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Original Contribution

Ascorbate enhances the toxicity of the photodynamic action of Verteporfin in HL-60 cells

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Abstract

As a reducing agent, ascorbate serves as an antioxidant. However, its reducing function can in some settings initiate an oxidation cascade, i.e., seem to be a "pro-oxidant." This dichotomy also seems to hold when ascorbate is present during photosensitization. Ascorbate can react with singlet oxygen, producing hydrogen peroxide. Thus, if ascorbate is present during photosensitization the formation of highly diffusible hydrogen peroxide could enhance the toxicity of the photodynamic action. On the other hand, ascorbate could decrease toxicity by converting highly reactive singlet oxygen to less reactive hydrogen peroxide, which can be removed via peroxide-removing systems such as glutathione and catalase. To test the influence of ascorbate on photodynamic treatment we incubated leukemia cells (HL-60 and U937) with ascorbate and a photosensitizer (Verteporfin; VP) and examined ascorbic acid monoanion uptake, levels of glutathione, changes in membrane permeability, cell growth, and toxicity. Accumulation of VP was similar in each cell line. Under our experimental conditions, HL-60 cells were found to accumulate less ascorbate and have lower levels of intracellular GSH compared to U937 cells. Without added ascorbate, HL-60 cells were more sensitive to VP and light treatment than U937 cells. When cells were exposed to VP and light, ascorbate acted as an antioxidant in U937 cells, whereas it was a pro-oxidant for HL-60 cells. One possible mechanism to explain these observations is that HL-60 cells express myeloperoxidase activity, whereas in U937 cells it is below the detection limit. Inhibition of myeloperoxidase activity with 4-aminobenzoic acid hydrazide (4-ABAH) had minimal influence on the phototoxicity of VP in HL-60 cells in the absence of ascorbate. However, 4-ABAH decreased the toxicity of ascorbate on HL-60 cells during VP photosensitization, but had no affect on ascorbate toxicity in U937 cells. These data demonstrate that ascorbate increases hydrogen peroxide production by VP and light. This hydrogen peroxide activates myeloperoxidase, producing toxic oxidants. These observations suggest that in some settings, ascorbate may enhance the toxicity of photodynamic action.

Keywords: Photosensitization; HL-60 cells; U937 cells; Ascorbic acid; Oxidative stress; Free radicals

Photodynamic therapy (PDT) is a novel treatment for cancer that induces oxidative stress primarily through the light-mediated production of singlet oxygen [1]. Singlet oxygen and related reactive oxygen species initiate oxidations that damage malignant tissue. The generation of singlet oxygen requires oxygen, visible light, and a photosensitizing drug. Light exposure activates the drug and in the presence of O_2 produces singlet oxygen through type II energy transfer processes [2]. Benzoporphyrin-derivative monoacid ring A (BPD-MA), the active component of Verteporfin (VP), is a second-generation photosensitizer that has been proposed for leukemic bone marrow purging [3] and for treatment of human psoriasis [4,5] and is in wide use for the treatment of age-related macular degeneration [6]. In animal models, Verteporfin shows promise for cancer treatment [7–9]. Verteporfin, as a lipid-formulated drug preparation, localizes mainly in lipophilic structures of the mitochondria and endoplasmic reticulum, with a small fraction distributed

Abbreviations: 4-ABAH, 4-aminobenzoic acid hydrazide; $AscH^-$, ascorbic acid monoanion; AT, 3-amino-1*H*-1,2,4-triazole; BPD-MA, benzoporphyrin derivate monoacid ring A, the active component of VP; DETAPAC, diethylenetriaminepentaacetic acid; DMSO, dimethyl sulfoxide; DTNB, 5,5′-dithio-*bis*(2-nitrobenzoic acid); FBS, fetal bovine serum; GPx, glutathione peroxidase; GR, glutathione disulfide reductase; GSH, glutathione; GSH_T, total glutathione; GSSG, glutathione disulfide; MPO, myeloperoxidase; NADPH, reduced nicotinamide adenine dinucleotide phosphate; PBS, phosphate-buffered saline; PDA, photodynamic action, i.e., VP + light; PDT, photodynamic therapy; ROS, reactive oxygen species; SOD, superoxide dismutase; t_d, doubling time; VP, verteporfin.

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within the cytoplasm [10]. The biological mechanism of phototoxicity in vitro seems in most cases to be mitochondrial-induced apoptosis [11], as seen in human promyelocytic leukemia HL-60 [12], mouse P815 mastocytoma [13], and HeLa epidermoid carcinoma [14].

Ascorbate (vitamin C) is a water-soluble, small-molecule antioxidant and reducing cofactor. As a vitamin, it must be taken up from the diet; it is found in almost all tissues [15]. Its concentration in the blood of normal individuals varies from 5 to 90 μM [16]. However, it is actively accumulated in human tissues to a concentration as much as 50-fold greater than in plasma [17]. In vitro cancer cells also accumulate ascorbate [18,19]. There are data demonstrating higher levels of vitamin C in neoplasms than in adjacent normal tissue [20]. Ascorbate can initiate new oxidation cascades [21] and thereby enhance the toxicity of photosensitization processes by forming H_2O_2 upon its reaction with singlet oxygen ($^1O_2$) [22]:

$k = 3 \times 10^8\ M^{-1}s^{-1}$ [23,24]

$$\text{Ascorbate} + {}^1O_2 \rightarrow H_2O_2 + \text{dehydroascorbate};$$

Singlet oxygen is thought to have a very short lifetime (≈100 ns) within lipid membranes [25] and thus a very limited diffusion distance compared to H_2O_2. Hydrogen peroxide is a highly diffusible oxidant that could expand the damage inflicted by $^1O_2$. It has been shown that $^1O_2$ will readily diffuse from the interior of, or across, a hydrophobic volume, such as a membrane, into the neighboring aqueous space [25–34]. Most of the $^1O_2$ generated in the membrane would diffuse from the membrane and react with components in the water space [28,30,31]. Recent results studying the decay of $^1O_2$ in single cells suggest its lifetime to be more than an order of magnitude greater in the water space of cells than previously thought [33,34]. Thus singlet oxygen would have a diffusion distance greater than currently accepted, although much less than H_2O_2. Using the simple diffusion equation ($r = (6Dt)^{0.5}$, where r is the diffusion distance, D the diffusion coefficient, and t the time, an increase in the lifetime of $^1O_2$ by a factor of 10 would increase the diffusion distance by $10^{0.5}$. This would result in the effective reaction volume of singlet oxygen increasing by a factor of ≈30 (i.e., $10^{0.5})^3$. Thus, singlet oxygen produced in one structure of a cell will diffuse a significant distance in the water space of cells [33,34]. Thus possible reactions with aqueous solutes such as ascorbate could be significant. The reaction of ascorbate with singlet oxygen could lead to two opposite effects:

(a) "Pro-oxidant" effect: ascorbate enhances the toxicity of photodynamic action because the highly diffusible H_2O_2 can damage targets in different locations. The H_2O_2 will react with a different set of targets and can also lead to hydroxyl radical formation via Fenton chemistry [35–37] as well as the initiation of oxidative cascades via activation of heme peroxidase enzymes [38] (Fig. 1A).

