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PHARMACOLOGIC DOSES OF ASCORBIC ACID REPRESS SPECIFICITY PROTEIN (Sp) TRANSCRIPTION FACTORS AND Sp-REGULATED GENES IN COLON CANCER CELLS

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Abstract

Ascorbic acid (vitamin C) inhibits cancer cell growth and there is a controversy regarding the cancer chemoprotective effects of pharmacologic doses of this compound which exhibits pro-oxidant activity. We hypothesized that the anticancer activity of pharmacologic doses of ascorbic acid (< 5 mM) is due, in part, to reactive oxygen species (ROS)-dependent downregulation of specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4 and Sp-regulated genes. In this study, ascorbic acid (1 – 3 mM) decreased RKO and SW480 colon cancer cell proliferation and induced apoptosis and necrosis and this was accompanied by downregulation of Sp1, Sp3 and Sp4 proteins. In addition, ascorbic acid decreased expression of several Sp-regulated genes that are involved in cancer proliferation [hepatocyte growth factor receptor (c-Met), epidermal growth factor receptor (EGFR) and cyclin D1], survival (survivin and bcl-2), and angiogenesis [vascular endothelial growth factor (VEGF) and its receptors (VEGFR1 and VEGFR2)]. Other pro-oxidants such as hydrogen peroxide exhibited similar activities in colon cancer cells and cotreatment with glutathione inhibited these responses. This study demonstrates for the first time that the anticancer activities of ascorbic acid are due, in part, to ROS-dependent repression of Sp transcription factors.

Keywords

Ascorbic acid; colon cancer; ROS; peroxide; Sp downregulation

INTRODUCTION

Vitamin C (ascorbic acid) is an essential dietary nutrient and highly effective antioxidant that is a critical cofactor required for the activity of multiple enzymes. As a pharmacological agent, vitamin C has a controversial history, particularly with respect to the effectiveness of high doses of this compound for treating the common cold and cancer (1–3). Cameron, Pauling and coworkers summarized a series of studies on the anticancer effects of high doses of ascorbic acid and concluded that this regimen improved the quality of life and extended the lifespan of cancer patients (4, 5). In contrast, results of two trials carried out by Moertel and coworkers at the Mayo Clinic did not observe any benefit for patients taking vitamin C versus patients who did not receive the drug (6, 7). Thus, the anticancer activity of

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vitamin C is controversial and it is possible that, in common with many other mechanism-based drugs, only select sub-sets of cancer patients may respond to ascorbic acid alone or in combination with other agents.

The use of vitamin C or any other drug for cancer chemotherapy is predicated, in part, by an understanding of the underlying mechanism of action in both *in vitro* and *in vivo* models. Several studies show that vitamin C inhibits growth of a number of cancer cell lines (6–13), and it was recently reported that EC_{50} values using the MTT reduction assays ranged from 3 to 7 mM in 5 different human cancer cell lines and that both intravenous and intraperitoneal administration of ascorbate in mice could result in blood levels of ascorbate as high as 20 mM (14). Moreover, intravenous administration of ascorbate in humans can also give low mM concentrations of this compounds in serum (15, 16). It was also reported that ascorbic acid-dependent decreases in cancer cell viability was attenuated after cotreatment with catalase and the antioxidant N-acetylcysteine (NAC) and this observation was consistent with the induction of reactive oxygen species (ROS) (14). The results also correlated with studies demonstrating the pro-oxidant activity of ascorbic acid and the identification of hydrogen peroxide in extracellular fluid of mice administered pharmacologic doses of ascorbic acid (9, 17–19).

The pro-oxidant activity induced by ascorbic acid is comparable to that observed for many other anticancer drugs such as arsenic trioxide which is currently being used for treatment of acute promyelocetic leukemia (APL) and is also being evaluated for treating solid tumors (20–22). Ongoing studies in this laboratory confirm that arsenic trioxide decreases mitochondrial membrane potential (MMP) and induces ROS in bladder and pancreatic cancer cell lines (23). Moreover, arsenic trioxide-induced ROS decreased expression of specificity protein (Sp) transcription factors Sp1, Sp3 and Sp4 and several Sp-regulated genes associated with cancer cell proliferation, survival and angiogenesis (23). We therefore hypothesized that the anticancer activity of ascorbic acid may also be related to ROS-dependent downregulation of Sp transcription factors. Using colon cancer cells as a model, we have now confirmed that ascorbic acid decreases expression of Sp1, Sp3, Sp4 and Sp-dependent genes and these responses are ROS-dependent and blocked by antioxidants such as glutathione. Similar effects were observed after treating colon cancer cells with other pro-oxidants such as hydrogen peroxide (H_2O_2) This study identifies for the first time an important ascorbic acid-induced pathway that contributes to the pro-oxidant anticancer activity of this compound.

MATERIALS AND METHODS

Cell lines, reagents and antibodies

RKO and SW480 human colon carcinoma cell lines were obtained from American Type Culture Collection (Manassas, VA). RKO and SW480 cells were maintained in Dulbecco's modified/Ham's F-12 (Sigma-Aldrich, St. Louis, MO) with phenol red supplemented with 0.22% sodium bicarbonate, 5% fetal bovine serum, and 10ml/L 100X antibiotic antimycotic solution (Sigma) containing 10,000 units penicillin, 10,000 μg streptomycin and 25 μg amphotericin B/ml, in 0.85% saline. The cells were grown in 150 cm^2 culture plates in an air/CO_2 (95:5) atmosphere at 37°C and passaged approximately every 3–5 days. All antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), except cleaved poly (ADP) ribose polymerase (PARP) and c-Met (Cell Signaling Technology, Danvers, MA), Sp1 and VEGF-R2 (Millipore, Temecula, CA), survivin (R&D Systems, Minneapolis, MN), VEGFR1 (Abcam Inc. Cambridge, MA), and β-actin antibodies (Sigma-Aldrich). L-Ascorbic acid (99%) and glutathione, 98% (γ-glu-cys-gly, GSH) were purchased from Sigma-Aldrich. Dithiothretol (DTT, 98%) was obtained from Boehringer Mannheim Corp, (Indianapolis, IN).

