BIOMEDICAL AND ENVIRONMENTAL SCIENCES **15**, 223-232 (2002)

Redifferentiation of Human Gastric Cancer Cells Induced by Ascorbic Acid and Sodium Selenite

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Objective To explore the effects and mechanisms of ascorbic acid (AA) and sodium selenite (SS) on growth inhibition and redifferentiation in human gastric cancer cells. **Methods** In the present study, trypan blue dye exclusion method was used to determine the cell growth curve and mitotic index, cell electrophoresis and colonogenic potential were used as the indexes of redifferentiation. In order to find out the mechanisms of redifferentiation, the activities of superoxide dismutase (SOD), glutathione peroxidase (GPX) and catalase (CAT) were assayed, the content of malondialdehyde (MDA), reduced glutathione (GSH) and H_2O_2 were evaluated. **Results** After treatment with AA 3 mol/L + SS 2μ mol/L, the growth rate and mitotic index of human gastric cancer cells (MGc-803) decreased remarkably. The indexes related with cell malignancy were alleviated. For example, cell surface charge was obviously decreased, the electrophoresis rate was dropped from 2.21 to 1.15 μ m ▪s^{-1} ▪V^{-1} ▪cm^{-1}. The indexes related with cell redifferentiation were promoted. For example, the colonogenic potential was decreased to 93.5%. These results indicated that redifferentiation of human gastric cancer cells was successfully induced by AA + SS. The activities of SOD and GPX were significantly higher, while the activity of CAT was slower in treated group than that in the control. The content of MDA was slightly decreased, GSH was sharply decreased, and H_2O_2 content was dramatically increased. **Conclusion** These results indicated that combination of ascorbic acid and sodium selenite may induce the redifferentiation of human gastric cancer cells and inhibit cell growth by virtue of enhancing the activities of antioxidative enzymes and inducing the formation of H_2O_2, and altering the cell redox status. Combination of ascorbic acid and sodium selenite may be a potent anticancer agent for human gastric cancer.

Key words: Ascorbic acid; Sodium selenite; Gastric cancer; Malondialdehyde; Catalase; Superoxide dismutase

INTRODUCTION

Many studies have shown that redifferentiation of malignant tumor cells could be induced. Some of these inducers such as retinoic acid[1], dimethyl sulfoxide[2], superoxide dismutase (SOD)[3], verbascoside[4], DL-α-tocopherol[5] are effective antioxidants. In our laboratory, redifferentiation of human hepatoma cells was successfully induced with several antioxidants[4, 6, 7]. An increasing amount of experimental and epidemiological evidences implicated the involvement of free radicals in every step of cell carcinogenesis, such as initiation, promotion and metastasis.

Ascorbic acid (AA), an essential nutrient in human is involved in many cellular

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0895-3988/2002
CN 11-2816

functions. The epidemiological evidence and laboratory studies showed that AA is a powerful plasma antioxidant[8] that can protect lipids, proteins, and cell membranes from oxidative damage by scavenging oxygen radicals. AA has been found to be able to inhibit cell growth and induce cell redifferentiation by our[6] and other's laboratories[9], and can be used as a leukemia therapeutic agent. There has been a considerable discussion on AA and cancer since last decade. Although some authors believed that AA could protect human against cancers, or at least, against some types of cancer, the others seemed to be inclined to doubt such functions. Pauling *et al.* [10] found that an overload of AA (600 mg /d) can prevent human against cancers. But physiologic mechanisms of AA absorption, tissue uptake, metabolism, and elimination support the theory that an overload of AA is unlikely to occur in man[11]. Podmore *et al.* [12] recently found that AA administered as a dietary supplement to healthy human at a high dose (500 mg/d) exhibits a pro-oxidant effect and lead to DNA damage *in vivo.* Therefore, it becomes necessary to find a way to treat gastric cancer with a relatively low dose of AA.

