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Ascorbate (ascorbic acid, vitamin C), in pharmacologic concentrations easily achieved in humans by i.v. administration, selectively kills some cancer cells but not normal cells. We proposed that pharmacologic ascorbate is a prodrug for preferential steady-state formation of ascorbate radical ($Asc^{\bullet -}$) and H_2O_2 in the extracellular space compared with blood. Here we test this hypothesis *in vivo*. Rats were administered parenteral (i.v. or i.p.) or oral ascorbate in typical human pharmacologic doses (≈0.25–0.5 mg per gram of body weight). After i.v. injection, ascorbate baseline concentrations of 50–100 μM in blood and extracellular fluid increased to peaks of >8 mM. After i.p. injection, peaks approached 3 mM in both fluids. By gavage, the same doses produced ascorbate concentrations of <150 μM in both fluids. In blood, $Asc^{\bullet -}$ concentrations measured by EPR were undetectable with oral administration and always <50 nM with parenteral administration, even when corresponding ascorbate concentrations were >8 mM. After parenteral dosing, $Asc^{\bullet -}$ concentrations in extracellular fluid were 4- to 12-fold higher than those in blood, were as high as 250 nM, and were a function of ascorbate concentrations. By using the synthesized probe peroxyxanthone, H_2O_2 in extracellular fluid was detected only after parenteral administration of ascorbate and when $Asc^{\bullet -}$ concentrations in extracellular fluid exceeded 100 nM. The data show that pharmacologic ascorbate is a prodrug for preferential steady-state formation of $Asc^{\bullet -}$ and H_2O_2 in the extracellular space but not blood. These data provide a foundation for pursuing pharmacologic ascorbate as a prooxidant therapeutic agent in cancer and infections.

ascorbic acid | cancer | vitamin C | pharmacokinetics

Ascorbic acid (ascorbate, vitamin C) has a controversial history in cancer treatment (1). Observational studies, initiated by Cameron and Campbell (2) and expanded in collaboration with Pauling (3, 4), suggested that ascorbate doses of 10 g daily prolonged survival. These studies had multiple uncertainties, including use of retrospective controls, lack of independent pathologic confirmation, and no blinding or placebo use (1, 5–7). In two double-blind, placebo-controlled trials, investigators at the Mayo Clinic found that 10 g of ascorbate had no effect on cancer survival (5, 6). Ascorbate was dismissed as a therapeutic agent in cancer treatment (7), but its use continues by practitioners of complementary and alternative medicine (8–10).

Emerging evidence indicates that ascorbate in cancer treatment deserves reexamination. Pharmacokinetics studies in healthy men and women show that ascorbate concentrations in plasma and tissue are tightly controlled as a function of oral dose (11–13). Intravenous injection of ascorbate bypasses tight control and produces plasma concentrations as much as 70-fold greater than those produced by maximal oral dosing (13). These data demonstrate that oral and i.v. ascorbate administration are not comparable. Surprisingly, it was unrecognized for years that the observational Cameron studies used both oral and i.v. administration, but the Mayo Clinic studies used oral dosing only. Thus, these outcome studies are also not comparable (1, 8, 13). New *in vitro* studies using ascorbate at pharmacologic concentrations only achievable by i.v. administration show that ascorbate is selectively toxic for some cancer but not normal cells (14). Clinical case reports also suggest that i.v. ascorbate might have a role in treating some cancers and that further investigation is warranted (9, 10).

Although many hypotheses could be tested to explain ascorbate action on cells, it is an essential prerequisite to investigate whether reaction products obtained from ascorbate *in vitro* are found *in vivo*. *In vitro*, pharmacologic ascorbate concentrations mediated selective cancer cell toxicity via formation of $Asc^{\bullet -}$ and H_2O_2 in cell culture media, with minimal $Asc^{\bullet -}$ and no H_2O_2 detectable in blood (14). H_2O_2 concentrations ≥25 μM *in vitro* were toxic to cancer cells (14). Based on these data, we propose *in vivo* (Fig. 1) that pharmacologic ascorbate concentrations selectively generate $Asc^{\bullet -}$ in extracellular fluid but not in blood. The electron lost from ascorbate would reduce a protein-centered metal, selectively driving H_2O_2 formation in extracellular fluid. In contrast, in blood pharmacologic ascorbate concentrations would produce low $Asc^{\bullet -}$ concentrations compared with extracellular fluid, whereas any H_2O_2 formed in blood would be immediately destroyed (14–18). Based on the proposed reactions, if the predicted products are formed *in vivo*, then many next steps are justified, including determining molecular mechanisms of ascorbate action, isolation of proteins that mediate H_2O_2 formation, full characterization of ascorbate's preferential action on malignant but not normal cells, and animal and clinical trials. If the predicted reaction products are not formed *in vivo*, then a potential role of ascorbate in cancer treatment would require an entirely new explanation or may have to be discarded.