Cell proliferation assays

RKO and SW480 cancer cell lines were plated (3×10^4 per well) using DMEM:Ham's F-12 medium containing 2.5% charcoal stripped fetal bovine serum (FBS) in 12-well plates and left to attach for 24 hr. Cells were then treated with either vehicle or the indicated concentrations of ascorbate (pH 7) for 3 hr and washed and further grown for additional 24 hr in growth medium in the absence of ascorbate. Ascorbic acid was neutralized to pH 7.0 with sodium hydroxide and prepared immediately before use. After 24 hr, cells were counted using a Coulter Z1 particle counter. Each experiment was done in triplicate and results are expressed as means ± SE for each determination.

WST-1 cell survival assay

RKO or SW480 cells were seeded in 96-well plates and then treated with H_2O_2 or *t*-butylhydroperoxide (T-BOOH) (Sigma) alone or co-treated with GSH for 24 hr. The WST-1 assay kit was obtained from Roche (Indianapolis, IN) and the assay was carried out according to the manufacturer's instructions. Cell viability was determined by the absorbance of the formazan product at 440 nm. The rate of cell survival was calculated as the percentage of the absorbance of the treated samples divided by the controls. All experiments were determined in triplicates and repeated at least two times and results are expressed as means ± SD for each treatment group.

Apoptotic and Necrotic assays

RKO and SW480 cells (10×10^4) were seeded in two chambered glass slides and left to attach overnight. The apoptotic and necrotic assay kit was obtained from Biotium, Inc. (Hayward, CA) and contained FITC-Annexin V, ethidium homodimer III and Hoechst 3342. Cells were treated with ascorbate for 3 hr and further grown for additional 12 hr, and the apoptotic, necrotic and healthy cell detection kit was used according to the instructions provided by the manufacturer for analysis of adherent cells.

Western blot assays

RKO and SW480 cancer cells were seeded in DMEM:Ham's F-12 medium containing 2.5% charcoal-stripped FBS. After 24 hr, cells were treated with either vehicle or ascorbate for 3 hr, media was changed, and cells were incubated for 24 hr. Western blot analysis on whole cell lysates was determined using β-actin as a loading control. Cells were cotreated with dithiothreitol (DTT) and glutathione (GSH) in the presence or absence of ascorbate, H_2O_2 or T-BOOH for the indicated times and harvested 24 hr after treatment, and whole cell lysates were obtained for analysis by western blots as previously described (24–26). Briefly, cells were collected using high-salt buffer (50 mmol/L HEPES, 0.5 mol/L NaCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EGTA, 10% glycerol, and 1% Triton-X-100, pH 7.5) and 10μL/mL of Protease Inhibitor Cocktail (Sigma Aldrich). Protein lysates were incubated for 3 min at 100°C and then separated by electrophoresis on 10% SDS-PAGE 120 V for 3 to 4 hr. Protein lysates were transferred onto polyvinylidene difluoride (PVDF) membranes by wet electroblotting in a buffer containing 25 mmol/L Tris, 192 mmol/L glycine, and 20% methanol for 1.5 hr at 180 mÅ. Membranes were blocked for 30 min with 5% TBST-Blotto [10 mmol/L Tris-HCl, 150 mmol/L NaCl (pH 8.0), 0.05% Triton X-100, and 5% nonfat dry milk] and incubated in fresh 5% TBST-Blotto with 1:500 primary antibody overnight with gentle shaking at 4°C. The PVDF membrane was washed with TBST for 10 min and then incubated with secondary antibody (1:5000) in 5% TBST-Blotto for 2 hr by gentle shaking. After washing with TBST for 10 min, the membrane was incubated with 6 mL of chemiluminescence substrate for 1 min and exposed to Kodak image station 4000 mm Pro (Carestreamhealth, Woodbridge, CT).

ROS estimation

Cellular ROS levels were evaluated with the cell permeant probe CM-H_2DCFDA (5-(and-6)-chloromethyl-2′,7′dichlorodihydrofluorescein diacetate acetyl ester). CM-H_2DCFDA is nonfluorescent until removal of the acetate groups by intercellular esterases and oxidation occurs within the cell. Following treatment for 12 hr, 48-well cell culture plates containing cells were loaded with 10 μM CM-H_2DCFDA for 30 min, washed once with serum free medium, and analyzed for ROS levels using Bio Tek Synergy 4 plate reader (Bio Tek Instruments, Inc., Winooski, VT) set at 480 nm and 525 nm excitation and emission wavelength, respectively. Cells were then washed twice with PBS and fixed with methanol for 3 min at room temperature. Methanol was then completely removed and 1 mg/ml Janus green was added to the cultures for 3 min. Following removal of Janus green, cultures were washed twice with PBS and 100 μl of 50% methanol was added to each well. Cell counts were then determined with the plate reader set to an absorbance of 654 nm, and ROS intensities were then corrected accordingly. Three experiments were performed and analyzed on different days using 8 wells per treatment group.

Statistical analysis

All statistical tests were two-sided and statistical significance in the differences between treatment groups was determined by analysis of variance and Student's t-test. Results are expressed as means ± SD for replicate (at least three) experiments and levels of significance are noted.

