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Original Research Communication

The *In Vitro* Cytotoxicity of Ascorbate Depends on the Culture Medium Used to Perform the Assay and Involves Hydrogen Peroxide

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ABSTRACT

Reports about the effects of ascorbate (vitamin C) on cultured cells are confusing and conflicting. Some authors show inhibition of cell death by ascorbate, whereas others demonstrate that ascorbate is cytotoxic. In this report, using three different cell types and two different culture media (Dulbecco's modified Eagle's medium and RPMI 1640), we show that the toxicity of ascorbate is due to ascorbate-mediated production of H_2O_2, to an extent that varies with the medium used to culture the cells. For example, 1 m*M* ascorbate generates 161 ± 39 μM H_2O_2 in Dulbecco's modified Eagle's medium and induces apoptosis in 50% of HL60 cells, whereas in RPMI 1640 only 83 ± 17 μM H_2O_2 is produced and no apoptosis is detected. Apoptosis is prevented by catalase, and direct addition of H_2O_2 at the above concentration to the cells has similar effects to ascorbate. These results show that ascorbate itself is not toxic to the cell lines used and that effects of ascorbate *in vivo* cannot be predicted from studies on cultured cells. The ability of ascorbate to interact with different cell culture media to produce H_2O_2 at different rates could account for many or all of the conflicting results obtained using ascorbate in cultured cell assays. Antioxid. Redox Signal. 3, 157–163.

INTRODUCTION

THE BENEFICIAL ROLE OF ASCORBATE (vitamin C) in the human diet has been recognized for many years. Numerous epidemiological studies have suggested the importance of ascorbate (or foods rich in ascorbate) in the prevention of various types of cancer (6). Ascorbate is an essential cofactor for several enzymes, but also appears to be an important antioxidant *in vivo* (4, 5). Several articles have shown that ascorbate inhibits tumor cell death induced by oxidative stress (2, 21, 26–28). Paradoxically, however, multiple studies using cultured cells have reported that ascorbate is cytotoxic (17, 19, 20). Hence, reports on the effect of ascorbate on cultured cells are confusing and contradictory.

In this report, using three different cell lines and two different culture media, we show that the *in vitro* toxicity of ascorbate is due to ascorbate-mediated production of H_2O_2, at rates that vary with the medium used to culture the cells. These data raise the possibility that the conflicting results found in the literature arise from the different cell culture conditions used and that effects of ascorbate on cells in culture should be interpreted with caution, and may

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not be indicative of the effect of ascorbate *in vivo*.

MATERIALS AND METHODS

Tumor cell line

The human promyelocytic leukemia cell line HL60 and human bladder carcinoma T24 were obtained from ATCC (Rockville, MD, U.S.A.), and the human melanoma M14 cells were a gift from Dr. Armando Bartolazzi (Oncologia Clinica e Sperimentale, Rome, Italy). HL60 cells were maintained in culture in RPMI 1640 (Hyclone, Irvine, CA, U.S.A.), T24 in McCoy's media (GibcoBRL, Gaithersburg, MD, U.S.A.), and M14 in Dulbecco's modified Eagle's medium (DMEM; Hyclone). All media were supplemented with 5% fetal bovine serum (FBS), 1% glutamine, and 0.5% gentamicin (GibcoBRL).

Reagents

L-Ascorbic acid sodium salt (tissue culture tested), propidium iodide (PI), RNase A, catalase (type C-40), aprotinin, pepstatin A, leupeptin, and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma–Aldrich Pte Singapore and DMEM from Hyclone. PI stock solution was prepared as 0.5 mg/ml PI in 0.38 m*M* sodium citrate, pH 7. RNase A stock solution is a 10 mg/ml solution in 10 m*M* Tris-HCl, pH 7.5, 15 m*M* NaCl.

