Role of Hydrogen Peroxide in the Inhibitory Effect of Ascorbate on Cell Growth

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Summary Organ cultures have become an important method for the study of some of the biochemical reactions in which ascorbate takes part, and there are conflicting views on the stimulatory and inhibitory actions of ascorbate on cell growth. This study aimed to clarify the inhibitory mechanism of ascorbate using 3T6 fibroblasts. Cells were exposed to ascorbate at various concentrations in medium. The results showed that 3T6 fibroblasts were killed in medium containing more than 0.3 mM ascorbate. This lethal effect of ascorbate on cells was inhibited by the addition of catalase, an enzyme that decomposes hydrogen peroxide (H$_2$O$_2$), to the medium. When the medium with ascorbate was incubated in the absence of cells, the amount of H$_2$O$_2$ generation depended on the ascorbate concentration, and decreased in inverse proportion to the serum concentration added to the medium. The addition of albumin, which is the main protein in serum, also inhibited H$_2$O$_2$ generation in the medium with ascorbate. However H$_2$O$_2$ generation was not inhibited completely by serum and albumin. These results indicate that cytotoxicity of ascorbate is induced by H$_2$O$_2$.

Key Words ascorbate, cell growth, catalase, hydrogen peroxide

Ascorbate is widely used in experiments employing cultured cells for metabolism, especially collagen studies. Many authors reported the stimulatory action of ascorbate on collagen synthesis, and consequently on DNA synthesis (1, 2). For example, after prolonged exposure to ascorbate at 0.25 mM, collagen synthesis in cultured human skin fibroblasts increased eightfold compared with that in experiments conducted without ascorbate (3). Ascorbate treatment at 0.1 mM resulted in a twofold stimulation of procollagen mRNA (4). Ascorbate at 0.2 mM stimulated matrix formation in rabbit chondrocytes (5). Ascorbate at 0.1 mM also played a role in glycosaminoglycan production (6). In contrast, an inhibitory effect of ascorbate on cell proliferation has been reported. Chick embryo fibroblasts were killed by the addition of ascorbate at 0.05 to 0.25 mM (7). With supplemental
ascorbate at 0.38 mM, the cells grew at a slower rate and had a shorter lifespan (8). Ascorbate at 1.1 mM, which was normally found in aqueous humor, killed fibroblasts derived from human Tenon's capsule (9). Ascorbate has been considered to be an anti-cancer agent since cytotoxic effects of malignant melanoma cells were determined at concentrations over 0.2 mM (10).

From these reports, it is suggested that high concentrations of ascorbate induce cytotoxicity while low concentrations of ascorbate have a stimulatory effect on growth. Some reports assessed \( \text{H}_2\text{O}_2 \) in the presence of metal ions to be toxic (7–11), but they barely measured \( \text{H}_2\text{O}_2 \) generated by ascorbate directly. Therefore, the current level of understanding regarding \( \text{H}_2\text{O}_2 \) generated by ascorbate remains limited with regard to the time course of generation, effect of temperature, dependence on concentration, and relationship with co-present components.

The purpose of this study was to clarify the cause of cytotoxicity by ascorbate. To precisely measure \( \text{H}_2\text{O}_2 \) generated by ascorbate, we used the oxygen electrode method for simple, rapid, and reproducible measurements of \( \text{H}_2\text{O}_2 \) (12). Our results identified \( \text{H}_2\text{O}_2 \) as one of the components responsible for cytotoxicity induced by ascorbate. We also discuss the toxicity of ascorbate against cultured cells.

**MATERIALS AND METHODS**

**Materials.** Dulbecco’s modified Eagle’s medium (DMEM) from Nissui Pharmaceutical Co. (Tokyo, Japan), fetal bovine serum (FBS) and trypsin (1:250) from GIBCO, N.Y., penicillin G and kanamycin sulfate from Meiji Seika (Tokyo, Japan), \( \text{NaHCO}_3 \) solution from Otsuka Co. (Tokyo, Japan), L-glutamine from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and culture plates from Corning were used. L-Ascorbic acid sodium salt and catalase were obtained from Kanto Chemical Co. (Tokyo, Japan). Albumin from Sigma (St. Louis, MO). Purified water from Kosakai Co. (Tokyo, Japan).

**Cell culture.** 3T6 fibroblasts, a mouse cell line, were purchased from Dainippon Co. (Osaka, Japan). Cells (5 to 8 \( \times \) 10^4) were plated onto 60-mm dishes and grown in 3 ml of DMEM supplemented with penicillin G (5,000 U/liter), kanamycin sulfate (50 mg/liter), \( \text{NaHCO}_3 \) (0.017 M), L-glutamine (4 mM), and 5% FBS (DMEM-5) in the absence or presence of ascorbate and catalase.

