Vitamin C is a well known antioxidant whose precise role in protecting cells from oxidative challenge is uncertain. In vitro results have been confounded by pro-oxidant effects of ascorbic acid and an overlapping role of glutathione. We used HL-60 cells as a model to determine the precise and independent role of vitamin C in cellular protection against cell death induced by oxidative stress. HL-60 cells do not depend on glutathione to transport or reduce dehydroascorbic acid. Depletion of glutathione rendered the HL-60 cells highly sensitive to cell death induced by H$_2$O$_2$, an effect that was not mediated by changes in the activities of glutathione reductase, glutathione peroxidase, catalase, or superoxide dismutase. The increased sensitivity to oxidative stress was largely reversed when glutathione-depleted cells were preloaded with ascorbic acid by exposure to dehydroascorbic acid. Resistance to H$_2$O$_2$ treatment in cells loaded with vitamin C was accompanied by intracellular consumption of ascorbic acid, generation of dehydroascorbic acid, and a decrease in the cellular content of reactive oxygen species. Some of the dehydroascorbic acid generated was exported out of the cells via the glucose transporters. Our data indicate that vitamin C is an important independent antioxidant in protecting cells against death from oxidative stress.

Oxidative stress occurs in aerobic organisms because of the generation of reactive oxygen species (ROS) during respiratory energy production. Mammalian cells therefore have evolved effective antioxidant defense systems to cope with the toxic ROS generated in the course of aerobic ATP generation. The health of cells in tissues is influenced by the balance of antioxidants and ROS (1, 2). Oxidative damage has been linked to many disease states (3, 4) and to the development of cancer via the oxidation of DNA bases (5, 6). The role of antioxidant defenses in tumor cells themselves, however, has been poorly studied, and the role of antioxidants in neoplastic cell responses to radiation and chemotherapy is not understood completely (7–10).

We used a model system to analyze the independent effects of glutathione (GSH) and vitamin C in cellular defense against an antioxidant stress induced by H$_2$O$_2$. We selected HL-60 cells because we showed previously that the reduction of newly transported dehydroascorbic acid (DHA) in these cells was not dependent on the GSH concentration, and the HL-60 cells only transport vitamin C in the form of DHA through the facilitative glucose transporters (11–13). Cells with normal or depleted GSH were loaded with vitamin C by exposing them to DHA, and the effect of oxidative challenge was studied. We found that vitamin C protected cells from death induced by H$_2$O$_2$ in GSH-depleted cells. In the course of its antioxidant action, ascorbic acid was converted to DHA intracellularly and subsequently effluxed from the cells. The efflux of DHA is mediated by the glucose transporters, and the DHA generated was related to the cellular GSH content. We also show that GSH protects HL-60 cells from oxidative stress independently of ascorbic acid. These results indicate that ascorbic acid and GSH are individually important in protecting cells from oxidative challenge.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Human myeloid HL-60 cells were cultured in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% l-glutamine.

**Depletion of Glutathione**—Cells were incubated with L-buthionine-(S,R)-sulfoximine (Sigma) or diethyl maleate (Sigma) for the time indicated in the respective figure legends. Cells were resuspended in incubation buffer (15 mM Heps (pH 7.5), 150 mM NaCl, 5 mM KCl, 1.8 mM CaCl$_2$, 0.8 mM MgCl$_2$) at 1–6 x 10$^6$ cells/ml for efflux essays or processed directly for GSH determination. For glutathione assays, the method of Meister was used as described previously (9).

**Ascorbic Acid Efflux**—For ascorbic acid efflux, control cells and cells with different GSH content were incubated in buffer containing 25 μM-1 mM ascorbic acid, 0.2 μCi of L-[1-14C]ascorbic acid (PerkinElmer Life Sciences), and 2–5 units of ascorbate oxidase (Sigma) at 37 °C for 30 min. Cells were washed twice and resuspended in complete Iscove’s modified Dubelcco’s medium. H$_2$O$_2$ was added in a time course or dose response manner at room temperature. The cellular pellet and supernatant were used to determine $^{14}$C/ascorbic acid by scintillation counting. Cytochalasin B and E were used at 20 μM to inhibit the exit of ascorbic acid from the cells. These reagents were added after DHA loading and before incubation with H$_2$O$_2$.