Sodium selenite (Na_2SeO_3) (SS) is an effective free-radical scavenger[13], and is able to enhance the activity of glutathione peroxidase (GPX)[14]. It has also been found that Na_2SeO_3 can induce redifferentiation of tumor cells[14], however high-concentration SS is cytotoxic. By this means a clinically viable method could be offered to combat cancer by normalizing tumor cells with a low dose of AA and SS combination instead of a high dose of AA or SS alone. In order to find out whether the redifferentiation induced by AA+SS is connected with the decreasing of reactive oxygen species (ROS), regulating the activities of antioxidant enzymes and altering the cell redox status, the activities of SOD, CAT, GPX and the content of MDA, H_2O_2, GSH were assayed.

MATERIALS AND METHODS

Reagents

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 regents were analytical purities.

Cell Culture

Human gastric cancer cells were grown in DMEM medium containing 100 g/L inactivated bovine serum, streptomycin (100 μg/mL), penicillin (100 units/mL), and $NaHCO_3$ 2.0 g/L, and were maintained at 37℃ in a humidified atmosphere of 5% CO_2. After the cells (1×10^8 cells/mL) were cultured for 24 h, the culture medium was aspirated and replaced with the culture medium containing AA 3 mmol/L alone, or SS 2 μ mol/L alone, or AA 3 mmol/L combined with SS 2 μ mol/L. After adding AA to medium, it was adjusted to pH7.1 with 1 mol/L NaOH.

Determination of Cell Growth Curve and Mitotic Index

The viable cells were counted every day in the first 8 days by trypan blue dye exclusion method. The cell doubling time was calculated as following: $T_D=0.693\ (T_2-T_1)/\ln(N_2/N_1)$, 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[15]. Three cover slips, on which cells were cultured, were fixed in Bouin-Hollonde solution and stained with hematoxylin-eosin. The mitotic cells were counted from 1 000

cells every day. Mitotic index is defined here as the fraction of gastric cancer cells in metaphase, anaphase, or telophase.

Cell Electrophoresis

The cells were collected and washed with D-Hanks' solution twice, then re-suspended at a density of 1×10^9 cells/L. The cell electrophoresis determination was performed with a round plastic tube electric-bridge filled with NaCl 100 g/L- agar 10 g/L and Ag-AgCl electrodes at a direct current voltage 40V, room temperature of 24℃, taking sucrose 90 g/L as the electrophoretic medium. The results were expressed by the average time(s) during which a cell moved over a distance of 120 μm and 40 cells in each group were determined. The experiments were repeated three times with similar results. Thus, the results of one experiment were used as the criterion.

Colonogenic Assay

48 h after treatment, the cells were washed with DMEM medium containing 100 g/L heat-inactivated bovine serum. Cell counts were performed by hemocytometer, and viable cells were assayed by trypan blue exclusion method. The cells were plated at 24-well culture plate in a double layer nutrient agar system[16]. The medium was DMEM with a final concentration of 100 g/L bovine serum. Cells were routinely plated at a concentration of 6×10^6 cells/mL (0.5 ml for each well in 24-well culture plate). The plates were incubated for 21 days at 37℃ in a humidified air of 5% CO_2. A colony was defined to be an aggregate of >50 cells[17].

Assays for SOD, GPX and CAT

Cellular lysate was prepared by sonication of scraped cells in phosphate-buffered saline (PBS), pH 7.4, containing 0.5 g/L deoxycholate. An aliquot of cell lysate was used for protein estimation. CAT activity was measured by the method of Aebi[18] with some modification[19]. SOD activity was measured according to Sun and Zigman[20]. GPX activity was measured by the method of Flohe and Gunzler[21]. Protein content was measured with Folin phenol reagent by Lowry's method, using BSA as a standard.

Assays for the Content of MDA and H_2O_2

Cell lipid peroxides were determined by measuring malondialdehyde (MDA) formation according to Wong *et al*.[22]. H_2O_2 in cells was determined fluorometrically on 0.15 aliquots of the supernatant, using 25 μg *p*-hydroxyphenyl acetate (PHPA) and 80 μg horse-radish peroxidase (HRP)[23] with the excitation and emission wavelengths of 300 nm and 420 nm. The non-fluorescent substrate PHPA was oxidized to the stable fluorescent product, tentatively named as 2,2'-dihydroxybiphenyl-5,5'-diacetate [$(PHPA)_2$], via the enzymatic reduction of H_2O_2 by horse-radish peroxidase (HRP).