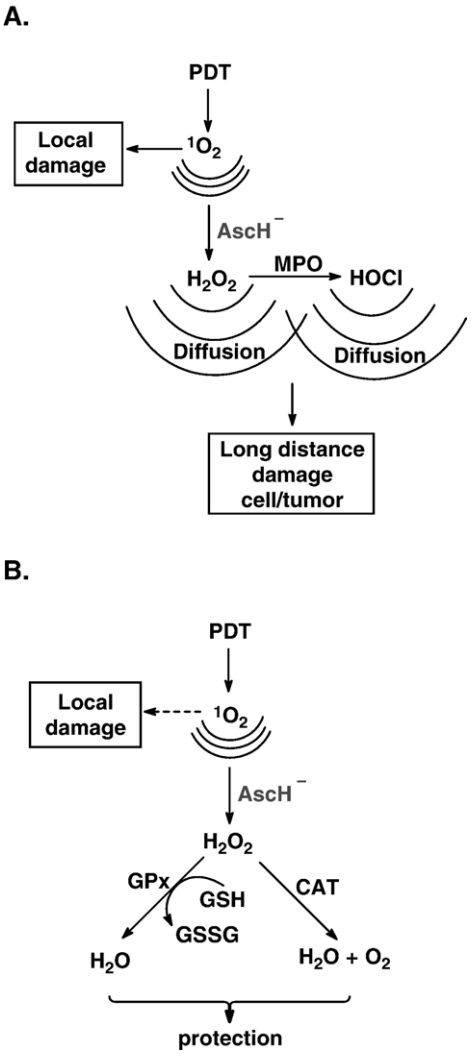

Fig. 1. $AscH^-$ can enhance or protect against the oxidations of PDA. A large percentage of the $^1O_2$ produced in or near a membrane will diffuse out of the membrane and react with electron-rich molecules in the water space [28,30,31]. Ascorbate ($AscH^-$) can react with part of the singlet oxygen that diffuses out of the membrane into the aqueous phase, converting it into H_2O_2. (A) Ascorbate can enhance the damage initiated by PDA. H_2O_2 can produce damage different from that of $^1O_2$ and can diffuse farther, thereby spreading the damage throughout the tumor (bystander effect). Some of this H_2O_2 can be a substrate for myeloperoxidase, resulting in the formation of hypochlorous acid. Thus, by converting part of the $^1O_2$ to H_2O_2 ascorbate creates additional reactive oxygen species that can enhance the destruction of the tumor. (B) Ascorbate can protect against the damage initiated by PDA. Converting some of the singlet oxygen to H_2O_2 can result in a decrease in local damage, i.e., oxidation of lipids or proteins. Further, H_2O_2 can be converted to water by peroxide-removing systems. These scenarios would lead to protection against photodynamic action.

(b) Antioxidant effect: ascorbate can chemically quench singlet oxygen in the water space, thereby protecting aqueous targets that are sensitive to $^1O_2$; this quenching would also limit the diffusion of $^1O_2$ back into the membrane, thereby protecting membrane lipids and proteins. Although H_2O_2 is highly diffusible, protection would imply that the H_2O_2 formed would be less toxic than singlet oxygen, perhaps because of a robust antioxidant network that removes hydroperoxides (Fig. 1B).

Which of these two scenarios is dominant will probably depend on the activity of the peroxide-removing enzyme systems as well as the levels of the small-molecule antioxidants ascorbate and glutathione. Both of these antioxidants are present at high levels in cells in vivo. Glutathione is key to the removal of H_2O_2.

Combining PDT with high concentrations of $AscH^-$ might enhance the efficacy of PDT in certain settings. Evidence that ascorbate can increase the toxicity induced by photodynamic action with different photosensitizers has been demonstrated with both in vitro [39–41] and in vivo models [42–45]. For example, ascorbate enhanced phthalocyanine-sensitized photohemolysis of human erythrocytes [39]. It has also been shown that phthalocyanine and sodium ascorbate significantly slowed the growth of Ehrlich carcinoma in mice in comparison with the suppressive effect of PDT only [46]. But it is still unclear why and in what settings ascorbate realizes its pro- or antioxidant capacities. To expand our knowledge of the conditions that allow ascorbate to initiate new oxidation cascades during photosensitization, we incubated leukemia cells with $AscH^-$ and a photosensitizer (Verteporfin) and upon exposure to light determined $AscH^-$ uptake, changes in membrane permeability, cell growth, and toxicity.

Materials and methods

Chemicals

Verteporfin is produced by Novartis (Switzerland) as a lipid-formulated photosensitizer (BPD-MA or VP) containing 13% BPD-MA. Propidium iodide, catalase, 3,3′,5,5′-tetramethylbenzidine, dimethylformamide, acetic acid, sodium acetate, and 4-ABAH were from Sigma (St. Louis, MO, USA); annexin V–FITC reagent and annexin binding buffer were from Biovision, Inc. (Mountain View, CA, USA). Ascorbic acid (EMD Chemical, Inc., Merck, Gibbstown, NJ, USA) was dissolved in phosphate buffer (KH_2PO_4, 1.15 mM; Na_2HPO_4, 2.88 mM; NaCl, 154 mM), pH 7.4, just before the start of the experiments. Adventitious transition metals were removed from PBS with chelating resin (Sigma) using the batch method [47].

Cell culture

The human promyelocytic leukemia cell lines (HL-60 and U937) were obtained from ATCC (Rockville, MD, USA). Both cell lines were cultured in RPMI 1640 medium (without phenol red) supplemented with 10% (v/v) FBS (medium-10), 2 mM glutamine, penicillin (100 U/ml), streptomycin (100 μg/ml), all from GIBCO (Grand Island, NY, USA). Both cell lines were incubated at 37°C in a 95% humidity atmosphere under 5% CO_2. Cells from passages 7–25 were used for the experiments. In cell growth experiments cells were counted daily using a Coulter Counter Z2 (Beckman Coulter, Inc., Fullerton, CA, USA). When cells were incubated with ascorbate or with Verteporfin, agents were added to the cell suspension (density 1×10^6/ml) in medium-2 (growth medium with 2% FBS) and incubated for 1 h, 37°C. Medium-2 was used because the uptake of VP was higher than in medium-10 [12,13].

Photodynamic treatment

Aliquots of Verteporfin (2 mg/ml) in DMSO were stored at −80°C. For PDA experiments Verteporfin was added to medium-2 (10 ng/ml, equivalent to 1.8 nM) with or without $AscH^-$ and cells were incubated for 1 h at 37°C. Cells were washed twice with RPMI 1640 and 7.5×10^6 cells were resuspended in 11 ml RPMI 1640 and placed into 100-mm dishes. Cells were then exposed to white light on a light box equipped with two linear 30-W daylight fluorescent bulbs and a UV-light-removing surface. The intensity of the white light was measured with a NIST traceable radiometer photometer with the probe for visible light from International Light, Inc. (Newburyport, MA, USA). The light intensity was measured for all areas of the light box surface and samples were placed only in areas that had the same fluence rate, 2.2 mW cm^{-2}. Cells were exposed to light for 10–20 min translating into a light dose of 1.32–2.62 J cm^{-2}. Temperature in the cell suspensions increased no more than 1.5°C during treatment.

Verteporfin (BPD-MA) uptake

Fluorescence measurements were performed to determine the uptake of Verteporfin by leukemia cells. Cells were incubated with Verteporfin (1.8 nM) in medium-2 for 1 h [48]. Cells (5×10^7/ml) were then washed twice with PBS and the cell pellet was resuspended in 0.75 ml PBS containing 2% Triton X-100. To lyse the cells, the cell suspension was freeze–thawed (three times) in dry ice/EtOH and water (37°C). Methanol (0.5 ml) was then added to the cell suspension to precipitate the protein. The suspension was centrifuged and the supernatant stored at −20°C. Fluorescence of BPD-MA was measured using a Perkin–Elmer LS50B luminescence spectrometer. The BPD-MA was excited at 430 nm and the emission spectrum was recorded, 650–750 nm. The concentration of standards of Verteporfin in PBS was determined with UV–Vis, $\varepsilon_{689} = 1.35 \times 10^4\ M^{-1}\ cm^{-1}$ [49].

Trypan blue assay for membrane permeability

Membrane permeability was determined using trypan blue exclusion. Trypan blue dye solution (0.2%) was added to the cell suspension (1:1 v/v) and stained cells counted under a light microscope using a hemacytometer.

