

## Mechanism of Ascorbic Acid-induced Reversion Against Malignant Phenotype in Human Gastric Cancer Cells

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**Objective** To find out the mechanisms of redifferentiation and reversion of malignant human gastric cancer cells induced by ascorbic acid. **Methods** Human gastric cancer cells grown in the laboratory were used. The Trypan blue dye exclusion method was used to determine the cell doubling time. The electrophoresis rate and colonogenic potential were the indices used to measure the rate of redifferentiation. The content of malondialdehyde (MDA) was measured using the thiobarbituric acid (TBA) method. The activities of superoxide dismutase (SOD), catalase (CAT) and the content of H<sub>2</sub>O<sub>2</sub> were evaluated by spectrophotography. **Results** Six mmol/L ascorbic acid was used as a positive control. Human gastric cancer cells were treated with 75 μm hydrogen peroxide, which alleviated many of the malignant characteristics. For example, the cell surface charge obviously decreased and the electrophoresis rate dropped from 2.21 to 1.10 μm·s<sup>-1</sup>·V<sup>-1</sup>·cm<sup>-1</sup>. The colonogenic potential, a measure of cell differentiation, decreased 90.2%. After treatment with ascorbic acid, there was a concentration- and time-dependent increase in hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) and the activity of superoxide dismutase (SOD). However, the activity of catalase (CAT) resulted in a concentration- and time-dependent decrease. SOD and 3-amino-1,2,4-triazole (AT) exhibited some effects, but there were statistically significant differences between the SOD and AT group and the H<sub>2</sub>O<sub>2</sub> group. **Conclusions** Ascorbic acid induces growth inhibition and redifferentiation of human gastric cancer cells through the production of hydrogen peroxide.

**Key words:** Aminotriazole; Ascorbic acid; Catalase; Gastric cancer; Hydrogen peroxide; Redifferentiation; Superoxide dismutase

### INTRODUCTION

In this laboratory, we have successfully induced human hepatoma cells and gastric cancer cells to redifferentiate using an ascorbic acid treatment<sup>[1-2]</sup>. The antioxidant properties of ascorbic acid (AA) are well known. Moreover, cytosolic ascorbate plays a significant role in cell defense against the toxic effects of free radicals and reactive oxygen species, although this protective role is not yet fully understood<sup>[3]</sup>. On the other hand, ascorbate also behaves as a pro-oxidant compound. Thus, an ascorbate-dependent, iron-catalyzed formation of peroxide has been described<sup>[4]</sup>. Furthermore, ascorbate accelerates the release of iron from ferritin, stimulating its pro-oxidant effects<sup>[5]</sup>. However, it is not clear whether ascorbate acts as a pro-oxidant under normal physiological conditions<sup>[6-8]</sup>.

Podmore *et al.*<sup>[9]</sup> found that AA administered as a dietary supplement to healthy humans exhibits a pro-oxidant as well as an antioxidant effect *in vivo*. Lee *et al.*<sup>[10]</sup> ascertained that AA induces lipid hydroperoxide decomposition to the DNA-reactive bifunctional electrophiles 4-oxo-2-nonenal, 4,5-epoxy-2 (*E*)-decenal, and 4-hydroxy-2-nonenal. The compound 4,5-epoxy-2 (*E*)-decenal is a precursor of etheno-2'-deoxyadenosine, a highly mutagenic chemical found in human DNA.

Ascorbate incubated in buffered solution undergoes auto-oxidation in the presence of oxygen at 37°C<sup>[11]</sup>. The first product of this oxidation is the intermediate free radical (AFR), which behaves both as one-electron oxidant and as one-electron reductant<sup>[12]</sup>, explaining both the anti-oxidative and pro-oxidant effects described for ascorbate. AFR is a relatively stable, non-hazardous biological free

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radical. However, ascorbate oxidation seems to contribute to the generation of other free radicals and reactive oxygen species such as hydroxyl, a superoxide radical, and hydrogen peroxide<sup>[5]</sup>. The low levels of catalase and peroxidase in cancer cells render them particularly sensitive to ascorbate toxicity<sup>[13-14]</sup>. In fact, ascorbic acid has been reported to be cytotoxic to Ehrlich ascites tumor cells<sup>[15]</sup> and some leukemia and pediatric tumors in humans<sup>[16-18]</sup>.