Here, we tested *in vivo* the hypothesis that ascorbate is a prodrug for selective delivery of $Asc^{\bullet -}$ and H_2O_2 to the extracellular space. Ascorbate in rats was administered parenterally (by i.v. or i.p. injection) or by oral gavage, and extracellular fluid was obtained by microdialysis. Ascorbate and $Asc^{\bullet -}$ were measured in blood and extracellular fluid by using HPLC with coulometric electrochemical detection or EPR, respectively. H_2O_2 formation in extracellular fluid was measured as a function of time and administered ascor-

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The authors declare no conflict of interest.

Abbreviations: $Asc^{\bullet -}$, ascorbate radical; GSH, glutathione; GSSG, GSH disulfide; PARP, polyADP-ribose polymerase; PX1, peroxyxanthone.

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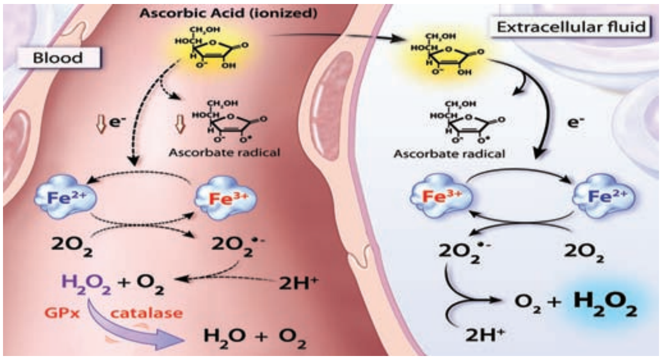

Fig. 1. Proposed mechanism of preferential formation of $Asc^{\bullet-}$ and H_2O_2 in extracellular fluid compared with blood. After oral and parenteral administration, ascorbic acid is proposed to achieve equivalent concentrations in blood (left side) and extracellular fluid (right side). In extracellular fluid, pharmacologic concentrations of ascorbic acid lose one electron and form $Asc^{\bullet-}$. The electron reduces a protein-centered metal: An example reaction is shown as reduction of Fe^{3+} to Fe^{2+}. Fe^{2+} donates an electron to oxygen, forming active oxygen including superoxide ($O_2^{\bullet-}$) with subsequent dismutation to H_2O_2 (17). In blood (left side), it is proposed that these reactions are damped or inhibited (dashed lines). $Asc^{\bullet-}$ appearance will be inhibited by red blood cell membrane-reducing proteins (18) and/or by large plasma proteins that do not distribute to the extracellular space. Any formed H_2O_2 will be immediately destroyed by plasma catalase and red blood cell GSH peroxidase, so that no H_2O_2 will be detectable (14–16). The identities of the metal-centered proteins are unknown.

bate. Because ascorbate interferes with most peroxidase-based detection methods, we used an assay based on a modified synthesis of peroxyxanthone (PX1) (19). The data show that pharmacologic ascorbate concentrations produced $Asc^{\bullet-}$ selectively in extracellular fluid compared with blood and that H_2O_2 formation occurred when $Asc^{\bullet-}$ concentrations were >100 nM in extracellular fluid. These data validate the hypothesis that ascorbate is a prodrug for selective delivery of reactive species to the extravascular space (Fig. 1) and provide the foundation for rational exploration of pharmacologic ascorbate as a prooxidant drug for therapeutic use.

Results

Pharmacologic Ascorbate Concentrations Achieved in Blood and in Extracellular Fluid by i.v. or i.p. Administration. We first tested whether parenteral (i.v. or i.p.) injection but not oral administration by gavage could achieve pharmacologic ascorbate concentrations *in vivo* both in blood and in extracellular fluid. Rats received doses similar to pharmacologic doses in humans (0.5 mg per gram of body weight) (8–10, 13). Blood was sampled at 0, 5, 15, 30, 60, 90, and 120 min, and plasma was separated for ascorbate analyses using HPLC with coulometric electrochemical detection. Extracellular fluid was collected by microdialysis at 30-min intervals before and after ascorbate administration, and ascorbate was determined at the end of each time period. With oral (gavage) dosing, initial plasma ascorbate concentrations of 50 μM did not increase to >100 μM, similar to findings of tight control of ascorbate concentrations in humans mediated by intestinal absorption (11–13). Intravenous administration of the same dose resulted in peak plasma concentrations of >8 mM, 80-fold higher than that produced by oral dosing. Intraperitoneal injection achieved peak concentrations of ≈3 mM, 30-fold higher than concentrations produced by oral dosing (Fig. 2*A*). In extracellular fluid, ascorbate concentrations produced by the different administration routes mirrored the findings for plasma. Both i.v. and i.p. administration produced pharmacologic ascorbate concentrations, whereas oral dosing did not (Fig. 2*B*). For all dose routes and all time points, ascorbate concentrations in extracellular fluid were a highly correlated function of ascorbate concentrations in plasma (Fig. 2*C*). Furthermore, the correlation underestimated the coupled relationship between plasma and extracellular fluid concentrations as a consequence of extracellular fluid collection. To obtain enough fluid for analyses, it was necessary to collect it over 30-min intervals; values are for the whole collection time. Plasma values are point values at the end of 30-min intervals. Considered together, these data show that parenteral injection bypassed the tight control of oral ascorbate administration, pharmacologic concentrations were established both in blood and in extracellular fluid by parenteral injection but not oral dosing, and ascorbate was distributed similarly in plasma and extracellular fluid for all dosing routes.

