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Role of Intracellular Ca^{2+} Signal in the Ascorbate-Induced Apoptosis in a Human Hepatoma Cell Line

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Although ascorbate (vitamin C) has been shown to have anti-cancer actions, its effect on human hepatoma cells has not yet been investigated, and thus, the exact mechanism of this action is not fully understood. In this study, the mechanism by which ascorbate induces apoptosis using HepG2 human hepatoblastoma cells is investigated. Ascorbate induced apoptotic cell death in a dose-dependent manner in the cells, was assessed through flow cytometric analysis. Contrary to expectation, ascorbate did not alter the cellular redox status, and treatment with antioxidants (*N*-acetyl cysteine and *N,N*-diphenyl-*p*-phenylenediamine) had no influence on the ascorbate-induced apoptosis. However, ascorbate induced a rapid and sustained increase in intracellular Ca^{2+} concentration. EGTA, an extracellular Ca^{2+} chelator did not significantly alter the ascorbate-induced intracellular Ca^{2+} increase and apoptosis, whereas dantrolene, an intracellular Ca^{2+} release blocker, completely blocked these actions of ascorbate. In addition, phospholipase C (PLC) inhibitors (U-73122 and manoalide) significantly suppressed the intracellular Ca^{2+} release and apoptosis induced by ascorbate. Collectively, these results suggest that ascorbate induced apoptosis without changes in the cellular redox status in HepG2 cells, and that the PLC-coupled intracellular Ca^{2+} release mechanism may mediate ascorbate-induced apoptosis.

Key words: Ascorbate, Apoptosis, HepG2 cell, Intracellular Ca^{2+}

INTRODUCTION

Ascorbate (vitamin C), a water-soluble chain-breaking antioxidant and enzyme cofactor, appears to play an essential role in maintaining vital functions, such as biosynthesis of collagen, carnitine, catecholamine, and peptide neurohormones (Bendich, 1997; Wilson, 2002). Reduced serum concentration of ascorbate in humans seems to cause neurological problems, eventually leading to scurvy (Richardson *et al.*, 2002). In addition, ascorbate has been examined in various epidemiologic studies as a potential chemopreventive agent for cancer (Lee *et al.*, 2003). Moreover, ascorbate could inhibit cancer cell growth in a variety of cancer cells, for examples, in lymphoma and leukemia cells (Kao *et al.*, 1993), melanoma cells (Gardiner and Duncan, 1989), brain tumor cells (Baader *et al.*, 1994; Lee and Wurster, 1994), and prostate cancer cells (Maramag *et al.*, 1997; Menon *et al.*, 1998). The mechanism by which ascorbate inhibits tumor cell growth has been postulated to be caused by its paradoxical prooxidant property (Menon *et al.*, 1998). Reactive oxygen species (ROS), particularly superoxide anions, appear to be produced during autooxidation of ascorbate by molecular oxygen (Scarpa *et al.*, 1983). However, the exact mechanism of ascorbate-induced inhibition of tumor cell growth has not been fully elucidated.

Apoptosis is characterized by the condensation of nuclear chromatin, loss of plasma membrane phospholipid asymmetry, activation of proteases and endonucleases, enzymatic cleavage of the DNA into oligonucleosomal fragments, and segmentation of the cells into membrane-bound apoptotic bodies (Kidd, 1998). Apoptosis plays a critical role in the maintenance of tissue homeostasis through the selective elimination of excessive cells (Song and Steller, 1999). In particular, genetic mutations culminating in the disturbance of apoptosis or derangement of apoptosis-signaling pathways seem to be an essential factor of carcinogenesis (Lowe and Lin, 2000; Wang, 1999). On the other hand, the induction of apoptosis of cancer cells is regarded as one of the most important methods for cancer treatment (Kornblau, 1998). Additionally, many anti-cancer agents have been reported to

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induce apoptosis of cancer cells (Kamesaki, 1998), and radiation therapy for cancer seems to be based on apoptosis induction of cancer cells (Crompton, 1998). Although apoptotic signaling pathways are not completely known, perturbation of intracellular Ca^{2+} appears to be a common mechanism of apoptosis (McConkey and Orrenius, 1997).