**Measurement of Intracellular Ascorbic Acid**—HL-60 cells (5 x 10$^6$) were lysed in 60% methanol and 1 mM EDTA (pH 8.0). Lyesates were stored at -70 °C until use. High performance liquid chromatography (HPLC) analyses were performed as described previously (9).

**Determination of Cell Viability upon Oxidative Stress**—Cells were washed twice with phosphate-buffered saline (PBS) and resuspended in complete medium. Glutathione-depleted or control cells were incubated with or without DHA (Aldrich) at different concentrations and treated with H$_2$O$_2$ (Fisher). Dose-response and time course experiments for DHA, GSH, and H$_2$O$_2$ were carried out (see figure legends). GSH content and cell viability by trypan blue exclusion were measured.

**Oxidation of Ascorbic Acid by Hydrogen Peroxide**—In a cell-free system, H$_2$O$_2$ was tested for its ability to oxidize ascorbic acid (Sigma) in vitro. Briefly, 100 μl of ascorbic acid was added to start the reaction.
in a prewarmed cuvette containing 880 μl of incubation buffer (pH 7.4) and 20 μl of H₂O₂. Kinetics of oxidation of 0.01, 0.025, 0.05, 0.1, and 0.25 mM ascorbic acid by 0–50 mM H₂O₂ were monitored at 266 nm for 10 min at 37 °C.

Measurement of Hydrogen Peroxide Removal by HL-60 Cells—The method used was that described by Makino et al. (14) with slight modifications. In brief, cells were washed twice with PBS, 5.6 mM glucose, resuspended in the same solution, and incubated at 37 °C. Cells were treated with different concentrations of H₂O₂ in a time-dependent manner to measure the removal of H₂O₂ from the medium. For each time point, 100 μl of cells was separated and analyzed by chemiluminescence.

Determination of Hydrogen Peroxide—The method of Williams et al. described by Makino et al. (14) using peroxynitrate luminescence was applied with some modifications. In a 6-ml glass tube containing 60 μl of water, 90 μl of PBS, and 10 μl of 0.005% (v/v) triethylamine solution in methanol, 50 μl of a test solution was added. The vial was placed in a chemiluminescence detector (Berthold luminometer LB 9501), and the intensity of the chemiluminescence was measured in an interval of 10 s after the reaction was started. A standard curve for H₂O₂ solutions was used to have quantitative results.

Determination of Catalase Activity—HL-60 cells were incubated with 100 mM catalase inhibitor 3-amino-1,2,4-triazole (Sigma) for 4 h at 37 °C, washed, and resuspended in medium. Cells were treated with increasing concentrations of H₂O₂. Cell viability and H₂O₂ removal capacity were measured as described above. For the catalase activity assay, 1 × 10⁷ HL-60 cells were solubilized with 0.5% Triton X-100 and 4 mM EDTA in 0.1 M phosphate buffer (pH 7.4), 0.5 ml final volume. Supernatant (30 μl) was added to 50 mM potassium phosphate buffer (pH 7.0) containing 10 mM H₂O₂ in a final volume of 1 ml. The absorbance at 240 nm was recorded for 10 min, and the final absorbance was obtained by the addition of 40 units of catalase (Sigma).

Measurement and Assay for Glutathione Peroxidase Activity—HL-60 cells were washed with PBS, pelleted, and resuspended in 50 mM Tris-HCl (pH 7.5), 5 mM EDTA, and 1 mM diethiothreitol. Samples were sonicated and centrifuged at 10,000 × g for 20 min at 4 °C. Supernatants were separated and stored at −70 °C until assay. Glutathione peroxidase determination was carried out according to kit protocol (Calbiochem), and sample absorbance values were read.