Cells were cultured at 37°C in an atmosphere of 5% \( \text{CO}_2 \) in air. The medium was changed daily. Ascorbate was freshly prepared immediately before addition to the medium.

**Cell analysis.** After removal of the medium, the cell layer was rinsed twice with 1 ml of phosphated-buffered saline (PBS(−)). The cells were dispersed by trypsin-EDTA, after which the cell number was determined by hemocytometer.

**Determination of hydrogen peroxide.** The concentration of \( \text{H}_2\text{O}_2 \) was determined by using the oxygen electrode (Oritector, Oriental Electric Co. Tokyo), based on measurement of the \( \text{H}_2\text{O}_2 \) decomposition rate at which released oxygen is
detected (12). The samples were taken into the reaction cell of a dissolved oxygen meter equipped with a sensitive oxygen electrode, and N₂ gas was bubbled into the cell to remove dissolved oxygen. Ascorbate which was confirmed not to contain catalase activity was added to the medium. After the addition of ascorbate, the dishes were incubated under the same conditions with cell culture. Every 10 min after the addition of ascorbate, H₂O₂ generation in the medium was measured. To investigate the effect of ascorbate concentration, effect of temperature and effect of serum on H₂O₂ generation, aqueous ascorbate solution dissolved in purified water or medium containing ascorbate were incubated in the absence of cells.

RESULTS

Figure 1 shows the effect of ascorbate at concentrations of 0.1 and 5 mM on the morphology of 3T6 fibroblasts. Cells cultured in 0.1 mM ascorbate proliferated actively and maintained an elongated shape (Fig. 1a). In contrast, the addition of 5 mM ascorbate influenced the shape of the cells (Fig. 1b). The number of the cells did not increase further.

The lethal effects of ascorbate were prevented by the addition of catalase, which decomposes H₂O₂ to give H₂O and O₂ to the medium (Fig. 2). This indicates that the cytotoxic effect of ascorbate is exerted extracellularly since large proteins such as catalase can not penetrate the cell membrane. Consequently, the mechanism responsible for ascorbate cytotoxicity may involve H₂O₂ generation in the
Fig. 1b

Fig. 1. Monolayers of 3T6 fibroblasts cultured for 2 days in the presence of 0.1 mM (a) or 5 mM (b) ascorbate. Scale = 20 μm.

Fig. 2. The effect of catalase on the toxicity of ascorbate in the growth of 3T6 fibroblasts. Cells were cultured in the following medium. ○, DMEM-5; ▲, DMEM-5 + 0.5 mM ascorbate; ●, DMEM-5 + 0.5 mM ascorbate + 400 U/ml catalase.

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medium in the presence of ascorbate. Accordingly, the characteristics of \( \text{H}_2\text{O}_2 \) generation in ascorbate solution were examined.

Figure 3 shows the changes in \( \text{H}_2\text{O}_2 \) concentration when various concentrations of ascorbate solution in the absence of cells were incubated at room temperature. \( \text{H}_2\text{O}_2 \) generated in ascorbate solution increased with increasing concentration of ascorbate. In the case of 40 mM ascorbate, \( \text{H}_2\text{O}_2 \) concentration increased linearly up to about 2 h of incubation and remained at the maximum up to 9 h.

Fig. 3. Effect of various concentrations of ascorbate on \( \text{H}_2\text{O}_2 \) generation. Ascorbate in purified water at concentrations of 10 mM (○), 20 mM (■), and 40 mM (▲) were incubated at room temperature.

Fig. 4. Changes in \( \text{H}_2\text{O}_2 \) concentration in medium and purified water. Ascorbate (5 mM) in purified water (○) or DMEM-5 (□) were incubated at 37°C.
H$_2$O$_2$ generation in the medium containing ascorbate at 37°C was examined (Fig. 4). H$_2$O$_2$ was generated in the purified water immediately after the addition of ascorbate, but not in the medium. It began to increase after 6 h. H$_2$O$_2$ was generated later in the medium than in the water; this may be due to serum, which contains many kinds of components, such as H$_2$O$_2$ scavengers and inhibitors for

Fig. 5. Effect of serum on H$_2$O$_2$ generation. Ascorbate (1 mM) in purified water containing 70% (○), 60% (■), 50% (▲), 10% (●), and 5% (△) serum was incubated at 37°C.

