Selective Ascorbate Toxicity in Malignant Mesothelioma
A Redox Trojan Mechanism

Elia Ranzato[1,2], Stefano Biffo[1,2], and Bruno Burlando[1]

[1]Department of Environment and Life Sciences, DiSAV, University of Piemonte Orientale Amedeo Avogadro, Alessandria, Italy; and [2]Laboratory of Molecular Histology and Cell Growth, San Raffaele Science Institute, Milan, Italy

We studied the mechanism of ascorbate toxicity in malignant mesothelioma (MMe) cells. Neutral red uptake showed that ascorbate, but not dehydroascorbate, was highly toxic in the MMe cell lines REN and MM98, and less toxic in immortalized (human mesothelial cells-htert) and primary mesothelial cells. Ascorbate transport inhibitors phloretin, sodium azide, and ouabain did not reduce ascorbate toxicity. Ascorbate promoted the formation of H_2O_2 in the cell medium, and its toxicity was suppressed by extracellular catalase, but the concentration of endogenous catalase was higher in MMe cells than in normal cells. The confocal imaging of cells loaded with the dihydrhodamine 123 reactive oxygen species probe showed that ascorbate caused a strong increase of rhodamine fluorescence in MMe cells, but not in mesothelial cells. MMe cells showed a higher production of superoxide and NADPH oxidase (NOX)4 expression than did mesothelial cells. Two inhibitors of cellular superoxide sources (apocynin and rotenone) reduced ascorbate toxicity and the ascorbate-induced rise in rhodamine fluorescence. NOX4 small interfering RNA also reduced ascorbate toxicity in REN cells. Taken together, the data indicate that ascorbate-induced extracellular H_2O_2 production induces a strong oxidative stress in MMe cells because of their high rate of superoxide production. This explains the selective toxicity of ascorbate in MMe cells, and suggests its possible use in the clinical treatment of malignant mesothelioma.

Keywords: catalase; hydroxyl radical; malignant mesothelioma cells; normal mesothelial cells; NOX4

CLINICAL RELEVANCE

This study provides information about differences in the redox processes of malignant mesothelioma cells with respect to their normal counterparts. Data also indicate that these differences can be exploited for cytotoxic treatments that may offer new possibilities in the therapeutic strategies against this drug-resistant cancer.

Ascorbate is an essential nutrient in the human diet, but is also widely used as a medicinal product, and has long been held as a remedy for various diseases (1). About 50 years ago, it was hypothesized that ascorbate could be used in cancer therapy (2, 3). This gave rise to a first wave of research, suggesting that ascorbate could act by reinforcing the extracellular matrix and preventing its degradation, thereby hindering cancer cell migration and potential for invasion (4, 5).

Clinical studies on the chemotherapeutic use of ascorbate showed beneficial effects in some cases (6, 7) although no benefit was observed in others (8, 9). These discrepant results possibly derived from intravenous versus oral ascorbate administration (10, 11). Further studies showed that the oral administration of ascorbate leads to plasma concentrations of $\sim$ 0.1 mmol/L (12), whereas intravenous administration leads to plasma concentrations up to 5.0 mmol/L (13), with rare adverse effects (14).

These latter data are of particular interest, because concentrations approaching the millimolar range were shown to be toxic in a variety of cancer cell lines, suggesting that the use of ascorbate in cancer treatment deserves in-depth investigation (15–17). Studies focusing on the mechanism of ascorbate toxicity reported the induction of apoptosis through cell cycle arrest in melanoma and hepatoma cells (18, 19), activation of the apoptosis-inducing factor (AIF) factor in human breast cancer cells (20), and interference with iron uptake in neuroblastoma and melanoma cells (17, 21). However, ascorbate is known to act as an electron donor in redox reactions (22), and a body of evidence supports the idea that oxidative stress plays a major role in the mechanism of ascorbate toxicity in tumor cells (23). Moreover, the toxicity of ascorbate was shown to result mainly from extracellular activity, possibly depending on the formation of H_2O_2 in the extracellular space (24).

Malignant mesothelioma (MMe) is a lethal tumor arising from the mesothelium of serous cavities. This cancer shows a close relationship with asbestos exposure, and its incidence has been increasing in several countries as a result of the widespread use of asbestos. The delay between the first exposure and the appearance of illness is 20–40 years, and patients generally die within 4–18 months after diagnosis, because of the involvement of vital thoracic organs, especially the lungs (25). The treatment of MMe is extremely problematic (26, 27), and an urgent need exists to find new therapeutic approaches.

To date, the sensitivity of MMe cells to ascorbate was unknown. Therefore we explored the possibility of using ascorbate as a chemotherapeutic agent. We first evaluated the cytotoxicity of ascorbate in different human MMe cell lines by using neutral red uptake (NRU), and compared these results with those obtained in normal mesothelial cells. By using the confocal imaging of reactive oxygen species (ROS) production and the cytochrome c superoxide assay, we also tried to reveal the mechanism of ascorbate toxicity and of its possible selectivity toward tumor cells. Our results showed that ascorbate is more toxic in MMe cells than in normal mesothelial cells, and revealed for the first time, to the best of our knowledge, that ascorbate-selective toxicity is attributable to a redox Trojan mechanism involving extracellular H_2O_2 production, combined with higher rates of intracellular superoxide production in MMe cells than in mesothelial cells.

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Correspondence and requests for reprints should be addressed to Elia Ranzato, Ph.D., Department of Environment and Life Sciences, DiSAV, University of Piemonte Orientale Amedeo Avogadro, Viale T. Michel 11, 15121 Alessandria, Italy. E-mail: ranzato@mfn.unipmn.it

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MATERIALS AND METHODS

Reagents

Unless otherwise specified, all reagents were from Sigma (St. Louis, MO).

Cell Lines and Culture

Two MMe cell lines were used: REN cells, a p53 mutant epithelial subtype (28), and MM98 cells, established from pleural effusion of a sarcomatous mesothelioma (29). MMe cells were cultured in DMEM supplemented with 10% FBS (Euroclone, Pero, Italy) and 1% antibiotic mixture (Gibco, Invitrogen Life Technologies, S. Giuliano Milanese, Milan, Italy). The immortalized human mesothelial cell line (HMC-htert) was generously donated by Prof. G. Gaudino (Department of Chemical, Food, Pharmaceutical and Pharmacological Sciences, University of Piemonte Orientale, Novara, Italy) and was maintained in Medium 199 supplemented with 10% FBS, 200 nM L-glutamine, 3.3 nM epidermal growth factor, 400 nM hydrocortisone, and 870 nM insulin. Primary mesothelial cells were established by scraping the inner surface of the wall of surgically removed hernial sacs. Cells were grown in F10-Ham medium supplemented with 10% FBS and 1% antibiotic mixture (29).

Neutral Red Uptake Assay

The NRU assay is a cell viability test based on the incorporation of neutral red dye into the lysosomes of viable cells after incubation with test agents. Briefly, cells were seeded (20,000 cells/well) on 96-well plates, grown for 24 hours before the experiments, and then exposed to ascorbate at various concentrations for 24 hours or for 1 hour, followed by 23 hours of recovery. After removing the medium, a 0.05% solution of neutral red was added to each well, followed by incubation for 3 hours at 37°C. Cells were then washed with 1 × PBS, followed by the addition of a solution of 1% glacial acetic acid in 50% ethanol to fix the cells and extract the neutral red incorporated into lysosomes. Plates were shaken, and the absorbance was measured at 540 nm in a plate reader (Sirio S; SEAC, Florence, Italy). NRU tests were performed at least in duplicate, with a minimum of five replicates for each condition.