DNA fragmentation

HL60, T24, and M14 cells (10^6) were treated with increasing concentrations of ascorbate. After 18 h of incubation time, cells were washed with phosphate-buffered saline (PBS) and pelleted. Cell pellets were resuspended in 0.5 ml of PBS/1% FBS and immediately fixed by adding 7 ml of 75% (vol/vol) ethanol while vortexing to avoid clumping. Fixed cells were left at 4°C for 15 min, centrifuged at 1,000 *g* for 5 min, and washed once with PBS/1% FBS. Cell pellets were then suspended in a PI/RNase A solution prepared by adding 1/50 volume PI stock and 1/40 volume of RNase A stock of PBS/1% FBS and incubated for 30 min at 37°C. Stained cells were analyzed by flow cytometry using a coulter Epics Elite ESP flow cytometer (Coulter Corp., Miami, FL, U.S.A.) at excitation 488 nm and emission 610 nm. Flow cytometry data analysis was performed using the WINMDI software.

Determination of caspase 3 activity

HL60 cells (10^6) were exposed to increasing concentrations of ascorbate for 6 h before being resuspended in 50 μl of chilled cell lysis buffer (10 m*M* HEPES, pH 7.4, 2 m*M* EDTA, 0.1% CHAPS, 5 m*M* dithiothreitol, 1 m*M* PMSF, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 20 μg/ml leupeptin) and incubated on ice for 10 min. Fifty microliters of 2× reaction buffer (10 m*M* HEPES, 2 m*M* EDTA, 10 m*M* KCl, 1.5 m*M* $MgCl_2$, 1 m*M* PMSF, 10 μg/ml aprotinin, 10 μg/ml pepstatin A, 20 μg/ml leupeptin) containing 10 m*M* dithiothreitol and 5 μl of 1 m*M* of the synthetic oligopeptide substrate DEVD-(7-amino-4-trifluoromethyl coumarin) was then added to each sample and incubated at 37°C for 30 min. Detection of caspase 3 activity was performed by measuring the relative fluorescence intensity at 505 nm following excitation at 400 nm using a spectrofluorimeter (Luminescence Spectrometer LS50B, Perkin–Elmer, Bucks, U.K.). Data are shown as DEVDase activity expressed as fold increase over cells left in medium without ascorbate for the same period of time.

O_2 electrode assay

Ascorbate was found to interfere with many of the conventional methods used to assay H_2O_2, especially peroxidase-based methods (data not shown). Hence, we used a method not subject to such interference, based on a Hansatech O_2 electrode (Hansatech, Norfolk, U.K.) (9). The electrode was stabilized for 30 min with 1.5 ml of air-saturated PBS, pH 7.4, in the chamber. The buffer was then replaced by 1.5 ml of culture medium containing various concentrations of ascorbate (or added H_2O_2 for calibration purposes) and the recorder pen adjusted to a position ~50% of full-scale deflection. Catalase solution (100 μl; containing 1,000 units) in PBS was injected through the cap and H_2O_2 concentration calculated from the "spike" of O_2 evolution. The electrode was calibrated for O_2 evolution using freshly prepared solu-

tions of H_2O_2 at various concentrations in PBS or in the culture medium being used. Control experiments showed that H_2O_2 added to the culture media could be quantitatively detected, i.e., the media showed no significant ability to scavenge H_2O_2.

RESULTS

The cytotoxicity of ascorbate is dependent on the culture medium used for the assay