Fig. 6. Effect of albumin added to medium on H$_2$O$_2$ generation. Ascorbate (5 mM) in DMEM in the absence (○) or presence (●) of 50 μM albumin was incubated at 37°C.
H₂O₂ or H₂O₂ generation. To clarify the effect of serum, we determined H₂O₂ generation in ascorbate solution containing serum (Fig. 5). In high concentrations of serum (80%, 90%), H₂O₂ was not detected. As the serum concentration was lowered, H₂O₂ generation increased in proportion, indicating that serum may have some components which inhibit H₂O₂ generation.

Albumin was used to test whether or not protein inhibited the generation of H₂O₂ in a high concentration of serum, since albumin is the major component in serum. H₂O₂ concentration in the medium supplemented with ascorbate and albumin is shown in Fig. 6. Albumin inhibited H₂O₂ generation.

**DISCUSSION**

Although it is well known that dietary ascorbic acid is required for the prevention of scurvy (6–10 mg/day), a definitive daily requirement to optimize the functions of ascorbic acid other than for prevention of scurvy is uncertain and continues to be controversial. Recommendations for intake of vitamin C range from 50 mg/day based on Recommended Dietary Allowance (RDA) to megadose intakes (>1 g/day) based on saturating tissues and fluids to maximize these functions. In order to define the human requirement of ascorbic acid, further studies regarding the functions of ascorbic acid other than the prevention of scurvy are needed at the cellular level. In humans, the concentration of ascorbic acid depends on the amount of uptake from foods; in general, the normal concentration is 0.06–0.08 mM in blood and 1.0–1.5 mM in tissues. However, 1 mM ascorbate led to cytotoxicity on monolayer cultures of fibroblasts (7).

Ascorbate, which is well known to be a cofactor of the hydroxylation enzyme of procollagen, is often used in studies on collagen synthesis. Many workers have reported that more than 0.5 mM ascorbate was less effective than 0.1 mM for activation of collagen synthesis (13), while more than 0.2 mM damaged DNA synthesis (14), and more than 0.1 mM inhibited total protein synthesis, followed by late neuronal death (15).

The present report also shows cytotoxicity of ascorbate. Our results indicate the inhibition of cell growth by the addition of ascorbate at excess concentrations and are consistent with those of many other workers who have used cultured cells with ascorbate (7–9,15).

Since the addition of catalase to the medium prevented the lethal effect of ascorbate, we assume that the cytotoxicity of ascorbate is due to H₂O₂ generated from ascorbate in the medium. The amounts of H₂O₂ were dependent on ascorbate concentration, that is, a high concentration of ascorbate led to the generation of large amounts of H₂O₂.

H₂O₂ generation was inhibited by medium for 6 h, and gradually increased until 48 h. Therefore, cells exposed to ascorbate might eventually show cytotoxicity. Further, the higher the amounts of serum added to the medium, the less H₂O₂ was generated. Since same components in serum seem to inhibit H₂O₂ generation,
the effect of albumin, the major protein in serum, was examined. The addition of albumin to the medium inhibited H$_2$O$_2$ generation. Albumin is known to be a scavenger of ·OH (16) with the following mechanism. Albumin reacts with H$_2$O$_2$ and produces ·OH, after which albumin scavenges ·OH. Consequently, H$_2$O$_2$ generation seems to be inhibited by albumin.

$\text{Albumin} + \text{H}_2\text{O}_2 \rightarrow \cdot\text{OH},$

$\cdot\text{OH} + \text{albumin} \rightarrow \text{H}_2\text{O}.$

Also, proteins like albumin may play a role in stabilizing ascorbate by binding ascorbate (17).

There may also be another reason for the inhibition of H$_2$O$_2$ by serum, i.e. serum contains some enzyme such as catalase, which decomposes H$_2$O$_2$.

Since catalase decomposes H$_2$O$_2$, the addition of catalase in the medium completely prevents cytotoxicity by H$_2$O$_2$ or ·OH (18). Rowley reported that a mixture of ascorbate, iron salt, and H$_2$O$_2$ produced ·OH, and the detected radicals can be decreased by catalase but not by bovine serum albumin (19).

In vivo investigations to evaluate the effects of megadoses of ascorbate suggest that megadoses of ascorbate may have clinical significance. The effects of high concentrations of ascorbate should also be investigated at the cellular level. However, in a cell culture system, since high concentrations of ascorbate have an inhibitory effect on cell growth, the ascorbate effect in excess physiological concentrations is still unknown.

In the present study, we found cytotoxicity by ascorbate to be induced by H$_2$O$_2$. Taking into account the fact that enzymes such as catalase, which decompose H$_2$O$_2$, are present in large amounts in living tissue, cytotoxicity on experimental cell growth by ascorbate may not accurately reflect the effect of ascorbate in vivo.

We thus recommend the addition of catalase or some peroxidase into the medium when cultured cells are used to study the physical functions of high concentrations of ascorbate.

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