Measurement and Assay for Superoxide Dismutase Activity—HL-60 cells (1 × 10⁷ cells) were washed with PBS, pelleted, and resuspended in 100 μl of water. Samples were sonicated and centrifuged at 15,000 × g for 10 min at 4 °C. Samples were treated with a mix of ethanol/chloroform 2:1 v/v, and the resulting supernatants were stored at 4 °C until assay. Superoxide dismutase determination was carried out according to kit protocol (Calbiochem). Samples were read at 525 nm at 37 °C.

Measurement of Reactive Oxygen Species Activity—Fluorescence-activated cell sorter analyses of the oxidation of 2,7’-dichlorofluorescein diacetate (DCFH-DA) (Molecular Probes, Eugene, OR) and hydroethidine (HE) (Polysciences, Warrington, PA) were performed in cells with normal and decreased contents of GSH. These fluorescent dyes have been used to determine the intracellular changes of ROS in several cell lines (15–18); DCFH-DA and HE measure H₂O₂ and O₂⁻, respectively (16). Cells were preloaded with ascorbic acid as above and stained with 5 μM DCFH-DA or HE at 37 °C. Cells were washed with PBS and treated with H₂O₂. The increase in fluorescence was measured using a FACScalibur (Becton/Dickinson, Franklin Lakes, NJ).

RESULTS

Effect of H₂O₂ on Cell Viability

Depletion of GSH prominently increased the sensitivity of HL-60 cells to the cytotoxic effect of H₂O₂ (Fig. 1). Greater than 90% of GSH-depleted cells died after a 24-h incubation with 10 μM H₂O₂. With the same conditions, however, there was nearly 100% viability in cells with a normal content of GSH (Fig. 1, A and B). Cellular toxicity was dependent on the concentration and time of exposure to the oxidant, as shown in Fig. 1, C and D. Higher concentrations of H₂O₂ were required to decrease cell viability in control cells (Fig. 1C). After a 4-h incubation with 500 μM H₂O₂, cell viability was reduced to 50%. Lower
concentrations of \( \text{H}_{2}\text{O}_{2} \) do not show deleterious effects on cell survival or proliferative capacity. In GSH-depleted cells, however, there was a rapid increase in cell death (Fig. 1D), and 100% cell killing was observed at concentrations of 50 \( \mu \text{M} \) \( \text{H}_{2}\text{O}_{2} \) or higher after a 6-h incubation. These results show that GSH is a quantitatively important antioxidant, and its absence leaves the cells poorly protected from the oxidative damage induced by \( \text{H}_{2}\text{O}_{2} \). The variability of the GSH content in control cells, as shown in Fig. 1E, is an aspect already described (11), but these cells are able to maintain their GSH content even when they are exposed to high doses of \( \text{H}_{2}\text{O}_{2} \). In the case of GSH-depleted cells (Fig. 1F), the cells are unable to recover the GSH content mainly because the \( \text{H}_{2}\text{O}_{2} \) produces a rapid cell death. With lower concentrations of \( \text{H}_{2}\text{O}_{2} \) these cells show recovery of their GSH content.

**Vitamin C Protects HL-60 Cells from Oxidative Stress**

Glutathione-depleted and control HL-60 cells were preloaded with vitamin C, by incubation with graded concentrations of DHA, and cell viability was measured after incubating the cells with increasing concentrations of \( \text{H}_{2}\text{O}_{2} \). Vitamin C protects cells from killing induced by \( \text{H}_{2}\text{O}_{2} \). Cells without the vitamin were substantially more sensitive to cell death than those loaded with ascorbic acid. A major role in cell protection by vitamin C was demonstrated in GSH-depleted cells because the ascorbate-loaded cells were more resistant to oxidative damage. A 5-fold higher concentration of \( \text{H}_{2}\text{O}_{2} \) was required to kill half of the cells preloaded with vitamin C compared with nonloaded cells (Fig. 2A). Control (GSH-containing) cells did not show that level of difference, although it was possible to observe that vitamin C-loaded control cells were more resistant to \( \text{H}_{2}\text{O}_{2} \) than nonloaded cells. About 50 \( \mu \text{M} \) \( \text{H}_{2}\text{O}_{2} \) killed all of the GSH-depleted cells; however, when these cells were preloaded with ascorbic acid, a 10-fold higher concentration of \( \text{H}_{2}\text{O}_{2} \) was unable to kill all of the cells present in the assay.