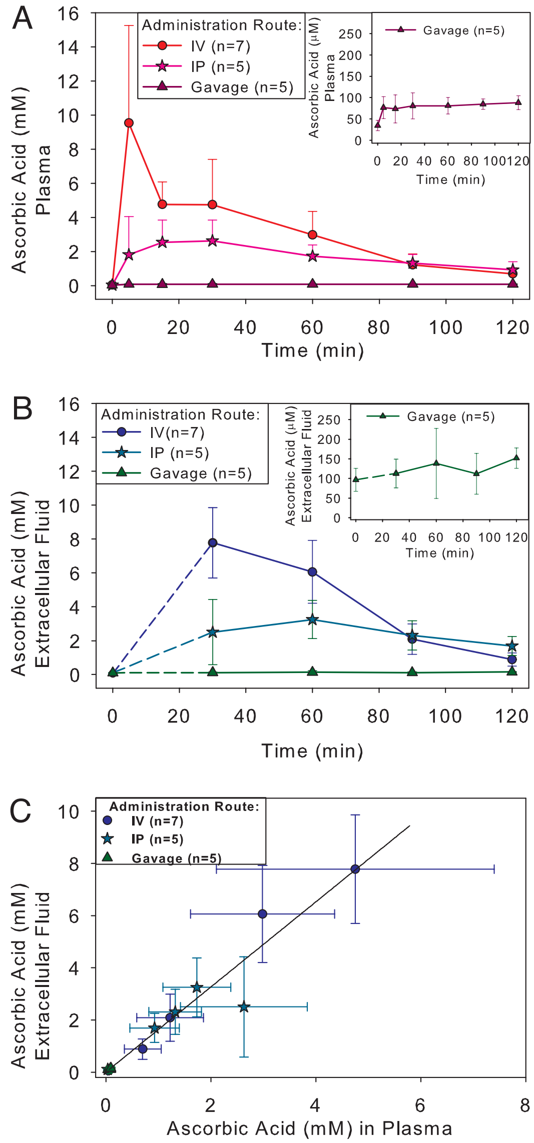

Fig. 2. Parenteral administration of ascorbic acid bypasses tight control of its intestinal absorption. A total dose of 0.5 mg of ascorbate per gram of body weight was given to rats by i.v. injection (circles) (two-thirds of the dose at 0 min and one-third at 30 min); by i.p. injection (stars) at 0 min; or by gavage (oral administration) (triangles) at 0 min. Blood was taken at each indicated time point. Extracellular fluid at the end of 30-min intervals was collected for ascorbic acid measurement (see *Materials and Methods*). Numbers of rats for each administration route are indicated. All data are displayed ± SD. (*A* and *B*) Ascorbic acid concentration in plasma (*A*) and extracellular fluid (*B*), measured in millimolar as a function of time in minutes. (*A Inset* and *B Inset*) Gavage administration of ascorbic acid, displayed as plasma concentration (micromolar) as a function of time (minutes). (*C*) Ascorbic acid concentration in extracellular fluid (millimolar) as a function of ascorbic acid concentration in plasma (millimolar) for all administration routes, all animals, and all time points (R^2 = 0.93, $P < 0.0001$).

Preferential Formation of Asc$^{\bullet-}$ in Extracellular Fluid Compared to Blood with Parenteral Administration of Ascorbate. Before and after i.v. administration of ascorbate, we investigated whether Asc$^{\bullet-}$ formation occurred in blood and extracellular fluid. Asc$^{\bullet-}$ was measured by EPR in whole blood and extracellular fluid obtained by microdialysis. As a control, Asc$^{\bullet-}$ measurements were performed after ascorbate was added to the perfusate buffer used with the microdialysis pump, with no extracellular fluid present. After i.v. administration of ascorbate at 0.5 mg per gram of body weight, EPR spectra showed preferential formation of Asc$^{\bullet-}$ in extracellular fluid compared to blood, and the signal was present for at least 2 h (Fig. 3*A*). The spectra shown are a 4-fold underestimate of the difference in the magnitude of the Asc$^{\bullet-}$ signal from extracellular fluid compared with blood. The spectra for extracellular fluid are underestimates because extracellular fluid had to be diluted 1:1 with buffer for collection and because efficiency of Asc$^{\bullet-}$ transit across the microdialysis membrane was 50% (data not shown). In contrast, blood and saline samples were measured without dilution. The Asc$^{\bullet-}$ signal was not an artifact, because no Asc$^{\bullet-}$ was observed when ascorbate was added to the perfusate buffer without extracellular fluid present.