Detection of Apoptosis and Necrosis

Cell apoptosis and necrosis assays were performed on REN cells seeded in 12-well plates and exposed to increasing concentrations of ascorbate for 1 hour, followed by 6 hours of recovery.

Cells were examined for apoptosis by terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine, 5′-triphosphate (dUTP) nick-end labeling (TUNEL; Promega Corporation, Madison, WI) and counterstained with propidium iodide. Labeled cells were acquired with an Axiovert fluorescence microscope (Zeiss, Oberkochen, Germany), equipped with a high-resolution digital camera controlled by Axiovision software (Zeiss).

Caspase-3 activity was determined in cell lysates by using a colorimetric assay kit (CASP-3-C, Sigma), according to the manufacturer's instructions. The chromophore p-nitroaniline cleaved from labeled substrate Ac-DEVD-p-nitroaniline was detected at 405 nm in a plate reader.

Cell necrosis was detected by the lactate dehydrogenase assay performed on cell supernatants, according to the method of Legrand and colleagues (30).

Western Blotting

Amounts of 100 μg of protein were loaded on gel, subjected to SDS-PAGE (12% gel), and transferred to a nitrocellulose membrane, using a Bio-Rad Mini Trans Blot electrophoretic transfer unit (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked for nonspecific protein with 5% nonfat dry milk in PBS and then probed at room temperature for 1 hour, or at 4°C overnight, with a primary antibody against NADPH oxidase (NOX)–4 (ab60940; dilution, 1:500; AbCam, Cambridge, UK). Membranes were then washed three times (10 minutes per wash) with PBS supplemented with 0.05% Tween-20, to remove unbound antibodies, and further incubated with appropriate horseradish peroxidase–conjugated secondary antibodies (dilution, 1:1,000; Bethyl Laboratories, Montgomery, TX). Membranes were developed using an enhanced chemiluminescence kit (Millipore, Billerica, MA), according to the manufacturer's protocol, and digitized with Quantity One Image Software (ChemiDoc XRS; Bio-Rad). Equal loadings were confirmed with an anti-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Detection of Intracellular Reactive Oxygen Species Production

The fluorescent dye precursor dihydrorhodamine (DHR)-123 was used to estimate intracellular levels of ROS (31). DHR-123 is converted to fluorescent rhodamine 123 upon reaction with ROS. Cells were plated on fibronectin-coated, glass-base cell culture dishes (Iwaki Glass, Inc., Tokyo, Japan), allowed to settle overnight, and then loaded in the dark at room temperature for 30 minutes with DHR-123 (30 μM) in a loading buffer consisting of (in mM) 10 Hepes, 140 NaCl, 10 glucose, 1 $MgCl_2$, 2 $CaCl_2$, and 5 KCl, pH 7.4. After probe loading, cells were washed thoroughly with loading buffer and examined through confocal time-lapse analysis at 25°C, using a Zeiss LSM 510 confocal system interfaced with a Zeiss Axiovert 100M microscope (Carl Zeiss, Inc., Thornwood, NY). Excitation was obtained by the 488-nm line of an argon laser, and emission was collected using a 505–550 bandpass filter. The laser power was reduced to 15% to lower probe bleaching. Confocal imaging was performed at a resolution of 512 × 512 pixels at 256 intensity values, with a framing rate of 1 frame/60 seconds. Several cells were viewed together through a ×20 Plan-Neofluar Zeiss objective (0.5 NA). Increases in average fluorescence through time were recorded by confocal image analysis of single cells, and taken as a measure of ROS production rates.

H_2O_2, Catalase, and Superoxide Assay

The formation of H_2O_2 attributable to ascorbate was determined in tissue culture medium by the Colorimetric Hydrogen Peroxide kit (Assay Designs, Ann Arbor, MI). Cells were exposed for 1 hour to a range of ascorbic acid concentrations, and measurement was performed at 540 nm with the Sirio S plate reader, using a color reagent. The dye contains xylenol orange, which in acidic solution with sorbitol and ammonium iron sulfate reacts to produce a purple color proportional to the concentration of H_2O_2 in the sample.

For the assay of catalase (CAT), cells were cultured as already described until confluence, and then lysed in a buffer consisting of 200 mM Tris HCl, pH 7.4, 1 mM PMSF, and 1 μg/ml each of aprotinin, leupeptin, and pepstatin. CAT activity was analyzed in total cell lysates by following the consumption of H_2O_2, as described by Greenwald (32). The final reaction mixture contained 50 mM H_2O_2 in 50 mM phosphate buffer, pH 7.0. Absorbance was monitored at 240 nm (extinction coefficient of 40 M^{-1} cm^{-1}) in a Cary 50 spectrophotometer (Varian, Inc., Palo Alto, CA).

Superoxide generation in total cell lysates was determined from the rate of NADPH–dependent, superoxide dismutase (SOD)–inhibitable cytochrome c reduction (33). The reaction mixture consisted of 50 mM KH_2PO_4-K_2HPO_4 buffer, pH 7.6, 300 μM bovine-heart cytochrome c, and 100 μM EDTA. The reaction was started by adding 200 μg protein of lysate, followed by the addition of 100 μM NADPH and then of 200 U/ml SOD. Absorbance was monitored at 550 nm in a Cary 50 spectrophotometer. Absorbance at 550 nm (ΔA_{550}) values were converted to nmol cytochrome c reduced (mg protein)$^{-1}$ minute^{-1}, using a specific absorbance of 21.1 mM^{-1} cm^{-1} (34).

Inhibition of NOX4 by RNA Interference

REN cells were transfected or not with small interfering RNA (siRNA) oligonucleotides (5 μM), or with equimolar concentrations of a control scramble siRNA, by using the N-ter Nanoparticle siRNA Transfection System (Sigma-Aldrich, St. Louis, MO) according to the manufacturer's instructions. For anti-NOX4 RNA interference, we used a commercial (Sigma-Aldrich) siRNA specific to human NOX4. The sequences consisted of 5′-GAAUGAGUGCAAUUUCUAA-3′ (sense) and 5′-UCCCAUAUGAGUUCUG-3′ (antisense). For nonspecific siRNA (scramble), we used a commercially available nontargeting siRNA (MISSION siRNA Universal Negative Control; Sigma-Aldrich). Cells were harvested at 24, 48, and 72 hours after transfection and used for Western blot analysis and dose–response experiments, as already described.

Statistics

Data were analyzed using the Instat Software package (GraphPad Software, Inc., La Jolla, CA). Fifty percent effective concentrations (EC_{50}s) and their 95% confidence intervals (95% CIs) were determined by using a downhill logistic dose–response curve:

$$y = T/(1 + \exp(-S(\mathrm{Log}(A) - \mathrm{Log}(E))))$$

where T = top, S = Hill's slope, E = EC_{50}, and (A) = ascorbate concentration in mmol/L. Statistical comparisons between EC_{50} values were based on overlapping or nonoverlapping 95% CIs.