Vitamin C was found to protect GSH-depleted cells in a dose-dependent manner (Fig. 2B), and increasing the dose of intracellular ascorbic acid rendered cells more resistant to oxidative damage. This effect was observed at all time points (Fig. 2C) and indicates that vitamin C is able to subsume the antioxidant role of GSH in protecting the cells from \( \text{H}_{2}\text{O}_{2} \). When GSH-depleted cells were exposed to high concentrations (50 \( \mu \text{M} \)) of \( \text{H}_{2}\text{O}_{2} \), however, cell survival decreased to 50% after 4 h of incubation (data not shown). Concentrations of \( \text{H}_{2}\text{O}_{2} \) higher than 100 \( \mu \text{M} \) induced rapid cell death, and vitamin C had little effect on cell survival (Fig. 2D). The extent of cell protection depended on the intracellular concentration of vitamin C and the degree of oxidative challenge.

**Oxidative Stress Induces DHA Efflux from HL-60 Cells**

Preliminary data indicated that in \( \text{H}_{2}\text{O}_{2} \)-treated cells an efflux of vitamin C occurred to the extracellular environment. To characterize the exit of vitamin C and understand its role in the mechanism of cellular protection, we performed experiments to ascertain the nature of the efflux and its relationship to the intracellular GSH content and the level of oxidative challenge.

**Role of Glutathione—**Cells with different GSH content were preloaded with a low concentration of ascorbic acid and then treated with a high concentration of \( \text{H}_{2}\text{O}_{2} \). We observed a rapid efflux of vitamin C, with kinetics of efflux which were linear during the first 2 min, and at 10 min almost all of the radiolabeled vitamin C was present in the medium (Fig. 3A). The efflux of vitamin C was GSH-dependent; at higher cellular GSH content, less efflux of vitamin C was observed. The rate of efflux as shown in Fig. 3B was higher at a lower GSH concentration. These inverse relationships reach a plateau when the intracellular content of GSH was at m\( \text{M} \) levels. The rate of efflux decreased from 0.3 nmol/10^5 cells/min in the absence of GSH to 0.17 nmol/10^5 cell/min at 1 m\( \text{M} \) GSH. Only a minor change (to 0.12 nmol/10^5 cells/min) was observed when intracellular GSH was 10 m\( \text{M} \). A similar result is shown in a plot of total efflux after a 1-h incubation with \( \text{H}_{2}\text{O}_{2} \) (Fig. 3C). The data indicate that only a fraction (about 30%) of vitamin C exits the cells when the GSH content is in the m\( \text{M} \) range. In GSH-depleted cells, the rapid efflux of the vitamin likely reflects oxidation of intracellular ascorbic acid as it functions to protect the cell.
Role of H$_2$O$_2$—The efflux kinetics of vitamin C were studied in GSH-depleted cells preloaded with a high concentration of ascorbic acid and then treated with H$_2$O$_2$ in a time- and dose-dependent manner. Fig. 4A shows that the efflux of vitamin C increased proportionally with the concentration of H$_2$O$_2$ exposure. At concentrations greater than 250 $\mu$M H$_2$O$_2$ almost all of the ascorbic acid was found outside the cells at 10 min, whereas only 25% exited the cell at 25 $\mu$M H$_2$O$_2$. Efflux was linear during the first 2 min and reached a plateau after this time. During the first min, the efflux rate of vitamin C increased with the concentration of H$_2$O$_2$ (Fig. 4B), reaching a plateau at a concentration of 250 $\mu$M H$_2$O$_2$. The 50% efflux rate in these cells was seen at about 50 $\mu$M H$_2$O$_2$. When total efflux was measured after 1 h of incubation with H$_2$O$_2$ (Fig. 4C), the sigmoidal shape of the curve indicated that at concentrations of H$_2$O$_2$ lower than 20 $\mu$M, there was less than a 20% exit of ascorbic acid from the cells. These results indicate that ascorbic acid exited the cells in a manner that was a complex function of H$_2$O$_2$ concentration and cannot be described by a simple linear relationship.

