Inhibitory Effect of Ascorbate on Cell Growth: Relation to Catalase Activity

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Summary The effect of ascorbate on cell growth was examined using primary cultured hepatocytes and chondrocytes elicited from guinea pigs and six kinds of cell lines derived from the tissue and blood of mammals. Cells were cultured in medium supplemented with or without ascorbate at various concentrations for 24 and 48 h. There were differences among the cells used here in the effect of ascorbate on growth, and also in the concentrations of ascorbate required to lower cell viabilities. This indicates that different cell species have varying sensitivities to ascorbate in medium. On the other hand, cells such as HL-60, which showed growth inhibition at higher concentrations of ascorbate in medium among observed cells, were damaged by the exposure to higher concentrations of hydrogen peroxide (H_2O_2). Furthermore, there was a positive correlation between the activity of catalase in cells that decomposed H_2O_2 and the concentration of ascorbate required to lower cell viability ($p < 0.01$). These results indicate that the concentration of ascorbate in medium required to inhibit cell growth depends on the activity of catalase in the cells.

Key Words ascorbate, cell growth, catalase, hydrogen peroxide

Many authors have reported that ascorbate has stimulatory (1–3) and inhibitory effects (4–6) on cell growth *in vitro*. The redox properties of ascorbate are considered to be important in these conflicting results. It has been assumed that H_2O_2 derived from an aqueous solution of ascorbate in the presence of metal ions is responsible for the inhibitory effect of ascorbate (7–9). Our previous paper (10) revealed that H_2O_2 generated in ascorbate solution increased with increasing concentrations of ascorbate. However, little is known of the cytotoxic mechanism of ascorbate in medium.

Alcain et al. (11) reported that the stimulatory effects of ascorbate might be due to the enhancement of transmembrane redox enzyme activity by converting ascorbate to an ascorbate free radical. It is of interest that both the stimulatory and inhibitory effects of ascorbate can be explained by the same process. Kao et al. (12)

also reported that the cytotoxic effects of ascorbic acid on lymphocyte cell lines sensitive to ascorbic acid were time- and dosage-dependent.

In this study, using primary cultured cells elicited from guinea pigs and cell lines derived from the tissue and blood of mammals, we investigated the concentration of ascorbate and H_2O_2 in medium that affected cell growth, and catalase activity in the cells. We also discuss the relationship between ascorbate concentration inhibiting cell growth and catalase activity in cultured cells.

MATERIALS AND METHODS

Cell culture. HL-60 (derived from human blood, human promyelo leukemia cells), LT4Tr (derived from mouse spleen) and RCR1 (derived from rat brain) were obtained from Riken Cell Bank (Tsukuba, Japan), while 3T6 (derived from mouse embryo), PC12 (derived from rat pheochromocytoma) and UMR106 (derived from rat bone) were obtained from ATCC. Primary cultured hepatocytes and chondrocytes were prepared from guinea pigs according to the methods of Seglen (13) and Shimomura et al. (14), respectively. Hepatocyte, chondrocyte, and cell lines of 3T6, PC12 and UMR106 were cultured in Dulbecco's modified Eagle's medium (Nissui Pharmaceutical Co., Tokyo). The cell lines of HL-60 and LT4Tr were cultured in RPMI 1640 medium (Nissui), while RCR1 cells were cultured in DM-160AU medium (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo). These media were supplemented with 5% fetal bovine serum, penicillin (Meiji Seika, Tokyo), kanamycin sulfate (Meiji Seika), NaHCO_3 (Otsuka Co., Tokyo), and L-glutamine (Wako Pure Chemical Industries, Ltd., Osaka, Japan). To culture of LT4Tr cells, 0.05 mM 2-mercaptoethanol (Tokyo Kasei, Tokyo) was also added to the medium. The cells ($1 \times 10^5/3$ ml) were placed into 60-mm dishes and incubated at 37°C in a 5% CO_2 environment. To investigate the effect of ascorbate on cell growth, 0.1 mM L-ascorbic acid sodium salt (Kanto Chemical Co., Tokyo) was used, and medium was changed every two days. The final concentrations of 0.1–15 mM L-ascorbic acid sodium salt and 0.0001–2 mM H_2O_2 (Kanto Chemical) dissolved in each medium were freshly prepared immediately before use.

Measurement of viability. The cells were incubated in each medium with ascorbate or H_2O_2 for 24 or 48 h. After removal of the medium, the cell layer was rinsed twice with phosphate-buffered saline (PBS(–), Nissui Pharmaceutical). The adhesive cells were dispersed by EDTA and trypsin (GIBCO, NY, USA), for cell counts using a hemocytometer. Values are $M \pm \text{SD}$ of triplicate counting from 3 dishes.