Exposure of HL60 cells to 4 m*M* sodium ascorbate in RPMI 1640 medium was found to induce cell death, in agreement with the report of Sakagami *et al.* (15). The cells showed shrinkage, nuclear fragmentation, and DNA laddering, consistent with death by apoptosis (data not shown). Apoptosis involves activation of a family of proteases known as caspases (24). Hence, to verify if ascorbate could indeed activate the apoptotic machinery, we assayed the activity of caspase 3 after 6 h of incubation of HL60 cells with increasing concentrations of ascorbate. As shown in Fig. 1, incubation of HL60 cells with 4 m*M* ascorbate in RPMI 1640 induced a 3.1-fold increase in caspase 3 activity (DEVDase activity) compared with HL60 cells maintained in RPMI 1640 alone for the same time, confirming the ability of ascorbate to induce apoptosis in HL60 cells. However, when the same experiments were performed in DMEM, 0.5 m*M* ascorbate was sufficient to induce an increase in caspase 3 activity similar to that obtained with 4 m*M* ascorbate in RPMI 1640 (Fig. 1). In addition, Fig. 1 summarizes data at different ascorbate concentrations that show clearly that the ability of ascorbate to induce caspase 3 activation depends on the cell culture medium used. Control experiments show that no increase in caspase activity was detected in either RPMI or DMEM medium without ascorbate over the incubation time used. Moreover, the difference in cell death was confirmed using DNA staining with PI and analysis of the percentage of HL60 cells in Sub-G1 phase (apoptotic cells) after 18 h of incubation with increasing concentrations of ascorbate in either DMEM or RPMI 1640. As shown in Fig. 2, 0.5 m*M* ascorbate was sufficient to induce apoptosis in >30% of HL60 cells in DMEM, whereas 4 m*M* ascorbate was required for similar killing in RPMI 1640. It was noted that concentrations of ascorbate above 4 m*M* in DMEM induced necrotic cell death in HL60 cells that was not detected by our cell death assay. Similar results were obtained using two other tumor cell lines, namely, the bladder carcinoma T24 and the human melanoma cell line M14 (data not shown).

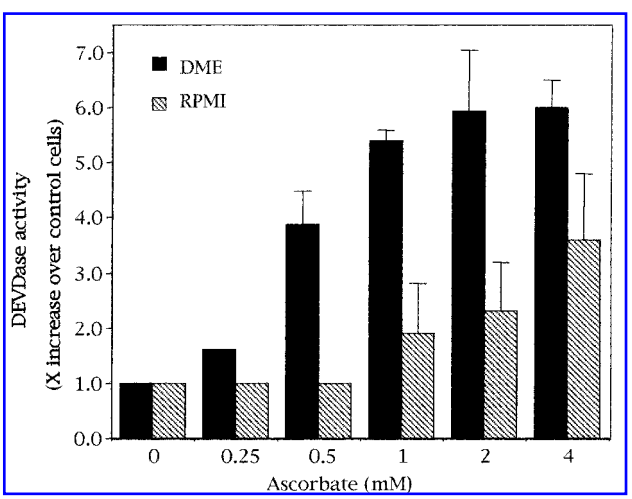

FIG. 1. Caspase 3 activity induced by increasing concentrations of ascorbate in RPMI 1640 (RPMI) and DMEM (DME). Caspase 3 activity (DEVDase activity) in HL60 cells was assessed after 6 h of incubation with increasing concentrations of ascorbate as described in Materials and Methods. Data are shown as fold increase in caspase activity over the activity found in HL60 cells left in medium alone for the same time (X increase over control cells). X = 1 represents no caspase activity. Concentrations of ascorbate stated are the final concentrations in the medium. Data presented are from one representative experiment out of three.

Effect of catalase on ascorbate-mediated caspase activation and HL60 killing in DMEM and RPMI

Figure 3 shows that ascorbate-induced caspase 3 activity (Fig. 3A) and killing of HL60 cells (Fig. 3B) in either medium could be inhibited in the presence of 750 U/ml catalase, showing that ascorbate toxicity in both media involves H_2O_2. Incubation of HL60 cells with catalase for 18 h does not induce caspase activity or cell death. Similar results were obtained using two other tumor cell lines, namely, the bladder carcinoma T24 and the human melanoma cell line M14 (data not shown).