RESULTS

Ascorbate Cytotoxicity in Mesothelioma and Mesothelial Cells

The cytotoxicity of ascorbate was measured by the highly sensitive NRU assay in two MMe cell types (REN and MM98), in an immortalized human mesothelial cell line (HMC-htert), and in primary mesothelial cells. The toxicity of ascorbate was significantly higher in MMe than in normal mesothelial cells, as shown by the EC_{50} values obtained from dose–response curves (Table 1). Even more pronounced differences between MMe cell types and mesothelial cells were found by using 1 hour of exposure followed by 23 hours of recovery, a treatment that mimics clinical intravenous use (35). Under these conditions, the toxicity of ascorbate to MMe cells was only minimally reduced with respect to 24-hour treatments, whereas a marked decrease of toxicity (significantly higher EC_{50}s) was evident in mesothelial cells (Table 2). Such a result indicates that selective ascorbate toxicity depends on a rapid triggering mechanism, and therefore in subsequent NRU assays, we used 1-hour exposures and 23 hours of recovery.

The assay of lactic dehydrogenase release in the supernatant of REN cells exposed to ascorbate for 1 hour + 6 hours of recovery showed a dose-dependent increase of cell necrosis up to a dose of 10 mM ascorbate. Conversely, the TUNEL and caspase-3 assays consistently showed a maximum of apoptosis at 1 mM ascorbate, followed by a significant decrease (Figure 1). These data indicate that ascorbate induces cell death attributable to both necrosis and apoptosis at concentrations around the EC_{50} (1 mM), whereas at higher doses, necrosis tends to prevail.

In these NRU experiments, cells were exposed to ascorbate in their proper medium. However, HMC-htert and primary mesothelial cells were also exposed for 1 hour to ascorbate in DMEM, followed by 23 hours of recovery in Medium 199 and F10-Ham, respectively. These tests yielded EC_{50} values that were not significantly different from those derived by exposing these cells to ascorbate in their own medium (Table 2). We also checked the possible role of ascorbate redox status in the mechanism of ascorbate toxicity by using the oxidized analogue dehydroascorbate (DHA). The DHA showed almost no toxicity in REN cells, with an EC_{50} of 33 mM (95% CI, 31–36 mM).

TABLE 1. ASCORBATE TOXICITY IN MESOTHELIOMA AND NORMAL MESOTHELIAL CELLS EXTRAPOLATED ACCORDING TO NEUTRAL RED UPTAKE INDEX AFTER 24-HOUR EXPOSURES

Cells	Cell Type	Culture Medium	EC_{50}
REN	Tumor	DMEM	1.2 (1.1–1.3)
MM98	Tumor	DMEM	1.0 (0.8–1.1)
HMC-htert	Normal immortalized	M199	9.1 (8.8–9.3)
Mesothelial	Primary	F10-Ham	4.3 (2.5–7.6)

EC_{50} values and their 95% confidence intervals are expressed in mM.

Effect of Ascorbate Transport Inhibition on Ascorbate Toxicity

Two different mechanisms of cellular ascorbate transport have been described. First, the facilitated glucose transporter (GLUT-1) mediates the import of DHA, followed by DHA reduction to ascorbate (36). Mesothelioma tissue differs from normal mesothelium in exhibiting high expression levels of the GLUT-1 carrier (37). We tested ascorbate cytotoxicity in the NRU assay using phloretin, a GLUT-1 inhibitor (38) that was also reported to inhibit the sodium–ascorbic acid cotransporter (39). In the presence of 100 μM phloretin, the EC_{50} of ascorbate was 0.9 mM (95% CI, 0.01–2.5 mM) for REN, and 0.4 mM (95% CI, 0.2–0.6 mM) for MM98, showing that toxicity levels were not significantly different from those obtained using ascorbate alone (Table 2).

Second, ascorbate is transported by the sodium–ascorbic acid cotransporter (40), which can be inhibited by sodium transport inhibitors such as sodium azide and ouabain (41). In the presence of 0.5 mM sodium azide, the EC_{50} of ascorbate was 1.4 mM (95% CI, 1.0–1.8 mM) for REN, and 0.12 mM (95% CI, 0.10–0.13 mM) for MM98, i.e., slightly higher and lower, respectively, than those derived from ascorbate alone. In the presence of 1 mM ouabain, the EC_{50} of ascorbate in MM98 was 1.7 mM (95% CI, 1.6–1.8 mM). Taken together, these results unequivocally show that the cytotoxicity of ascorbate in MMe cells does not depend, or only minimally depends, on ascorbate uptake by cells.

Role of H_2O_2 in Ascorbate Toxicity

To verify if ascorbate cytotoxicity is attributable to the formation of H_2O_2 in the extracellular environment, we first evaluated the formation of the compound in DMEM medium in the presence or absence of REN cells, after 1 hour of incubation with different ascorbate concentrations. The data showed that in the presence of cells, ascorbate induced a dose-dependent increase of H_2O_2 production (Figure 2A). In contrast, in the absence of cells (medium only), ascorbate also caused the formation of H_2O_2, but such production was significantly lower

TABLE 2. ASCORBATE TOXICITY IN MESOTHELIOMA AND NORMAL MESOTHELIAL CELLS EXTRAPOLATED ACCORDING TO NEUTRAL RED UPTAKE INDEX AFTER 1 HOUR OF EXPOSURE, FOLLOWED BY 23 HOURS OF RECOVERY

Cells	Cell Type	Culture Medium	EC_{50} (95% Confidence Interval)
REN	Tumor	DMEM	0.81 (0.75–0.89)
MM98	Tumor	DMEM	0.6 (0.4–0.7)
HMC-htert	Normal immortalized	DMEM	32 (26–38)
HMC-htert	Normal immortalized	M199	22 (16–30)
Mesothelial	Primary	DMEM	10 (9.4–10.8)
Mesothelial	Primary	F10-Ham	9.0 (7.6–10.7)

EC_{50} values and their 95% confidence intervals are expressed in mM.