We studied Asc$^{\bullet-}$ formation in extracellular fluid and blood (Fig. 3*B*) as a function of time. Each dosing route was used to administer a pharmacologic dose of ascorbate, 0.5 mg per gram of body weight. For extracellular fluid, Asc$^{\bullet-}$ concentrations approaching 250 nM were detected after i.v. administration, corresponding to peak ascorbate concentrations of 6–8 mM in extracellular fluid (see Fig. 2 for ascorbate concentrations). With i.p. injections, peak Asc$^{\bullet-}$ concentrations in extracellular fluid were $\approx$150 nM, corresponding to lower peak ascorbate concentrations of $\approx$3 mM. With gavage, Asc$^{\bullet-}$ concentrations in extracellular fluid did not exceed 50 nM, consistent with minimal elevations in ascorbate concentrations from oral dosing. Findings for Asc$^{\bullet-}$ in extracellular fluid after ascorbate administration stand in contrast to findings in blood. With i.v. or i.p. injection, Asc$^{\bullet-}$ concentrations in blood maximized at $\approx$30–40 nM. With oral administration, Asc$^{\bullet-}$ in blood was undetectable.

Data were combined from all doses, time points, and administration routes. Asc$^{\bullet-}$ formation in extracellular fluid was expressed as a function of ascorbate concentration in this fluid, and Asc$^{\bullet-}$ formation in blood was expressed as a function of ascorbate concentration in plasma (Fig. 3*C*). Asc$^{\bullet-}$ formation in extracellular fluid was exponentially correlated with ascorbate concentration in this fluid. For example, with 6 mM ascorbate in plasma, 220 nM of Asc$^{\bullet-}$ was formed in extracellular fluid. However, in blood, Asc$^{\bullet-}$ concentrations were all <50 nM, even when plasma ascorbate concentration was 9 mM. As expected, Asc$^{\bullet-}$ formation in extracellular fluid was similarly correlated with ascorbate concentration in plasma (data not shown). Together, these findings support the hypothesis that pharmacologic ascorbate concentrations from parenteral administration selectively generate Asc$^{\bullet-}$ in extracellular fluid, with minimal formation of Asc$^{\bullet-}$ in blood.

H_2O_2 Formation in Extracellular Fluid with Parenteral Administration of Ascorbate. H_2O_2 in extracellular fluid was measured by an assay using a synthesized boronate fluorophore PX1 (see *Materials and Methods* and Fig. 4*A*) (19). PX1 reacted with H_2O_2 and produced blue fluorescent 3,6-dihydroxyxanthone, with peak fluorescence at an emission wavelength of 450 nm. Catalase was added to samples to account for H_2O_2-specific fluorescence and background. With background subtraction, H_2O_2 concentrations were linearly correlated with fluorescence intensity. This assay was used to measure H_2O_2 in extracellular fluid before and after ascorbate administration by all dose routes as a function of time (Fig. 4*B*). After i.v. administration, H_2O_2 concentrations increased from undetectable to $\approx$20 μM, but no change occurred after oral administration. With i.p. administration, H_2O_2 concentrations were lower than those