GSH measurements

GSH and GSSG were measured as described by Baker et al. [50]. GSSG is reduced by glutathione disulfide reductase and the total GSH of the sample is oxidized with DTNB (5,5′-dithio-*bis*(2-nitrobenzoic acid)) (2GSH + DTNB → GSSG + 2TNB). Formation of TNB is measured at 405 nm with a microtiter plate reader. To determine [GSSG], the sample is first

treated with 2-vinylpyridine, a compound that conjugates with GSH, inhibiting its reaction with DTNB. The GSSG in the sample is then reduced by glutathione disulfide reductase in the presence of NADPH and oxidized with DTNB. Standards were made in 5% sulfosalicylic acid. GSH standards (10–100 μM), blank, or samples (30 μl) were added to a 96-well flat-bottom microtiter plate (Costar 3596). Triethanolamine (20 μl of a 0.657 M solution) was added to each well. Two hundred microliters of enzyme cocktail (NADPH (0.298 mM) prepared in sodium-phosphate buffer, DTNB (6 mM), GR equivalent to 125 units) was added to each well. The plate was read at 405 nm with a microtiter plate reader (Tecan Spectrafluor Plus) using a kinetic analysis protocol (10 cycles). The GSH_T concentration was calculated from the GSH standard curve and normalized against cell number. GST_T was used because the level of GSSG was always low in comparison to GSH and changes were small after all treatments. GSH_T was calculated as [GSH]+ 2[GSSG], the 2 being present to convert to equivalents of GSH.

Intracellular hydrogen peroxide

Steady-state levels of intracellular H_2O_2 were estimated using a sensitive assay based on the rate of inhibition of catalase by 3-amino-1*H*-1,2,4-triazole (AT; 20 mM) [51–53]. For catalase-activity assays cell pellets were thawed rapidly [54] and then homogenized in a Vibra Cell sonicator (Sonics and Materials, Inc., Danbury, CT, USA). Spectrophotometric catalase activities were run as described [55]. For each assay the lysate of 1.0×10^6 cells was introduced into the cuvette. The kinetic reaction was initiated by the addition of 500 μl of a 30 mM H_2O_2 stock solution in phosphate buffer, pH 7.0, and the loss of absorbance at 240 nm over a 2-min period was monitored. Catalase activities were calculated as a first-order reaction rate constant (k) and expressed as milli k units/10^6 cells. Cellular H_2O_2 concentrations were then determined by the kinetic analysis of the rate of decrease in catalase activity [52].

Cell survival by flow cytometry

Cell survival was determined with propidium iodide (PI) and annexin V–FITC by flow cytometry. A stock solution of PI (1 mg/ml) was prepared in distilled water and stored at 4°C in the dark. Annexin V (0.8 μg/ml final) and PI (1 μg/ml final) were added to 0.5 ml of cell suspension (5×10^5) in 1× annexin binding buffer and incubated for 5 min in the dark. Cell survival was determined with a FACScan flow cytometer from BD Biosciences (San Jose, CA, USA) equipped with an argon laser (488 nm). Data handling was performed by CellQuest software from BD Biosciences. Because the border/area between annexin staining and PI staining was not very distinct, we did not determine mode of cell death but rather combined both areas to determine overall killing.

Intracellular vitamin C by HPLC

Intracellular ascorbate concentrations were determined with HPLC following the protocol from Levine et al. [56]. Briefly, cells were incubated with ascorbate and washed twice with PBS. Cells were counted and then 3–4 × 10^6 cells were extracted with 0.50 ml methanol/H_2O (60%/40%) containing 250 μM DETAPAC. The mixture was kept on ice for 5 min to precipitate the protein, then centrifuged, and the supernatant filtered with a 0.22-μm syringe filter and stored frozen (−80°C) until the day of measurement. HPLC analysis was performed with a PhotoDiodeArray detector from the Thermo Electron Corp. (Waltham, MA. USA) using a Luna (2) C-18, 5-μm, 250 × 4.6-mm column from Phenomenex, Inc. (Torrance, CA. USA). The mobile phase consisted of 15% organic phase (36.6 μM tetraoctylammionium bromide in methanol) and 85% aqueous phase (189 μM dodecyltrimethylammionium chloride, 0.05 M sodium phosphate monobasic, and 0.05 M sodium acetate HPLC grade, pH adjustment to 4.8 with ortho-phosphoric acid). Standards of vitamin C were prepared fresh using the same extraction solution as for the samples; for quality control a standard sample was run every fifth injection.

Heme peroxidase activity

The activity of myeloperoxidase was determined by the ability of the enzyme to oxidize tetramethylbenzidine [57]. The MPO activity is expressed as change in absorbance units at 655 nm in 3 min. Briefly, cell homogenate ($0.1–0.5 \times 10^6$ cells) was placed in 3.05 ml 50 mM sodium acetate assay buffer, pH 5.4. The assay was initiated with 200 μl of 5.25 mM H_2O_2 followed by the addition of 50 μl of 100 mM 3,3′,5,5′-tetramethylbenzidine in dimethylformamide. After a 3-min incubation of samples at 25°C the enzymatic reaction was stopped by addition of 100 μl of a catalase solution (100 U/ml) and then 3.4 ml ice-cold acetic acid (2 mM).

To inhibit myeloperoxidase cells were incubated with VP and ascorbate in medium-2 in the presence of 200 μM 4-ABAH (MPO inhibitor). Cell growth and trypan blue permeability of cells were checked at different times after light treatment.

Statistical analysis

Data are expressed as means ± SD. Statistical significance of differences between paired data was determined either by Student's test (single comparisons) or by one-way analysis of variance for multiple comparisons. Difference among means were considered to be significant at $p < 0.05$.

Results and discussion

Cell lines

Because the reducing nature of ascorbate leads to its function as an antioxidant as well as the initiator of oxidation cascades in some settings, we chose to work with two cell lines to determine if the effect of ascorbate on PDA is similar in somewhat different cellular environments. The two human leukemia cell lines were chosen because they are similar as both were cancer cells originating from the same myelomonocytic lineage. However, HL-60 cells have structure and properties of

myelocytes, whereas U937 originated from monocytic progenitors. Both cell lines were grown in the same medium. Because these two cell lines are closely related, we hypothesized that they would behave similarly in response to ascorbate and PDA treatment.

Cell survival after PDA and ascorbate treatment

To investigate the effect of ascorbate on PDA we measured cell survival, cell growth, and membrane permeability after PDA and ascorbate treatment. Verteporfin (benzoporphyrin derivative) is a photosensitizer that accumulates mainly in mitochondria [10]. Upon light exposure, VP produces singlet oxygen that can react with unsaturated fatty acids and proteins, leading to radical formation and membrane leakage. Some of the $^1O_2$ produced diffuses out of the membrane into the cytosol where it can react with proteins and other electron-rich molecules, such as $AscH^-$, and produce diffusible H_2O_2 (Fig. 1). We hypothesized that ascorbate will react with some of the $^1O_2$ produced during PDA and thereby enhance cellular damage. To test this hypothesis we adjusted PDA conditions so that approximately 40% of the HL-60 cells survived (Fig. 2A, VP+ light). We added 200 μM ascorbate during incubation with VP to determine the effect of ascorbate on cell survival. Cells were harvested every 24 h for 3 days and cell death was determined using FACS analysis with PI and annexin V–FITC. When HL-60 cells were exposed to ascorbate and light, a slight (10%) decrease in cell survival was observed compared to cells exposed to light alone. Incubation with Verteporfin and ascorbate in the dark decreased survival by approximately 30% ($p < 0.01$). After PDA with Verteporfin and light, survival of HL-60 cells decreased by 65% (24 h; $p < 0.01$). Incubation with 200 μM ascorbate enhanced cell killing by an additional three-fold 3 days after PDA. Thus ascorbate enhances the toxicity of PDA in HL-60 cells.