*In vitro* treatment of PC3 and MLL with sodium ascorbate (0-10 mmol/L) results in a decrease in cell viability and thymidine incorporation into DNA through the production of hydrogen peroxide<sup>[19]</sup>. In this laboratory, we have successfully induced human gastric cancer cells to redifferentiate with 6 mmol/L of AA. We also found that the H<sub>2</sub>O<sub>2</sub> content increases during redifferentiation of human gastric cancer cells<sup>[2]</sup>, but the mechanism remains unclear.

The aim of this study was to find out the mechanism of redifferentiation and growth inhibition of human gastric cancer cells induced by AA.

## MATERIALS AND METHODS

### Reagents

Dulbecco's modified eagle medium (DMEM) was purchased from Gibco Laboratories (Santa Clara, CA). Bovine serum was obtained from Si-Ji-Qing Biotechnology Co (Hangzhou, China). Trypsin was obtained from Sigma (St Louis, MO, USA). All other reagents were produced on site and tested for purity.

### Cell Culture

Human gastric cancer cells were grown in DMEM containing 10% inactivated bovine serum, 100 µg/mL streptomycin, 100 units/mL penicillin, and 2.0 g/L NaHCO<sub>3</sub>, and were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. The cells were held in solution at a concentration of 1×10<sup>8</sup> cells/mL and cultured for 24 h. Then the culture medium was aspirated and replaced with a culture medium containing one of the following: 2, 4, 6, or 8 mmol/L of AA, 75 µmol/L H<sub>2</sub>O<sub>2</sub>, 200 U·mL<sup>-1</sup> SOD or 1.5 mmol/L AT alone. After AA was added to the medium, the pH was adjusted to 7.1 using 1 mmol/L NaOH.

### Superoxide Dismutase (SOD) Assay

For measurement of SOD activity, cells were washed in PBS and resuspended in a solution containing 10 mmol/L Tris-HCl (pH 7.5), 0.25 mol/L sucrose, 1 mmol/L EDTA, 0.5 mmol/L DL-dithiothreitol, and 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF)<sup>[20]</sup>. Triton X-100 was added to a final

concentration of 1%. The sample was then incubated for 30 min at 4°C. The solution was then centrifuged at 3000×g for 15 min. The supernatant fractions were separated and assayed for enzyme activity. The activity of SOD was measured at 550 nm. The rate of suppression of nitrotetrazolium blue was reduced by the superoxide anion radical generated during oxidation of xanthine by xanthine oxidase<sup>[21]</sup>. The reaction mixture contained 50 mmol/L sodium carbonate dissolved in 50 mmol/L K, Na-phosphate buffer (pH 7.8, at 25°C), 0.1 mmol/L EDTA, 0.1 mmol/L xanthine, and 0.025 mmol/L nitrotetrazolium blue. The activity of SOD was calculated according to the standard curve of SOD and expressed as U·mg<sup>-1</sup> protein. The protein content was measured by Lowry's method<sup>[23]</sup> with BSA as a standard.

### Assay of Catalase (CAT)

For measurement of catalase activity, cell homogenate was made in a solution containing 10 mmol/L Tris-HCl (pH 7.5), 0.25 mol/L sucrose, 1 mmol/L EDTA, 0.5 mmol/L DL-dithiothreitol, and 0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF)<sup>[20]</sup>. Triton X-100 was added to a final concentration of 1% and the sample was then incubated for 30 min at 4°C. The solution was then centrifuged at 3000×g for 15 min. The supernatant fractions were separated and assayed for enzyme activity. The activity of catalase was determined by measuring the rate of disappearance of 15 mmol/L hydrogen peroxide at 240 nm in phosphate buffer, pH 7.0<sup>[22]</sup>. The molar extinction coefficient of H<sub>2</sub>O<sub>2</sub> was 43.6 mol/L<sup>-1</sup>·cm<sup>-1</sup><sup>[20]</sup>. The amount of the enzyme utilizing 1 µmol H<sub>2</sub>O<sub>2</sub> per min was taken as 1 activity unit.