Role of Ascorbic Acid—The time course and dose-response experiments with ascorbic acid-loaded cells exposed to 50 $\mu$M H$_2$O$_2$ (which produces a 50% efflux in GSH-depleted cells) show that efflux was dependent on the ascorbic acid concentration in the cells. There was a direct correlation between the exit of ascorbic acid from the cells and the amount of vitamin C present in the cells (Fig. 5A). The rate of efflux was slow at ascorbic acid concentrations under 10 mM but increased rapidly at higher concentrations (Fig. 5B). There was four times more efflux of ascorbic acid when 50 $\mu$M H$_2$O$_2$ was used compared with 10 $\mu$M H$_2$O$_2$; however, there was a fixed efflux of vitamin C from the cell independent of the intracellular ascorbic acid concentration (Fig. 5C). At 10 $\mu$M H$_2$O$_2$ only a 20% efflux occurred and increased to 60% at a concentration of 50 $\mu$M. These data indicate that H$_2$O$_2$ induces only partial exit of the vitamin to the extracellular medium.

Ascorbic Acid Oxidation in Vitro

The proposed mechanism for the efflux of vitamin C observed in our experiments is the intracellular oxidation of ascorbic acid to DHA, which is able to exit the cell. To test this hypothesis we first studied the oxidation of ascorbic acid in a cell-free system to detect the generation of DHA induced directly by H$_2$O$_2$. Fig. 6A shows the initial velocity of ascorbic acid oxidation induced by H$_2$O$_2$. Oxidation was very slow during the 1st min of incubation at H$_2$O$_2$ concentrations under 100 $\mu$M. An exponential increase in oxidation occurred at H$_2$O$_2$ concentrations of 1 mM and above. The shape of the curve did not change when ascorbic acid was increased from 10 to 100 $\mu$M. Although the initial velocity of oxidation increased with the H$_2$O$_2$ concentration, the efficiency of oxidation of ascorbic acid in vitro was very low compared with the effect of H$_2$O$_2$ on the HL-60 cells. At 50 $\mu$M H$_2$O$_2$, the rate of oxidation of 100 $\mu$M ascorbic acid is 0.160 nmol/µl/min, which is almost 2 orders of magnitude lower than the rate observed in the HL-60 cells under similar conditions (~4.5 nmol/106 cells/min, which translate in 13 nmol/µl/min). Total oxidation depends on the ascorbic acid and H$_2$O$_2$ concentrations in solution (Fig. 6B). 50% oxidation of ascorbic acid required an increase of 2 orders of magnitude for H$_2$O$_2$ (0.1–10 mM) when ascorbic acid increased from 10 to 100 $\mu$M. Total oxidation of 30 $\mu$M or lower ascorbic acid occurs only if 10 mM H$_2$O$_2$ is present in the assay. If ascorbic acid increases to 100 $\mu$M, about 60% oxidation occurs at the higher dose of H$_2$O$_2$ shown (10 mM). The requirement of high levels of H$_2$O$_2$, which are 2–3 orders of magnitude in excess of the ascorbic acid concentrations, implies that the intracellular efflux of ascorbic acid cannot be explained by a direct chemical reaction between H$_2$O$_2$ and ascorbic acid. The efflux of ascorbic acid shown then could depend on its interaction with intermediate molecules, ROS produced by H$_2$O$_2$.