Using the same PDA conditions as were used for the HL-60 cells, minimal changes in survival of U937 cells were observed (Fig. 2B, VP and light). This demonstrates that U937 cells are more resistant to PDA compared to HL-60 cells. Even when the dose of ascorbate was increased to 600 μM no decrease in cell survival was observed. When the light dose was increased from 1.32 to 1.98 J cm^{-2}, PDA alone decreased U937 survival to 10% ($p < 0.01$) 2 days after light exposure (Fig. 2C). In contrast to HL-60 cells, ascorbate showed a protective effect in U937 cells; cell survival was twofold higher than that of VP + light.

Growth after PDA and ascorbate treatment

In parallel with measurement of the influence of ascorbate and PDA on cell survival, we examined the growth rate of HL-60 and U937 cells after various treatments (Fig. 3). Incubation of HL-60 cells with VP or ascorbate (200 μM) in the dark had minimal effect on the growth rate compared to cells with no treatment ($p < 0.01$). Interestingly, when HL-60 cells were incubated with VP and ascorbate with or without light a

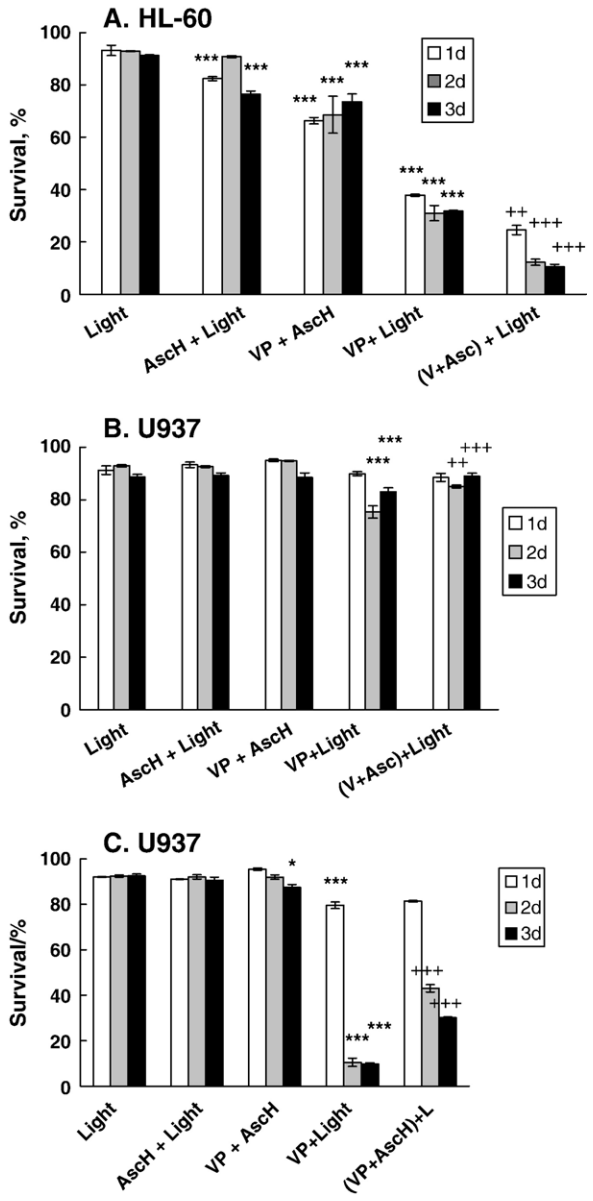

Fig. 2. Ascorbate decreased survival of HL-60 cells after photodynamic treatment but protected U937 cells. Cells (1×10^6/ml) were incubated with Verteporfin (1.8 nM) in the presence of $AscH^-$ (200 (A) and 600 μM (B, C)) for 1 h in medium containing 2% FBS. Cells were washed and placed into RPMI 1640 for light exposure. (A) HL-60 cells were exposed to light, 1.32 J cm^{-2}. (B) U937 cells were exposed to light, 1.32 J cm^{-2}. (C) U937 cells were exposed to light, 1.98 J cm^{-2}. After light exposure, cells were washed and placed into regular growth medium. Cells were harvested every 24 h for 3 days and cell survival was determined using propidium iodide and annexin V–FITC. Each point and bar represent the mean and standard error of three independent determinations. *$p < 0.05$, ***$p < 0.01$ represent a significant difference of survival with respect to light-treated cells. ++$p < 0.02$, +++$p < 0.01$ represent a significant difference of survival with respect to cells after PDA.

significant delay (3 days) in cell growth was observed. This delay was not seen with $AscH^-$ or VP alone. Thus, VP and $AscH^-$ produce some toxicity in HL-60 cells as seen in both survival and growth.

Exposure of HL-60 cells to VP plus light delayed growth by approximately 2 days and the growth rate was slower than in

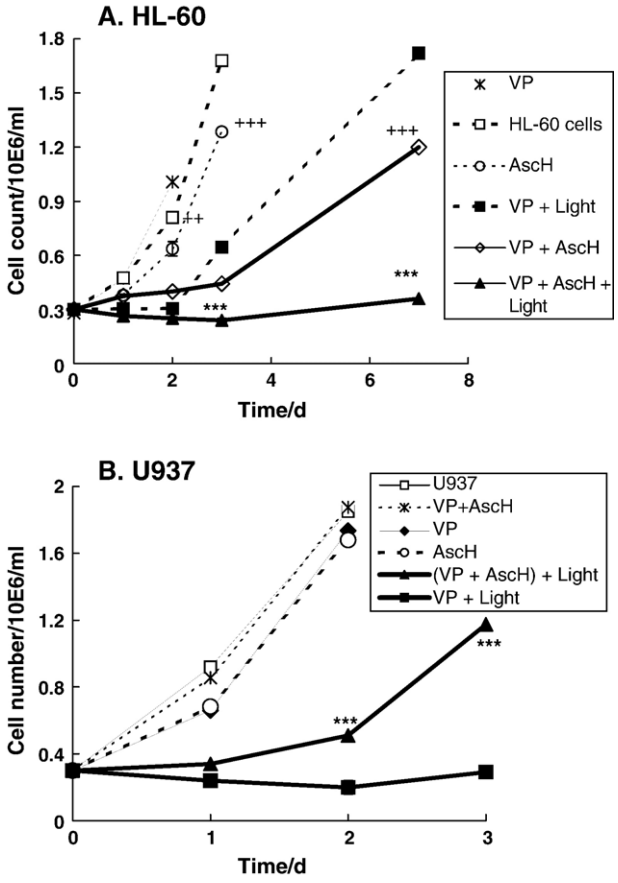

Fig. 3. Ascorbate inhibits cell growth after PDA treatment. Cells (1×10^6/ml) were incubated with Verteporfin (1.8 nM) in the presence of $AscH^-$ (200 (A) and 600 μM (B)) for 1 h in medium-2. Cells were washed and placed into RPMI 1640 for light exposure. (A) HL-60 cells were exposed to light (1.32 J cm^{-2}). (B) U937 cells were exposed to light (1.98 J cm^{-2}). Cells were then transferred into medium-10 and grown at 37°C and 5% CO_2. Cells were counted every 24 h. Each point and bar represent the mean and standard error of three independent determinations. ***$p < 0.01$ represents a difference between data of VP + light and VP + $AscH^-$ + light groups. ++$p < 0.02$, +++$p < 0.01$ represent a significant difference of cell growth rate with respect to cells in medium-2.

cells without treatment (t_d = 48.0 vs 28.4 h,[1] Table 1A and Fig. 3A). When these cells were co-incubated with 200 μM ascorbate ($AscH^-$ + VP + light) the doubling time increased compared to VP plus light (t_d = 155 vs 48 h). These results are in agreement with the survival assays of Fig. 2. Thus, for HL-60 cells, ascorbate in the presence of VP slows cell growth both in the dark and with light.