Thus, the purpose of this study was to elucidate the mechanism by which ascorbate induces apoptosis in tumor cells. Specifically, the roles of ROS and intracellular Ca^{2+} were investigated in the mechanism of ascorbate-induced apoptosis using HepG2 human hepatoblastoma cells as a model cellular system.

MATERIALS AND METHODS

Materials

The HepG2 human hepatoblastoma cell line was purchased from American Type Culture Collection (Rockville, MA). Powdered Eagle's minimum essential medium (MEM) and Earle's basal salt solution (EBSS), trypsin solution, sodium pyruvate, sodium ascorbate, *N*-acetyl cysteine (NAC), *N,N*-diphenyl-*p*-phenylenediamine dantrolene (DPPD), manoalide, 1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1*H*-pyrrole-2,5,-dione (U-73122), ethylene glycol-bis-(aminoethylether)-ethane-*N,N,N',N'*-tetraacetic acid (EGTA), propidium iodide (PI), ribonuclease A, and all salt powders were obtained from Sigma Chemical CO. (St. Louis, MO). 2',7'-Dichlorofluorescin diacetate (DCFH-DA), bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid/acetoxymethyl ester (BAPTA/AM), and 1-(2,5-carboxyoxazol-2-yl-6-aminobenzfuran-5-oxyl)-2-(2'-amino-methylphenoxy)-ethane-*N,N,N',N'*-tetraacetoxylmethyl ester (Fura-2/AM) were purchased from Molecular Probes, Inc. (Eugene, OR). Fetal bovine serum (FBS) and antibiotics (penicillin and streptomycin mixture) were purchased from GIBCO (Grand Island, NY). DCFH-DA, BAPTA/AM, and Fura-2/AM were prepared as stock solutions in dimethyl sulfoxide (DMSO), and then, they were diluted with aqueous medium to the desired concentrations. The stock solution of drugs was sterilized by filtration through 0.2 μm disc filters (Gelman Sciences: Ann Arbor, MI).

Cell culture

HepG2 cells were grown at 37°C in a humidified incubator under a 5% CO_2/95% air mixture in MEM that was supplemented with 10% FBS, 200,000 IU/L penicillin, 200 mg/L of streptomycin, and 1 mM sodium pyruvate. Culture medium was replaced every other day. After attaining confluence, the cells were subcultured following trypsinization treatment.

Intracellular Ca^{2+} measurement

Aliquots of the HepG2 cells were washed in EBSS. Then, 5 μM Fura-2/AM was added, and the cells were incubated for 30 min at 37°C. Unloaded Fura-2/AM was removed by centrifugation at 150×*g* for 3 min. Cells were resuspended at a final density of 2×10^9 cells/L in Krebs-Ringer buffer (KRB) containing 125 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 5 mM $NaHCO_3$, 25 mM HEPES, 6 mM glucose, and 2.5 mM probenecid (pH 7.4). Fura-2-loaded cells were maintained at 25°C for 90 min prior to undergoing fluorescence measurement. For each experiment, 0.5 mL aliquot of Fura-2-loaded cells was equilibrated to 37°C in a stirred quartz cuvette. Fluorescence emission (510 nm) was monitored with the excitation wavelength cycling between 340 and 380 nm using a Hitachi F4500 fluorescence spectrophotometer. At the end of an experimental run, maximum and minimum fluorescence values at each excitation wavelength were obtained by first lysing cells with 20 mg/L digitonin for the maximum fluorescence value and then adding 10 mM EGTA for the minimum fluorescence value. After obtaining these maximum and minimum values, the 340:380 nm fluorescence ratios were converted into free Ca^{2+} concentrations using the software, F-4500 Intracellular Cation Measurement System, which was provided by Hitachi.