Assay for Intracellular Reduced Glutathione (GSH)

GSH was assessed with DTNB [5,5'-dithio-bis(2-nitrobenzoic)] based on the method described by Jocelyn[24]. The cells (1×10^8/mL) were sonicated for 30 s in 300 μL 50 g/L 5-sulfosalicylic acid and centrifuged for 10 min at 1 000×g. The resultant acid thiol extract was assayed for nonprotein sulfhydryls by quantifying the reduction of DTNB through its conversion to 5-thiol-2-nitrobenzoic acid (TNB) at 412 nm by using a spectrophotometer.

Sample values were then calculated from a standard curve generated with known amounts of GSH.

Statistics

The results were presented as $\bar{x} \pm s$ and Student's *t* test was used to evaluate the statistical significance.

RESULTS

Effect on Cell Proliferation

The cell doubling time was retarded from 27.3 h in control to 38.5 h in AA + SS group. However, the cell doubling time was slightly retarded in AA 3 mmol/L group or in SS 2 μmol/L group. The cell inhibition was 60.4% in AA + SS group, but only 17.9% in AA 3 mmol/L group and 19.8% in SS 2 μmol/L group (Table 1).

TABLE 1

Effect of Ascorbic Acid and Sodium Selenite on Human Gastric Cancer Cell Proliferation

	Cell Doubling Time (h)	$10^5 \times$Number of Cells		Inhibition (%)
		Original	Final	
Control	27.3 ± 1.7	2.0	106 ± 18	
AA (3 mmol/L) (a)	29.1 ± 1.8	2.0	87 ± 12*	17.9
SS (2 μmol/L) (b)	30.3 ± 1.8	2.0	85 ± 13*	19.8
a+b	38.5 ± 2.1	2.0	42 ± 6**	60.4

Note. n =3 experiments, each experiment contains 3 cultures ($\bar{x} \pm s$). *P<0.05, **P<0.01 *vs* control group.

TABLE 2

Effect of Ascorbic Acid and Sodium Selenite Treatment for 48 h and 72 h on the Survival Rate of Gastric Cancer Cells (Total Cells=1000 in Each Group)

	Dead Cells/1000 Cells		Survival Rate (%)	
	48 h	72 h	48 h	72 h
Control	18 ± 4	23 ± 5	98.2	97.7
AA (3 mmol/L) (a)	32 ± 6	40 ± 6	96.8	96.0
SS (2 μmol/L) (b)	36 ± 7	42 ± 7	96.4	95.8
a+b	54 ± 9	58 ± 7	94.6	94.2

Note. n =3 experiments, each experiment contains 3 cultures($\bar{x} \pm s$).

Effect on the Survival Rate of Human Gastric Cancer Cells

In order to find out whether the inhibitory effect on cell growth was due to cytotoxicity, the survival rate of treated cells was evaluated by trypan blue dye exclusion method.

Although, the inhibitory effect on human gastric cancer cell proliferation was exacerbated by AA + SS combination, but the survival rate of cells showed no obvious difference among different groups (Table 2). Therefore, the inhibitory effect of AA + SS combination was not due to their cytotoxicity.

Effect on Mitotic Index

To further study the characteristics of inhibition and proliferation, the mitotic index was investigated in various groups. The mitotic index showed that untreated gastric cancer cells exhibited vigorous proliferating capability with a division peak on the fifth day. After treatment with AA + SS, the mitotic index declined from 6.2% (control) to 3.0% , while after treatment with AA 3 mmol/L or SS 2μmol/L alone, it declined to 4.4 % and 4.3% respectively, and the division peak in SS alone, AA alone and AA+SS groups was shifted to the fourth day (Fig. 1).