RESULTS

RKO and SW480 cells were treated with 1 – 3 mM ascorbic acid for 3 hr and after 24 hr, cells were counted to determine effects on cell survival. The results (Fig. 1A) show that 1, 2 and 3 mM ascorbic acid significantly inhibited cell proliferation and the growth inhibitory concentrations were similar to those previously reported in other cancer cell lines (9, 14). Growth inhibitory IC_{50} values were 1.62 and 1.64 mM in RKO and SW480 cells, respectively The effects of ascorbate on caspase-dependent PARP cleavage were cell context-dependent (Fig. 1B). In RKO cells, 2 and 3 mM ascorbate increased PARP cleavage; however, at the higher concentrations, there was a decrease in PARP cleavage and similar results were observed in SW480 cells. These results are consistent with previous studies showing that ascorbate also induces necrosis in cancer cells and this is due, in part, to the induction of H_2O_2 which inhibits apoptosis (27, 28). The cell death pathways were further investigated using a kit containing FITC-Annexin V, ethidium homodimer III, and Hoechst 3342 that simultaneously stains apoptotic cells (green), necrotic cells (red), and healthy cells (blue). Compared to control (solvent-treated) RKO cells, ascorbate induced both apoptosis (green) and necrosis (red) and cotreatment with GSH decreased both death pathways (Fig. 1C). Ascorbate also induced apoptosis and necrosis in SW480 cells and these effects were also inhibited after cotreatment with GSH, demonstrating that ascorbate-induced cell death is associated with its pro-oxidant activity. Green staining (apoptosis) was more evident in SW480 cells, whereas necrosis (red staining) was more enhanced in RKO cells.

Since ascorbate inhibits cell proliferation and is cytotoxic to colon cancer cells, we also investigated expression of several genes that mediate these responses. For example, treatment of RKO and SW480 cells with 2 or 3 mM ascorbate resulted in decreased expression of EGFR, c-Met and cyclin D1 proteins (Figs. 2A and 2B). We also observed that ascorbate decreased levels of the survival genes bcl-2 and survivin in RKO and SW480 cells (Figs. 2C and 2D, respectively), and loss of these gene products is likely associated with induction of apoptosis in cancer cells as observed in the PARP cleavage and

fluorescent staining assays (Fig. 1B and 1C). Previous RNA interference studies in this laboratory indicate that individual knockdown of Sp1, Sp3 or Sp4 and their combination decreased expression of cyclin D1, c-Met, EGFR, bcl-2 and survivin (23, 25, 29–32), indicating that these genes are regulated by Sp1, Sp3 and Sp4 transcription factors (24, 25, 29–32). These results suggest that ascorbate may also affect expression of these proteins by downregulation of Sp transcription factors. Figure 3A demonstrates that after treatment of RKO and SW480 cells with 2 and 3 mM ascorbate, levels of Sp1, Sp3 and Sp4 proteins were decreased in both cells lines. The angiogenic proteins VEGF, VEGFR1 and VEGFR2 are Sp-regulated genes (31, 32) and ascorbate also decreased expression of these proteins in RKO and SW480 cells (Fig. 3B) further confirming that Sp1, Sp3, Sp4 and Sp-regulated genes are critical targets of ascorbate in colon cancer cells and similar results have been observed in other cancer cell lines including KU7 (bladder), L3.6pL (pancreatic), and LNCaP and PC3 (prostate) cancer cells (data not shown). Since ascorbate treatment results in formation of hydrogen peroxide (H_2O_2) in both *in vitro* and *in vivo* models (9, 17–19), we also investigated the effects of H_2O_2 and the pro-oxidant t-butyl hydroperoxide (T-BOOH) on Sp protein expression in RKO and SW480 colon cancer cells. Both H_2O_2 and T-BOOH decreased expression of Sp1, Sp3 and Sp4 proteins and induced PARP cleavage in RKO and SW480 cells (Figs. 3C and 3D) and these results are comparable to the effects of ascorbate on expression of these proteins (Fig. 3A).

We also investigated the induction of ROS 12 hr after incubation of RKO and SW480 cells with ascorbate for 3 hr. The results indicate that ROS was induced by ascorbate in RKO and SW480 cells at this time point and in cells cotreated with ascorbate plus the thiol antioxidants GSH and DTT, the ascorbate-induced ROS response was attenuated (Figs. 4A and 4B). Figures 4C and 4D show that GSH inhibited ascorbate-induced cell death and these results also correlated with the inhibitory effects of GSH on ascorbate-induced inhibition of RKO and SW480 cell proliferation (Figs. 4C and 4D).

Since the antioxidant GSH inhibited ascorbate-induced cytotoxicity, we also investigated the role of ROS in mediating downregulation of Sp1, Sp3 and Sp4 and Sp-regulated genes in colon cancer cells treated with 3 mM ascorbate. Figure 5A shows that ascorbate-induced downregulation of Sp1, Sp3 and Sp4 in RKO and SW480 cells was attenuated after cotreatment with GSH, whereas minimal effects were observed in cells cotreated with DTT. The effects of antioxidants on ascorbate-induced downregulation of growth inhibitory (Fig. 5B) and angiogenic/survival (Fig. 5C) gene products was also determined. GSH inhibited ascorbate-induced downregulation of EGFR, c-Met and cyclin D1 protein expression (Fig. 5C) and also VEGF, VEGFR1, VEGFR2 and survivin protein expression (Fig. 5B) in RKO and SW480 cells. DTT was relatively ineffective as an inhibitor in these assays and this was consistent with results summarized in Figure 5A.

The role of antioxidants in attenuating the effects of H_2O_2 and T-BOOH were also investigated. Figure 6A shows that H_2O_2 and T-BOOH inhibit growth of RKO cells and cotreatment with the antioxidants significantly blocks the growth inhibitory effects of the pro-oxidants. Similar results were observed in SW480 cells (Fig. 6A). Treatment of RKO and SW480 cells with 150 μM H_2O_2 (Fig. 6B) or 160 μM T-BOOH (Fig. 6C) decreased expression of Sp1, Sp3, Sp4 and cyclin D1 (an Sp-regulated gene); however, cotreatment with thiol antioxidants (GSH or DTT) inhibited H_2O_2-mediated effects and GSH blocked T-BOOH-dependent downregulation of Sp1, Sp3, Sp4 and cyclin D1 (Fig. 6C). Thus, ascorbate and prototypical pro-oxidants such as H_2O_2 and T-BOOH inhibit growth of colon cancer cells, and this is accompanied by decreased expression of Sp1, Sp3 and Sp4 transcription factors and Sp-regulated genes that are important for to cancer cell growth (c-Met, EGFR, cyclin D1) and angiogenesis (VEGF, VEGFR1 and VEGFR2). This suggests

that the anticancer activity of ascorbate is due, in part, to ROS-dependent downregulation of Sp transcription factors.