U937 cells were found to be more resistant to PDA with VP, thus the light dose was increased (from 1.32 to 1.98 J cm^{-2}) as well as the amount of ascorbate (from 200 to 600 μM). In the dark, incubation with VP, ascorbate, or both resulted in growth curves that were the same as U937 cells with no treatment. Exposure of U937 cells to VP + light suppressed growth by a factor of 4.6 compared to cells incubated with VP in the dark (Table 1B and Fig. 3B). In contrast to HL-60 cells inclusion of ascorbate during PDA resulted in protection of U937 cells (t_d = 19.2 h in presence of ascorbate; t_d = 87.1 h for cells after PDA only, Table 1B). Thus, ascorbate protects U937 cells against PDA; this is similar to the effect seen in the cell survival experiment with U937 cells (Fig. 2B).

Ascorbate enhances PDA in HL-60 cells, but protects U937 cells as seen by membrane permeability

Membrane integrity can be a marker of cell damage. Oxidative damage to cell membranes can breach this integrity. As a measure of the oxidative damage induced by PDA changes in membrane integrity were determined by trypan blue dye exclusion. To test the effect of PDA and ascorbate on the plasma membrane we subjected cells to conditions similar to those in Fig. 2. In both cell lines the permeability of the plasma membrane was enhanced 20 h after PDA (Fig. 4). In HL-60 cells 600 μM ascorbate enhanced PDA-induced membrane permeability ($p < 0.01$) (Fig. 4A). Incubation with 600 μM ascorbate alone or together with the photosensitizer in the dark did not enhance permeability of the plasma membrane (data not shown). This supports the viability results that the reducing nature of ascorbate results in the initiation of oxidation processes in HL-60 cells.

In contrast to HL-60 cells ascorbate seems to protect against PDA in U937 cells (Fig. 4B); the higher the ascorbate concentration the greater the protection against membrane permeability. However, when the light dose was increased the protective effect of ascorbate (600 μM) was lost (Fig. 4C), probably because the flux of oxidants overwhelms the antioxidant aspects of ascorbate.

These results suggest that in general ascorbate acts as a pro-oxidant in HL-60 cells and an antioxidant in U937 during PDA. To probe for the reason ascorbate behaves so differently in these two cells lines we investigated the following factors: uptake of VP, $AscH^-$ uptake and toxicity, redox environment via GSH and H_2O_2, and myeloperoxidase activity.

Uptake of VP

A possible reason for the differences seen in the sensitivity of the two cell lines to the combined treatment of VP and ascorbate could be a variation in cellular uptake of VP. We examined the uptake of VP by these two leukemia cell lines in the presence or absence of ascorbate. The cells were incubated with VP for 1 h in medium-2 as described under Materials and methods. We found no difference in the fluorescence intensity of BPD-MA in cell extracts (50×10^6 cells) from HL-60 and U937 cells after incubation with VP (n = 4). The presence of ascorbate during incubation with VP increased the fluorescence of BPD-MA by 10–30% in both HL-60 and U937 cell extracts. Thus, possible differences in uptake of VP are not factors between the two cell lines and their response to PDA.

[1] Here t_d is not the traditional doubling time for cell growth [58] because at time 0 the cells were a mixture of dying and surviving cells. Thus, t_d is a value for the growth rate of this mixture.

Table 1a
Doubling time (t_d) of proliferating HL-60 cells after combined ascorbate treatment with VP+light

	Medium-2	VP	$AscH^-$ + light	VP + light	VP + $AscH^-$	VP + $AscH^-$ + light
t_d/h	28.4 ± 0.2 [a]	30.2 ± 0.1	23.4 ± 1.0	48.0 ± 1.8	66.7 ± 1.2	154.8 ± 24.9
t test		$p < 0.01$ vs medium-2	$p < 0.01$ vs medium-2	$p < 0.01$ vs VP	$p < 0.01$ vs VP	$p < 0.01$ vs VP+$AscH^-$

[a] Each result represents the mean and standard error of three independent determinations.

Ascorbate slows cell growth in vitro

It has been shown that prolonged exposure of cells to ascorbate in culture medium can be cytotoxic due to the production of H_2O_2 [59]; the rate of H_2O_2 production from oxidation of ascorbate is dependent on the medium [60]. To determine how ascorbate will influence the growth rate of HL-60 and U937 cells we incubated these cell lines with various concentrations of ascorbate (0–3000 μM). To allow cells to accumulate ascorbate and avoid prolonged exposure, cells were incubated for only 1 h with ascorbate in medium-2. Ascorbate-containing medium was then removed and replaced with medium-10 and cells were incubated at 37°C. Cells were counted every 24 h. Ascorbate inhibited the growth of HL-60 and U937 cells in a concentration-dependent manner. HL-60 cells were more sensitive to ascorbate compared to U937 cells (Fig. 5). Medium concentrations of ascorbate ≈200 μM resulted in significant delay in cell growth in HL-60 cells ($p < 0.01$), whereas U937 cells tolerated ≥600 μM $AscH^-$ without a significant change in cell growth. Thus, HL-60 cells are more sensitive to the oxidation initiated by $AscH^-$ than U937 cells. To minimize direct influence of ascorbate no more than 200 μM $AscH^-$ was used for most of the PDA experiments with HL-60 cells.

Cellular uptake of $AscH^-$ is higher in U937 cells compared to HL-60 cells

To determine if ascorbate uptake varies between HL-60 and U937 cells, intracellular concentrations of $AscH^-$ were measured using HPLC/EC. To determine how the intracellular levels of ascorbate varied with the concentration in the medium, ascorbate (0–3000 μM) was added to cells in medium-2 for 1 h and then intracellular ascorbate was measured. Ascorbate uptake by HL-60 cells increased as ascorbate was varied from 20 to 400 μM; relative uptake plateaued when medium concentrations were greater than ≈400 μM (Fig. 6). In contrast to HL-60 cells, U937 cells accumulated higher levels of intracellular $AscH^-$ (two- to four-fold). $AscH^-$ uptake in U937 cells also seems to have two different phases. Like HL-60 cells, when $AscH^-$ in the medium ranged from 20 to 400 μM, intracellular changes in the uptake of ascorbate were steep. At concentrations higher than ≈400 μM, relative uptake decreased. These results are consistent with the known active accumulation of ascorbate by cells to concentrations many times higher than in the extracellular space [17,61,62]. The ascorbate levels in human neutrophils and monocytes in vivo [15] are similar to the levels of ascorbate taken up by HL-60 and U937 cells using our approach. Although the molar concentration of ascorbate is higher in the cells compared to the medium, at 1×10^6 cells ml^{-1} there will be ≈50–200 times more moles of ascorbate in the medium than in the cells. Thus, if there is significant oxidation of the ascorbate in the medium, the H_2O_2 produced outside the cells could dominate the level of H_2O_2 in the cells. This would be in accordance with data demonstrating that the growth-inhibitory effect of ascorbate is dependent on the concentration of ascorbate in the culture medium and not its intracellular concentration (compare Figs. 5 and 6) [59,63].

The presence of Verteporfin had no effect on intracellular ascorbate accumulation in U937 cells, but there was somewhat less ascorbate uptake (<15%) in HL-60 cells at higher levels of ascorbate (Fig. 6). From Fig. 6 we can infer that in the experiments of Figs. 2–4 the intracellular concentration of ascorbate in HL-60 cells was three times lower (0.3 mM) than that of the U937 cells (1.0 mM). Therefore the effects of ascorbate on HL-60 cells during PDA must be due to factors other than the levels of intracellular ascorbate.