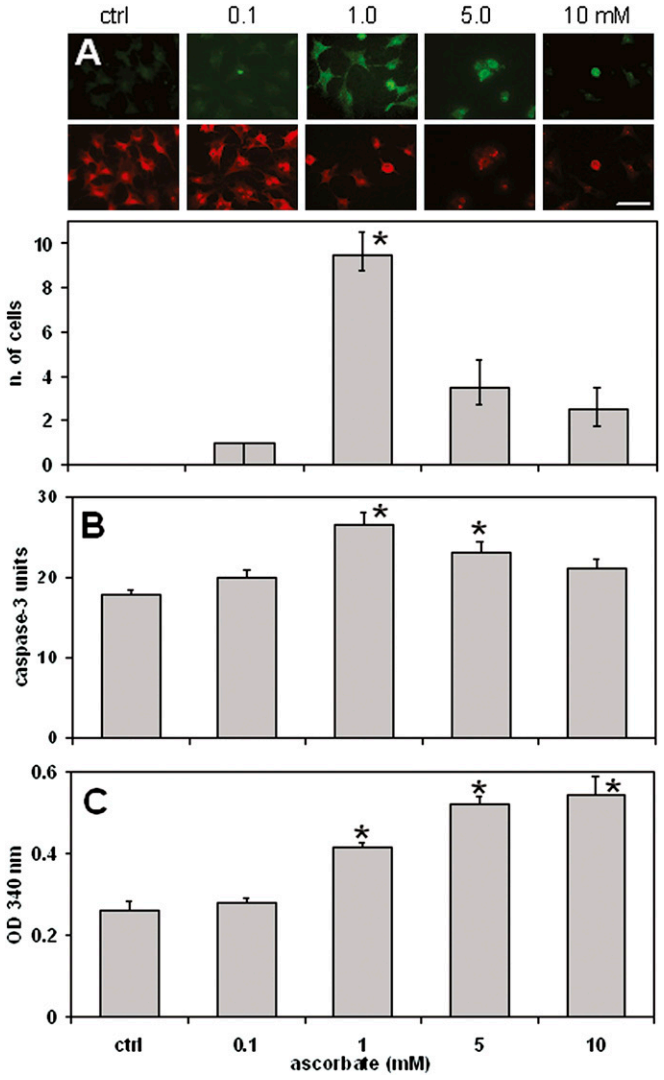

Figure 1. (*A*) Detection of apoptosis in REN cells exposed to increasing concentrations of ascorbate for 1 hour, followed by 23 hours of recovery. Cells were stained with the terminal deoxynucleotidyl transferase-mediated 2′-deoxyuridine, 5′-triphosphate nick-end labeling (TUNEL) technique (*upper series*), and counterstained with propidium iodide (*lower series*). *Bar* = 40 μm. Data plotted in the graph are median numbers of apoptotic cells ± interquartile range, observed in microscopic fields of 120 × 100 μm (n = 5–6). *$P < 0.01$, with respect to control cells according to Kruskal-Wallis statistic. (*B*) Caspase-3 activity detected in lysates of cells exposed to ascorbate, as described. Activity was determined by the amount of DEVD-p-nitroaniline converted to p-nitroaniline (*pNA*). Liberated pNA was determined colorimetrically by absorbance at 405 nm (see Materials and Methods). Data are means ± SD (n = 3) of caspase-3 units, expressed as (μmol pNa) (ml cell lysate)$^{-1}$. (*C*) Lactate dehydrogenase (*LDH*) release in the supernatant of REN cells after exposure to ascorbate, as described. Data are means ± SD (n = 3), expressed as absorbances at 340 nm (see Materials and Methods). *$P < 0.01$, with respect to control sample according to Dunnett test. OD, optical density.

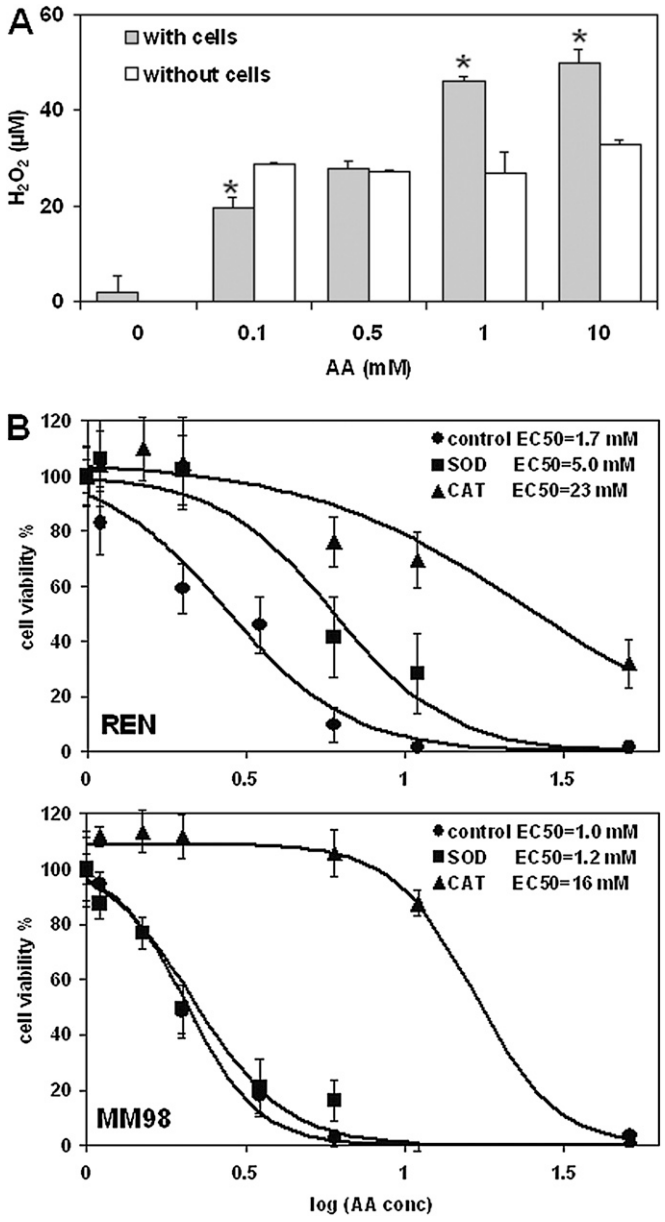

Figure 2. (*A*) Production of H_2O_2 in DMEM medium after 1 hour of incubation with increasing ascorbate concentrations, in the presence or absence of cells. Data are means ± SD (n = 3) of H_2O_2 concentrations, determined as described in Materials and Methods. *$P < 0.01$, in comparison with sample without cells at same ascorbate concentration. (*B*) Concentration–response curves were obtained by plotting the percent viability of REN and MM98 cells exposed to increasing concentrations of ascorbate for 1 hour, followed by 23 hours of recovery. Where indicated, ascorbate treatments were performed in the presence of superoxide dismutase (*SOD*) or catalase (*CAT*) at a concentration of 500 U/ml. Values are extrapolated by the neutral red uptake (*NRU*) viability index, and are expressed as means ± SD (n = 12 replicates from two different experiments). Regression lines were derived using a downhill logistic dose–response curve. Data are representative of all NRU experiments in this study.

than in the presence of cells at concentrations of 1.0 and 10 mM ascorbate (Figure 2A).

REN and MM98 cells were also preincubated at 37°C with the H_2O_2-degrading enzyme CAT (500 U/ml), and the effect of ascorbate was then evaluated by NRU. These experiments showed that CAT reduced the toxicity of ascorbate in both REN and MM98 cells, as shown by a more than 10-fold increase of EC_{50} (Figure 2B). Conversely, REN cells exposed to ascorbate in the presence of CAT (500 U/ml) inactivated by heating for 60 minutes at 60°C (42) showed no reduction in ascorbate toxicity (EC_{50}, 1.7 mM; 95% CI, 0.7–4.0 mM).

Preincubation of REN cells with SOD (500 U/ml) also reduced ascorbate toxicity, but to a significantly lower extent than did CAT (Figure 2B). In MM98 cells, SOD preincubation did not induce any significant variation in ascorbate toxicity with respect to ascorbate alone (Figure 2). These data indicate that ascorbate toxicity is mainly mediated by extracellular H_2O_2 production, and allow us to set a lower bound for CAT-inhibitable ascorbate toxicity, at an EC_{50} of $\sim$ 20 mM.

