Pharmacologic ascorbic acid concentrations selectively kill cancer cells: Action as a pro-drug to deliver hydrogen peroxide to tissues

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Human pharmacokinetics data indicate that i.v. ascorbic acid (ascorbate) in pharmacologic concentrations could have an unanticipated role in cancer treatment. Our goals here were to test whether ascorbate killed cancer cells selectively, and if so, to determine mechanisms, using clinically relevant conditions. Cell death in 10 cancer and 4 normal cell types was measured by using 1-h exposures. Normal cells were unaffected by 20 mM ascorbate, whereas 5 cancer lines had EC50 values of <4 mM, a concentration easily achievable i.v. Human lymphoma cells were studied in detail because of their sensitivity to ascorbate (EC50 of 0.5 mM) and suitability for addressing mechanisms. Extracellular but not intracellular ascorbate mediated cell death, which occurred by apoptosis and pyknosis/necrosis. Cell death was independent of metal chelators and absolutely dependent on H2O2 formation. Cell death from H2O2 added to cells was identical to that found when H2O2 was generated by ascorbate treatment. H2O2 generation was dependent on ascorbate concentration, incubation time, and the presence of 0.5–10% serum, and displayed a linear relationship with ascorbate radical formation. Although ascorbate addition to medium generated H2O2, ascorbate addition to blood generated no detectable H2O2 and only trace detectable ascorbate radical. Taken together, these data indicate that ascorbate at concentrations achieved only by i.v. administration may be a pro-drug for formation of H2O2, and that blood can be a delivery system of the pro-drug to tissues. These findings give plausibility to i.v. ascorbic acid in cancer treatment, and have unexpected implications for treatment of infections where H2O2 may be beneficial.

Abbreviations: MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PI, propidium iodide.

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assessment of apoptosis, necrosis, and pyknosis/necrosis by fluorescence microscopy as described in ref. 19. Briefly, 2.5 × 10^5 cells per ml were incubated with ascorbate or H_2O_2 for 1 h, washed with PBS, and suspended in fresh media. After 18–22 h, at least 200 cells were stained with Hoechst/PI and visualized under fluorescence microscopy.

MTT was used as a screening assay and performed as described in ref. 20. Cells in 96-well plates were treated with ascorbate (0.1–20 mM) for 1 h, washed, and incubated for an additional 24 h. The EC_{50} value was the concentration that reduced survival by 50%.

For colony formation on agar plates, cells were treated with 5 mM ascorbate for 1 h, washed, and plated. A two-layer agar system was used, and colonies were visualized after 10–14 days (21).

To determine the effects of red blood cells on ascorbate-induced cell death, red blood cells were prepared by centrifugation of heparinized human blood at 500 × g for 30 min. Human Burkitt’s lymphoma cells at 2.5 × 10^5 cells per ml were mixed with red blood cells, 25% or 50% hematocrit. Cell mixtures were treated with 2 mM ascorbate for 1 h. Lymphoma cells were recovered by using Vacutainer CPT tubes (Becton Dickinson) according to the manufacturer’s instructions. After washing, lymphoma cells were returned to fresh medium and assessed after 18 h by nuclear staining as above.

Quantitative Procedures. Catalase activity was determined by using Amplex Red (Molecular Probes) (22). Glutathione was detected by using 5,5’-dithio bis-2-nitrobenzoic acid, and glutathione peroxidase activity was measured by a coupled reaction with glutathione reductase (Cayman Chemical, Ann Arbor, MI), according to the manufacturer’s instructions.

Ascorbate radical in culture media and blood was detected by using electron paramagnetic resonance (23, 24). Spectrometer (E9 series, Varian) settings were as follows: microwave power, 20 mW; modulation amplitude, 1.0 G; time constant, 0.25 s; scan range, 4 × 10 G; and scan time, 4 min. Radical quantitation was performed by using 3-carboxyproxyl as a standard (23).