DISCUSSION

Sp/Krüppel-like factors (KLFs) are an important family of transcription factors that regulate mammalian and viral genes through high affinity interactions with GC-rich promoter elements (33–35). Sp/KLF proteins contain three zinc fingers in their C-terminal DNA-binding domains and Sp1 was the first transcription factor identified (36). Sp1 and other Sp transcription factors are critically important in embryonic development (33–35); however, their expression decreases with age (37–39). Studies in this laboratory have demonstrated high expression of Sp1, Sp3 and Sp4 proteins in cancer cell lines and in tumors (xenografts) but minimal levels were detected in non-tumor tissues in rodent models (24, 26, 40, 41) and in cells (42), and the differential expression contributes to the selectivity of agents that target Sp transcription factors. Lou and coworkers showed differences in expression of Sp1 in normal human fibroblasts (low), a non-transformed immortalized cell line (medium), and an H-ras transformed cell line (high), and oncogene- or carcinogen-induced transformation of human fibroblasts resulted in an 8- to 18-fold increase in Sp1 protein (43). Moreover, knockdown of Sp1 in these transformed fibroblasts markedly decreased their tumorigenicity in mouse xenografts (43). RNA interference studies in which Sp1, Sp3 or Sp4 are knocked down individually or in combination also decreased expression of several Sp-regulated genes involved in cancer cell growth (c-Met, EGFR, cyclin D1), survival (survivin and bcl-2), and angiogenesis (VEGF, VEGFR1 and VEGFR2) (23–25, 29–32). These results clearly demonstrate the pro-oncogenic activity of Sp transcription factors and their potential importance as a drug target for cancer chemotherapy.

Ongoing studies with arsenic trioxide show that this anticancer drug induced ROS and ROS-dependent downregulation of Sp1, Sp3, Sp4 and Sp-regulated genes in several different cancer cell lines including RKO and SW480 colon cancer cells (23). Ascorbate has also previously been identified as a pro-oxidant drug that inhibits growth of multiple cancer cell lines; however, the underlying mechanisms of action of this compound are unclear. Using RKO and SW480 colon cancer cells as models, it was apparent that ascorbate inhibited cell proliferation and induced cell death was due to both necrosis and apoptosis (Fig. 1), and these results are consistent with previous studies (8–14). A survey of 43 cancer cell lines demonstrate variability in the IC_{50} values for growth inhibition by ascorbate (low mM to > 10 mM) (9) and our results suggest that RKO and SW480 cells are among the more sensitive cancer cell lines (9). Induction of apoptosis was somewhat variable between the cell lines, even though ascorbate significantly decreased expression of the antiapoptotic genes bcl-2 and survivin (Fig. 2) in both cell lines and this may be due, in part, to the reported inhibitory effects of H_2O_2 on apoptosis (9, 18, 19). However, the effects of ascorbate on bcl-2, survivin, cyclin D1, c-Met, EGFR, VEGF, VEGFR1 and VEGFR2 suggested that the underlying anticancer activity of this compounds may be due, in part, to targeting downregulation of Sp1, Sp3 and Sp4 transcription factors that are overexpressed in cancer cell lines and tumors (26, 40–43). Other compounds such as arsenic trioxide, curcumin and some triterpenoid anticancer agents (23, 24, 26, 41, 42) decrease expression of many of the same gene products downregulated by ascorbate (Fig. 2), and RNA interference studies show that expression of these genes is regulated by Sp1, Sp3 and Sp4 in cancer cells (24, 25, 29–32). Not surprisingly, we also observed that ascorbate decreased expression of Sp1, Sp3 and Sp4 proteins in RKO and SW480 cells (Fig. 3), and this response correlated with the downregulation of several Sp-regulated genes (Fig. 2). However, it should also be noted that 1 mM ascorbate decreased colon cancer cell growth, whereas ≥ 2 mM ascorbate was required for decreased expression of Sp1, Sp3 and Sp4 proteins. Thus, ascorbate-induced Sp

downregulation is not the only pathway associated with the anticancer activity of this compound.

Ascorbate-induced cytotoxicity has been associated with generation of H_2O_2 (9, 18–23, 27, 28), and our results with the pro-oxidants H_2O_2 and T-BOOH show that like ascorbate, these compounds are also cytotoxic and decrease proliferation and downregulate Sp1, Sp3 and Sp4 expression in RKO and SW480 cells (Figs. 3 and 6). The linkage between the pro-oxidant activities of ascorbate, H_2O_2 and T-BOOH and their effects on cell proliferation and expression of Sp transcription factors was confirmed in studies showing that these effects were partially reversed in cells after cotreatment with the antioxidant GSH which also exhibits many other activities (Figs. 5 and 6). Previous studies with hydrogen peroxide in normal cells and in cancer cell lines were mixed and have reported both increased and decreased effects on Sp1 and/or Sp-regulated genes (44–48). However, these studies primarily attributed these effects to modulation of Sp1 phosphorylation or Sp1 DNA binding. Results in this paper clearly demonstrate that ascorbate and pro-oxidants induced downregulation of Sp1, Sp3 and Sp4 proteins and Sp-regulated gene products, and we have observed similar results for arsenic trioxide (23). The effectiveness of DTT as an antioxidant was variable since GSH but not DTT inhibited ascorbate-induced downregulation of Sp1, Sp3 and Sp4 proteins (Fig. 5A), whereas both GSH and DTT blocked downregulation of Sp proteins by H_2O_2 (Fig. 6B). The specificity of DTT and other antioxidants for inhibiting drug-induced Sp downregulation has previously been observed (23).