Cytochalasin B Blocks the Efflux of Vitamin C in Cells Treated with H$_2$O$_2$

HPLC data indicate that vitamin C is oxidized to DHA when HL-60 cells are treated with H$_2$O$_2$ (data not shown). In the oxidized form, DHA, vitamin C is able to exit the cell through the facilitative glucose transporters. Cytochalasin B is a specific inhibitor of the transport of glucose and DHA through the facilitative transporters. Fig. 7 shows that the efflux of vitamin C in GSH-depleted cells treated with H$_2$O$_2$ occurs as described above. With cytochalasin B treatment, 80% of the efflux was blocked. Control experiments using the analog cytochalasin E, which does not inhibit the glucose transporter functionally, shows no inhibition of vitamin C efflux. Cells not treated with H$_2$O$_2$ do not show any efflux when treated with both cytochalasins. These data demonstrate that the efflux of vitamin C occurs through the glucose transporter.

Antioxidant Enzymatic Activities in HL-60 Cells

Hydrogen peroxide is quite stable and has a high diffusion rate in solution. To avoid oxidative damage induced by H$_2$O$_2$, the cells must have mechanisms that block the influx of H$_2$O$_2$ into the cells or produce antioxidants that neutralize its deleterious effects. We studied how depletion of GSH changes the enzymatic activities involved with the removal of oxidants. Control and GSH-depleted HL-60 cells decomposed extracellular H$_2$O$_2$ rapidly. By 5 min of incubation with 1 mM H$_2$O$_2$, 50% of the oxidant was removed from the medium (Fig. 8A). More
respectively, of data from panel A.

Concentration of H\textsubscript{2}O\textsubscript{2} was measured at 266 nm at 37°C. Mean ± S.D. of three experiments, each performed three times.

Depletion of GSH does not change the activities of GSH peroxidase or superoxide dismutase (Fig. 8B). Control and GSH-depleted cells show similar enzymatic levels, indicating that they are not involved in the changes observed in cell survival when GSH-depleted cells were exposed to oxidative stress. GSH function in cell protection could then be related largely to the neutralization of reactive oxygen intermediates produced from H\textsubscript{2}O\textsubscript{2}. These potentially more destructive molecules could induce cellular damage rapidly if an antioxidant is not present in sufficient concentration in the cells. GSH and vitamin C have the ability to quench ROS and prevent damage.

Vitamin C Inhibits the Formation of Reactive Oxygen Species

Cells undergoing oxidative stress generate ROS as a mechanism of detoxification and cell defense. The use of nonfluorescent compounds that turn fluorescent upon oxidation by ROS is a convenient tool to measure ROS in cells. We used two compounds, DCFH-DA and HE, which measure intracellular H\textsubscript{2}O\textsubscript{2} and O\textsubscript{2}° respectively. Fig. 9 shows the oxidation in DCFH-DA for 30 min at 37°C, to reach an intracellular ascorbic acid concentration of more than 10 mM. After washing with PBS, cells were incubated at 37°C and centrifuged at high speed for 1 min. The supernatant was separated, and [14C]DHA in the pellet and supernatant was evaluated by scintillation counter. Data represent the mean ± S.D. of three experiments, each performed three times.

Vitamin C Inhibits the Formation of Reactive Oxygen Species

FIG. 6. Ascorbic acid oxidation induced by H\textsubscript{2}O\textsubscript{2} in a cell-free system. Oxidation of ascorbic acid (AA) in solutions containing variable concentrations of H\textsubscript{2}O\textsubscript{2} was measured at 266 nm at 37°C. Panel A, kinetics of the initial velocity of oxidation of 10 μM, 25 μM, 50 μM, and 100 μM H\textsubscript{2}O\textsubscript{2}. Panel B, ascorbic acid oxidation after a 10-min incubation with 0.1 μM, 1 μM, or 10 μM H\textsubscript{2}O\textsubscript{2}. Data represent the mean ± S.D. of three experiments, each performed twice.