Intracellular GSH, $AscH^-$, and PDA

Ascorbate uptake in leukemia cells, such as HL-60 and U937, occurs mainly through the oxidized form DHA [61,64,65]. DHA is transported into cells by sodium-independent glucose transporters. Once inside the cell, DHA is reduced to $AscH^-$. Reduction of DHA to $AscH^-$ can be both GSH-dependent and GSH-independent [61,66]. Glutathione is considered to be the principal redox buffer in the cell [67]. Depletion of GSH has been shown to increase the vulnerability of leukemia cells to exposure to ROS [68,69]. To determine if ascorbate influences the levels of GSH, ascorbate (200–3000

Table 1b
Doubling time (t_d) of proliferating U937 after combined ascorbate treatment with VP + light

	Medium-2	VP	$AscH^-$+ light	VP + light	VP + $AscH^-$	VP + $AscH^-$ + light
t_d/h	19.1 ± 1.1 [a]	19.0 ± 0.4	19.2 ± 0.6	87.1 ± 5.1	21.2 ± 0.2	39.9 ± 3.3
t test				$p < 0.01$ vs VP	$p < 0.02$ vs VP	$p < 0.01$ vs VP + $AscH^-$

[a] Each result represents the mean and standard error of three independent determinations.

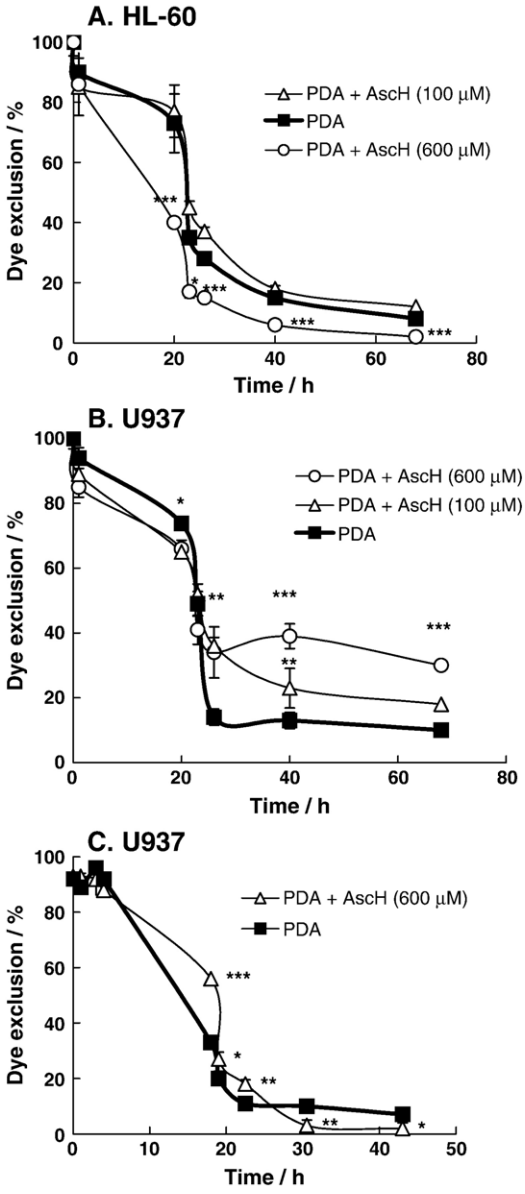

Fig. 4. Ascorbate changes membrane permeability after PDA. HL-60 or U937cells (1×10^6/ml) were incubated with Verteporfin (1.8 nM) (filled square) in the presence of $AscH^-$ (100 μM, open triangle, and 600 μM, open circle) for 1 h in medium containing 2% FBS. Cells were washed and placed into RPMI 1640 for light exposure. (A) HL-60 cells were exposed to light (1.32 J cm^{-2}); (B) U937 cells were exposed to light (1.32 J cm^{-2}); (C) U937 cells were exposed to light (1.98 J cm^{-2}). After light exposure cells were washed and placed into regular growth medium. Membrane permeability was tested at different times (h) after light exposure by trypan blue exclusion. Each point and bar represents the mean and standard error of three independent determinations; some error bars are within the symbol. *$p < 0.05$, **$p < 0.02$, ***$p < 0.01$ represent comparisons between experiments with and without PDA.

μM) was added to the culture medium. The level of GSH_T in HL-60 cells incubated in medium-2 was somewhat lower compared to that from incubation in medium-10 (Fig. 7A). The level in U937 cells in medium-2 was similar to that in medium-10. The GSH_T levels in the two cell lines were the same when incubated in medium-10. In addition, the intracellular ascorbate levels in both cell lines were similar in medium-10 (data not

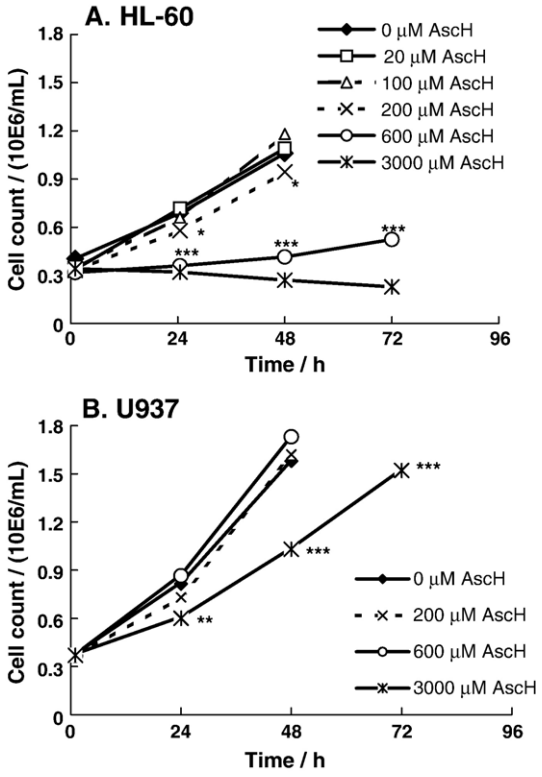

Fig. 5. Ascorbate slows cell growth. (A) HL-60 cells and (B) U937 cells were incubated with ascorbate (0–3000 μM) in medium-2 for 1 h and then washed and placed in medium-10 at a density of $0.35–0.40 \times 10^6$ cells/ml. Cells were counted every 24 h. Each point represents the mean and standard error of three independent determinations. *$p < 0.05$, **$p < 0.02$, ***$p < 0.01$ represent a significant difference in cell count after incubation of HL-60 cells in the presence of 200, 600, or 3000 μM compared to 0 μM $AscH^-$; for U937— in the presence of 3000 μM with respect to cells incubated in medium-2.

shown). Incubation of the two cell lines with 200 or 600 μM ascorbate in medium-2 resulted in minor changes in the intracellular concentration of GSH_T. The intracellular GSH_T in

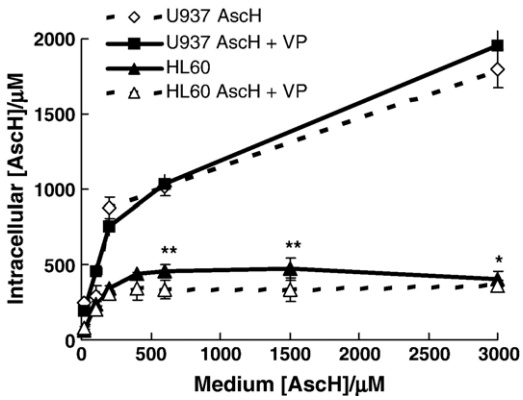

Fig. 6. Uptake of ascorbate is higher in U937 cells compared to HL-60 cells. HL-60 and U937cells (1×10^6/ml) were incubated with $AscH^-$ (0–3000 μM) with or without Verteporfin (1.8 nM) for 1 h in medium-2. Cells were counted and intracellular [$AscH^-$] was determined by HPLC. Each point and bar represent the mean and standard error of three independent determinations. *$p < 0.05$, **$p < 0.02$ represent a significant difference with respect to HL-60 cells after treatment with $AscH^-$+ VP.