Because ascorbate interferes with most peroxidase-based detection methods, H_2O_2 was measured by using a Clark-type oxygen electrode (5/6 Oxigraph, Gilson Medical Electronics, Middleton, WI). Oxygen evolution was measured upon introduction of catalase: 2H_2O_2 → 2H_2O + O_2. Calibration was performed with freshly prepared solutions of H_2O_2 (10–200 μM) (25).

Ascorbate was measured by HPLC with coulometric electrochemical detection (26). Protein was determined by using biocinchonic acid (27). Cell volumes were determined by using a Coulter Multisizer II cell counter. Intracellular ascorbate concentrations were calculated by converting cell protein to a measured intracellular volume (18).

Results

Effects of Ascorbic Acid in Pharmacologic Concentrations on Survival of Tumor and Normal Cells. We first investigated whether ascorbate in pharmacologic concentrations selectively affected the survival of cancer cells by studying nine cancer cell lines, four normal cell types, and clinically relevant conditions. Clinical pharmacokinetics analyses show that pharmacologic concentrations of plasma ascorbate, from 0.3 to 15 mM, are achievable only from i.v. administration (7). These concentrations are cleared within hours by renal filtration and excretion. In contrast, plasma ascorbate concentrations from maximum possible oral doses cannot exceed 0.22 mM because of limited intestinal absorption, which is bypassed with i.v. administration (7–9). To mimic potential clinical i.v. use, tested cells were incubated for 1 h with either pharmacologic ascorbate concentrations (0.3–20 mM) or a high physiologic concentration (0.1 mM) as control. Once ascorbate was removed, cell survival was determined by nuclear staining or MTT after 24 h (Fig. 1A). For five of the nine cancer cell lines, ascorbate concentrations causing a 50% decrease in cell survival (EC_{50} values) were less than 5 mM, a concentration easily achievable from i.v. infusion (7). All tested normal cells were insensitive to 20 mM ascorbate.

Colonies formation assays were used as an additional means to determine cell survival (21). Four cancer cell lines were incubated with 5 mM ascorbate or untreated media for 1 h. Cells were diluted and plated and growth assessed after 14 days (Fig. 1B). All four untreated cell lines grew in soft agar, whereas three of four exposed to ascorbate displayed at least 99% growth inhibition.

Effects of Ascorbic Acid on Death of Human Lymphoma Cells. Human lymphoma cells (JLP-119) were studied in detail to determine the effects of ascorbate on cell death. Lymphoma cells were selected because of their sensitivity to ascorbate (Fig. 1A), the suitability of these cells for nuclear staining to characterize the mode of cell death (16, 19, 28), and the report of a positive clinical response of lymphoma to i.v. ascorbate (14) (unpublished work). Cells were incubated for 1 h with 0.1–5 mM ascorbate and washed, and Hoechst/PI nuclear staining was performed 18 h later to determine the amount and type of cell death (Fig. 2A). Ascorbate induced concentration-dependent cell death, which was nearly 100% at 2 mM. As ascorbate concentration increased, the pattern of death changed from apoptosis to pyknosis/necrosis, a pattern suggestive of H_2O_2-mediated cell death (19). We determined the time necessary for cell death after exposure to 2 mM ascorbate for 1 h (Fig. 2B). Apoptosis occurred by 6 h after exposure, and cell death by pyknosis was ~90% at 14 h after exposure. In contrast to lymphoma cells, there was little or no killing of normal lymphocytes and monocytes by ascorbate (Fig. 2C).