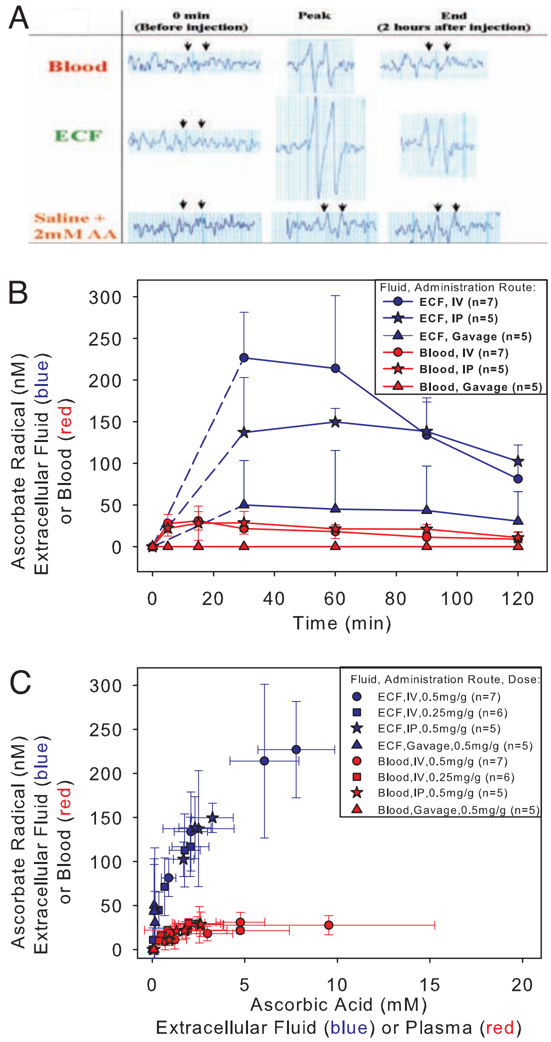

Fig. 3. Asc$^{\bullet-}$ concentrations in extracellular fluid and in blood measured by EPR, before and after i.v., i.p., and gavage (oral) ascorbic acid administration. Ascorbic acid dose was 0.5 mg per grams of body weight unless otherwise indicated and administered as described for Fig. 2. Numbers of animals for each condition are indicated. (*A*) EPR spectra of Asc$^{\bullet-}$ in blood and in extracellular fluid before i.v. ascorbic acid injection at the time corresponding to peak plasma ascorbic acid (see Fig. 2) and 2 h after injection. Blood samples and normal saline samples with added ascorbic acid (2 mM) were measured without dilution. Extracellular fluid samples were diluted 4-fold due to microdialysis sample collection procedures and microdialysis membrane throughput. Normal saline with 2 mM ascorbic acid was a control for baseline Asc$^{\bullet-}$ formation from possible ascorbate oxidation in the microdialysis perfusate buffer. Arrows indicate the expected positions of Asc$^{\bullet-}$ doublet signal. (*B*) Asc$^{\bullet-}$ concentrations (nanomolar) in extracellular fluid (blue) and blood (red) as a function of time (minutes) before and after ascorbate administration i.v., i.p., or by gavage. (*C*) Asc$^{\bullet-}$ concentrations (nanomolar) in extracellular fluid (blue) or blood (red) and as function of ascorbic acid concentration (millimolar) in extracellular fluid (blue) or plasma (red) from all animals, all doses, and all time points. Ascorbate doses were 0.5 mg per gram of body weight for i.p. and gavage administration and 0.5 mg per gram of body weight and 0.25 mg per gram of body weight for i.v. administration. Two-thirds of the i.v. dose was given at time 0, and one-third was given at 30 min. For Asc$^{\bullet-}$ in extracellular fluid, $R^2 = 0.96$ and $P < 0.0001$.

from i.v. administration, consistent with concentrations of ascorbate and Asc$^{\bullet-}$ produced by i.p. dosing.

H_2O_2 concentrations in extracellular fluid produced by all dosing routes and at all time points are displayed as functions of Asc$^{\bullet-}$ concentration in extracellular fluid (Fig. 4*C*), ascorbate concentration in extracellular fluid (Fig. 4*D*), and ascorbate concentration in