Flow cytometry assays

For flow cytometry analysis, HepG2 cells were collected and washed twice with PBS buffer, pH 7.4. After fixing in 80% ethanol for 30 min, cells were washed twice, and resuspended in PBS buffer (pH 7.4) that contained 0.1% Triton X-100, 5 mg/L PI, and 50 mg/L ribonuclease A for DNA staining. Then the cells were analyzed by a FACScan (BIO-RAD, Hercules, CA), and at least 20,000 events were evaluated. All histograms were analyzed using the WinBryte software (BIO-RAD, Hercules, CA) to determine the percentage of nuclei with a hypodiploid content that is indicative of apoptosis (Milner *et al.*, 1998).

The normal lipid organization of the plasma membrane is altered soon after apoptosis is initiated. Thus, to demonstrate the loss of phospholipid asymmetry and the presence of phosphatidylserine on the outer layer of the plasma membrane annexin-V binding was also employed as an indicator of apoptosis (Vermes *et al.*, 1995). Annexin-V binding was analyzed using a commercial kit (Boehringer Mannheim Biochemicals, Mannheim, Germany). The cells were washed in cold PBS and were then resuspended in binding buffer. An alliquot of cell suspension (500 μL) was exposed to Annexin-V-FLUOS. The cells were gently vortexed, incubated at room temperature for 20 min in the dark, and then analyzed by FACScan within 1 h of staining.

Measurement of intracellular ROS

Relative changes in intracellular ROS in HepG2 cells were monitored using a fluorescent probe, namely 2',7'-dichlorofluorescin diacetate (DCFH-DA) (LaBel *et al.*, 1992). DCFH-DA can diffuse through the cell membrane readily and can be hydrolyzed by intracellular esterases to nonfluorescent 2',7'-dichlorofluorescin (DCFH), which is then rapidly oxidized to highly fluorescent 2',7'-dichlorofluorescein (DCF) in the presence of ROS. The DCF fluorescence intensity is proportional to the amount of ROS that has been formed intracellularly (Shen *et al.*, 1996). Cells were washed twice and resuspended at a concentration of 4×10^5 cells/mL in Hanks solution. For loading DCFH-DA into the cells, cells were incubated with the dye for 2 hrs at a final concentration of 5 μM at 37°C. Fluorescence (excitation wavelength set at 485 nm and the emission wavelength at 530 nm) was monitored in a well-stirred cuvette.

Data analysis

All experiments were performed four times. The data were expressed as means ± standard error of the mean (SEM), and the results were analyzed using one way analysis of variance (ANOVA) and the Student-Newman-Keul's test for individual comparisons. *P* values less than 0.05 are considered statistically significant.

RESULTS

Induction of apoptotic cell death by ascorbate

The effect of ascorbate on apoptotic cell death of HepG2 cells was examined using two independent methods. The ascorbate-induced apoptosis was identified by using the annexin-V binding assay, which detected the loss of phospholipid asymmetry, and the presence of PS on the outer layer of the plasma membrane occurred at an early stage of apoptosis (Vermes *et al.*, 1995), as shown in Fig. 1A. Ascorbate also induced DNA fragmentation, a hallmark of apoptosis (Kidd, 1998), in a concentration-dependent manner, which was studied by flow cytometry through the determination of hypodiploid DNA content stained with PI (Milner *et al.*, 1998) as depicted in Fig. 1B and 1C. The prominent apoptotic effect of ascorbate was initially detected at the concentration of 3 mM. These results clearly demonstrate that ascorbate induced apoptotic cell death in the HepG2 cells.

No involvement of ROS in the ascorbate-induced apoptosis

Although ascorbate has been well known to act as an antioxidant, the inhibitory action of ascorbate on cancer cell growth appears to be associated with its pro-oxidant property (Menon *et al.*, 1998). Thus, the possible involve-