FIG. 7. Efflux of DHA is blocked by cytochalasin B. Time course for DHA efflux in GSH-depleted cells (0.2 mM GSH) preloaded with 500 μM [14C]DHA for 30 min at 37°C, to reach an intracellular ascorbic acid concentration of more than 10 mM. After washing with PBS, cells were incubated with untreated (○), 20 μM cytochalasin B (CytB) and 500 μM H\textsubscript{2}O\textsubscript{2} (●), 20 μM cytochalasin E (CytE) and 500 μM H\textsubscript{2}O\textsubscript{2} (●), or 500 μM H\textsubscript{2}O\textsubscript{2} (□). Cells were incubated at 37°C and centrifuged at high speed for 1 min. The supernatant was separated, and [14C]DHA in the pellet and supernatant was evaluated by scintillation counter. Data represent the mean ± S.D. of three experiments, each performed three times.
ROS in both normal and GSH-depleted cells. As expected, cells with low GSH content produce higher amounts of ROS than control cells. When GSH and vitamin C are present, the generation of ROS is reduced significantly, showing that both antioxidants are involved in the inhibition of ROS formation. The data obtained using HE show the same pattern, although this compound was more sensitive to the conditions of the assay (data not shown).

**DISCUSSION**

The precise role of vitamin C in protecting normal and neoplastic cells against oxidative challenge is unclear. *In vitro* studies have been confounded by the pro-oxidant effects of ascorbic acid *in vitro* and the overlapping antioxidant activities of glutathione. DHA enters cells through the glucose transporters and is reduced to ascorbic acid intracellularly (12, 13, 19). Extracellular oxidative events lead to the generation of DHA from ascorbic acid, which can then enter cells rapidly and be reduced back to ascorbic acid (20, 21). We showed recently that cancer cells accumulate vitamin C in a superoxide dismutase-inhibitable manner (22). We have used ascorbic acid loading of HL-60 cells to assess directly the role of vitamin C in protecting normal and neoplastic cells against oxidative challenge is unclear. *In vitro* studies have been confounded by the pro-oxidant effects of ascorbic acid *in vitro* and the overlapping antioxidant activities of glutathione. DHA enters cells through the glucose transporters and is reduced to ascorbic acid intracellularly (12, 13, 19). Extracellular oxidative events lead to the generation of DHA from ascorbic acid, which can then enter cells rapidly and be reduced back to ascorbic acid (20, 21). We showed recently that cancer cells accumulate vitamin C in a superoxide dismutase-inhibitable manner (22). We have used ascorbic acid loading of HL-60 cells to assess directly the role of vitamin C in protecting these cells from an oxidative challenge induced by H$_2$O$_2$. Glutathione is an important antioxidant and has been associated with the cellular recycling of vitamin C and the maintenance of the vitamin in its reduced state (23, 24, 31–34).

We found that GSH-depleted cells can be made resistant to H$_2$O$_2$ by preloading the cells with vitamin C via exposure to DHA. The effect was dose-dependent, and some protection was also seen in cells with normal GSH content. HL-60 cells accumulate millimolar concentrations of vitamin C, and their resistance to H$_2$O$_2$ increases in proportion to the intracellular concentration of ascorbic acid in a dose-dependent manner. Similar results have been observed in other cell lines where preloading with ascorbic acid was shown to protect from H$_2$O$_2$ generated by xanthine oxidase (35) and by direct treatment with H$_2$O$_2$ or radiation (7, 8, 36).

Under the conditions studied, vitamin C had an antioxidant role parallel to that of GSH. Both are primary antioxidants able to protect HL-60 cells from oxidative stress. Depletion of GSH did not change the cell’s enzymatic antioxidant activities. Catalase, GSH peroxidase, and superoxide dismutase were functionally normal in both untreated and GSH-depleted cells. Because cell death increased in GSH-depleted cells, these enzymes would not be the primary cell components responsible for the differential in cell death induced by H$_2$O$_2$. Preloading with vitamin C, however, rendered the cells resistant to H$_2$O$_2$, an effect mediated by inhibition of ROS. This response points to vitamin C as a primary antioxidant responsible for neutralizing ROS that induce cellular damage. Because catalase and GSH peroxidase were fully active in both GSH-depleted and untreated cells, removal of H$_2$O$_2$ occurred efficiently. Accordingly, cell killing induced by H$_2$O$_2$ is likely because of generated intermediate free radicals, and vitamin C has its major quenching effect at this level.