### Assay of MDA Content

Malondialdehyde (MDA), a terminal product of lipid peroxidation, was measured to estimate the extent of lipid peroxidation. The MDA level in human gastric cancer cells was determined using the thiobarbituric acid (TBA) method<sup>[24]</sup> with some minor modifications. Briefly, the cell homogenate was prepared in 0.5 mL of PBS with 1% SDS, and then mixed with 3 mL of 1% phosphoric acid and 1 mL of 0.67% thiobarbituric acid reagent (TBA), which was prepared by dissolving 0.67 g thiobarbituric acid in 100 mL double distilled water and adding 0.5 g solid NaOH and 100 mL glacial acid. The tubes were covered with foil, incubated at 95°C for 60 min, and then cooled. In order to extract the MDA, 375 µL N-butanol was added to each sample after cooling. The tubes were vortexed vigorously for 10 seconds, and then centrifuged at 5000×g for 10

min. The upper N-butanol layer, along with the extract of MDA, was transferred to a glass tube. The absorbance of the butanol phase was 532 nm. The MDA concentration was expressed as nmol/mg protein.

#### Assay of $H_2O_2$ Content

The amount of  $H_2O_2$  in the cells was determined fluorometrically in a 0.15 aliquot of the supernatant. Twenty-five  $\mu\text{g}$  *p*-hydroxyphenyl acetate (PHPA) and 80  $\mu\text{g}$  horseradish peroxidase (HRP)<sup>[25]</sup> with the excitation and emission wavelengths of 300 and 420 nm, respectively were added to the supernatant. The non-fluorescent substrate PHPA was oxidized to the stable fluorescent product *via* the enzymatic reduction of  $H_2O_2$  by horseradish peroxidase (HRP). It was determined to be 2,2'-dihydroxybiphenyl-5,5'-diacetate [(PHPA)<sub>2</sub>].

#### Determination of Cell Doubling Time

The viable cells were counted every day in the first 8 days by trypan blue dye exclusion method. The cell doubling time was calculated using the formula  $T_D = 0.693 (T_2 - T_1) / \ln (N_2/N_1)$ , where  $T_D$  is the cell doubling time from  $T_1$  to  $T_2$ ,  $N_1$  and  $N_2$  are the cell number at  $T_1$  and  $T_2$ <sup>[26]</sup>.

#### Cell Electrophoresis

The cells were collected and washed twice with D-Hanks' solution, and re-suspended at a density of  $1 \times 10^9$  cells/L. Cell electrophoresis was performed with a round plastic tube electric-bridge filled with NaCl, 10% agar and Ag-AgCl electrodes at a direct current voltage 40 V at 24°C. Nine percent sucrose was used as the electrophoretic medium. The average time for each of 40 cells in a group to move over a distance of 120  $\mu\text{m}$  was then calculated<sup>[27]</sup>. The experiments were repeated three times with similar results, and the results of the first experiment were used in later calculations.

**Colonogenic assay** After a 48-hour treatment, cells were washed with DMED medium containing 10% heat-inactivated bovine serum. Cell counts were performed by a hemocytometer, and viable cells were assayed using the trypan blue exclusion method. The cells were plated in a 24-well culture plate using a double layer nutrient agar system<sup>[28]</sup>. The culture medium was DMEM with a final concentration of 10% bovine serum. Cells were plated at a concentration of  $6 \times 10^6$  cells/mL, which was calculated to be 0.5 mL of the suspension in each well of the 24-well culture plate. The plates were then incubated for 21 days at 37°C in a humidified air containing 5% carbon dioxide. A colony was defined

to be any aggregate of more than 50 cells<sup>[29]</sup>.