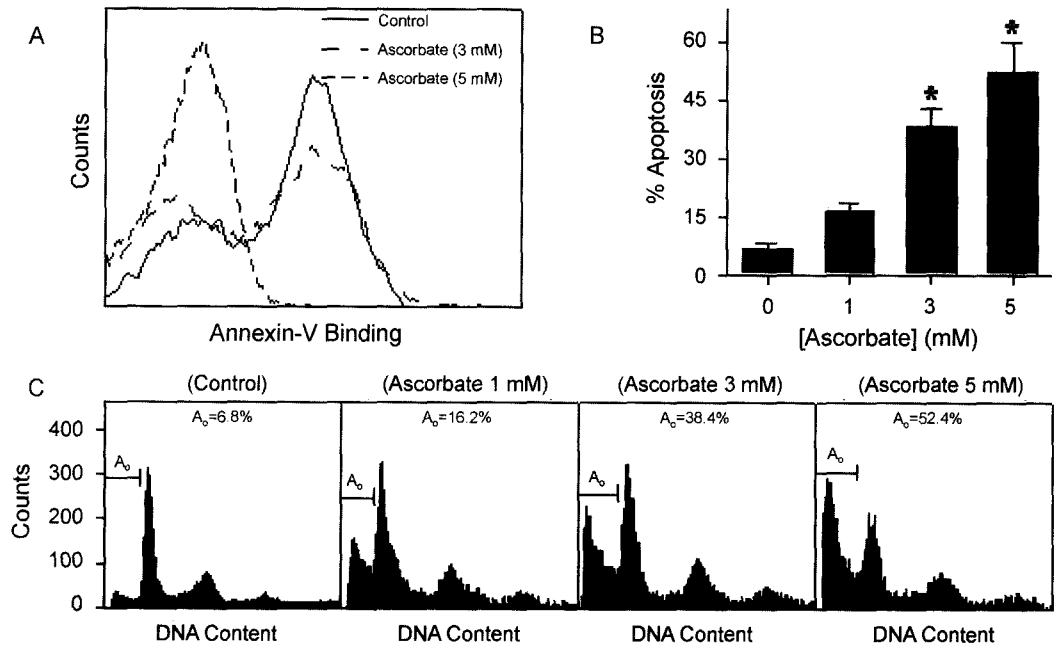

Fig. 1. Ascorbate induces a concentration-dependent apoptotic cell death in HepG2 human hepatoblastoma cells. In the experiments of (A), the cells were incubated in the absence (control) or in the presence of ascorbate (3 and 5 mM) for 48 h. Cells were stained with Annexin-V-FLUOS and analyzed by flow cytometry. Note that in the presence of ascorbate there is a shift in annexin-V-FLUOUS fluorescence. This is due to the binding of annexin-V to membrane phospholipids of cells undergoing apoptosis. Results are representative of four experiments. In the experiments of (B and C), cells were treated for 48 h with or without each concentration of ascorbate. The number of apoptotic cells was measured by flow cytometry. The region to the left of the G_0/G_1 peak, designated A_0, was defined as cells undergoing apoptosis-associated DNA degradation. In bar graphs (B), the data represent the mean values of four replications with bars indicating SEM. *$p<0.05$ compared to control.

ment of ROS in the ascorbate-induced apoptosis was investigated. Ascorbate did not significantly enhance the basal generation of ROS in the HepG2 cells, as illustrated in Figs. 2A and 2B. Moreover, treatment with antioxidants (NAC and DPPD) did not affect the induction of apoptosis by ascorbate, as shown in Fig. 2C. These results imply that ascorbate did not act as a pro-oxidant in the HepG2 cells, and that ROS may not be involved in the mechanism of ascorbate-induced apoptosis.

Role of intracellular Ca^{2+} signal in the ascorbate-induced apoptosis

Since the intracellular Ca^{2+} signal appears to be commonly involved in the mechanism of apoptosis (McConkey and Orrenius, 1997), we examined whether ascorbate alters intracellular Ca^{2+} concentration using the Fura-2 fluorescence technique (Grynkiewicz *et al.*, 1985). At the concentration that apoptosis is induced (5 mM), ascorbate rapidly increased intracellular Ca^{2+} concentration, as shown in Fig. 3A. Treatment with BAPTA/AM, an intracellular Ca^{2+} chelator, significantly suppressed the ascorbate-induced apoptosis as shown in Figs. 3B and 3C. These results suggest that ascorbate-induced apoptosis may be caused by the increase in intracellular Ca^{2+}.