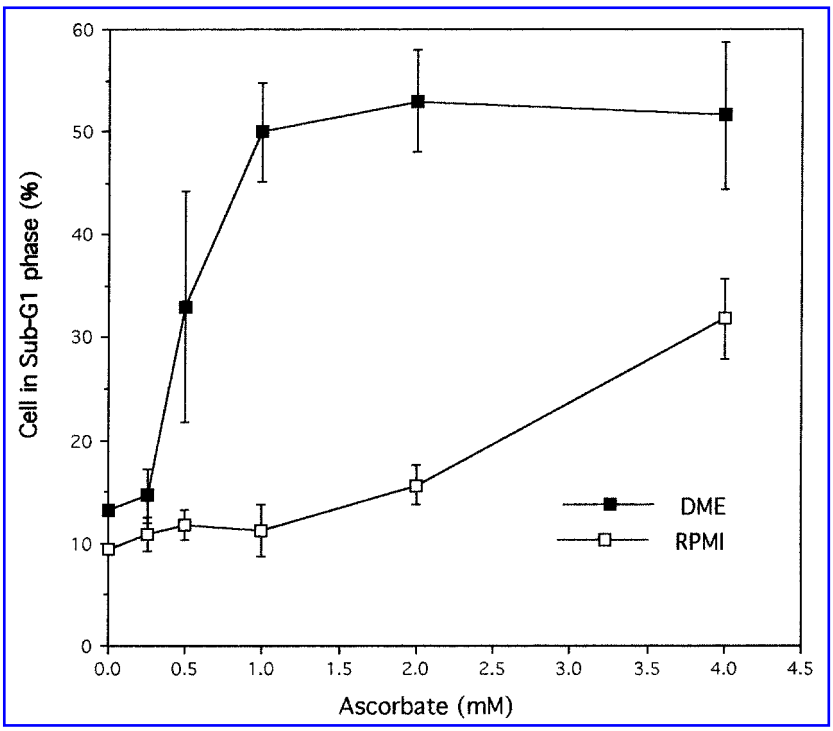

FIG. 2. HL60 cell killing by increasing concentrations of ascorbate in RPMI 1640 (RPMI) and DMEM (DME). The same experiment as in Fig. 1 was continued for 18 h, HL60 cells were stained with PI, and the percentage of cells in Sub-G1 phase was determined as described in Materials and Methods. Concentrations of ascorbate stated are the final concentrations in the medium. Data presented are from one representative experiment out of three.