In previous studies, the triterpenoid methyl 2-cyano-3,11-dioxo-18β-olean-1,12-dien-30-oate (CDODA-Me) decreased expression of Sp1, Sp3, Sp4 and Sp-regulated genes through induction of an Sp-repressor protein ZBTB10 in RKO and SW480 cells (26). The effects of CDODA-Me were dependent on downregulation of microRNA-27a (miR-27a) which suppresses ZBTB10 (26, 40). Ascorbate did not affect expression of miR-27a or ZBTB10 in RKO and SW480 cells, and other possible downstream targets are currently being investigated. In summary, results of this study show for the first time that the anticancer activity of ascorbate is due, in part, to downregulation of Sp1, Sp3 and Sp4 transcription factors and Sp-regulated genes. Many Sp-dependent genes such as EGFR, c-Met, bcl-2, VEGF and its receptors are themselves drug targets in specific cancers because of their critical role in tumor growth, survival and angiogenesis. Results of this study with ascorbate and previous reports with arsenic trioxide, curcumin, betulinic acid and CDODA-Me (23, 24, 26, 41, 42) demonstrate that many of these genes can be repressed at the same time by targeting Sp transcription factors, and we are currently developing this approach for single agent and combined therapies for cancer treatment.

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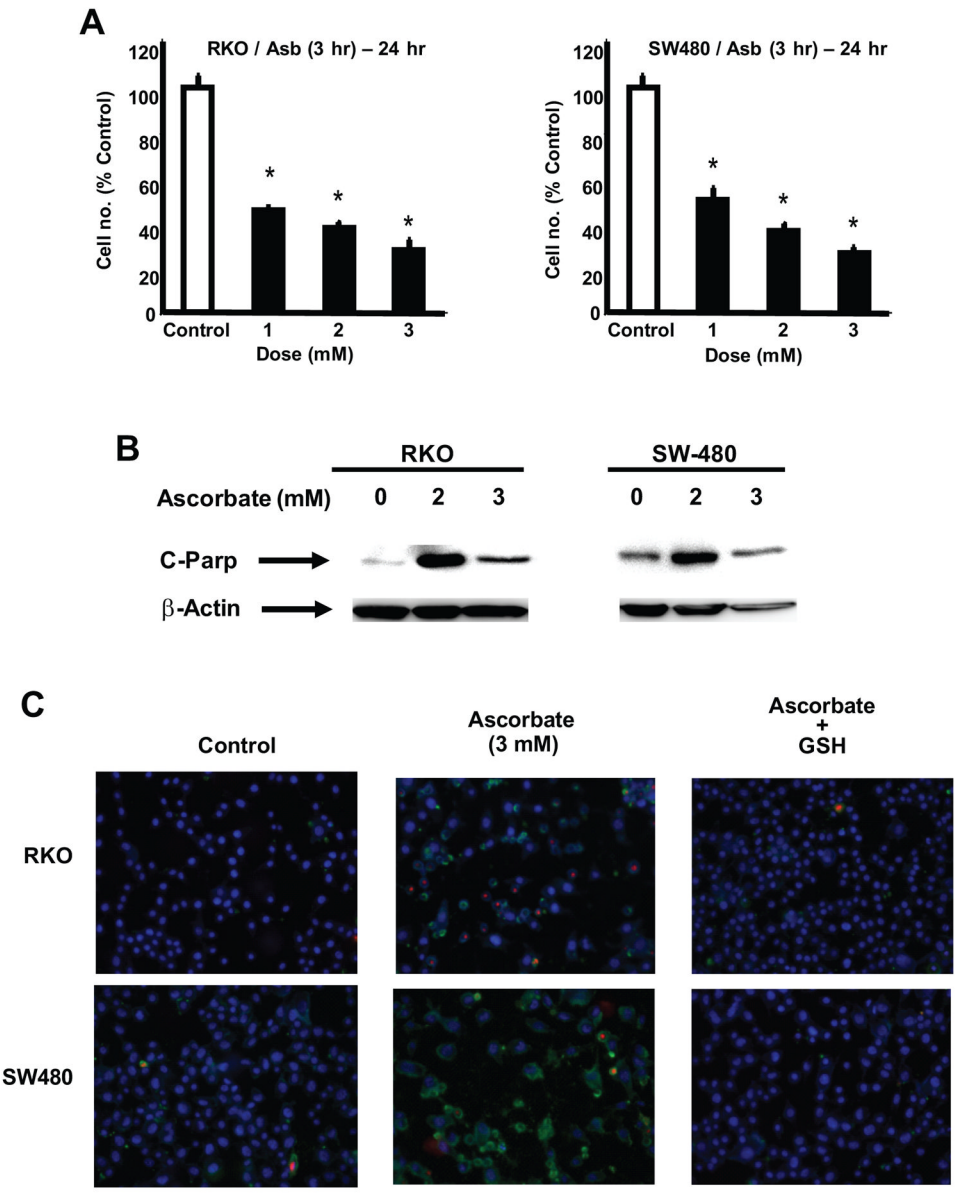

Fig. 1.

Ascorbate is cytotoxic to colon cancer cells. (*A*) Cell growth inhibition. RKO and SW480 cells were treated with 1 – 3 mM ascorbate for 3 hr and, after 24 hr, cell number was determined as described in the Materials and Methods. Results are expressed as means ± SE for 3 replicate determinations and significant ($P<0.05$) growth inhibition is indicated (*). Induction of PARP cleavage (*B*) and necrosis and apoptosis (C) in RKO and SW480 cells. RKO and SW480 cells were treated with 3 mM ascorbate for 3 hr and, after 24 hr, cell lysates were analyzed by western blots for PARP cleavage (B) or were stained with the fluorescent dye kit containing FITC-Annexin V, ethidium homodimer III and Hoechst 3342 and analyzed for apoptosis and necrosis as described in the Materials and Methods.