In order to determine the source of the ascorbate-induced intracellular Ca^{2+} increase, intracellular Ca^{2+} concentration was measured using a nominal Ca^{2+}-free medium containing 1 mM EGTA. This experimental protocol can effectively reduce extracellular free Ca^{2+} concentration, and in tern, blunt the available Ca^{2+} influx. Under these conditions, the response of cellular Ca^{2+} to ascorbate was not significantly altered as illustrated in Fig. 4. Treatment with dantrolene, an inhibitor of intracellular Ca^{2+} release from inositol 1,4,5-trisphosphate (IP_3)-sensitive stores (Ehrlich *et al.*, 1994), completely inhibited the Ca^{2+}-increasing effect of ascorbate. These results indicate that

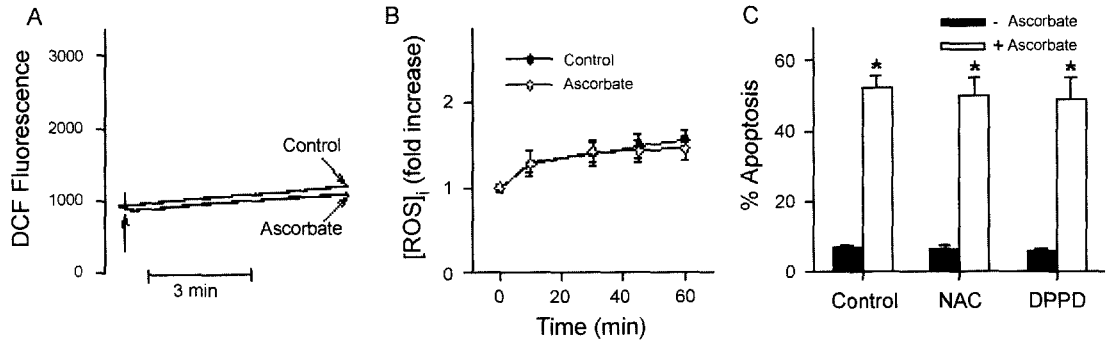

Fig. 2. No involvement of the cellular redox status in the ascorbate-induced apoptosis in HepG2 human hepatoblastoma cells. Data (A) show changes in ROS levels as a function of time, which was measured using the DCF fluorescence method. The arrow shows the time point for the addition of ascorbate (5 mM). In data (B), results are expressed as a fold increase compared to the initial DCF fluorescence intensity. Data points represent the mean values of four replications with bars indicating SEM. Note that ascorbate did not significantly alter the cellular ROS level. In the experiments of (C), the cells were incubated with or without ascorbate (5 mM) for 48 h. NAC (50 mM) and DPPD (10 μM) were given 30 min before ascorbate application. The number of apoptotic cells was measured by flow cytometry. The data represent the mean values of four replicates with bars indicating SEM. *P<0.05 compared to the control condition in which the cells were incubated with ascorbate-free medium.