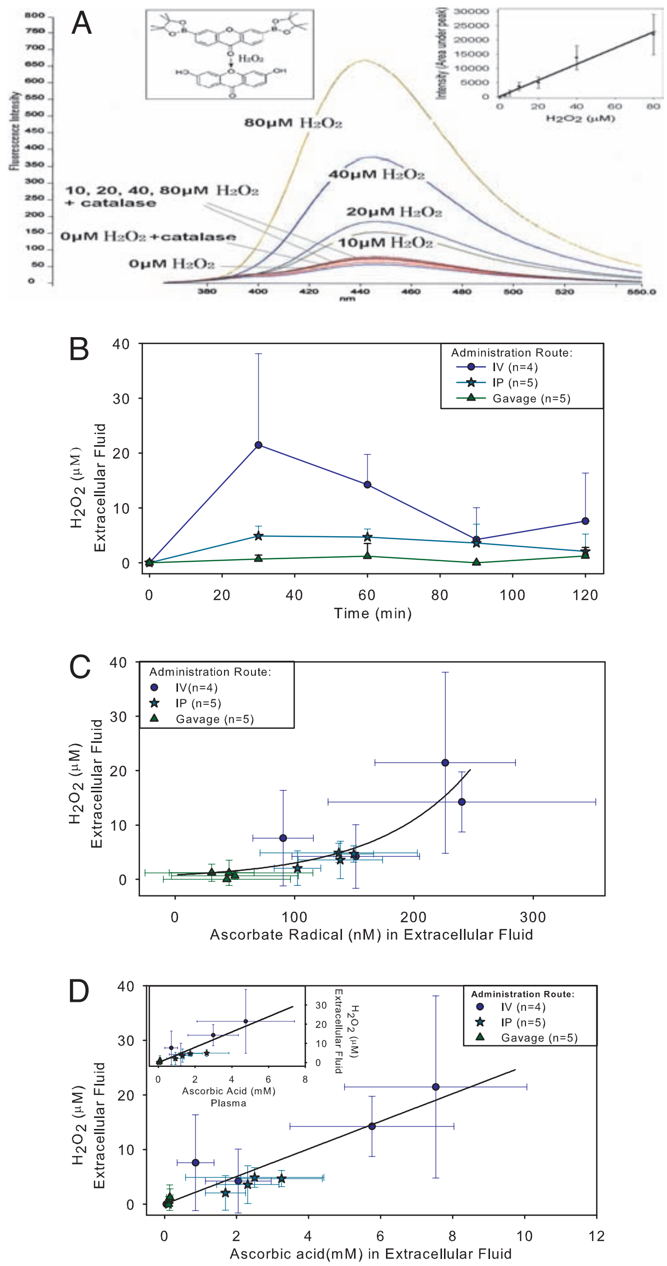

Fig. 4. H_2O_2 formation in extracellular fluid with parenteral administration of ascorbic acid. Administered doses were 0.5 mg per gram of body weight for all routes and were given as described for Fig. 2. Extracellular fluid at the end of 30-min intervals was collected for H_2O_2, ascorbic acid, and $Asc^{\bullet-}$ measurement (see Fig. 2 and *Materials and Methods*). Numbers of rats for each administration route are indicated. All data are displayed ± SD. (*A*) Fluorescent spectra of PX1 in normal saline with added H_2O_2 at the indicated concentrations. Catalase (600 units/ml) was added to parallel samples to account for non-H_2O_2 fluorescence background. (*Left Inset*) Chemical structure of PX1 and its product after reaction with H_2O_2. (*Right Inset*) Typical H_2O_2 standard curve. (*B*) H_2O_2 formation in extracellular fluid as a function of time before and after i.v. (circles), i.p. (stars), and oral (triangles) ascorbic acid administration. (*C*) Correlation of H_2O_2 concentration with $Asc^{\bullet-}$ concentration in extracellular fluid ($R^2 = 0.77$, $P < 0.0015$). (*D*) Correlation of H_2O_2 concentration with ascorbic acid in extracellular fluid ($R^2 = 0.84$, $P < 0.0001$) or in plasma (*Inset*; $R^2 = 0.87$, $P < 0.0001$). Ascorbic acid concentrations in plasma were determined as in Fig. 2.

plasma (Fig. 4*D Inset*). H_2O_2 formation in extracellular fluid required a threshold $Asc^{\bullet-}$ concentration of at least ≈100 nM (Fig. 4*C*). These concentrations only occurred with parenteral administration. Furthermore, there was a linear correlation between H_2O_2 concentrations in extracellular fluid and ascorbate concentrations in blood or in extracellular fluid (Fig. 4*D*). These data indicate that the higher the ascorbate concentration in either fluid, the higher the attained H_2O_2 concentration from parenteral administration.

In control animals, pharmacologic ascorbate concentrations added exogenously to collected extracellular fluid but not buffer induced formation of $Asc^{\bullet-}$ and H_2O_2 (data not shown), indicating that extracellular fluid components are required for reactions.

Discussion

Recently we reported that pharmacologic ascorbic acid concentrations produced H_2O_2 concentrations of ≥25 μM, causing cancer cell death *in vitro* (14). Building on these results, here we tested and validated *in vivo* the hypothesis that parenteral administration of ascorbate in pharmacologic doses produces millimolar concentrations in blood and extracellular fluid, with preferential generation of $Asc^{\bullet-}$ and H_2O_2 in extracellular fluid but not blood. We found that H_2O_2 concentrations generated *in vivo* were those that caused cancer cell death *in vitro* (14). When ascorbate was given parenterally, $Asc^{\bullet-}$, the product of a loss of one electron from ascorbate, was detected preferentially in extracellular fluid compared with blood. $Asc^{\bullet-}$ generation in extracellular fluid depended on the ascorbate dose and the resulting concentrations. With i.v. administration of ascorbate, $Asc^{\bullet-}$ concentrations were as much as 12-fold greater in extracellular fluid compared to blood and approached 250 nM. $Asc^{\bullet-}$ concentrations of >100 nM in extracellular fluid were the threshold concentration for detectable production of H_2O_2. In blood, such $Asc^{\bullet-}$ concentrations were never produced and were always <50 nM. Even if $Asc^{\bullet-}$ concentrations had reached the 100 nM threshold for H_2O_2 production, H_2O_2 in blood is immediately destroyed by plasma and red blood cell proteins (14–16). These data are all consistent with the hypothesis that pharmacologic ascorbate concentrations *in vivo* serve as a prodrug for selective delivery of H_2O_2 to the extracellular space.

The experiments in this paper are based on principles of tight control of ascorbate in humans (11–13). After oral ingestion, control of intracellular and extracellular ascorbate concentrations is mediated by three mechanisms: intestinal absorption, tissue transport, and renal reabsorption. First, intestinal absorption, or bioavailability, declines at doses >200 mg, corresponding to plasma concentrations of ≈60 μM. Second, at approximately this concentration, the ascorbate tissue transporter SVCT2 approaches V_{max}, and tissues appear to be saturated (11, 12, 20). Third, also at ≈60 μM, renal reabsorption approaches saturation, and excess ascorbate is excreted in urine (11, 12). These three mechanisms work coordinately, ensuring that ascorbate is tightly controlled. Parenteral administration bypasses tight control, which is restored as kidneys excrete ascorbate when concentrations are more than those corresponding to V_{max} of the reabsorptive transporters. Tight control principles in humans are based both on clinical and modeled data (11–13, 21). Here, we provide *in vivo* evidence that validates the modeled values and confirms principles of tight control. With an oral pharmacologic dose of 0.5 mg per gram of body weight in animals, plasma concentrations and extracellular fluid concentrations did not exceed 150 μM, whereas the same i.v. pharmacologic dose produced plasma concentrations up to 60-fold higher.