Given the role of extracellular H_2O_2 in ascorbate toxicity, we explored the causes of cell sensitivity to ascorbate by measuring intracellular CAT activity. These data demonstrated the highest levels of CAT in the immortalized, nonneoplastic HMC-htert cells (Figure 3), which could explain their low sensitivity to ascorbate, as evidenced by the highest EC_{50} values (Tables 1 and 2). However, primary mesothelial cells showed significantly lower levels of CAT with respect to MMe cell lines (Figure 3), whereas their resistance to ascorbate was higher (Tables 1 and 2). Taken together, our data raise an apparent paradox, because they indicate an essential role of H_2O_2 in ascorbate toxicity, but cells with lower CAT activity (primary mesothelial) are less sensitive to ascorbate than are cells with higher CAT activity (REN and MM98). Hence, our data suggest that the mechanism of action of ascorbate is not a simple effect of H_2O_2. Instead, some cell systems should be involved that are able to interact with H_2O_2, and that are differentially expressed in MMe.

Effect of Intracellular Ascorbate in Extracellular Ascorbate Toxicity

In the above experiments, we found similar results for both REN and MM98 cells. We therefore used REN cells for experiments aimed at elucidating the mechanism of ascorbate toxicity because of their higher *in vitro* growth rate.

In different studies, intracellular ascorbate was shown to either enhance or decrease the activity of various chemotherapeutic agents (43). We therefore tried to verify whether cell loading with ascorbate could interfere with extracellular ascorbate toxicity. REN, HMC-htert, and primary mesothelial cells were loaded overnight with DHA, which is known to be reduced intracellularly to ascorbate (44), by using a nontoxic concentration of 5 mM. After DHA preloading, cells were exposed to ascorbate and then analyzed according to the NRU assay. In REN cells, DHA preloading induced an increase of ascorbate EC_{50}, but it did not abolish CAT-inhibitable ascorbate toxicity, because the EC_{50} remained much lower than 20 mM (Table 3). In primary mesothelial cells, DHA completely abolished CAT-inhibitable ascorbate toxicity, whereas in HMC-htert cells, the EC_{50} of ascorbate was as high as 22 mM, both in the absence and presence of DHA preloading (Table 3). Hence, although DHA preincubation resulted in a general reduction of ascorbate toxicity, it did not abolish the selective toxicity of ascorbate in MMe cells.

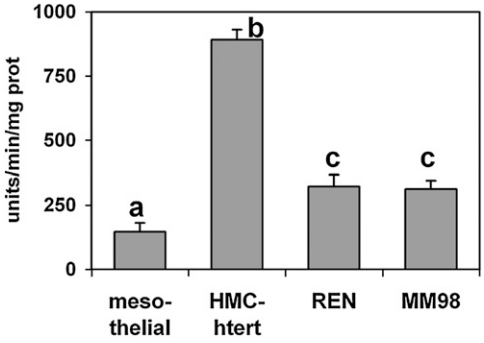

Figure 3. Evaluation of CAT activity in total lysates of primary mesothelial, HMC-htert, REN, and MM98 cells (see MATERIALS AND METHODS). Data are means ± SD (n = 5–9 different cell lysates) expressed as units of CAT minute^{-1} mg protein^{-1}. Letters *a–c* above bars indicate significant differences with respect to other groups, according to Tukey test ($P < 0.01$).

Intracellular ROS Production Induced by Ascorbate

We tested whether extracellular H_2O_2 production by ascorbate caused intracellular ROS production. Intracellular ROS production in cells exposed to ascorbate was evaluated in REN and primary mesothelial cells by loading them with the cell-permeant probe DHR-123, followed by monitoring rhodamine-123 fluorescence under a confocal microscope. The confocal imaging of REN cells revealed a rapid increase of the fluorescent signal after exposure to 1 or 5 mM ascorbate (Figure 4). In mesothelial cells, incubation with 1 or 5 mM ascorbate elicited almost no increase in fluorescence, whereas the subsequent bulk addition of H_2O_2 did elicit increased fluorescence, showing that the rhodamine-123 probe was still functional (Figure 4). Similar experiments showed that the intracellular ROS production induced by 1 mM ascorbate in REN cells was blocked by preincubation with 500 U/ml CAT, and that no ROS production was evident after exposing REN to 1 mM dehydroascorbate (Figure 5).

Spontaneous Cellular Superoxide Production

To explain the selectivity of ascorbate toxicity in MMe cells, we verified the rates of cellular superoxide production in mesothelial, MM98, and REN cells. Superoxide production was determined in total cell lysates by the rates of NADPH-dependent, SOD-sensitive cytochrome c reduction. Basal cytochrome c reduction rates were higher in REN cells than in mesothelial cells (Figure 6A). After the addition of NADPH, followed by the addition of SOD, the differences between NADPH-dependent and SOD-independent cytochrome c reduction rates were assessed as estimates of superoxide production rates. Based on these data, MMe cells produced significantly higher amounts of superoxide

TABLE 3. EFFECT OF DEHYDROASCORBATE PRELOADING ON TOXICITY INDUCED BY EXTRACELLULAR ASCORBATE

Cells	Cell Type	Dehydroascorbate Preloading, EC_{50} (95% Confidence Interval)	None, EC_{50} (95% Confidence Interval)
REN	Tumor	6.3 (5.4–7.4)	0.81 (0.75–0.89)
HMC-htert	Normal immortalized	22 (14–33)	22 (16–30)
mesothelial	Primary	22 (19–26)	9.0 (7.6–10.7)

Cells were preloaded (or not) overnight with 5 mM dehydroascorbate and then exposed to ascorbate for 1 hour, followed by 23 hours of recovery. Ascorbate EC_{50}s were extrapolated according to neutral red uptake index. EC_{50} values and their 95% confidence intervals are expressed in mM.