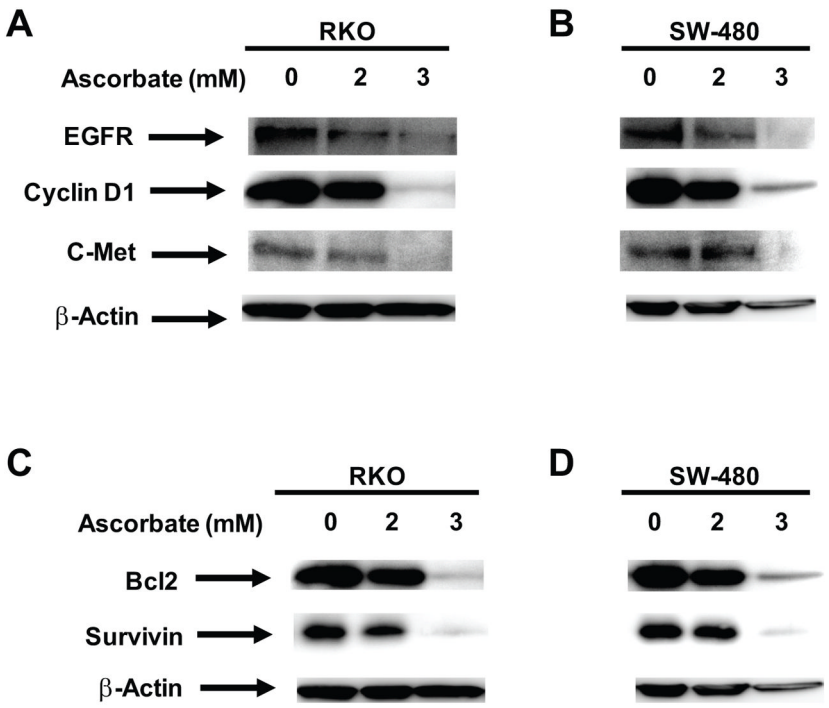

Fig. 2.

Ascorbate-induced effects on protein involved in cell growth and cell death. The effects of ascorbate on expression of EGFR, c-Met and cyclin D1 proteins in RKO (*A*) and SW480 (*B*) cells and also on levels of survivin and bcl-2 and cleaved PARP in RKO (*C*) and SW480 (*D*) cells were determined on whole cell lysates by western blots as described in the Materials and Methods. Results are typical of duplicate experiments.

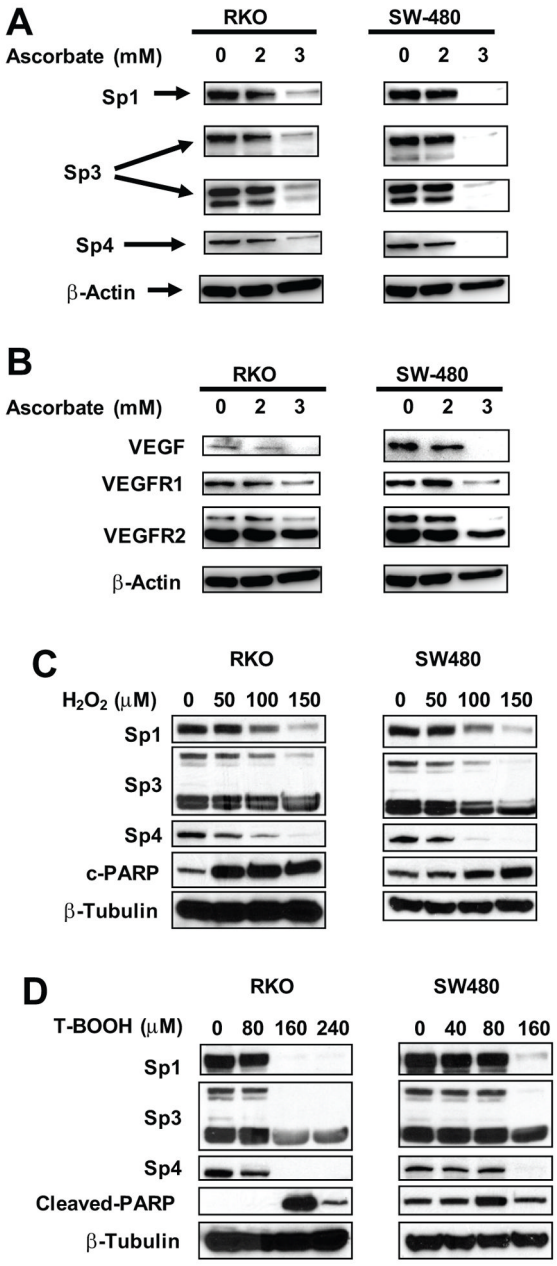

Fig. 3.

Ascorbate, H_2O_2 and T-BOOH downregulate Sp1, Sp3 and Sp4 proteins. RKO and SW480 cells were treated with ascorbate (*A, B*), H_2O_2 (*C*) and T-BOOH (*D*), and whole cell lysates were analyzed for Sp1, Sp3 and Sp4 proteins or VEGF, VEGFR1 and VEGFR2 proteins by western blots as described in the Materials and Methods. Gels are typical of results obtained in multiple (2 – 3) experiments. Cleaved PARP was also determined in cells treated with H_2O_2 or T-BOOH.