The data here offer an attractive explanation as to why tight control occurs. When tight control is bypassed, H_2O_2 forms in the extracellular space. As tight control is restored, H_2O_2 formation ceases. If tight control did not exist, H_2O_2 formation and exposure could be constant, with untoward consequences on cell division and growth (22–24). Tight control provides a mechanism preventing continuous tissue exposure to high concentrations of H_2O_2. Temporarily bypassing tight control with parenteral administration of ascorbate allows H_2O_2 to form in discrete time periods only, decreasing likelihood of harm, and provides a pharmacologic basis for therapeutic use of i.v. ascorbate.

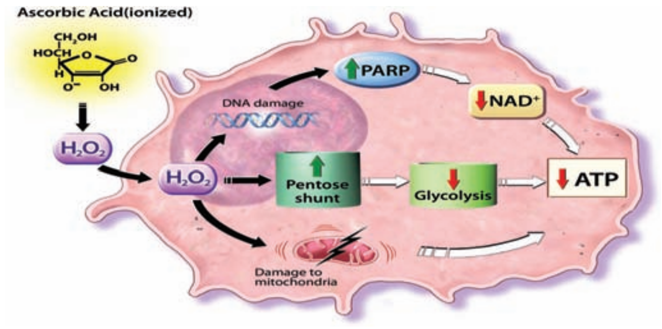

Fig. 5. Pharmacologic ascorbic acid concentrations: mechanisms for selective cell death. Pharmacologic ascorbic acid concentrations produce extracellular H_2O_2, which can diffuse into cells, deplete ATP in sensitive cells, and thereby cause cell death. ATP may be depleted by three mechanisms. (*i*) DNA damage induced by H_2O_2 activates PARP. Activated PARP catabolizes NAD^+, thereby depleting substrate for NADH formation and consequent ATP synthesis. (*ii*) H_2O_2 is catabolized by concurrent oxidation of GSH to GSSG. To reduce GSSG back to GSH, GSH reductase utilizes NADPH, which is provided by the pentose shunt from glucose. Glucose used to reduce $NADP^+$ to NADPH cannot be used for glycolysis or NADH production so that ATP generation is decreased. (*iii*) H_2O_2 may directly damage mitochondria, especially ATP synthase, so that ATP production falls. Some cancer cells rely primarily on glycolysis rather than on oxidative phosphorylation respiration for ATP production (the Warburg effect). Compared with oxidative phosphorylation, ATP generation by glycolysis is inefficient. In glycolysis-dependent cancer cells, decreased glycolysis may lower intracellular ATP. Cancer cells that are glycolysis-dependent may be particularly sensitive to pharmacologic ascorbic acid concentrations, compared with cells that use oxidative phosphorylation. See text for additional details.

We also provide data about ascorbate distribution in plasma and extracellular fluid with both oral and parenteral dosing. Although ascorbate extracellular fluid values were somewhat higher than those for plasma, this is likely due to the collection procedure. Plasma values, collected at the end of each 30-min collection period, are point values. To obtain enough volume for analyses of extracellular fluid, it was necessary to collect it for 30 min. Reported values represent averages for this time. Due to pharmacokinetics of ascorbate renal clearance, average values in extracellular fluid should be higher than point values in plasma at the end of each collection period. We interpret the data to show that *in vivo* extracellular fluid and plasma ascorbate concentrations are similar and that ascorbate diffuses from plasma to the extravascular space.

Pharmacologic ascorbate concentrations in extracellular fluid are stable for at least 1 h at 4°C (data not shown). Ascorbate must oxidize because $Asc^{\bullet -}$ is detected. The concentration of the latter is 10^{-3} to 10^{-4} less than the former, and ascorbate oxidation is only detectable by measuring $Asc^{\bullet -}$. $Asc^{\bullet -}$ measurements reflect a dynamic process of formation by ascorbate oxidation and disappearance by either dismutation or reduction. The lifetime of $Asc^{\bullet -}$ depends on its own concentrations and on ascorbate concentrations and the milieu. In extracellular fluid, $Asc^{\bullet -}$ likely degrades by dismutation, whereas blood red cells reduce $Asc^{\bullet -}$. As shown in Fig. 1, it is likely that the electron from ascorbate reduces a protein-centered metal and $Asc^{\bullet -}$ appearance is a reaction indicator, although it remains possible that $Asc^{\bullet -}$ itself provides an electron (25). *In vitro*, killing is mediated by H_2O_2 rather than $Asc^{\bullet -}$. H_2O_2 formation results in selective cytotoxicity. Tumor cells are killed with exposure to H_2O_2 for $\leq$30 min (26–30).