## RESULTS

#### Effect of AA on $H_2O_2$ Content

As shown in Fig. 1, different concentrations of AA resulted in a concentration- and time-dependent increase in the  $H_2O_2$  content of human gastric cancer cells.

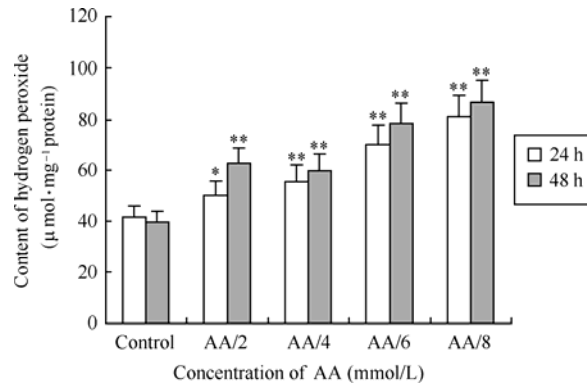


FIG. 1. Effect of ascorbic acid on  $H_2O_2$  content in gastric cancer cells. \* $P < 0.05$ , \*\* $P < 0.01$  vs control group.

#### Effect of AA on MDA Content

After treatment with 2 or 4 mmol/L of AA, the content of MDA slightly decreased. But in groups treated with 6 or 8 mmol/L of AA, the content of MDA increased in a time- and concentration-independent manner (Fig. 2).

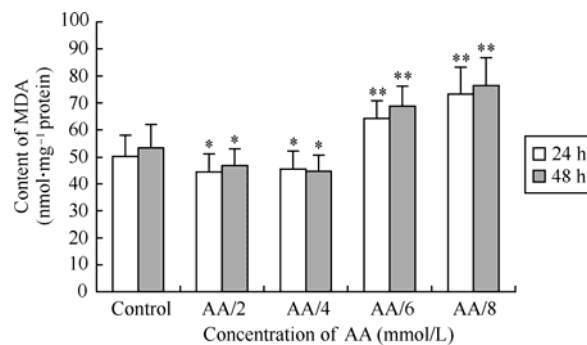


FIG. 2. Effect of ascorbic acid on MDA content in gastric cancer cells. \* $P < 0.05$ , \*\* $P < 0.01$  vs control group.

#### Effect of AA on CAT Activity

The AA treatment also resulted in a concentration- and time-dependent decrease of CAT activity of human gastric cancer cells (Fig. 3).

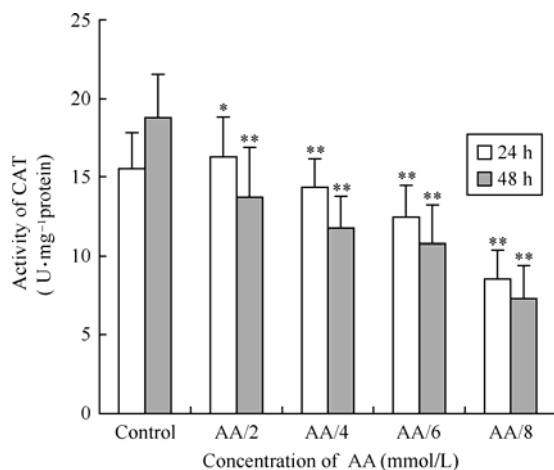


FIG. 3. Effect of ascorbic acid on CAT activity in gastric cancer cells. \* $P < 0.05$ , \*\* $P < 0.01$  vs control group.

#### Effect of AA on SOD Activity

After the treatment of human gastric cancer cells with AA, the activity of SOD increased in concentration- and time-dependent manner (Fig. 4).