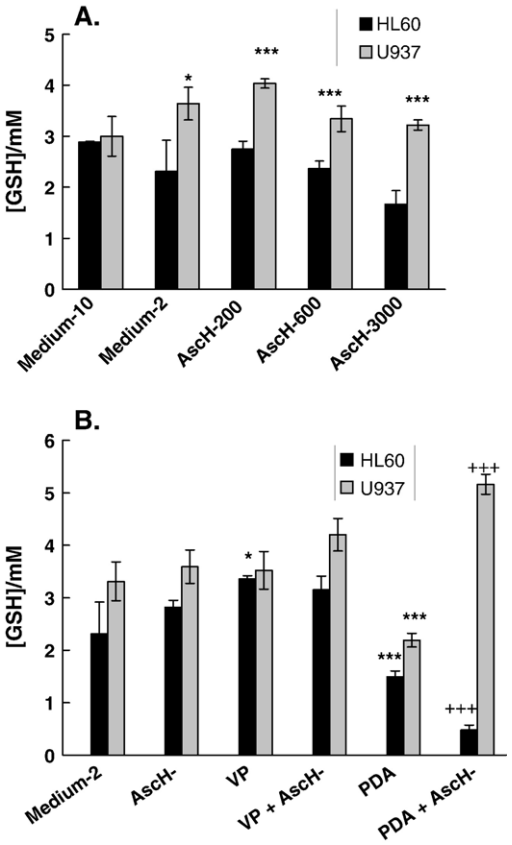

Fig. 7. Influence of ascorbate on intracellular [GSH]. (A) HL-60 and U937 cells (1×10^6/ml) were incubated with $AscH^-$ (0–3000 μM) for 1 h in medium-2. Cells were washed, counted, and lysed, and intracellular [GSH] was determined by a GSH recycling assay. Controls in medium-10 represent the GSH levels for the cells in their normal growth medium. Each bar represents the mean and standard error of at least three independent determinations. *$p < 0.05$, ***$p < 0.01$ represent a significant difference in the level of GSH in U937 cells compared to HL-60 cells incubated in medium-2. (B) Influence of ascorbate and PDA combined treatment on intracellular [GSH]. HL-60 and U937 cells (1×10^6/ml) were incubated with Verteporfin (1.8 nM) in the presence of $AscH^-$ (200 μM, HL-60, and 600 μM, U937) for 1 h in medium-2. Cells were washed and placed into RPMI 1640 for light exposure. HL-60 cells were exposed to a light dose of 1.32 J cm^{-2}, whereas U937 cells were exposed to a light dose of 1.98 J cm^{-2}. Cells were washed, counted, and lysed, and intracellular [GSH] was determined by a GSH recycling assay. Control represents the GSH levels for cells incubated in medium-2 for 1 h. Each bar represents the mean and standard error of at least three independent determinations. *$p < 0.05$, ***$p < 0.01$ represent a significant difference in GSH levels with respect to cells incubated in medium-2. +++$p < 0.01$ represents a significant difference in GSH with respect to cells after PDA.

HL-60 cells was lower than that found in U937 cells. GSH_T had a tendency to decrease with increasing ascorbate. However, compared to the control (medium-2), ascorbate did not change the level of GSH_T. The lower levels of GSH_T and ascorbate in HL-60 cells compared to U937 cells indicates that the redox environment [67] of HL-60 is more oxidized than in U937 cells, suggesting that HL-60 cells may have a lower capacity to buffer an increased flux of ROS and thus they would have a higher sensitivity to ROS.

Changes in GSH_T levels could be a marker of toxicity induced by PDA and ascorbate. Thus, we checked for changes in GSH_T after treatment with PDA/ascorbate (Fig. 7B). U937 cells have higher levels of GSH_T than HL-60 cells in all experimental groups, including after PDA. The GSH_T level in U937 cells did not change after treatment with ascorbate, VP, or VP plus ascorbate. The GSH_T level of HL-60 cells in all control groups was also the same, except for the group with VP. PDA induced significant loss of GSH_T ($p < 0.001$) in both cell lines. However, ascorbate in combination with PDA resulted in further loss of GSH_T in HL-60 cells ($p < 0.001$) but increased GSH_T in U937 cells ($p < 0.001$). These changes in GSH_T induced by PDA and ascorbate correlated with the cell survival of Fig. 2. Thus the sensitivity of these cells to PDA may be related in part to their levels of GSH_T.

Table 2
Catalase activity and H_2O_2 level of leukemia cells

	Total catalase activity (k mU/10^6 cells)	Catalase activity in presence of AT (k mU/10^6 cells)	$[H_2O_2]_{ss}$/pM [a]
HL-60	0.80 ± 0.15 [b]	0.24 ± 0.004	39.2 ± 0.6
U937	1.74 ± 0.35	0.93 ± 0.24	14.2 ± 0.7
t test [c]	$p < 0.01$	$p < 0.01$	$p < 0.01$

[a] An estimate of the intracellular steady-state level of hydrogen peroxide.
[b] Each result represents the mean and standard error of three independent determinations.
[c] Each test is a comparison between cell lines.

Intracellular H_2O_2 is higher in HL-60 cells compared to U937 cells

Another indicator for the intracellular redox environment is the steady-state level of H_2O_2. We examined the ambient intracellular levels of H_2O_2 via inhibition of catalase by aminotriazole [52,53]. We found that untreated U937 cells have lower steady-state levels of intracellular H_2O_2 compared to HL-60 cells, 14.2 ± 0.7 pM vs 39.2 ± 0.6 pM, respectively (Table 2). U937 cells have over twice the catalase activity of HL-60 cells. This can explain the lower intracellular hydrogen peroxide level in U937 cells and also our findings that U937 cells are more resistant to PDA ascorbate treatment than HL-60

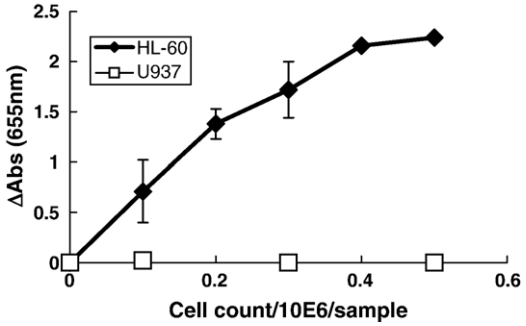

Fig. 8. HL-60 cells have MPO activity, whereas U937 cells are void of MPO activity. For the MPO assay cell homogenates from 0.1–0.5 × 10^6 HL-60 or U937 cells were placed in 3.05 ml of 50 mM sodium acetate buffer, pH 5.4. The MPO activity was expressed as change in absorbance at 655 nm. Data are presented as means ± SD ($n = 6$).

cells. Catalase can remove hydrogen peroxide formed by ascorbate and singlet oxygen, thereby preventing cellular damage.

The higher level of hydrogen peroxide is also consistent with the intracellular redox environment of HL-60 cells being more oxidizing than in U937 cells. This is also in line with the relative GSH levels in these two cell lines (Fig. 7), as well as the lower levels of ascorbate (Fig. 6). Unfortunately, the assay for intracellular hydrogen peroxide cannot be used in experiments with added ascorbate because ascorbate would interfere with the kinetics of the inactivation of catalase by AT. These observations are consistent with the formation of H_2O_2 by $AscH^-$ and the notion that cells having a lower peroxide-removing capacity are sensitized to PDA.

