowered, to 60% of the control value. The hepatic mRNA level of Apo E was not affected by ascorbic acid deficiency.

In the case of the spleen total RNA, we were not able to observe reliable signals of CINC-1 mRNA in northern blot analysis.

The lung mRNA level of CINC-1 in the ascorbic acid-deficient group was not different from the control value (Fig. 4). The lung mRNA level of GAPDH was not

| Table 2. Initial body weight, final body weight, organ weight, serum and tissue ascorbic acid concentrations, and serum alkaline phosphatase activity in the control and ascorbic acid-deficient groups.1 |
|-----------------|-----------------|-----------------|
| Group           | Control          | Ascorbic acid-deficient |
| Initial body weight (g) | 139.1±3.4        | 138.9±2.3        |
| Final body weight (g) | 204.8±4.4        | 196.6±3.4        |
| Organ weight (g/100 g body weight) | | |
| Liver           | 3.88±0.06        | 4.07±0.10        |
| Spleen          | 0.52±0.02        | 0.46±0.02        |
| Lung            | 0.23±0.01        | 0.26±0.01        |
| Ascorbic acid concentration | | |
| Serum (μg/mL)   | 3.90±0.36        | 0.84±0.05**      |
| Liver (μg/g)    | 136±5            | 8±1**            |
| Spleen (μg/g)   | 469±15           | 53±2**           |
| Lung (μg/g)     | 251±10           | 27±1**           |
| Serum alkaline phosphatase activity (units/L) | 348±6 | 327±14 |

1 Values are means±SE, n=5. From day 12 to 14, the control group was pair-fed the amount consumed by the ascorbic acid-deficient group. Means that are significantly different from controls are denoted; **p<0.01 by Student’s t test.
affected by ascorbic acid deficiency, either.

The Apo E mRNA level in the liver and GAPDH mRNA level in the lungs were measured as control genes whose expressions were not affected by ascorbic acid deficiency.

**DISCUSSION**

In ODS rats, we demonstrated in both the previous study (9) and the present study that ascorbic acid deficiency without obvious signs of scurvy stimulated the hepatic expressions of positive acute phase protein genes and decreased the hepatic expressions of negative acute phase protein genes, as seen in acute inflammation. From these results, we assumed that the production of inflammatory cytokines and chemokines are stimulated in ascorbic acid deficiency. In the present study, it was found that the serum concentration of CINC-1, which is an inflammatory chemokine and a member of IL-8, was significantly elevated in the ascorbic acid-deficient ODS rats. Moreover, we are also planning to analyze neutrophil infiltration into the liver by histological examination.

The expressions of inflammatory cytokine and chemokine genes are regulated by some inflammation-specific transcription factors. Nuclear factor-κB (NF-κB) is one of the transcription factors which are activated under acute inflammation. NF-κB plays an important role in the activation of cytokine expression. The expression of the CINC-1 gene is also NF-κB dependent. It is known that reactive oxygen intermediates are second messengers in the activation of NF-κB (21) and included in the stimulation of inflammatory cytokine gene expressions. Ascorbic acid has a capacity to scavenge reactive oxygen intermediates, and is a physiological compound exhibiting this capacity (22). In the liver of ascorbic acid-deficient ODS rats, it is speculated that the increase in the amount of reactive oxygen intermediates might cause the activation of NF-κB, leading to

Fig. 3. Hepatic mRNA levels of CINC-1, haptoglobin, apolipoprotein A-I (Apo A-I) and apolipoprotein E (Apo E) on day 14 in the control (Cont.) and ascorbic acid-deficient (Def.) groups. Values are means±SE, n=5. Values are presented as a percentage of the mean of each control group. **Significantly different (**p<0.01; *p<0.05) from the value of the control group by Student’s t test.

Fig. 4. Lung mRNA levels of CINC-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) on day 14 in the control (Cont.) and ascorbic acid-deficient (Def.) groups. Values are means±SE, n=5. Values are presented as a percentage of the mean of each control group.
the stimulation of CINC-1 gene expression.

In conclusion, it was demonstrated for the first time that ascorbic acid deficiency elevated the serum concentration of inflammatory chemokine, CINC-1, in ODS rats, and suggested that ascorbic acid deficiency stimulated the hepatic CINC-1 gene expression. Taken together with previous results (9), ascorbic acid deficiency seems to cause physiological changes similar to those caused by acute inflammation. Thus, these results suggest that ascorbic acid might have a physiological potency to suppress inflammatory responses.