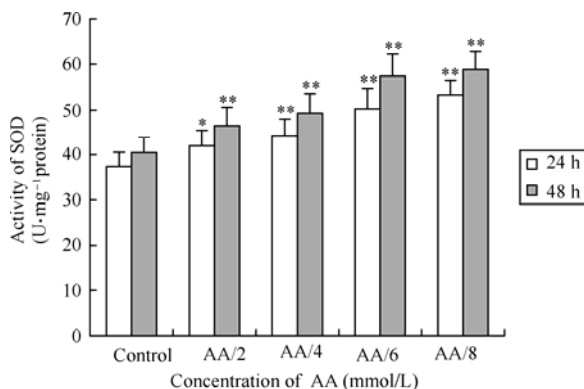


FIG. 4. Effect of ascorbic acid on SOD activity in gastric cancer cells. \* $P < 0.05$ , \*\* $P < 0.01$  vs control group.

#### Effects of AA, H<sub>2</sub>O<sub>2</sub>, SOD, or AT on Cell Proliferation

The cell doubling time slowed down at hour 27.1 in the control group, at hour 39.2 in the 6 mmol/L AA group, and at hour 32.7 in the 75  $\mu\text{mol/L}$  H<sub>2</sub>O<sub>2</sub> group. The cell doubling time of SOD 200 U·mL<sup>-1</sup> was slowed down at 30.8 h. Finally, in the 1.5 mmol/L aminotriazole (AT) groups, cell began to grow at 29.1 h (Table 1).

#### Effects of AA, H<sub>2</sub>O<sub>2</sub>, SOD, or AT on Cell Electrophoresis Rate

The net charges at tumor cell surface are generally more than those in corresponding normal cells, so the electrophoresis rate of tumor cells should be higher than that of normal cells. The decrease in cell electrophoresis rate has therefore been taken as an indicator of tumor cell differentiation<sup>[27]</sup>. After six days of treatment with 6 mmol/L AA or 75  $\mu\text{mol/L}$  H<sub>2</sub>O<sub>2</sub>, the electrophoresis rate slowed down. The percentages of retardation were 41.6% and 50.2%, respectively (Table 2).

#### Effects of AA, H<sub>2</sub>O<sub>2</sub>, SOD, or AT on Colonogenic Potential

The colonogenic potential (CP) is a general index of cell differentiation, so inspecting the CP of cultured cells in soft agar is an important means for distinguishing malignant from benign and normal cells<sup>[30]</sup>. The CP of cells treated with AA or H<sub>2</sub>O<sub>2</sub> in soft agar decreased significantly in a concentration-dependent manner (Table 3).

## DISCUSSION

Our results showed that treatment with 6 mmol/L AA and 75  $\mu\text{mol/L}$  H<sub>2</sub>O<sub>2</sub> alleviates the malignant characteristics of human gastric cancer cells and enhances the properties related to cell normalization,

TABLE 1

Effects of AA, H<sub>2</sub>O<sub>2</sub>, SOD, or AT on Human Gastric Cancer Cell Proliferation

	Cell Doubling Time (h)	10 <sup>-5</sup> Cells Original	Final	Inhibition (%)
Control	27.1±2.7	1.8	144±21	
AA (4 mmol/L)	32.4±2.4	1.8	101±27**	29.9
AA (6 mmol/L)	39.2±3.1	1.8	86±8**	40.2
AA (8 mmol/L)	41.6±5.2	1.8	88±11**	38.9
H <sub>2</sub> O <sub>2</sub> (75 $\mu\text{mol/L}$ )	32.7±2.9	1.8	92±9**	36.1
SOD (200 U·mL <sup>-1</sup> )	30.8±3.5	1.8	104±9**	27.8
AT (1.5 mmol/L)	29.1±2.7	1.8	109±8**	24.3

Note. \*\* $P < 0.01$  vs control group. \*\* $P < 0.01$  vs positive control group.