The toxicity of ascorbate is enhanced by MPO

Heme peroxidases, such as MPO, are activated by H_2O_2 to make more reactive oxidants, such as HOCl. To understand a possible role for MPO in the difference in the sensitivity of

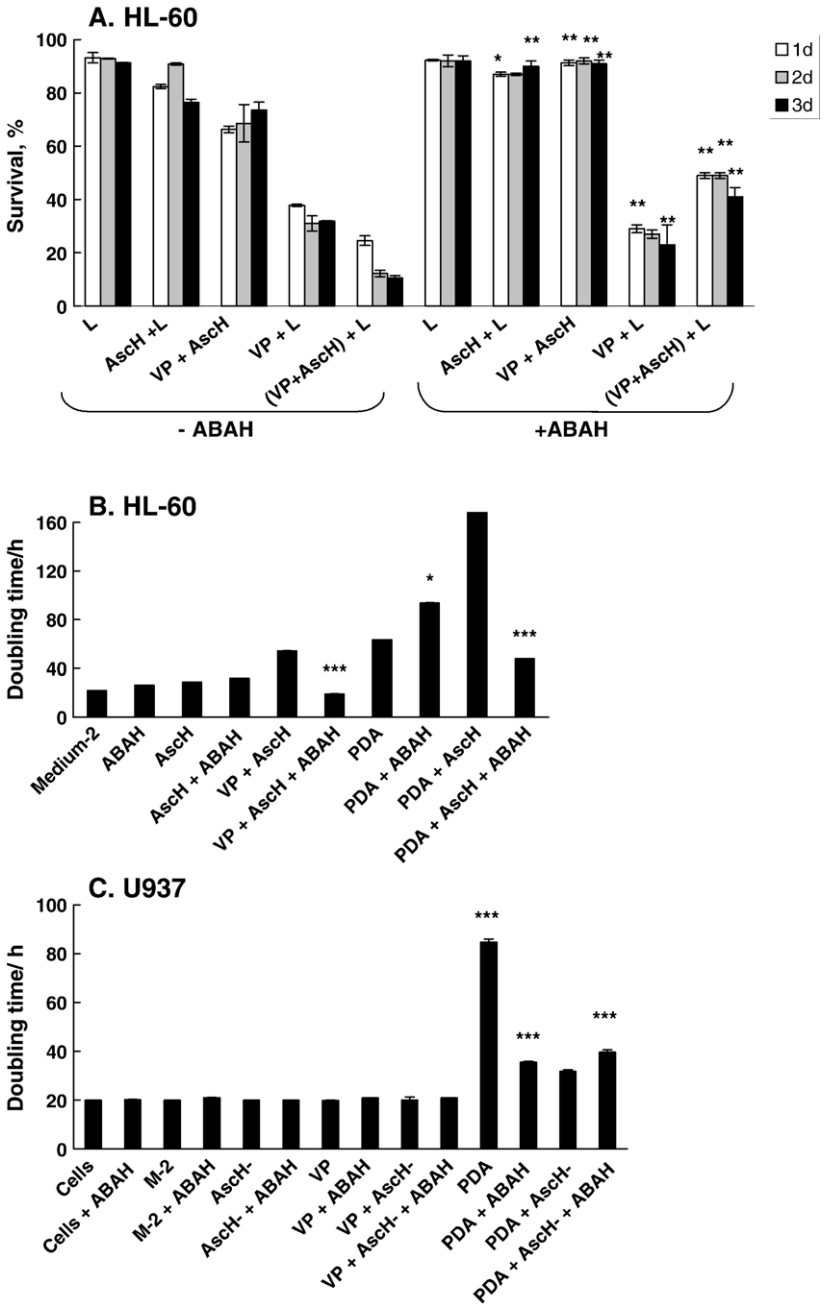

Fig. 9. Myeloperoxidase inhibitor (4-ABAH) protected HL-60 cells against ascorbate but not PDA toxicity. Cells (1×10^6/ml) were incubated with Verteporfin (1.8 nM) and $AscH^-$ (200 μM) in the presence or absence of 4-ABAH (200 μM) for 1 h in medium-2. Cells were washed and placed into RPMI 1640 for light exposure. HL-60 cells were then exposed to light, 1.32 J cm^{-2}. After light exposure, cells were washed and placed into regular growth medium. Cells were harvested every 24 h for 3 days and cell survival was determined using propidium iodide and annexin V–FITC. Each point and bar represent the mean and standard deviation of three independent determinations. *$p < 0.05$, **$p < 0.02$, ***$p < 0.01$ represent comparison between groups of data (−ABAH vs +ABAH).

HL-60 and U937 cells to PDA the activity of MPO was determined (Fig. 8). The activity of MPO in U937 cells was below the limit of detection, whereas HL-60 cells had high activity levels. This peroxidase activity in HL-60 cells was effectively inhibited by 200 μM 4-ABAH, an inhibitor of MPO (84 ± 2% with a 30-min incubation, $n = 3$), supporting the specificity of the assay. To examine if active MPO in HL-60 cells could be one of the factors responsible for the ascorbate toxicity we determined the effects of ascorbate on the growth rate and survival of HL-60 and U937 cells in the presence of 4-ABAH.

Cells were treated with ascorbate and PDA with or without 200 μM 4-ABAH. 4-ABAH maintained the survival of HL-60 cells treated with $AscH^-$ or with $AscH^-$ and VP (Fig. 9A). Interestingly, 4-ABAH did not affect the survival of the group treated with VP + light, in which singlet oxygen would be the major initiating oxidant. However, when $AscH^-$ was present during treatment with VP + light, 4-ABAH provided protection. This protective effect was also reflected in cell growth (Fig. 9B). 4-ABAH increased the rate of growth (shorter doubling time) of HL-60 cells treated with PDA + $AscH^-$, resulting in a doubling time similar to the doubling time of control cells (VP + $AscH^-$). These data suggest that the H_2O_2 produced by $AscH^-$ and $^1O_2$ is a substrate for MPO in HL-60 cells, enhancing the toxicity of PDA. As expected, 4-ABAH did not influence the antioxidant effect of ascorbate on U937 cells under the same experimental conditions (Fig. 9C). These data clearly show that PDA in the presence of ascorbate increases the production of H_2O_2. This H_2O_2 can induce additional toxicity by initiating the two-electron oxidation of myeloperoxidase forming the highly oxidizing compound I [70]. MPO compound I will in turn initiate detrimental oxidations.

Summary

This work clearly demonstrates that ascorbate can have quite different effects on the phototoxicity of two closely related human cell lines. U937 and HL-60 cells are derived from a promyelocytic progenitor; HL-60 cells have structure and properties of myelocytes, whereas U937 cells have properties of monocytes. We hypothesized that ascorbate could increase the flux of hydrogen peroxide during photodynamic action. Thus, the peroxide-removing systems of the two cell lines would be central to the potential toxicity of this H_2O_2. We have found that U937 cells have a somewhat higher level of GSH and twice the catalase activity of HL-60 cells, consistent with the observations of Cai et al. [71]. They also found the GPx-1 and total SOD activity of these two cell lines to be similar. This difference in catalase activity could render HL-60 cells more vulnerable to an increased flux of H_2O_2. However, a key difference in these two cell lines is that HL-60 cells have a high level of MPO, whereas in U937 cells this enzyme activity is below the limit of detection. Upon exposure to H_2O_2, this MPO is activated, producing oxidants that are toxic to HL-60 cells [72]. The data of Fig. 9 clearly show that ascorbate enhances the production of H_2O_2 associated with the photodynamic action of VP + light and that this H_2O_2 can enhance the toxicity of PDA. Thus ascorbate could be an adjuvant for photodynamic therapy, but the capacity of the tumor to remove peroxide would be a central consideration in its use.

Acknowledgments

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