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REFERENCES

1) Konishi T, Makino S, Mizushima Y, Harauchi T, Hasegawa Y, Yoshizaki T, Kishimoto Y, Oohara T. 1990. What is the ODS rat? Historical description of the characterization studies. In: Vitamin C and the scurvy-prone ODS rat (Fujita T, Fukase M, Konishi T, eds), p 3–21. Elsevier Science Publishers, Amsterdam.
2) Kawai T, Nishikimi M, Ozawa T, Yagi K. 1992. A missense mutation of l-gulono-γ-lactone oxidase causes the inability of scurvy-prone osteogenic disorder rats to synthesize L-ascorbic acid. J Biol Chem 267: 21973–21976.
3) Horio F, Ozaki K, Yoshida A, Makino S, Hayashi Y. 1985. Requirement for ascorbic acid in a rat mutant unable to synthesize ascorbic acid. J Nutr 115: 1630–1640.
4) Horio F, Ozaki K, Kohmura M, Yoshida A, Makino S, Hayashi Y. 1986. Ascorbic acid requirement for the induction of microsomal drug-metabolizing enzymes in a rat mutant unable to synthesize ascorbic acid. J Nutr 116: 2278–2289.
5) Horio F, Ozaki K, Oda H, Makino S, Hayashi Y, Yoshida A. 1989. Effect of dietary ascorbic acid, cholesterol and PCB on cholesterol and bile acid metabolism in a rat mutant unable to synthesize ascorbic acid. J Nutr 119: 409–415.
6) Horio F, Takahashi N, Makino S, Hayashi Y, Yoshida A. 1991. Ascorbic acid deficiency elevates serum level of LDL-cholesterol in a rat mutant unable to synthesize ascorbic acid. J Nutr Sci Vitaminol 37: 63–71.
7) Ikeda S, Horio F, Yoshida A, Kakinuma A. 1996. Ascorbic acid deficiency reduces hepatic apolipoprotein A-I mRNA in scurvy-prone rats. J Nutr 126: 2505–2511.
8) Ikeda S, Takasu M, Matsuda T, Kakinuma A, Horio F. 1997. Ascorbic acid deficiency decreases the renal level of kidney fatty acid-binding protein by lowering the a2u-globulin gene expression in liver in scurvy-prone ODS rats. J Nutr 127: 2173–2178.
9) Ikeda S, Horio F, Kakinuma A. 1998. Ascorbic acid deficiency changes hepatic gene expression of acute phase proteins in scurvy-prone ODS rats. J Nutr 128: 832–838.
10) Cybulsky MI, McComb DJ, Movat HZ. 1989. Protein synthesis dependent and independent mechanisms of neutrophil emigration: different mechanisms of inflammation in rabbits induced by interleukin-1, tumor necrosis factor α or endotoxin versus leukocyte chemoattractants. Am J Pathol 135: 227–237.
11) Carlos TM, Harlan JM. 1994. Leukocyte-endothelial adhesion molecules. Blood 84: 2068–2101.
12) Baumann H, Gauldie J. 1994. The acute phase response. Immunol Today 15: 74–80.
13) American Institute of Nutrition. 1993. AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN76A rodent diet. J Nutr 123: 9–151.
14) Roe JH, Kuether CA. 1943. The determination of ascorbic acid in whole blood and urine through the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid. J Biol Chem 147: 399–407.
15) Chomczynski P, Sacchi N. 1987. Single step method of RNA isolation by acid guanidium thiocyanate-phenolchloroform extraction. Anal Biochem 162: 156–159.
16) Konishi K, Tanaka Y, Yamamoto M, Yokogawa K, Watanabe K, Tsurafuji S, Fujikoka M. 1993. Structure of the gene encoding rat neutrophil chemo-attractant Gro. Gene 126: 285–286.
17) McLean JW, Fukazawa C, Taylor JM. 1983. Rat apolipoprotein E mRNA. Cloning and sequencing of double-stranded cDNA. J Biol Chem 258: 8993–9000.
18) Eddy EM, Toshimori K, O’Brien DA. 2003. Fibrous sheath of mammalian spermatozoon. Microsc Res Tech 61: 103–115.
19) Ohira H, Ueno T, Shakado S, Sakamoto M, Torimura T, Inuzuka S, Sata M, Tanikawa K. 1994. Cultured rat hepatic sinusoidal endothelial cells express intercellular adhesion molecule-1 (ICAM-1) by tumor necrosis factor-α or interleukin-1α stimulation. J Hepatol 20: 729–734.
20) Stanley TP, Schmal H, Warner RL, Schmid E, Friedl HP, Ward PA. 1997. Requirement for C-X-C chemokines (macrophage inflammatory protein-2 and cytokine-induced neutrophil chemoattractant) in IgG immune complex-induced lung injury. J Immunol 158: 3439–3448.
21) Shea LM, Buehler C, Shnirman M, Shenker R, Tuder R, Abraham E. 1996. Hypoxia activates NF-κ B and increases TNF-α and IFN-γ gene expression in mouse pulmonary lymphocytes. J Immunol 157: 3902–3908.
22) Yamaguchi T, Hashizume T, Tanaka M, Nakayama M, Sugimoto A, Nakajima H, Horio F. 1997. Bilirubin oxidation provoked by endotoxin treatment is suppressed by feeding ascorbic acid in a rat mutant unable to synthesize ascorbic acid. Eur J Biochem 245: 233–240.