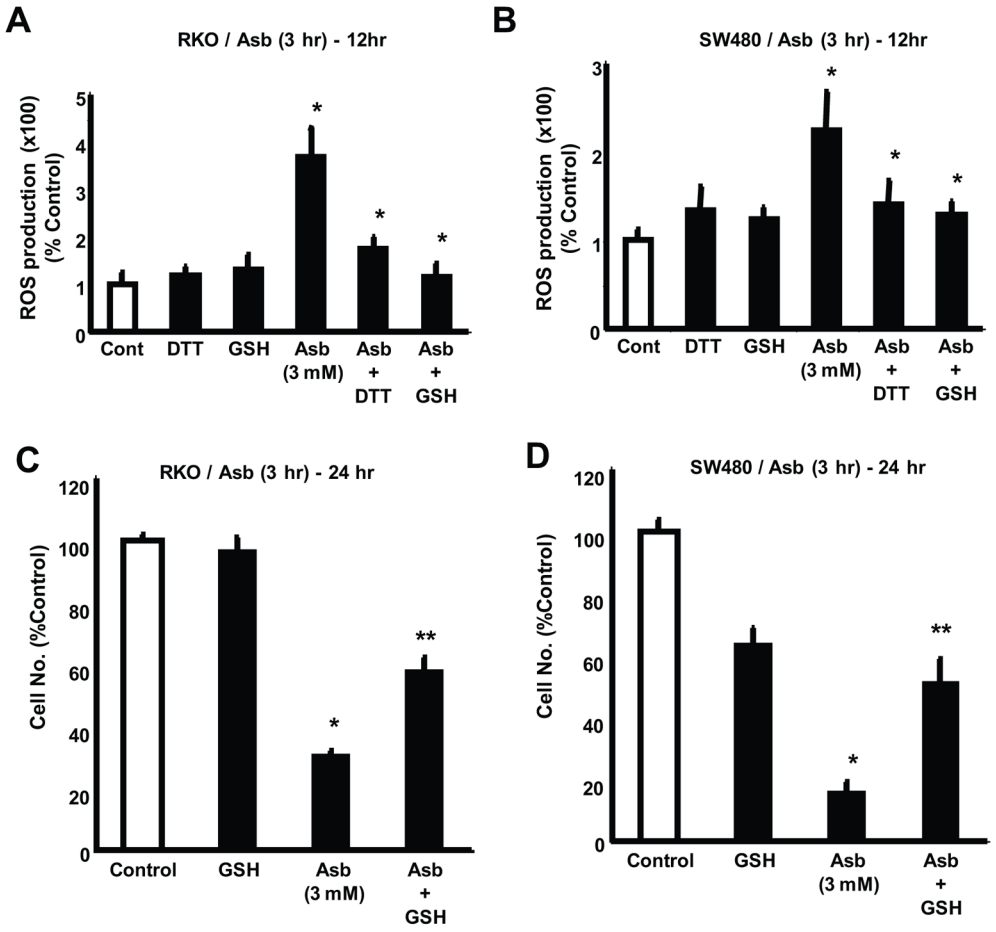

Fig. 4.

Antioxidants inhibit ascorbate-induced ROS and growth inhibition. Induction of ROS in RKO (*A*) and SW480 (*B*) cells. Cells were treated with DMSO, 3 mM ascorbate alone or in combination with antioxidants and, after 12 hr, ROS was determined as described in the Materials and Methods. Cell proliferation in RKO (*C*) and SW480 (*D*) cells. Cells were treated with DMSO, ascorbate alone, or in combination with antioxidants (3 hr) and, after 24 hr, cell numbers were counted as described in the Materials and Methods. Results (*A, B*) are means ± SE for at least 3 separate experiments per treatment group and significant ($P<0.05$) responses induced by ascorbate (*) and inhibited after cotreatment with antioxidants (**) are indicated.