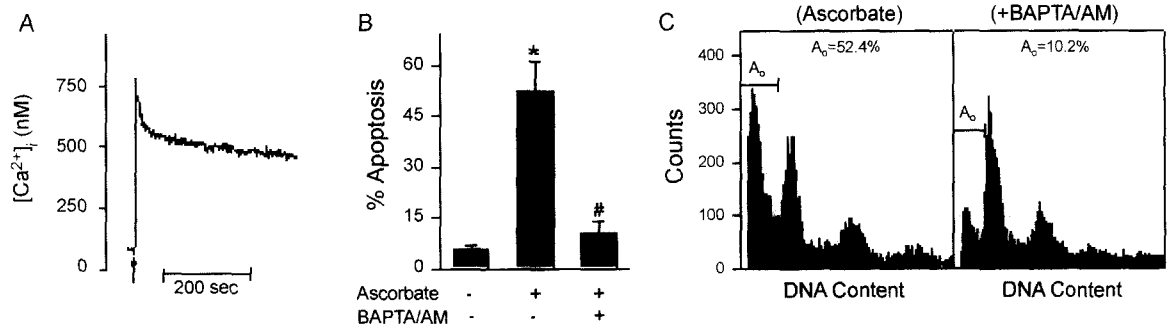

Fig. 3. Ascorbate-induced apoptosis is dependent on intracellular Ca^{2+} increase in HepG2 human hepatoblastoma cells. Intracellular Ca^{2+} concentration was assessed by Fura-2 fluorescence technique (A), and the data represent intracellular Ca^{2+} changes with time. The arrow shows the time point for the addition of ascorbate (5 mM). The number of apoptotic cells was measured by flow cytometry (B and C). In the data (C), the region to the left of the G_0/G_1 peak, designated A_0, was defined as cells undergoing apoptosis-associated DNA degradation. BAPTA/AM (1 μM) was added to the cells 4 h before treatment with ascorbate (5 mM). In bar graphs (B) the data represent the mean values of four replications with bars indicating SEM. *p<0.05 compared to the control. #p<0.05 compared to ascorbate alone.

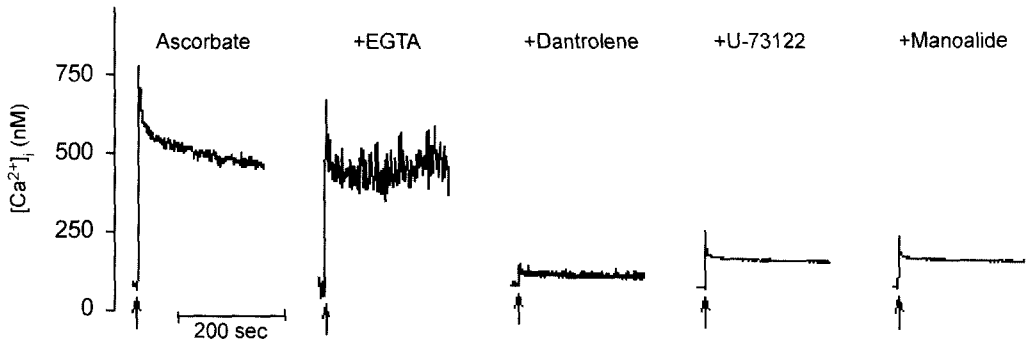

Fig. 4. Effects of various Ca^{2+} inhibitors on the ascorbate-induced intracellular Ca^{2+} increase in HepG2 human hepatoblastoma cells. Intracellular Ca^{2+} concentration was assessed by the Fura-2 fluorescence technique, and the data represent intracellular Ca^{2+} changes with time. The arrow shows the time points for addition of ascorbate (5 mM). In these experiments an intracellular Ca^{2+} release blocker (25 μM dantrolene) and PLC inhibitors (10 μM U-73122, 2 μM manoalide) were added to the cells 5 min before treatment with ascorbate. In the experiment with EGTA, an extracellular Ca^{2+} chelator, nominal Ca^{2+}-free medium containing 1 mM EGTA was used.