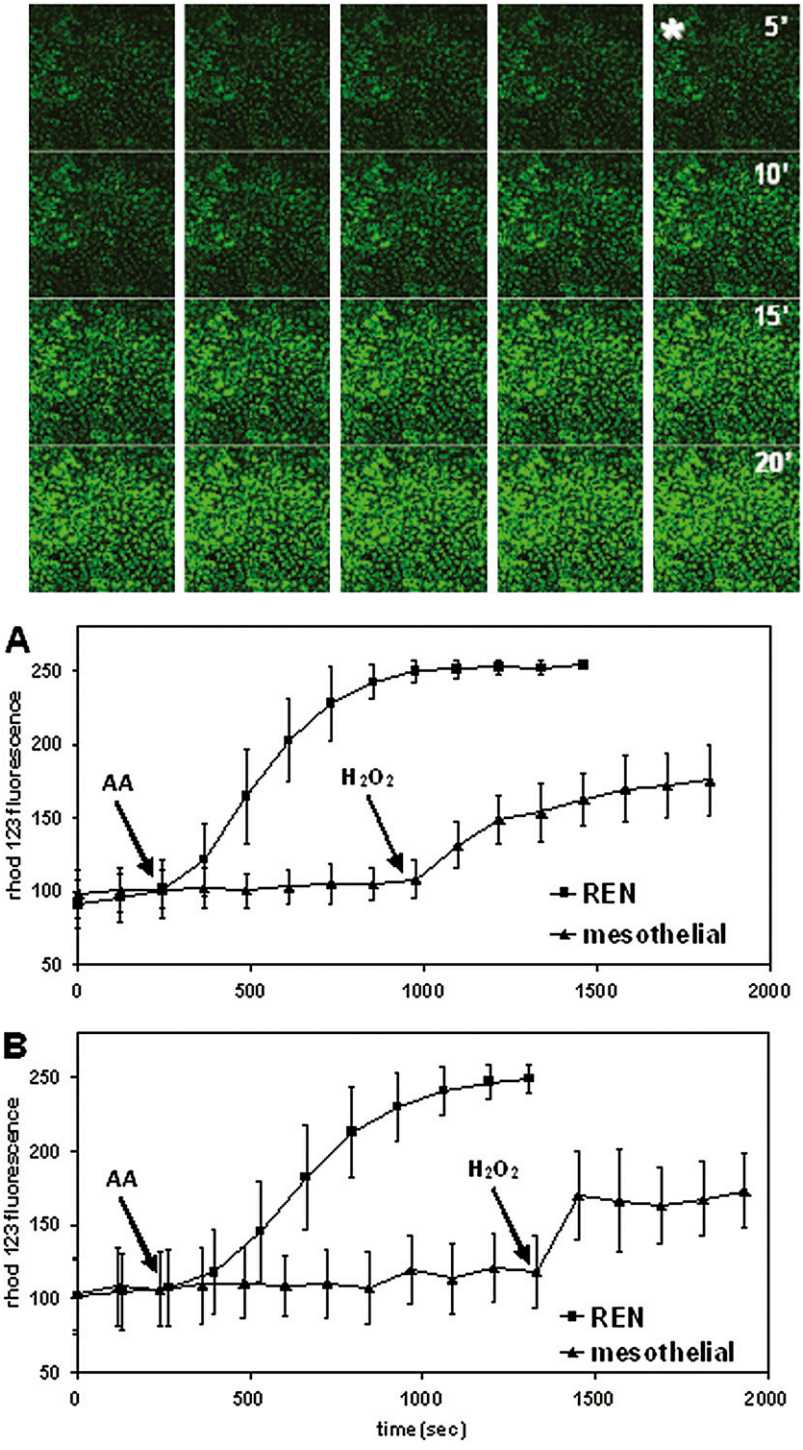

Figure 4. Reactive oxygen species (ROS) production detected in cells loaded with 30 μM dihydrorhodamine (DHR)-123. The probe is oxidized to rhodamine-123 (*rhod 123*), which is excitable at 488 nm and emits at 515 nm. *Top*: Time-lapse confocal images of REN cells (field size, 450 × 450 μm) acquired at 1-minute intervals. *Exposure to 1 mM ascorbate. *Below*: Time courses of ROS production in REN and primary mesothelial cells obtained from confocal imaging of rhodamine-123 fluorescent emission. *AA*, addition of 5 mM (*A*) or of 1 mM (*B*) ascorbate; H_2O_2, addition of 50 mM H_2O_2. Data are means ± SD of cell fluorescent emissions, expressed in arbitrary units (n = 30–60 cells in two different experiments).

with respect to non-neoplastic cells. The highest rates of production were found in REN cells (Figure 6B).

We also verified the expression of NOX in MMe and mesothelial cells, using Western blot analysis with an antibody against NOX4. NOX4 is a widespread member of the superoxide-generating NADPH oxidase enzymes (45), which was found to be upregulated in cancer cells (45). Western blot data showed a marked upregulation of NOX4 expression in both REN and MM98 cells with respect to mesothelial cells (Figure 6C). This result is quite consistent with the finding oh higher superoxide production in MMe cells.

Role of Cellular Superoxide in the Mechanism of Ascorbate Toxicity

Next, we evaluated the role of cellular superoxide in ascorbate toxicity by using different inhibitors of superoxide production in NRU experiments. Apocynin is a NOX inhibitor that prevents superoxide production, e.g., in white blood cells such as neutrophils (45). REN cells preincubated with 50 μM apocynin, washed, and exposed to ascorbate showed a complete abolition of CAT-inhibitable ascorbate toxicity, because an EC_{50} of around 20 mM was observed (Table 4).

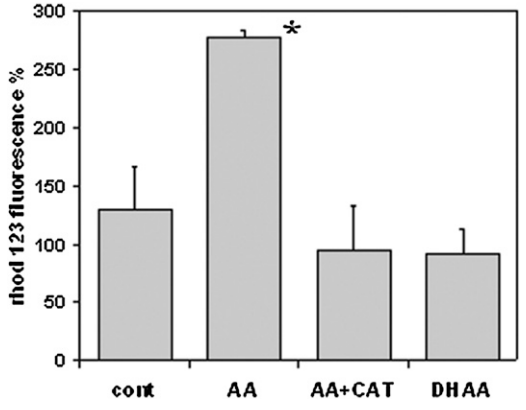

Figure 5. ROS production detected by confocal imaging of rhodamine-123 fluorescence in REN cells at 20 minutes after start of experiments. *cont*, untreated cells; *AA*, exposure to 1 mM ascorbate; *AA + CAT*, exposure to 1 mM ascorbate in the presence of 500 U/ml catalase; *DHAA*, exposure to 1 mM dehydroascorbate. Data were recorded as in Figure 2, and are means ± SD of percent cell fluorescence variations (n = 30–40) with respect to t = 0. *$P < 0.01$, according to Tukey test.

Another cellular source of superoxide is the electron transport chain of mitochondria. Preincubation with 1.0 μM rotenone, an inhibitor of mitochondrial complex I, was able to reduce the toxicity of ascorbate in REN cells, similarly to what we observed with apocynin (Table 4).

Superoxide production can also arise indirectly from the activity of NO synthase (NOS) (46). We therefore used the NOS inhibitor N(G)-nitro-L-arginine at a dose of 300 μM, but obtained a slight reduction in the toxicity of ascorbate (Table 4).

The role of intracellular superoxide production in ascorbate toxicity was also assessed through confocal ROS evaluation in REN cells. The incubation of DHR-123–loaded REN cells with 50 μM apocynin or 1 μM rotenone, followed by exposure to 5 mM ascorbate, resulted in a significant reduction of the increase in fluorescence with respect to ascorbate alone (Figure 7). These data indicate that inhibitors of superoxide production can prevent the burst of intracellular ROS caused by ascorbate in MMe cells. However, rotenone also lowered basal rhodamine fluorescence levels, whereas apocynin did not, possibly because of the reported ability of apocynin to elicit oxidative processes independent of superoxide production (47).

To achieve further evidence that cellular superoxide production is a determinant of ascorbate-induced toxicity, we studied the effects of ascorbate in REN cells after the siRNA-mediated knockdown of NOX4. Western blot analysis showed that cell transfection with NOX4 siRNA resulted in a sharply reduced detection of NOX4, whereas cells transfected with scramble siRNA showed the same expression level as control samples (Figure 8A). The cytotoxicity of ascorbate in cells undergoing RNA interference was measured by the NRU assay, to reveal a progressive shift of the EC_{50} toward higher levels in cells subjected to 24, 48, or 72 hours of siRNA, with respect to cells subjected to scramble siRNA (Figures 8B and 8C).