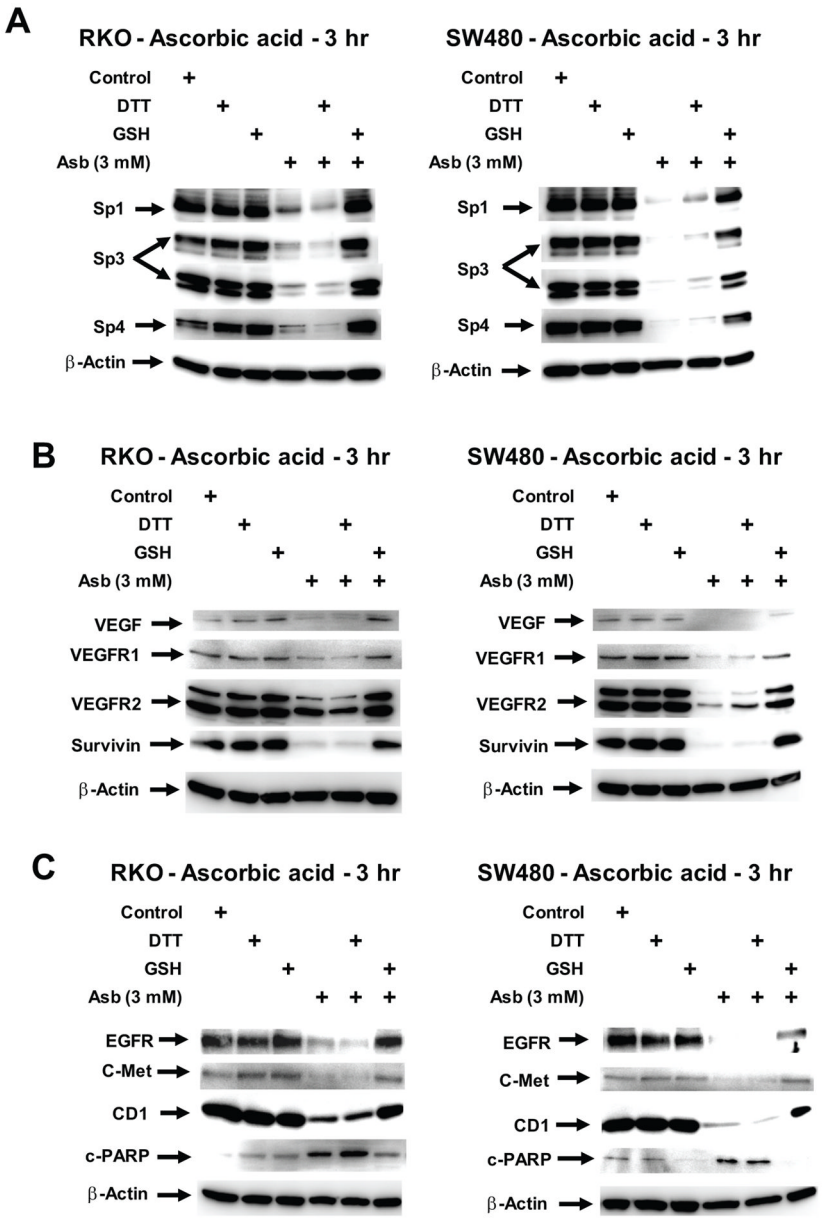

Fig. 5.

Antioxidants inhibit ascorbate-induced repression of Sp1, Sp3, Sp4 and Sp-regulated gene products. RKO and SW480 cells were treated with 3 mM ascorbate for 3 hr in the presence or absence of thiol antioxidants and, after 24 hr, the expression of Sp1, Sp3 and Sp4 proteins (*A*), Sp-regulated growth promoting (*B*), and angiogenic (*C*) proteins were determined by western blot analysis of whole cell lysates. The blots are representative of replicated (2 – 3) experiments.

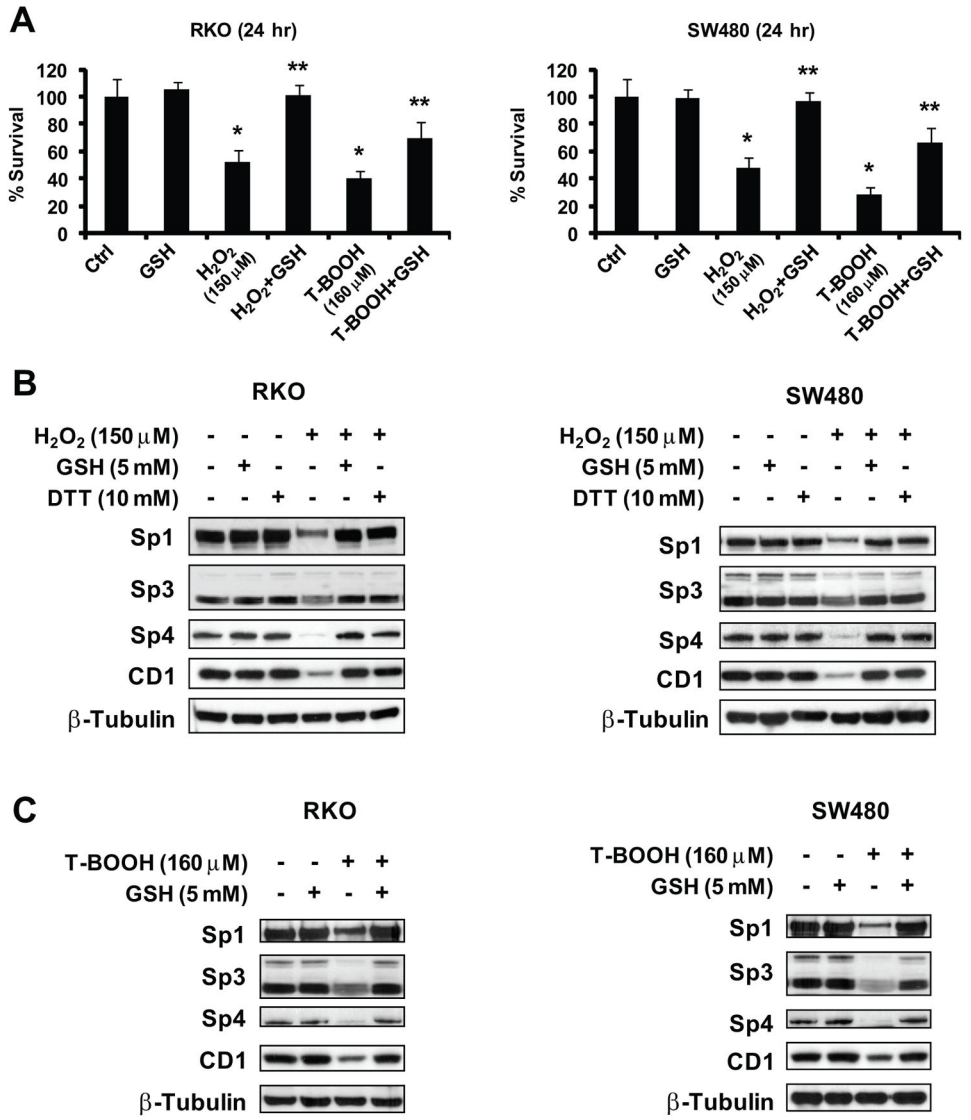

Fig. 6.

Antioxidants block H_2O_2- and T-BOOH-induced responses in colon cancer cells. (*A*) Cell proliferation. RKO and SW480 cells were treated with H_2O_2 (24 hr) or T-BOOH (24 hr) alone or in the presence of antioxidants. Cells were then counted as described in the Materials and Methods. Results are expressed as means ± SE for at least 3 replicate experiments for each treatment group and significant ($P<0.05$) H_2O_2- and T-BOOH-induced responses (*) and inhibition by antioxidant (**) are indicated. Effects of antioxidants on H_2O_2 (*B*) and T-BOOH (*C*)-dependent downregulation of Sp transcription factors. Cells were treated as described in (*A*) and whole cell lysates were analyzed by western blots. Gels are typical of replicate (at least 2) experiments.