The roles of intracellular versus extracellular ascorbate in causing cell death were examined, using ascorbate and its oxidized product dehydroascorbic acid. Ascorbate is transported into cells as such by sodium-dependent transporters, whereas dehydroascorbic acid is transported into cells by glucose transporters and then immediately reduced internally to ascorbate (29). By using either external ascorbate or external dehydroascorbic acid, lymphoma cells were loaded to equal internal concentrations of ascorbate over 1 h (data
Similar to most cultured cells, lymphoma cells contain no ascorbate unless the vitamin is added to the extracellular medium (data not shown) (17). In contrast, excepting red blood cells, all cells in vivo or acutely isolated contain ascorbate, usually in millimolar concentrations. We investigated whether the prior presence of intracellular ascorbate affected death mediated by extracellular ascorbate. Lymphoma cells were preloaded with physiologic concentrations of ascorbate to produce millimolar intracellular concentrations, similar to normal lymphocytes (8, 9). Their response to external ascorbate was compared with unloaded cells (Fig. 2E). Whether or not intracellular ascorbate was preloaded, extracellular ascorbate induced the same amount and type of death. Taken together, the data in Fig. 2A–E indicate that extracellular ascorbate in pharmacologic concentrations mediates death of lymphoma cells by apoptosis and pyknosis/necrosis, independently of intracellular ascorbate.

Mechanism of Ascorbate-Mediated Cell Killing. To determine the mechanism of ascorbate-mediated lymphoma cell death, we tested the effects of the membrane-impermeant H$_2$O$_2$-scavenger catalase, the membrane-permeant H$_2$O$_2$-scavenger tetrakis (4-benzoic acid) meso-substituted manganoporphyrin (MnTBAP) (30), and the thiol-reducing agent Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) (31). We also tested whether adventitious transition metal ions were responsible, by using the membrane impermeant chelator diethylenetriamine-pentaacetic acid (DTPA) (32) and the membrane permeant chelator N,N′-bis(2-hydroxybenzyl)ethylenediamine-N,N′-diacetic acid (HBED) (33–35) (Fig. 3A,E). The H$_2$O$_2$ scavengers were completely protective, identifying H$_2$O$_2$ as the effector species mediating pharmacologic ascorbate-induced cell death. The effect of ascorbate was not due to chelatable, trace redox-active metals, because the two chelators had no effect on preventing death. Superoxide dismutase was not protective (data not shown), consistent with its action in producing but not degrading H$_2$O$_2$ (36).

Because these data implicated H$_2$O$_2$ in cell killing, we added H$_2$O$_2$ to lymphoma cells and studied death patterns using nuclear staining (19, 28). The death patterns found with exogenous H$_2$O$_2$ exposure were similar to those found with ascorbate. For both ascorbate and H$_2$O$_2$, death changed from apoptosis to pyknosis as concentrations increased (Fig. 3B). As a specific test of ascorbate action, the amount of H$_2$O$_2$ formed in the presence of ascorbate was measured by using an oxygen electrode. We compared the effects on cell death of H$_2$O$_2$ amounts formed in the presence of ascorbate to effects from exogenously added H$_2$O$_2$. H$_2$O$_2$ generated by ascorbate oxidation and exogenously added H$_2$O$_2$ produced cell death curves that were indistinguishable (Fig. 3C).

Sensitivity to direct exposure to H$_2$O$_2$ was greater in lymphoma cells compared with normal lymphocytes and normal monocytes (Fig. 3D), consistent with the cytotoxicity pattern found above with pharmacologic ascorbate exposure. Taken together, these data are consistent with the conclusion that extracellular ascorbate induced cell death by formation of H$_2$O$_2$.

We investigated whether activities of intracellular H$_2$O$_2$-removal systems correlated with ascorbate-mediated cell death, for all cells studied. There was no association between the EC$_{50}$ for ascorbate-mediated cell death and intracellular glutathione concentrations, catalase activity, or glutathione peroxidase activity (data not shown).

Mediators and Inhibitors of H$_2$O$_2$ Generation. H$_2$O$_2$ concentrations generated by ascorbate were similar with tumor cells, normal cells, or in medium without cells (data not shown), as measured by using an oxygen electrode as above. H$_2$O$_2$ generation was dependent on time, ascorbate concentration, and the presence of trace amounts of serum in media (Fig. 4A and B).