DISCUSSION

Our experiments showed for the first time, to the best of our knowledge, that ascorbate is strongly cytotoxic *in vitro* to different MMe cell lines. Conversely, and most interestingly, ascorbate resulted in significantly less toxicity in primary and immortalized mesothelial cells. Moreover, the finding of low ascorbate toxicity in mesothelial cells, using either their proper medium or DMEM,

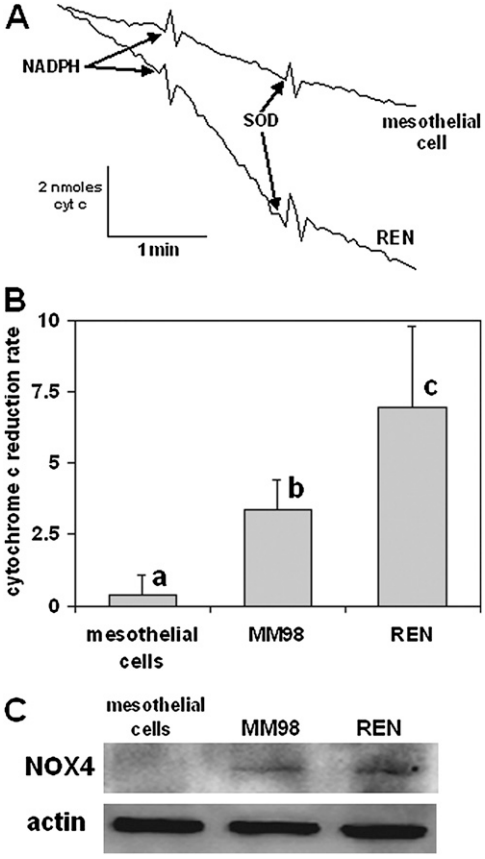

Figure 6. (*A*) Evaluation of superoxide production rates in lysates of REN and mesothelial cells, based on SOD-inhibitable, *NADPH*–dependent cytochrome c reduction. Representative traces of cytochrome c (*cyt c*) reduction were monitored at 550 nM. Data are expressed as nmol cytochrome c reduced minute^{-1} (see MATERIALS AND METHODS). (*B*) Superoxide production rates in lysates of REN, MM98, and mesothelial cells, obtained from differences between cytochrome c reduction rates after addition of 100 μM NADPH and 200 U/ml SOD. Data are means ± SD (n = 7), expressed as nmol cytochrome c reduced minute^{-1} (mg protein)$^{-1}$. Letters *a–c* above bars indicate significant differences with respect to other groups, according to Tukey test ($P < 0.01$). (*C*) NADPH oxidase (*NOX*)–4 expression in REN, MM98, and mesothelial cells visualized by Western blot analysis. Total cell lysates (100 μg protein) were separated on 12% SDS-PAGE, transferred to a nitrocellulose membrane, labeled with anti-NOX4, and then stripped and reprobed with anti–β-actin as loading control. Data are representative of three different experiments.

ruled out the possibility that the different ascorbate toxicities in mesothelioma and mesothelial cells depended on the use of different culture media, as reported by Young and Tsao for murine leukemia cells (48).

The finding that DHA is much less toxic than ascorbate in MMe cells, and the use of ascorbate transport inhibitors in cytotoxicity assay, consistently indicated that the toxic effect of ascorbate does not depend on cell ascorbate uptake, but is a consequence of extracellular effects, thus confirming previous findings (16). Furthermore, the use of CAT showed that ascorbate toxicity depends on extracellular H_2O_2 production. These data are consistent with current views, which assume that pharmacologic ascorbate concentrations would generate ascorbate radicals in the extracellular fluid, and that the electrons lost from ascorbate would reduce some organic-centered metal. This would lead, for example, to oxygen's reduction to superoxide, followed by extracellular H_2O_2 formation (16).

TABLE 4. ASCORBATE TOXICITY IN REN CELLS EXTRAPOLATED ACCORDING TO NEUTRAL RED UPTAKE INDEX IN THE PRESENCE OR ABSENCE OF INHIBITORS OF INTRACELLULAR SUPEROXIDE AND NITRIC OXIDE PRODUCTION

Treatment	EC_{50} (95% Confidence Interval)
None	0.81 (0.75–0.89)
Apocynin (50 μM)	18 (13–26)
Rotenone (1.0 μM)	15 (12–18)
N(G)-nitro-L-arginine (300 μM)	2.2 (1.8–2.9)

Cells were exposed to ascorbate for 1 hour, followed by 23 hours of recovery. EC_{50} values and their 95% confidence intervals are expressed in mM.

In our *in vitro* experiments, cell incubation with SOD exerted a significantly lower inhibition than did CAT in the cytotoxic effects of ascorbate. This indicates that superoxide does not play a direct role in the extracellular phase of ascorbate cytotoxicity, although a limited role of superoxide in the formation of extracellular H_2O_2 cannot be ruled out. Regardless of the mechanism of H_2O_2 production, our findings are in agreement with the interpretation that ascorbic acid administered intravenously in pharmacologic concentrations may serve as a prodrug for H_2O_2 delivery to the extracellular milieu.

The cellular consequences of the extracellular production of H_2O_2 were explored by confocal recordings of intracellular ROS production. Confocal data provided initial clues toward an understanding of the higher toxicity of ascorbate in MMe cells compared with normal mesothelial cells. The use of the DHR-123 probe indicated that pharmacologic doses of ascorbate induce a higher burst of ROS in REN than in HMC-htert cells. In these experiments, the kinetics of rhodamine fluorescence in REN cells showed that the effect of ascorbate is extremely rapid, and this finding correlates with cytotoxicity assays indicating that a 1-hour exposure to ascorbate followed by 23 hours of recovery is sufficient to induce approximately the same toxicity as a 24-hour exposure. Confocal data also showed that CAT can block the ascorbate-induced rise in rhodamine fluorescence, providing further confirmation of the role of H_2O_2 in ascorbate toxicity.

These data indicate that extracellularly produced H_2O_2 should produce an intracellular oxidative stress able to induce severe damage, ultimately leading to cell death. Such a mechanism is confirmed by the occurrence of both cell necrosis and apoptosis at lower ascorbate concentrations, and by the prevalence of necrosis at higher doses. However, this mechanism does not explain the selective ascorbate toxicity in MMe cells. Previous studies showed no correlation between ascorbate-induced cell death and glutathione, CAT, or glutathione peroxidase activity (16). Furthermore, ascorbate initiates the formation of H_2O_2 extracellularly, but the targets of H_2O_2 can be either extracellular or intracellular, because H_2O_2 is membrane-permeant.