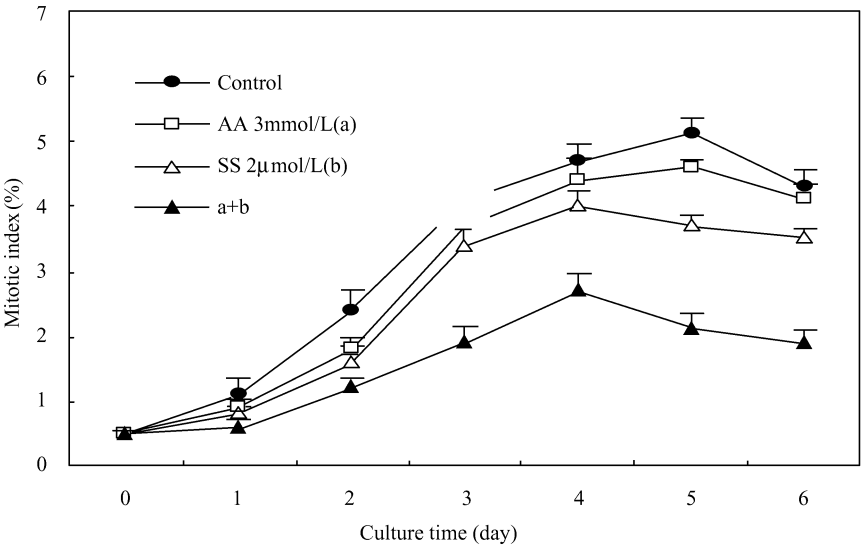

FIG. 1. Effects of ascorbic acid and sodium selenite on the mitotic index of gastric cancer cells. Cells were cultured at a density of 1×10^8 cells/mL. The cells were cultured in medium without ( ) or with AA 3 mmol/L alone ( ), or SS 2 ì mol/L alone (△), or AA 3 mmol/L + SS 1.5 ì mol/L (a+b)( ). Means of three parallel experiments. *n*=3 experiments × 3 cultures (± *s*).

Continuous division and constant multiplication are the essential characteristics of malignant tumor. Therefore, the inhibitory effect on multiplication of tumor cells is a significant appraisal of induced differentiation. The results in Tables 1 and 2 and Fig. 1 confirmed that AA + SS combination inhibited multiplication of gastric cancer cells without any cytotoxicity.

Effect on Cell Surface Charge

The net charges at the tumor cell surface are generally more than those in corresponding normal cells, thus, the cell electrophoresis rate of tumor cells should be higher than that of normal cells. Therefore the decrease of the cell electrophoresis rate has been taken as an appraisal of tumor cell redifferentiation[25]. After treatment with AA 3 mmol/L + SS 2μmol/L (a+b) for 6 days, the electrophoresis rate was apparently slowed down. The percentage of

retardation reached as high as 47.9% (Table 3).

TABLE 3

Effect of Ascorbic Acid and Sodium Selenite on Human Gastric Cell Electrophoresis Rate

	Electrophoresis Time (s)	Electrophoresis Rate ($\mu m \cdot s^{-1} \cdot V^{-1} \cdot cm^{-1}$)	Retardation (%)
Control	10.7 ± 1.2	2.21	
AA (3 mmol/L) (a)	12.5 ± 1.6	1.84	16.7
SS (2 μmol/L) (b)	13.2 ± 1.5	1.76	19.5
a + b	23.3 ± 2.1	1.15	47.9**

Note. n =3 experiments × 3 cultures ($\bar{x} \pm s$). **P<0.01 *vs* control group.

Effect on Colonogenic Potential

Colonogenic potential (CP) is a general index of cell redifferentiation, inspecting CP of cultured cells in soft agar is a very important marker for distinguishing malignant, benign and normal cells[26,27]. The colonogenic potential (CP) of treated cells in soft agar was significantly decreased (Table 4). Thus, AA+SS showed its inhibitory effect on cell colony formation.