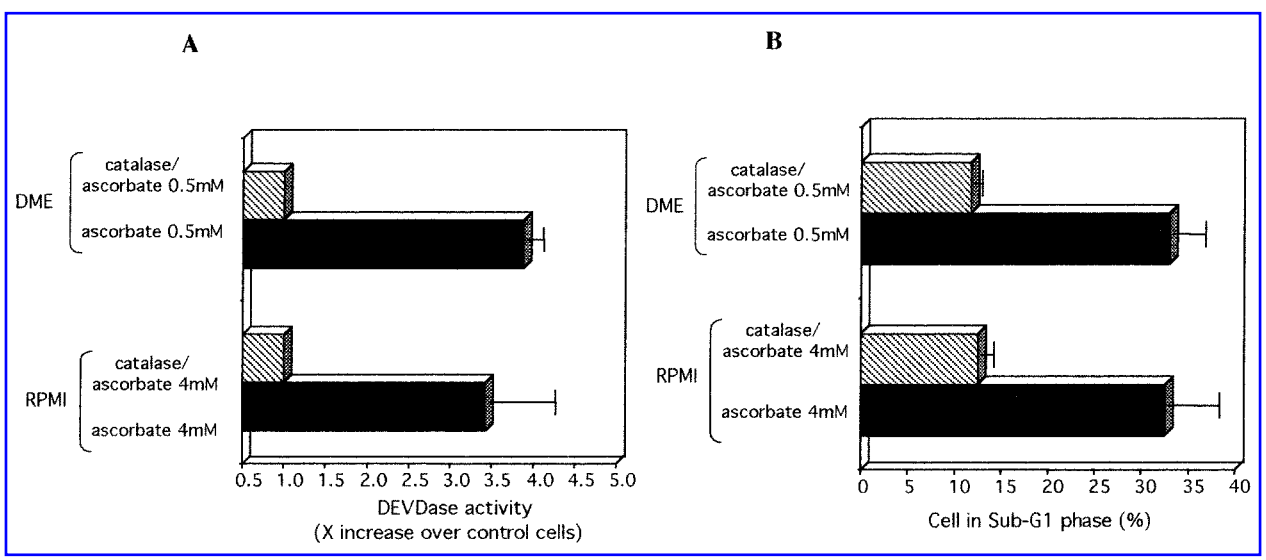

FIG. 3. Caspase 3 activity and HL60 apoptosis induced by ascorbate in DMEM and RPMI 1640 in the presence of catalase. HL60 cells were incubated in the presence of a toxic dose of ascorbate in RPMI 1640 (RPMI) or DMEM (DME) with or without 750 U/ml catalase. (**A**) Caspase 3 activity was determined after 6 h as described in Fig. 1. (**B**) Cell death was assessed after 18 h as in Fig. 2. Concentrations of ascorbate and catalase stated are the final concentrations in the medium. Data presented are from one representative experiment out of three.

Direct measurement of H_2O_2 generation in DMEM and RPMI media and effect of direct addition of H_2O_2 in DMEM and RPMI media on HL60 cell killing

H_2O_2 was measured using the O_2 electrode method (9, 10). Essentially, the medium is placed in an O_2 electrode and catalase injected through the cap. The "burst" of O_2 detected as H_2O_2 is decomposed is then used to calculate the level of H_2O_2 present in the medium as explained in Materials and Methods. Hence, increasing concentrations of ascorbate were dissolved in either DMEM or RPMI in the absence of cells, and H_2O_2 production was measured after various times. As shown in Fig. 4, H_2O_2 production increased with the concentration of ascorbate and was significantly greater in DMEM than RPMI. For example, 1 m*M* sodium ascorbate generates 161 ± 39 μM H_2O_2 in DMEM in 120 min, whereas the same concentration in RPMI only produces about half that amount (83 ± 17 μM). Moreover, we found that apoptosis could be induced in HL60 cells by direct addition of concentrations of H_2O_2 similar to the one measured in the presence of ascorbate, e.g., 125–250 μM H_2O_2 is toxic to HL60 cells in DMEM, whereas <125 μM in RPMI causes little or no apoptosis (data not shown).

DISCUSSION

Conflicting effects of ascorbate on tumor cells have been reported *in vitro*. Some author reported that ascorbate can inhibit tumor cell death induced by oxidative stress (2, 21, 26–28), whereas others showed that ascorbate kills tumor cells (17, 19, 20). Our data show that ascorbate-mediated killing of HL60 cells depends on the level of H_2O_2 produced by the reaction of ascorbate with the cell culture medium. For example, 1 m*M* ascorbate generated 161 ± 39 μM H_2O_2 in DMEM and induced cell death in 50% of HL60 cells, whereas in RPMI 1640 only 83 ± 17 μM H_2O_2 was produced and no toxicity was detected. Direct addition of H_2O_2 to the cells reproduced these results, e.g., 125–250 μM H_2O_2 is toxic to HL60 cells in DMEM, whereas <125 μM in RPMI causes little or no apoptosis. These data and the fact that killing is suppressed by catalase show that ascorbate itself is not toxic, but that killing by ascorbate is due to the production of extracellular H_2O_2, the level of which varies depending upon the cul-