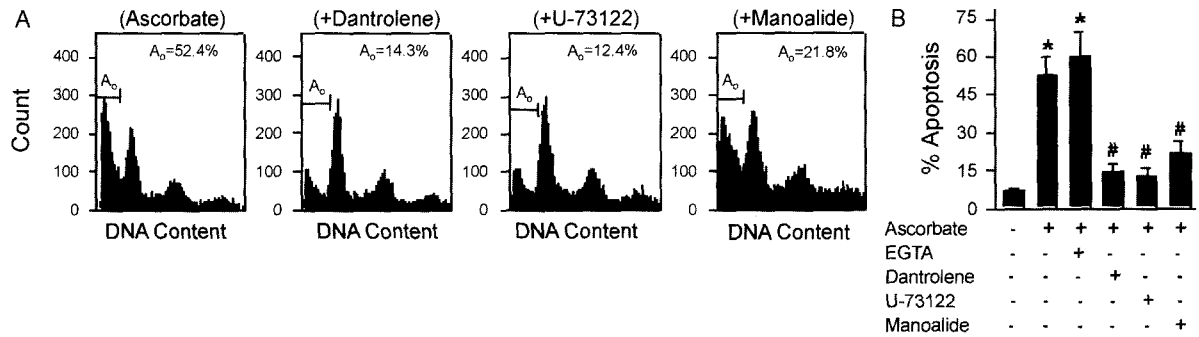

Fig. 5. Effects of various Ca^{2+} inhibitors on the ascorbate-induced apoptosis in HepG2 human hepatoblastoma cells. In the experiments, cells were treated for 48 h with ascorbate (5 mM). The number of apoptotic cells was measured by flow cytometry. The region to the left of the G_0/G_1 peak, designated A_0, was defined as cells undergoing apoptosis-associated DNA degradation. In these experiments, an extracellular Ca^{2+} chelator (1 mM EGTA), an intracellular Ca^{2+} release blocker (25 μM dantrolene) and PLC inhibitors (10 μM U-73122, 2 μM manoalide) were added 4 h before ascorbate application. In bar graphs (B) the data represent the mean values of four replications with bars indicating SEM. *$p<0.05$ compared to control. #$p<0.05$ compared to ascorbate alone.

the increased intracellular Ca^{2+} concentration by ascorbate may be due to Ca^{2+} release from IP_3-dependent internal stores. Since phospholipase C (PLC) seems to act as an enzyme to liberate IP_3 from the plasma membrane (Hughes and Putney, 1990)], the role of PLC in these actions of ascorbate was further examined. As illustrated in Fig. 4, treatment with U-73122, a specific PLC inhibitor (Jin *et al.*, 1994), or manoalide, a non-specific inhibitor of PLC and phospholipase A_2 (Bennett *et al.*, 1987), significantly inhibited the ascorbate-induced intracellular Ca^{2+} release. Moreover, treatment with these inhibitors (dantrolene, U-73122, manoalide) also significantly prevented the apoptosis that was induced by ascorbate, as depicted in Figs. 5A and 5B. These results strongly support that PLC may mediate the ascorbate-induced intracellular Ca^{2+} release through the liberation of IP_3, ultimately resulting in the induction of apoptosis.

DISCUSSION

As an antioxidant, ascorbate plays a broad spectrum of biological roles, one of which is to restrict the propagation of multiple reactive electrophilic end-products from free radicals produced within the cells either by normal cellular processes or from toxic exogenous precursors (Brown and Jones, 1996; Buettner and Jurkiewicz, 1996). Protective effects against ionizing radiation damage have been extensively documented in animals and in cultured cells (Konopacka *et al.*, 1998; Tauchi and Sawada, 1993; Yasulkawa *et al.*, 1989). In addition, ascorbate has been shown to have anti-cancer effects through inhibition of the growth of various tumor cells (Baader *et al.*, 1994; Gardiner and Duncan, 1989; Kao *et al.*, 1993; Lee and Wurster, 1994; Maramag *et al.*, 1997; Menon *et al.*, 1998; Tamayo and Richardson, 2003). Ironically, the mechanism of the anti-cancer actions of ascorbate appears to not be related with its well-known anti-oxidant effects. On the contrary, the increased production of oxidants (e.g., hydrogen peroxide) may mediate the growth-inhibitory action of ascorbate (Menon *et al.*, 1998). In addition, ascorbate-induced oxidative stress and ascorbate-induced apoptosis

have been effectively inhibited by antioxidants in B16F10 murine melanoma cells (Kang *et al.*, 2003) and PC12 cells (Song *et al.*, 2001). Although we do not know this discrepancy between the results of these cells and HepG2 cells, in the present study, we clearly demonstrated that ascorbate can induce apoptotic cell death independent of the production of oxidants in HepG2 human hepatoma cells (Figs. 1 and 2).

Accumulating evidence implies that intracellular Ca^{2+} is commonly involved in the mechanism of apoptosis (McConkey and Orrenius, 1997). Consistent to this implication, in this study, it was also shown that ascorbate appeared to induce apoptosis through the rise of intracellular Ca^{2+}, since ascorbate induced a rapid and sustained increase in intracellular Ca^{2+} concentration (Fig 3A), and intracellular Ca^{2+} chelation with BAPTA/AM effectively prevented ascorbate-induced apoptosis (Fig. 3B). In addition to apoptosis, cell proliferation and differentiation have been linked to the stimulation of the intracellular Ca^{2+} signal (Munaron *et al.*, 2004). Interestingly, Ca^{2+} channel antagonists have decreased cell proliferation in a variety of cancer cells *in vitro* (Yoshida *et al.*, 2003) and *in vivo* (Taylor *et al.*, 1992).