TABLE 2  
Effects of AA, H<sub>2</sub>O<sub>2</sub>, SOD, or AT on Cell Electrophoresis Rate

	Electrophoresis Time(s)	Electrophoresis Rate ( $\mu\text{m}\cdot\text{s}^{-1}\cdot\text{V}^{-1}\cdot\text{cm}^{-1}$ )	Retardation (%)
Control	11.2±1.6	2.21±0.26	
AA (4 mmol/L)	12.7±2.6	1.80±0.41**	18.5
AA (6 mmol/L)	24.5±3.2	1.29±0.21**	41.6
AA (8 mmol/L)	27.4±5.6	0.91±0.15**	58.8
H <sub>2</sub> O <sub>2</sub> (75 $\mu\text{mol/L}$ )	29.6±4.1	1.10±0.26**	50.2
SOD (200 U·mL <sup>-1</sup> )	13.1±2.6	1.57±0.28**	28.9
AT (1.5 mmol/L)	13.4±2.3	1.72±0.24**	22.2

Note. \*\* $P<0.01$  vs control group. \*\* $P<0.01$  vs positive control group.

TABLE 3  
Effects of AA, H<sub>2</sub>O<sub>2</sub>, SOD, or AT on Colonogenic Potential of Gastric Cancer Cells.

	Number of Colonies	Colonogenic Potential/%
Control	341±37	100
AA (4 mmol/L)	172±21**	50.4
AA (6 mmol/L)	98±10**	28.7
AA (8 mmol/L)	74±11**	21.7
H <sub>2</sub> O <sub>2</sub> (75 $\mu\text{mol/L}$ )	33±7**	9.8
SOD (200 U·mL <sup>-1</sup> )	202±18**	59.2
AT (1.5 mmol/L)	271±31**	79.5

Note. \*\* $P<0.01$  vs control group. \*\* $P<0.01$  vs positive control group.

suggesting that gastric cancer cells tend to become normal after treatment with AA or H<sub>2</sub>O<sub>2</sub>.

In our previous studies, we successfully induced redifferentiation of human gastric cancer and hepatoma cells using 6 mmol/L AA<sup>[1-2]</sup>. In this research, we found that treatment with 2, 4, 6, or 8 mmol/L AA increased the content of H<sub>2</sub>O<sub>2</sub> and the activity of SOD, and decreased CAT activity in a time- and dose-dependent manner. The amount of MDA was reduced when the concentrations of AA (2 and 4 mmol/L) were low, but was increased when the concentrations (6 and 8 mmol/L) were high, suggesting that these effects are not related to MDA alteration. In this study, our interest was focused on the increase of H<sub>2</sub>O<sub>2</sub> and SOD activity and the decrease of CAT activity. When using AT as an inhibitor of CAT activity, no ideal redifferentiation was observed, compared with the control. When SOD was added to the medium, its effects on inducing redifferentiation were insignificant. But treatment with H<sub>2</sub>O<sub>2</sub> successfully induced the redifferentiation of human gastric cancer cells.

The effects of SOD may be due to H<sub>2</sub>O<sub>2</sub>. AT may exert its effect by restraining the activity of CAT, leading to accumulation of H<sub>2</sub>O<sub>2</sub>. There was a significant difference between the SOD and AT groups or between AA and H<sub>2</sub>O<sub>2</sub> groups, confirming that AA induces the redifferentiation and the reversion of human gastric cancer cells by producing H<sub>2</sub>O<sub>2</sub>.

The elevation of H<sub>2</sub>O<sub>2</sub> induced by AA may be influenced by enhancing SOD activity and inhibiting CAT activity. For example, AA changes the activities of SOD and CAT on lipid peroxide levels in chicken erythrocytes. It was reported that SOD activity in AA group is increased by 20%, while CAT activity in the liver and heart of AA group is decreased by 32%<sup>[31]</sup>. Another study reported that the induction of SOD activity leads to cell differentiation<sup>[32]</sup>, which is consistent with our results.

Another study<sup>[19]</sup> has shown that AA treatment decreases cell viability and thymidine incorporation into the DNA in a dose- and time-dependent manner.

Auto-oxidation of ascorbic acid or thiols present with the guanylate cyclase preparation leads to generation of H<sub>2</sub>O<sub>2</sub>, and its metabolism by bovine liver catalase mediates the concomitant activation of guanylate cyclase, which is associated with the presence of compound I of catalase and is inhibited by a superoxide anion<sup>[33]</sup>.