Based on these data, the most cogent explanation of ascorbate

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Fig. 2. Effects of ascorbic acid on human Burkitt's lymphoma cells. Cells were treated for 1 h, washed, and recultured without ascorbate. Amounts and types of cell death were determined 18–22 h later by nuclear staining with Hoechst/PI. Types of cell death: necrosis (black), pyknosis/necrosis (gray), early apoptosis (blue), and late apoptosis (red). (A) Amount and type of cell death as a function of external ascorbate concentration. (B) Time course and type of cell death after 1 h external ascorbate (2 mM). (C) Cell death as a function of external ascorbate concentration in human Burkitt's lymphoma cells (■), normal lymphocytes (■), and normal monocytes (■). (D) Cell death as a function of external ascorbate (■) or dehydroascorbic acid (■) concentrations (1-h incubation). (E) Type and amount of cell death with 2 mM ascorbate treatment, in cells previously loaded to contain 3 mM ascorbate (right), compared with unloaded cells (left).

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not shown). Despite similar intracellular ascorbate concentrations under both conditions, cells died only when ascorbate was present externally (Fig. 2D).
action in forming H$_2$O$_2$ is that the first step is ascorbate oxidation to its radical. We measured H$_2$O$_2$ concentration as a function of ascorbate radical concentration and found a linear relationship (Fig. 4C). These data imply that ascorbate radical is a surrogate marker for H$_2$O$_2$ formation.

For ascorbate to be useful clinically, it should increase the steady-state concentration of H$_2$O$_2$ in the extracellular milieu but not in blood. We predicted that steady-state concentrations of H$_2$O$_2$ generated by ascorbate oxidation would be undetectable in blood for several reasons. First, if any ascorbate radical is generated in blood, only very low concentrations are expected, and such concentrations should be lower than that needed to form detectable steady-state concentrations of H$_2$O$_2$ (37). Second, whatever H$_2$O$_2$ is generated should be removed by glutathione peroxidase and catalase within red blood cells, because H$_2$O$_2$ is membrane permeable (38–41). These predictions were explored in the following experiments. First, ascorbate (0–10 mM) was added to whole blood and to medium, and ascorbate radical was measured by electron paramagnetic resonance. Ascorbate radical in whole blood was not detectable when ascorbate concentrations were <3 mM and was present at minimal concentrations thereafter. In contrast, there was robust ascorbate radical generation in medium, a surrogate for extracellular fluid (Fig. 5A). Second, as direct tests, H$_2$O$_2$ concentrations were measured under the following conditions: In whole blood in the presence of varying concentrations of ascorbate, in whole blood after exogenous H$_2$O$_2$ addition, and in medium with varying concentrations of ascorbate (Fig. 5B). H$_2$O$_2$ was not detected in whole blood under either condition, even in the presence of far higher added concentrations than could be gener-