Measurement of catalase activity. The cells were cultured for 3 days in medium without either ascorbate or H_2O_2 . For adhesive cells, the medium was removed from the culture dish and the cell layer was washed with PBS(–) three times. For non-adhesive cells, the cell suspension was centrifuged and the cells were washed with PBS(–) twice followed by the addition of PBS(–) containing 0.1% Triton X-100. After centrifugation, catalase activity of the supernatant was

determined from the spectrophotometric disappearance of H_2O_2 (15). The decomposition of 10 mM H_2O_2 was measured for 0–60 s at 240 nm. Activity is expressed in units per 10^8 cells according to the standard curve.

RESULTS

Figure 1 shows the growth curves of chondrocytes, HL-60, 3T6 and UMR106 when the cells were cultured either with or without ascorbate at 0.1 mM concentration. Two types of cells were observed: cell types susceptible to stimulation of growth by ascorbate (stimulating types), and insensitive to ascorbate (non-stimulating types). The cell count of the stimulating types, such as chondrocytes and HL-60, cultured with ascorbate was significantly higher than that without ascorbate. In contrast, the difference between cell growth with and without ascorbate was negligible in non-stimulating types such as 3T6 and UMR106. LT4Tr was a stimulating type, while other cells tested were non-stimulating types (data not shown).

Figure 2 shows the viabilities of cells cultured with various ascorbate concentrations for 24 h and 48 h without changing the medium. When the viability of each

Fig. 1. Effect of ascorbate on cell growth. Cells were cultured with (●) or without (○) 0.1 mM ascorbate. Values were $M \pm SD$. *Significantly different from without ascorbate ($p < 0.05$).

Fig. 2. Effect of ascorbate on cell growth. Cells were supplemented with ascorbate at various concentrations. The number of cells cultured for 24 h (■) and 48 h (▨) were counted. *Significantly different from the viability without AsA (0 mM) in 48 h culture ($p < 0.05$).

cell line cultured without ascorbate was expressed as 100% (control), the concentration of ascorbate lowering cell viability below 100% differed among the cells. The cell viabilities of non-stimulating types such as RCR1, PC12 and 3T6 decreased when cultured with more than 0.2 mM ascorbate for 48 h, whereas cell viabilities of stimulating types such as HL-60 and LT4Tr decreased significantly at the concentrations of 1 mM and 2 mM, respectively. When cultured with 2 mM ascorbate for 24 h, the viability of HL-60 was still over 200%, but that of 3T6, PC12, RCR1 and UMR106 was less than 50%. Hepatocytes, with cell viability decreasing at 4 mM, were the cells most resistant to ascorbate.

The minimum concentrations of ascorbate required to lower cell viability below 100% are summarized in Table 1. Table 1 also shows the concentrations of H_2O_2 needed for 50% inhibition of cell survival in the cells cultured for 48 h. The growth of cells with the highest level of H_2O_2 (chondrocyte and LT4Tr) was inhibited by relatively high concentrations of ascorbate (1 mM and 2 mM, respectively). In contrast, the growth of cells with lower levels of H_2O_2 (UMR106, RCR1, PC12 and 3T6) was inhibited by only 0.1 and 0.2 mM ascorbate. A positive

Table 1. Concentrations of H_2O_2 and ascorbate for inhibition of cell growth.

	Concentration of H_2O_2 for 50% inhibition of cell survival (mM)	Minimum concentration of ascorbate (mM)
Hepatocyte	—*	4
Chondrocyte	0.5	1
HL-60	0.05	1
LT4Tr	0.5	2
UMR106	0.1	0.1
RCR1	0.01	0.2
PC12	0.05	0.2
3T6	0.05	0.2

*not investigated.

Fig. 3. Catalase activity in various cell lines and primary cultured cells.

correlation between the concentrations of ascorbate lowering cell viabilities and the concentrations of H_2O_2 for 50% inhibition of cell survival at 48 h was observed ($r = 0.832$, $p < 0.05$).

Figure 3 shows the activity of catalase in the various species of cells. Hepatocytes, which showed reduced viability at more than 4 mM of ascorbate in medium, had the highest catalase activity of all cells used here. Moreover, HL-60, which also showed decreased viability at a relatively high ascorbate concentration (> 1 mM), similarly had high catalase activity. In contrast, cell species such as 3T6, RCR1, PC12 and UMR106, which showed reduced viability at less than 0.2 mM ascorbate, had low catalase activities. Viabilities of LT4Tr and chondrocyte decreased with supplementation of H_2O_2 or ascorbate at high concentrations, however, catalase activities of these cells were relatively low.