TABLE 4

Effect of Ascorbic Acid and Sodium Selenite on Colonogenic Potential of Gastric Cancer Cells

	Numer of Colonies	Colonogenic Potential (%)
Control	321 ± 34	100
AA (6 mmol/L) (a)	278 ± 23	86.6
SS (2 μmol/L) (b)	269 ± 21	83.8
a + b	21 ± 5**	6.5**

Note. n =3 experiments × 3 cultures ($\bar{x} \pm s$). **P<0.01 *vs* control group.

In a word, the results showed that with AA 3 mmol/L + SS 2 μmol/L treatment, the malignant characteristics of human gastric cells were alleviated, while the properties related with cell normalization were strengthened. All these changes suggested that human gastric cancer cells were inclined towards normalization, and confirmed that AA + SS combination could induce human gastric cancer cell redifferentiation and impel cell reversion against malignant phenotype.

Influence of AA and SS on the Activities of Antioxidant Enzymes

The results showed that the activities of SOD and GPX in treated groups were significantly higher than those in control, while the activity of CAT was decreased (Fig. 2).

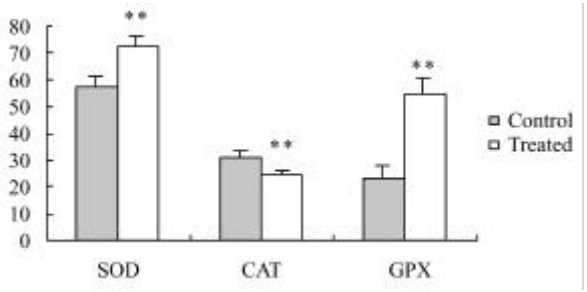

FIG. 2. Effects of ascorbic acid and sodium selenite on the activities of SOD, CAT and GPX in gastric cancer cells. Cells were incubated at a density of 1×10^8 cells/mL. The cells were cultured in medium without (control) or with AA 3 mmol/L + SS 2 μmol/L (treated). The activity of SOD is expressed by U·mg^{-1} protein, while CAT is K·10^4 · mg^{-1} protein and GPX is nmol·min^{-1}·mg^{-1} protein. Means of three parallel experiments. n=3 experiments × 3 cultures ($\bar{x} \pm s$). $^{**}P<0.01$ *vs* the control group.

Effect on Contents of H_2O_2, MDA, and GSH

After treatment with AA + SS, the content of MDA was slightly decreased, but the difference was not significant. H_2O_2 was dramatically increased, and GSH was sharply reduced (Fig. 3).

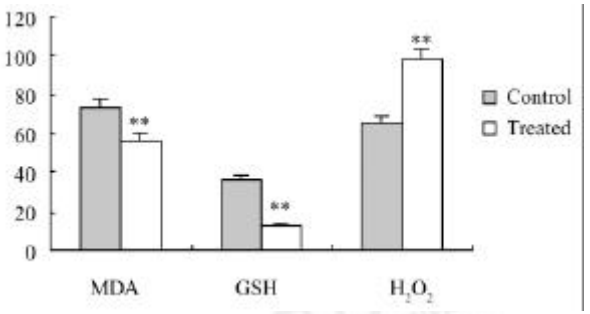

FIG. 3. Effects of ascorbic acid and sodium selenite on the contents of MDA, GSH and H_2O_2 in gastric cancer cells. Cells were incubated at a density of 1×10^8 cells/mL. The cells were cultured in medium without (control) or with AA 3 mmol/L + SS 2 μmol/L (treated). The content of MDA is expressed as nmol·mg^{-1} protein, while GSH is expressed $10 \times$ nmol mg^{-1} protein and H_2O_2 is expressed μmol mg^{-1} protein. Means of three parallel experiments. n =3 experiments × 3 cultures ($\bar{x} \pm s$). $^{**}P<0.01$ *vs* the control group.

DISCUSSION

Both AA and SS are famous antioxidants, and sodium selenite can enhance the activity of glutathione peroxidase (GPX), gastric cell redifferentiation induced by them may be related to their abilities of regulating the activities of antioxidant enzymes and altering the cell redox status.