Fig. 3. Extracellular ascorbate kills human Burkitt’s lymphoma cells by generating H$_2$O$_2$. Cell death determined and symbolized as in Fig. 2; H$_2$O$_2$ measured by oxygen electrode (see Materials and Methods). (A) Effects of reactive oxygen species quenchers/scavengers, reducing agent, and metal chelators on ascorbate-mediated cell death. The following (final concentrations) were preincubated with cells for 30 min before exposure to ascorbate (2 mM): catalase (100 μg/ml); tetrakis (4-benzoic acid) meso-substituted manganoporphyrin (MnTBAP) (100 μM); Tris (2-carboxyethyl) phosphine hydrochloride (TCEP) (500 μM); diethyleneetriamine-pentaacetic acid (DTPA) (1 mM); and N,N’-bis(2-hydroxybenzyl)ethylenediamine-N,N’-diacetic acid (HBED) (50 μM). (B) Type and amount of cell death as function of added H$_2$O$_2$ (final concentrations). (C) Cell death as a function of added H$_2$O$_2$ for 1 h (●) or mean H$_2$O$_2$ concentration generated by 0.2–2 mM ascorbate during a 1-h incubation (●). (D) Cell death in human Burkitt’s lymphoma cells (●), normal lymphocytes (■), and normal monocytes (●) as function of added H$_2$O$_2$ (final concentrations).
administration and conditions that reflect potential clinical use. The effect was due only to extracellular and not intracellular ascorbate, consistent with clinical i.v. dosing. Ascorbate-mediated cell death was due to protein-dependent extracellular \( \text{H}_2\text{O}_2 \) generation, via ascorbate radical formation from ascorbate in the electron donor. Like glucose, when ascorbate is infused i.v., the resulting pharmacologic concentrations should distribute rapidly in the extracellular water space (42). We showed that such pharmacologic ascorbate concentrations in media, as a surrogate for extracellular fluid, generated ascorbate radical and \( \text{H}_2\text{O}_2 \). In contrast, the same pharmacologic ascorbate concentrations in whole blood generated little detectable ascorbate radical and no detectable \( \text{H}_2\text{O}_2 \). These findings can be accounted for by efficient and redundant \( \text{H}_2\text{O}_2 \) catabolic pathways in whole blood (e.g., catalase and glutathione peroxidase) relative to those in media or extracellular fluid (38–41). The totality of the data are consistent with the interpretation that ascorbic acid administered i.v. in pharmacologic concentrations may serve as a pro-drug for \( \text{H}_2\text{O}_2 \) delivery to the extracellular milieu, but without \( \text{H}_2\text{O}_2 \) accumulation in blood.

Although it is possible that \( \text{H}_2\text{O}_2 \) might accumulate in blood, this would occur only under specific conditions that reflect on the general safety of i.v. ascorbate. Ascorbate administered i.v. is likely to be safe in most patients, with virtually no toxicity compared to most currently available cancer chemotherapeutic agents. The occurrence of one predicted complication, oxalate kidney stones, is controversial (13). In patients with glucose-6-phosphate dehydrogenase deficiency, i.v. ascorbate is contraindicated because it causes intravascular hemolysis (13). The mechanism of this previously unexplained observation is now straightforward, based on the results here. \( \text{H}_2\text{O}_2 \) generated in blood is normally removed by catalase and glutathione peroxidase within red blood cells, with internal glutathione providing reducing equivalents. The electron source for glutathione is NADPH from the pentose shunt, via glucose-6-phosphate dehydrogenase. If activity of this enzyme is diminished, the predicted outcome is impaired \( \text{H}_2\text{O}_2 \) removal causing intravascular hemolysis, the observed clinical finding.

Ascorbate as a potential cancer therapeutic agent has a controversial and emotionally charged past (1, 3–6). Clinical observational studies reported possible benefit in selected patients, but double-blind placebo-controlled studies reported no benefit, and ascorbate was discarded as a potential therapy by conventional practitioners. Only recently has it been understood that the discordant clinical findings can be explained by previously unrecognized fundamental pharmacokinetics properties of ascorbate (7). In vitro effects of ascorbate on death and survival of cell lines have been reported, but there are multiple experimental concerns. For example, reports compared an experimental condition to that with no ascorbate at all (43, 44), but such a condition has had unclear physiologic relevance, because ascorbate outside and inside cells is always present unless there is severe scurvy. It was unclear whether observed effects were due to extracellular or intracellular ascorbate, or both (12, 43–46). Some experiments have used widely varying incubation times and ascorbate concentrations that have had no corresponding clinical context, making interpretation difficult. \( \text{H}_2\text{O}_2 \) generation by ascorbate oxidation in culture media was variously interpreted as artifact (47, 48), even though chelators had no effect (49), or reported to mediate damage internally due to diminished intracellular ascorbate, but using an \( \text{H}_2\text{O}_2 \) assay in which ascorbate could interfere (43, 44).