Simultaneous addition of ascorbic acid and organic hydroperoxides to rat liver microsomes resulted in an approximately three-fold enhancement of lipid peroxidation, compared with incubation of organic hydroperoxides with microsomes alone. In the study, no lipid peroxidation was evident when ascorbate alone was incubated with microsomes. The stimulatory effect of ascorbate on linoleic acid hydroperoxide (LAHP)-dependent peroxidation was evident at all times. However, stimulation of cumene hydroperoxide (CHP)-dependent peroxidation occurred after a lag phase of up to 20 min. EDTA did

not inhibit CHP-dependent lipid peroxidation but completely abolished ascorbate enhancement of lipid peroxidation. Likewise, EDTA did not significantly inhibit peroxidation by LAHP but dramatically reduced ascorbate enhancement of lipid peroxidation. The results revealed a synergistic pro-oxidant effect of ascorbic acid on hydroperoxide-dependent lipid peroxidation, suggesting that endogenous metals play a role in mobilizing the hydroperoxide-dependent oxidations of microsomal components<sup>[34]</sup>. Lee *et al.*<sup>[8]</sup> recently found that AA generates bifunctional electrophiles, which explains why hydroperoxide-dependent lipid peroxidation is enhanced by AA *in vitro*.

Addition of reducing agents to commonly used cell-culture media leads to generation of H<sub>2</sub>O<sub>2</sub>. Some or all of the reported effects of ascorbic acid and polyphenolic compounds (e.g., quercetin, catechin, epigallocatechin, epigallocatechin gallate) on cells in culture may be due to H<sub>2</sub>O<sub>2</sub> generation by interaction with cell culture media<sup>[25]</sup>.

Some studies have shown that cell death is inhibited by ascorbate, whereas others demonstrated that ascorbate is cytotoxic. The toxicity of ascorbate is due to ascorbate-mediated production of H<sub>2</sub>O<sub>2</sub>, depending on the medium used to culture the cells. For example, 1 mmol/L ascorbate generates 161 μmol/L of H<sub>2</sub>O<sub>2</sub> in Dulbecco's modified Eagle's medium and induces apoptosis in 50% of HL60 cells. In RPMI 1640, only 83 μmol/L H<sub>2</sub>O<sub>2</sub> is produced, and no apoptosis is induced. Direct addition of H<sub>2</sub>O<sub>2</sub> to the cells has an effect similar to that of adding ascorbate, showing that ascorbate itself is not toxic to the cell lines used and effects of ascorbate *in vivo* cannot be predicted from studies on cultured cells. The capability of ascorbate to interact with different cell culture media to produce H<sub>2</sub>O<sub>2</sub> at different rates could account for many or all of the conflicting results obtained from cultured cell assays using ascorbate<sup>[35]</sup>.

Hydrogen peroxide is widely regarded as a cytotoxic agent when its levels is minimized by the action of antioxidant defense enzymes. Levels of H<sub>2</sub>O<sub>2</sub> in the human body may be controlled not only by catabolism but also by excretion. H<sub>2</sub>O<sub>2</sub> plays a role in the regulation of renal function and is used as an antibacterial agent in the urine. Recent research has also revealed that H<sub>2</sub>O<sub>2</sub> plays an important role in signal transduction<sup>[36]</sup> and regulation of gene expression<sup>[37]</sup>.

Although the pro-oxidant role of ascorbic acid cannot be neglected, under normal physiological conditions ascorbate mainly behaves as a first-order antioxidant. It protects cellular components from free radical-induced damage by directly quenching the soluble free radicals or by scavenging those radicals

that can initiate lipid peroxidation<sup>[38]</sup>. It is important to supplement ascorbic acid in the doses of less than 500 mg per day to achieve the desired antioxidant effect<sup>[7]</sup>.

Ascorbic acid induces growth inhibition and redifferentiation of gastric cancer cells through the production of H<sub>2</sub>O<sub>2</sub> and may be a potent anticancer agent for human gastric cancer cells.

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