DISCUSSION

In order to clarify the physiological functions of ascorbic acid other than the prevention of scurvy, many researcher performed experiments employing cultured cells (16–19). Some authors reported inhibitory effects of ascorbate on the cell growth (4–6), however, the concentrations of ascorbate inhibiting cell growth were not consistent (7,20,21): chick embryo fibroblasts were killed by the addition of ascorbate at 0.05 to 0.25 mM (7), the growth of corneal endothelial cells was affected at 0.38 mM (20), and the plating efficiency of fibroblasts derived from human Tenon's capsule decreased at 1.1 mM (21). Other reports indicated that excess concentrations of ascorbate in medium suppressed physiological functions in cell lines; the syntheses of collagen (4), DNA (22), and protein (5).

Our current data of cell viability shown in Fig. 2 revealed that the differences in the concentrations of ascorbate required to inhibit cell growth were due to differences in the sensitivity of different cell species to ascorbate. That is, cells susceptible to stimulation of growth by ascorbate, such as hepatocytes, HL-60 and LT4Tr, were resistant to ascorbate at concentrations 50- to 100-fold higher than physiological levels; the normal concentration is 0.02–0.08 mM in serum (23) and 0.2–3 mM in tissues (24), whereas cells insusceptible to stimulation of growth by ascorbate showed reduced viability at relatively low concentrations of ascorbate.

Our previous report (10) indicated that the addition of catalase in medium completely prevented the cells from being damaged by supplementation of high amounts of ascorbate, suggesting that the lethal effect was caused by H_2O_2 generated from ascorbate in medium. In order to identify why sensitivity to ascorbate is different among various cell species, we measured cytotoxicity of H_2O_2 and found a significantly positive correlation between the concentration of H_2O_2 needed for 50% inhibition of cell survival and the concentration of ascorbate reducing cell viability was obtained ($p < 0.05$). Therefore, the ability of cells to decompose H_2O_2 might account for its sensitivity to ascorbate.

Catalase is one of the enzymes decomposing H_2O_2 *in vivo*. The activities of catalase in cells differed greatly depending on the cell species; moreover, there was a significantly positive correlation between the concentration of ascorbate lowering cell viability and the activity of catalase in cells ($r = 0.879$, $p < 0.01$). Ascorbate-resistant cells such as hepatocyte had the highest catalase activities. These results suggest that the ascorbate concentration required to reduce cell viability depends on the catalase activity of the cell. As for a possible mechanism, Cherry and Wolin (25) reported that ascorbate activated soluble guanylate cyclase purified from bovine lung via H_2O_2 metabolism by catalase.

However, the assumption above does not apply to some of the cell lines we observed. Concerning the deviation of LT4Tr from the correlation line, we found that the activity of catalase decreased because of the addition of 2-mercaptoethanol to the medium. On the other hand, HL-60 was relatively resistant to the cytotox-

icity of ascorbate. Hur et al. (26) isolated unprocessed extracellular myeloperoxidase that was secreted into the medium from HL-60. Therefore, it is supposed that other peroxidases such as myeloperoxidase and glutathione peroxidase decomposing H_2O_2 are associated with the effect of ascorbate on cell growth.

Although this study assumed that the cytotoxicity of ascorbate in medium was mainly due to H_2O_2 , substances other than H_2O_2 may be related to this cytotoxicity. For example, Rowley and Halliwell (27,28) reported that hydroxyl radicals derived from ascorbate as well as H_2O_2 were toxic. Since catalase can decompose both H_2O_2 and ascorbate-dependent hydroxyl radicals (29), high catalase activity in cells may be favorable for preventing the cytotoxicity of ascorbate. It is of interest whether high doses of ascorbate induce cytotoxicity *in vivo*. It has been thought that the action of ascorbate as a pro-oxidant is unlikely to be important *in vivo* because other reductants are present (30). Further studies are required in order to clarify the relationship between catalase activities and the toxicity of ascorbate *in vivo*.

In this study using cultured cells, we found that the inhibitory concentration of ascorbate in medium for cell growth was closely associated with the concentration of H_2O_2 needed for 50% inhibition of cell survival and with catalase activity. These observations suggest that catalase activity in cultured cells is a dominant factor in the stimulatory and inhibitory effects of ascorbate in medium on cell growth.

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