The experiments presented here provide a clear clinical context for ascorbate action. Conditions were selected to reflect peak ranges of i.v. ascorbate concentrations, which clinically might last a few hours at most, depending on the infusion rate (7). Intracellular transport of ascorbate is tightly controlled in relation to extracellular concentration (8, 9, 29). Intravenous ascorbate infusion is expected to drastically change extracellular but not intracellular concentrations (8, 9). For i.v. ascorbate to be clinically useful in killing cancer cells, pharmacologic but not physiologic extracellular

Discussion
Our data show that ascorbic acid selectively killed cancer but not normal cells, using concentrations that could only be achieved by i.v.

Fig. 5. Human blood inhibits \( \text{H}_2\text{O}_2 \) and ascorbate radical generation from ascorbate. Ascorbate radical was measured by electron paramagnetic resonance. \( \text{H}_2\text{O}_2 \) was measured by oxygen electrode, and cell death was measured and displayed as in Fig. 2. (A) Ascorbate radical formation as function of ascorbate concentrations added to blood (●) or medium (▲). (B) \( \text{H}_2\text{O}_2 \) generated by ascorbate concentrations added to blood (●) or medium (▲) (1-h incubation), and \( \text{H}_2\text{O}_2 \) measured in blood immediately after the addition of indicated concentrations (●). (C) Human Burkitt’s lymphoma cell death in the presence or absence of red blood cells (RBC) at 25% or 50% hematocrit (HCT) (2 mM ascorbate, 1-h treatment).
lipids, forming hydroperoxides or reactive intermediates that are (38, 52). For example, extracellular H2O2 might target membrane H2O2 formation extracellularly, but H2O2 targets could be either peroxidase activity. The data here showed that ascorbate initiated given that extracellular milieu protein is as much as 20% of serum

It is unknown why ascorbate, via H2O2, killed some cancer cells but not normal cells. There was no correlation with ascorbate-induced cell death and glutathione, catalase activity, or glutathione peroxidase activity. The data here showed that ascorbate initiated H2O2 formation extracellularly, but H2O2 targets could be either intracellular or extracellular, because H2O2 is membrane permeant (38, 52). For example, extracellular H2O2 might target membrane lipids, forming hydroperoxides or reactive intermediates that are quenched or repaired in normal cells but not in sensitive cancer cells. In sensitive but not resistant cancer cells, intracellular H2O2 could target DNA, DNA repair proteins, or mitochondria because of diminished superoxide dismutase activity (53). New insights may follow from future studies of a very broad range of tumor cells or from microarray analysis of resistant and sensitive cells derived from the same genetic lineage.

H2O2 as the product of pharmacologic ascorbate concentrations, has potential therapeutic uses in addition to cancer treatment, especially in infections. H2O2 is a potent mammalian antimicrobial defense mechanism (54). Neutrophils generate H2O2 from superoxide, in turn formed by NADPH oxidase-catalyzed reduction of molecular oxygen. There may be particular therapeutic application in patients with chronic granulomatous disease who have diminished superoxide production (55). Old observational animal experiments, although uncontrolled, suggest that i.v. ascorbate is effective in some viral infections (56, 57). This finding is also consistent with in vitro experiments, in which H2O2 is toxic to hepatitis C (58). Use of ascorbate as an H2O2-delivery system against sensitive pathogens, viral or bacterial, has substantial clinical implications that deserve rapid exploration.

To proceed clinically with potential treatment of infectious diseases and cancer, clear safety documentation of i.v. ascorbate administration is necessary. More than 100 patients have been described, presumably without glucose-6-phosphate dehydrogenase deficiency, who received 10 g or more of i.v. ascorbate with no reported adverse effects other than tumor lysis (3, 4, 15, 59). However, these descriptions lack formal safety documentation. Complementary and alternative medicine practitioners worldwide currently use ascorbate i.v. in doses as high as 70 g over several hours (14, 15, 59). Because i.v. ascorbate is easily available to people who seek it, a phase I safety trial in patients with advanced cancer is justified and underway.

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