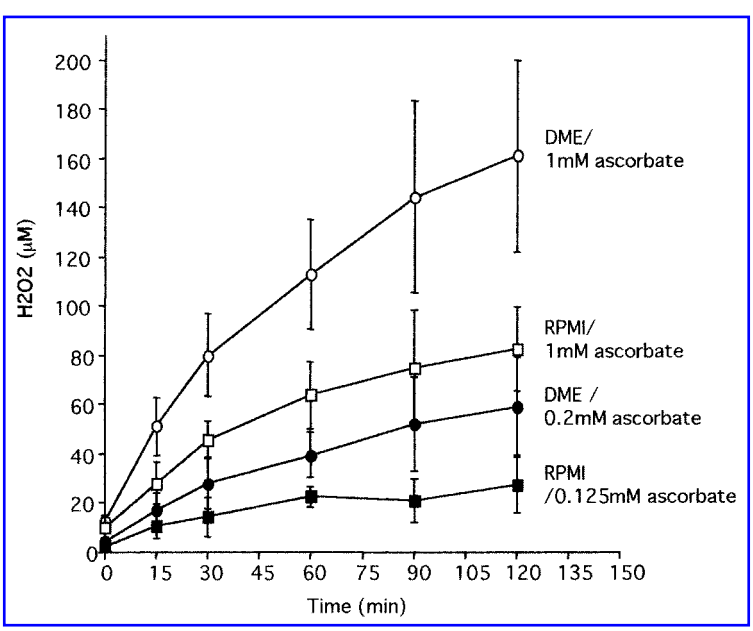

FIG. 4. Production of H_2O_2 in DMEM and RPMI 1640 by 1 m*M* and 0.125 m*M* ascorbate. Ascorbate at a final concentrations of 0.125, 0.2, or 1 m*M* was added to DMEM (DME) or RPMI 1640 (RPMI) without the cells and incubated at room temperature for various time points before H_2O_2 was measured as described in Materials and Methods. Data are presented as means ± SD of three independent experiments.

ture medium used. Several earlier articles had hinted that cellular effects of ascorbate may involve production of H_2O_2 (1, 7, 11, 12, 14, 16, 18, 19, 22, 25). However, our results directly illustrate the role of H_2O_2 as the mediator of ascorbate-induced apoptosis, and underline the critical role of the cell culture medium in the apparent tumor cell response to ascorbate. We show that performing the same assay on the same tumor cell line in two different culture media can lead to different conclusions.

How does ascorbate induce H_2O_2 production in tissue culture medium? A likely mechanism would be its interaction with iron ions in the culture media. Surprisingly, in agreement with the data from Sagakami *et al.* (17), the presence of deferoxamine (38–50 μM), a well known iron chelator, in DMEM inhibited neither cell death nor the production of H_2O_2 (data not shown). Further investigations will be required to elucidate the precise mechanism of ascorbate-induced H_2O_2 production in tissue culture medium.

In conclusion, our data show that interpretation of the effect of ascorbate upon cultured cells should take into account the interaction of ascorbate with cell culture media. A similar conclusion may apply to studies of other redox-active agents, such as phenolic compounds found in fruit, vegetables, wine, and green and black tea, on cells cultured *in vitro* (10).

PERSPECTIVE

It has been shown that expression of antioxidant enzymes, such as catalase and glutathione peroxidase, can be induced by non-toxic doses of H_2O_2 (8, 13, 23). In the light of our results, it is tempting to wonder if some of the alleged antioxidant effects of ascorbate in cells *in vitro* could be attributed to low-level H_2O_2 production in the medium and subsequent induction of antioxidant defenses. However, should ascorbate-dependent production of H_2O_2 be regarded exclusively as an *in vitro* artefact? Possibly not. Levels of ascorbate *in vivo* in humans vary from <100 μM in blood plasma to >1 mM intracellular concentrations in several cell types (for review, see 4 and 5). Perhaps, ironically the protective effects and health benefits attributed to ascorbate *in vivo* may be explained not only by its antioxidant activity, but also by its prooxidant property. Indeed, *in vivo* production of H_2O_2 by ascorbate could also be in agreement with the recently described promotional activity of ascorbate in DNA repair (3). However, we cannot use data on cell culture to predict actions of ascorbate *in vivo*.

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ABBREVIATIONS

DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PI, propidium iodide; PMSF, phenylmethylsulfonyl fluoride.

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