Reactive oxygen species (ROS), represented by superoxide, hydrogen peroxide and hydroxyl radicals, have been implicated in many diseases including cancer. ROS have been known to play an important role in initiating and promoting of multi-step carcinogenesis. The cellular antioxidants play a crucial role in protection against neoplastic disease.

The effects of AA and trace element Se on the activities of antioxidant enzymes and

lipid peroxide levels in chicken erythrocytes were investigated. SOD activity in AA group was increased by 20%. GPX activity was raised by 35% in Se group, and 33% in AA group, respectively[28]. An increase in GPX activities in Se and AA alone groups was also found, while CAT activities in the liver and heart of the AA group were significantly decreased (by 32%)[29]. Another study showed that the induction of SOD activity could lead to cell redifferentiation[30]. All these are consistent with our results. It was reported that GPX activity was increased during *in vitro*-induced monocytic or granulocytic redifferentiation of myeloid cell lines, and that increased expression of cellular GPX gene occurred through complex mechanisms such as transcriptional up-regulation[31].

In vitro treatment of PC3 and MLL cells with sodium ascorbate (0-10 mmol/L) resulted in a decrease in cell viability and thymidine incorporation into DNA[32]. Ascorbate induced these changes through the production of hydrogen peroxide by adding catalase (100-300 units/mL), an enzyme that degrades hydrogen peroxide, inhibits the effects of ascorbate on these cell lines. In contrast, superoxide dismutase, an enzyme that dismutates superoxide and generates hydrogen peroxide did not prevent ascorbate-induced changes, emphasizing the involvement of reactive oxygen species (ROS) in cellular damage. Singlet oxygen scavengers such as sodium azide and hydroquinone, hydroxyl radical scavengers such as D-mannitol and DL-alpha-tocopherol did not counteract the effects of ascorbate on thymidine incorporation. The increase of H_2O_2 was also observed in our studies (Fig. 3). The results suggested that AA can inhibit tumor growth and induce redifferentiation of human gastric cells by virtue of producing reactive oxygen species.

Glutathione is a major redox buffer in the secretory pathway[33], and reduced glutathione, a natural thiol antioxidant, maintains the redox potential and thus protects cells against oxidative damage[34]. During the cell redifferentiation induced by AA + SS, the activities of SOD and GPX and the amount of H_2O_2 increased, while the activity of CAT and the amount of GSH decreased, leading to the changes of redox. These results indicated that the redox status must be involved in cell redifferentiation.

Lee *et al.* [35] recentlydetermined that AA could induce 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 theno-2'-deoxyadenosine, a highly mutagenic lesion found in human DNA. At low concentrations of AA, the major products are *trans*-4,5-epoxy-2(*E*)-decenal, 4-hydroperoxy-2-nonenal, and 4-oxo-2-nonenal. As the concentration of AA increases, the amount of 4-hydroperoxy-2-nonenal decreases, with a concomitant increase in *trans*- and *cis*-4,5-epoxy-2(*E*)- decenal, 4-oxo-2-nonenal, and 4-hydroxy-2-nonenal. Maximal yields of 4-oxo-2-nonenal, 4,5-epoxy-2(*E*)-decenal, and 4-hydroxy-2-nonenal could be obtained with an excess of AA [28]. Thus, a relatively low dose of AA should be used for preventing and curing cancers.

AA-mediated formation of genotoxins from lipid hydroperoxides in the absence of transition metal ions could help explain why AA had no substantial efficacy in cancer therapy and prevention trials[36]. The finding that AA generates bifunctional electrophiles explains why hydroperoxide-dependent lipid peroxidation is enhanced by AA *in vitro*[37].

Although as an important antioxidant, AA possesses many physiological functions, it also has potential risk of DNA damage, so we must find out a way to treat cancers with a relatively low dose of AA.

These results suggest that combination of AA and SS may be a potent anticancer agent for human gastric cancer cells.

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(Received December 24, 2001 *Accepted May* 22, 2002*)*