Our results indicate that ascorbic acid and GSH protect cells against oxidative challenge in a dose-dependent manner and that vitamin C exited the cells after incubation with H$_2$O$_2$. The efflux of vitamin C was dependent on the concentration of H$_2$O$_2$ and the GSH content in the cells. This was a rapid event occurring over minutes. The ascorbic acid efflux appears to be a consequence of electron donation by ascorbic acid and conversion of ascorbic acid to DHA intracellularly. As H$_2$O$_2$ concentrations increased, there was an increase in the efflux of vitamin C. The kinetics of vitamin C efflux from cells were complex, and a general biphasic pattern could be related to compartmentalization of the vitamin in the cells. The oxidation of ascorbic acid to DHA implies that the major role of vitamin C is as an electron donor to quench oxidants intracellularly.
cellular content of GSH, pointing to the parallel role of both antioxidants. The result of the cytochalasin B inhibition experiments confirmed the hypothesis that vitamin C would exit the cell through the glucose transporter in the form of DHA, after ascorbic acid donated electrons. Although ascorbic acid accumulated intracellularly in HL-60 cells, only DHA exits into the medium after treatment of the cells with H$_2$O$_2$.

We asked if a direct chemical reaction between H$_2$O$_2$ and ascorbic acid inside the cells generated DHA that can exit the cell. Our in vitro data from a cell-free system clearly indicated that this is not the case. The concentration of H$_2$O$_2$ necessary to oxidize 50% of 100 mM ascorbic acid was 2 orders of magnitude higher than the concentration of ascorbic acid. Such concentrations of H$_2$O$_2$ are never reached inside the cell. Moreover, the rate of oxidation of ascorbic acid by the H$_2$O$_2$-treated HL-60 cells was 2 orders of magnitude greater than the in vitro oxidation rate. The explanation for the oxidation of ascorbic acid could be related to the generation of intermediates, such as the hydroxyl radical (‘OH), which have more potent oxidative power that could lead to a rapid generation of DHA, the chemical form that can exit the cells through the glucose transporters (12, 13).

Current evidence suggests that O$_2^\cdot$ and H$_2$O$_2$ injure cells as a result of the generation of more potent oxidizing species (6, 37). The hypothesis most consistent with the available data is that O$_2^\cdot$ reduces a cellular source of ferric to ferrous iron, and the latter then reacts with H$_2$O$_2$ to produce more potent oxidizing species, such as ‘OH. This more reactive species can be neutralized intracellularly by ascorbic acid generating DHA. If neutralization does not occur, ‘OH can initiate the peroxidative decomposition of phospholipids of cellular membranes. Hydroxyl radical also damages the inner mitochondrial membrane leading to a sequence of events that ends in cell death. DNA represents another cellular target for ‘OH, and, depending on the cell type, oxidative DNA damage can be coupled to cell killing through a mechanism related to the activation of poly(ADP-ribose) polymerase (6, 37, 38).

Our data suggest that DHA can be used to increase the intracellular concentration of ascorbic acid as a means to provide antioxidant defense to cells exposed to oxidative challenge. Cancer cells up-regulate the expression of the facilitative glucose transporters (39–41). This increase is also observed in tumors treated with radiation or chemotherapy, which induce ROS (42). Tumors can likely use the antioxidative properties of vitamin C as cell defense mechanisms to deal with oxidative challenge.

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Mechanism of Vitamin C Inhibition of Cell Death Induced by Oxidative Stress in Glutathione-depleted HL-60 Cells
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