In biological systems, H_2O_2 is known to react with superoxide through Fenton chemistry, giving rise to the highly reactive and injurious hydroxyl radical (49). Moreover, growing evidence indicates that cancer cells are subject to increased superoxide production compared with normal cells. The overexpression of various NOX isoforms and increased ROS generation was demonstrated in transformed human keratinocytes (50), prostate tumor cells (51), human colon cancer, and melanomas (51). Moreover, the suppression of ROS generation or treatment with NOX antisense RNA resulted in the inhibition of cell proliferation in melanoma and prostate cancer cells, suggesting a role for ROS in mitogenic signaling (51). This body of evidence indicates that the overexpression or dysregu-

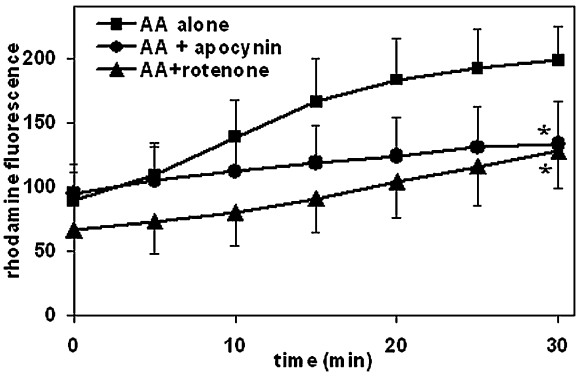

Figure 7. Time courses of ROS production in rhodamine-123–loaded REN cells, recorded by confocal imaging as in Figure 3. Cells were preincubated (or not) with 1.0 μM rotenone or 50 μM apocynin, and then exposed to 5 mM ascorbate at t = 0. Data are means ± SD (n = 45–60 cells in two different experiments). *$P < 0.01$, with respect to ascorbate alone, according to Tukey test.

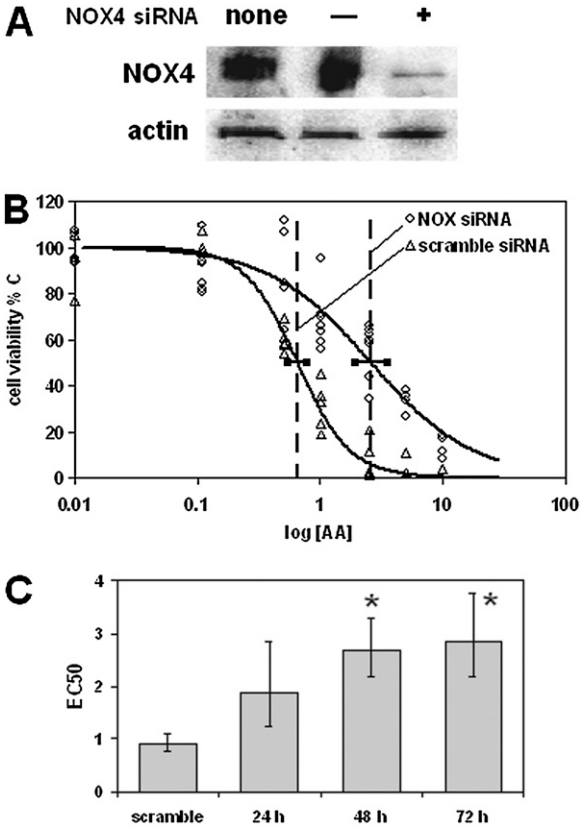

Figure 8. (*A*) RNA interference downregulates the expression of NOX4 in REN cells. REN cells were transfected or not (*none*) with 5 μM negative control small, interfering RNA (*siRNA*) (−) or specific NOX4 siRNA (+), with a 24-hour interval. Cell extracts were prepared after transfection and probed for NOX4 by Western blot analysis, as described in Figure 5. (*B*) Dose–response curves, derived from NRU endpoint, show a reduction of ascorbate toxicity in REN cells after 72 hours of NOX siRNA. The graph shows the experimental points, the regression lines obtained by fitting a downhill logistic dose–response curve, and for each curve, the position of the EC_{50} ± 95% confidence interval (*vertical dashed lines*). (*C*) Variation of the EC_{50} of ascorbate to REN cells, after 24, 48, or 72 hours of NOX siRNA, or 72 hours of scramble siRNA. Values of EC_{50} ± 95% confidence intervals were determined as already described. *$P < 0.05$, with respect to scramble siRNA.

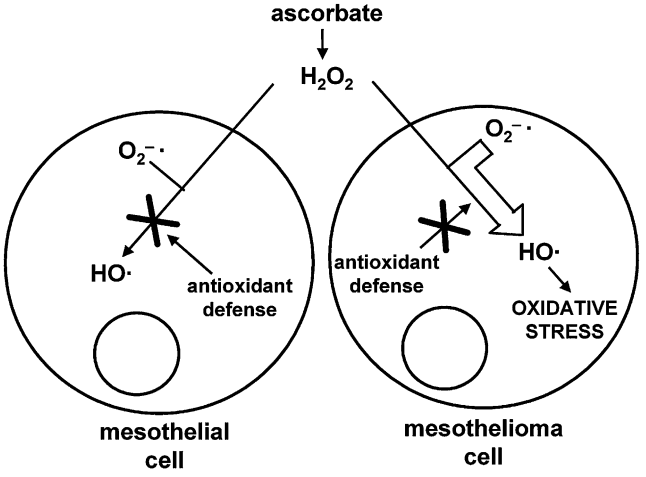

Figure 9. Diagram shows different outcomes of ascorbate-induced extracellular H_2O_2 production in normal mesothelial and malignant mesothelioma cells, attributable to different rates of superoxide production by NADPH oxidases or other cellular sources (see text for further explanation).

lation of superoxide-generating enzymes may play a role in the abnormal growth of cancer.

Hence, we hypothesized that a critical difference between MMe cells and their normal counterparts could result in higher rates of superoxide production in tumor cells. That assumption was confirmed by data showing higher superoxide production and NOX4 expression in MMe cells with respect to mesothelium. Further evidence came from the finding that inhibitors of the main sources of superoxide, i.e., apocynin and rotenone, abolished CAT-inhibitable ascorbate toxicity in REN cells. Consistent with these data, rotenone and apocynin were also able to reduce ascorbate-induced intracellular ROS production in REN cells. Finally, more specific evidence about the role of cellular superoxide derived from the finding that NOX4 siRNA was able to reduce significantly the toxicity of ascorbate in REN cells.

In conclusion, we showed that ascorbate promotes extracellular H_2O_2 production, which induces selective toxicity in MMe cells despite their high endogenous catalase activity. However, we also showed that MMe cells have high superoxide production rates, and that the inhibition of superoxide production systems results in lower ascorbate toxicity in these cells. Hence, our data allow us to explain the selective ascorbate toxicity in MMe cells by means of a redox Trojan mechanism. In normal cells, the H_2O_2 produced extracellularly by ascorbate would induce a limited oxidative stress, because of low cellular superoxide production. Conversely, in malignant cells, H_2O_2 would react with high amounts of superoxide, overwhelming cellular antioxidant defenses and eventually triggering hydroxyl radical production, causing strong oxidative stress (Figure 9). Such a view is in agreement with recent findings about the role of cell transition metals in ascorbate toxicity (35), because these metals are essential for the Fenton reaction at the basis of our mechanism of selective ascorbate toxicity. The elucidation of this mechanism provides a rationale for the use of ascorbate in the treatment of mesothelioma. Further animal studies are warranted, to assess the real potentialities of ascorbate, possibly combined with chemotherapeutic drugs, in the clinical treatment of malignant mesothelioma tumor masses.

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