Ascorbate appeared to elevate the level of intracellular Ca^{2+} through the release of internal Ca^{2+}, since this action of ascorbate was not altered by reducing extracellular Ca^{2+} concentration with a nominal Ca^{2+}-free medium containing 1 mM EGTA but was completely inhibited by the intracellular Ca^{2+} release blocker, dantrolene (Fig. 4). Significant inhibition of ascorbate-induced apoptosis by the Ca^{2+} release blocker (Fig. 5) implies that Ca^{2+} release from internal stores may act as a major trigger for the ascorbate-induced apoptotic cell death in the HepG2 cells. Although the intracellular Ca^{2+} store release was found to be the major mechanism of the ascorbate-induced Ca^{2+} increase, the possible involvement of other mechanisms cannot be completely excluded. Thapsigargin, a specific inhibitor of Ca^{2+}-ATPase of the endoplasmic reticulum (ER) that elevates intracellular Ca^{2+}, appeared to induce apoptosis in many cancer cells (Jackisch *et al.*, 2000). Thus, it is possible that the ascorbate-induced Ca^{2+} increase may be through Ca^{2+} release from the thapsigargin-sentive pools. In addition, the receptor-operated Ca^{2+} influx may possibly be involved in this process because the empty state of Ca^{2+} stores appeared to stimulate the Ca^{2+} influx pathway (Li *et al.*, 2002). Interestingly, Ca^{2+} influx through the receptor-operated Ca^{2+} channels has been shown to induce mitochondria-triggered cell death in Jarkat T cells (Jambrina *et al.*, 2003).

Members of the PLC family of enzymes hydrolyze phosphatidylinositol-(4,5)-bisphosphate (PIP_2), generating IP_3 and diacylglycerol (Hughes and Putney, 1990). The PLC inhibitors (U-73122 or manoalide) significantly prevented the ascorbate-induced elevation of intracellular Ca^{2+} (Fig. 4), which indicates that PLC may mediate the ascorbate-induced intracellular Ca^{2+} mobilization. In addition, the significant inhibition of ascorbate-induced apoptosis by these PLC inhibitors (Fig. 5) suggests that PLC activation may be necessary for this apoptotic process.

The inhibitory effect of ascorbate on tumor cell growth has been suggested to be independent of the intracellular incorporation of ascorbate (Amano *et al.*, 1998). The results of this study also suggest that the rise in intracellular Ca^{2+} and apoptotic effects of ascorbate may occur apart from ascorbate incorporation because ascorbate rapidly increased the intracellular Ca^{2+} level, and the major target of ascorbate appeared to be the PLC residing in the plasma membrane. The PLC family is further classified into three subgroups: β, γ, and δ. PLCγ is most commonly activated by protein tyrosine kinases, and PLCβ by heterotrimeric G-proteins (Singer *et al.*, 1997), which are composed of three subunits ($\alpha\beta\gamma$) and which include four families: G_s, G_i, G_q, and G_{12} (Sternweis and Smrcka, 1993). PLCβ can be activated by the α subunits of the G_q family, which includes the four members: G_q, G_{11}, G_{14}, and G_{15}/G_{16}, or by the $\beta\gamma$ subunits associated with any of the α subunit families (Singer *et al.*, 1997). At present, we do not exactly know how ascorbate activates the PLC. Although speculated, ascorbate may act on the putative membrane receptor coupled to the activation of PLC via stimulation of certain types of G proteins. This possibility should be determined in a future study.

In conclusion, ascorbate induced apoptosis in HepG2 human hepatoblastoma cells and the activation of PLC, and the subsequent release of intracellular Ca^{2+} from IP_3-sensitive stores may be essentially involved in the mechanism of this action. The results from this study further suggest that ascorbate may be valuable for the therapeutic intervention of human hepatomas.

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