With *in vivo* validation of ascorbate as a prodrug for selective H_2O_2 formation, we can now suggest mechanisms to account for selective ascorbate action. *In vitro* data indicate that external pharmacologic ascorbate concentrations are required for external H_2O_2 formation (14). We propose that external H_2O_2 formed from pharmacologic ascorbate concentrations diffuses into cells (31) and mediates toxicity in sensitive cells by ATP depletion (23) via one or more of three pathways (Fig. 5). First, H_2O_2 may cause DNA single-strand breaks, repaired by polyADP-ribose polymerase (PARP). Enhanced PARP activity may deplete NAD^+, resulting in ATP depletion (27, 29). Second, H_2O_2 removal within cells may be mediated in part by glutathione (GSH) peroxidase. GSH peroxidase has an essential requirement for GSH, which, upon enzyme activity, is oxidized to GSH disulfide (GSSG). GSSG is regenerated to GSH with reducing equivalents from NADPH, which in turn is regenerated from glucose via the pentose shunt. Glucose used to reduce $NADP^+$ to NADPH is not available for ATP generation (26). In cancer cells that depend on anaerobic metabolism for ATP generation (the Warburg effect), loss of glucose to the pentose shunt may result in decreased ATP, leading to cell death (32–35). Third, mitochondria in some cancer cells may have increased sensitivity to H_2O_2 (28, 34, 36). Mitochondria in such cells may be less efficient at baseline in generating ATP compared with normal cells. Enhanced mitochondrial sensitivity to H_2O_2, with or without inefficient generation of ATP at baseline, may result in decreased ATP production. These pathways for ATP depletion induced by H_2O_2 are independent, and more than one could be responsible for cell death in sensitive cells (28, 34). Pharmacologic ascorbate concentrations should not impair normal cells because their primary ATP generation is via aerobic metabolism and because their mitochondria may not be as sensitive to H_2O_2 as those in some cancer cells.

Accumulating evidence indicates that H_2O_2 is a signaling agent at intracellular concentrations of $<$1 μM (22, 24). Signaling actions result in proliferation and enhanced survival of some cells. However, H_2O_2 concentrations generated by pharmacologic ascorbate injection are greater than H_2O_2 concentrations that enhance survival and instead are in the range that induce cell death. Other consequences of such oxidative stress, distinct from effects on ATP concentrations (Fig. 5), might also induce selective H_2O_2 toxicity to cancer but not normal cells.

If pharmacologic parenteral ascorbate is a prodrug for selective H_2O_2 delivery to the extracellular space, then therapeutic use should consider more broadly H_2O_2 in applications where H_2O_2 may have clinical benefit. In addition to cancer treatment, another potential therapeutic use is for treatment of infections. H_2O_2 concentrations of 25–50 μM are bacteriostatic (37), and as we show here these concentrations are generated *in vivo* by pharmacologic ascorbate administration. We need to learn whether some bacteria are especially sensitive to clinically possible H_2O_2 concentrations, whether there is synergy with antibiotic therapy, and whether such synergy can be used to treat problematic resistant species, such as *Acinetobacter* or methicillin-resistant *Staphylococcal aureus*. H_2O_2 concentrations only slightly higher than those presented in this paper are selectively toxic to hepatitis C virus replication in cell culture models (38). Other virally infected cells may also be candidates (14) and should be investigated, particularly where there are no current therapies. Pharmacologic ascorbate as a prodrug for H_2O_2 generation offers potential promise in clinical treatment of some cancers and infections with minimal harm. We advocate enhanced basic and clinical research in these areas to advance possibilities quickly so that patients might benefit.

Materials and Methods

Animals. Using general anesthesia, terminal experiments were performed on Wistar rats (17 males; Charles River Laboratories, Wilmington, MA) and Sprague–Dawley rats (six females; Taconic Laboratories, Rockville, MD) at ages of 10–22 weeks. Animals were euthanized at the end of experiments, which were approved by the Animal Care and Use Committee.

Ascorbate Administration. Anesthesia was initiated by 5% isoflurane via nose cone and maintained by using inhaled 1–2% isoflurane (balance compressed air). Rats were supine and warmed on a 37°C water-jacketed heat pad. Ascorbate solutions were prepared for each experiment and adjusted to pH 7 with NaOH. Ascorbate dosages (≈0.25–5 mg per gram of body weight) were administered by tail vein injection, i.p. injection, or gavage. Doses for tail vein injections were divided to lessen osmotic load: two-thirds of the dose was given at 0 min, and the remaining third was given 30 min later. For i.p. injections and gavages, the full dose was given at 0 min.

Microdialysis and Blood Sampling. Microdialysis was performed as described (39). Briefly, after establishing maintenance isoflurane anesthesia, two CMA/20 microdialysis probes with 10-mm membrane lengths, 20-kDa cutoffs, and 0.5-mm outer diameters (CMA/Microdialysis, North Chelmsford, MA) were implanted into each hind limb by femoral muscle dissection. Each probe was connected to a pump for perfusion and sample collection. Before sample collection, implanted probes were equilibrated with normal saline for 30 min at a flow rate of 1 μl/min. Extracellular fluid was collected on ice at the same flow at 30-min intervals before and after ascorbate administration, analyzed immediately for H_2O_2 and $Asc^{\bullet -}$, and then frozen at -70°C for subsequent ascorbate analysis. Collection periods were 30 min because two microdialysis probes provided 60 μl, a sufficient sample volume for all analyses. Membrane efficiencies were as follows: ascorbate, 30%; $Asc^{\bullet -}$, 50%; H_2O_2, 85% (39).

Whole-blood samples were collected before and after ascorbate administration from femoral veins at the indicated time points. Whole blood was used immediately to measure $Asc^{\bullet -}$ and then centrifuged at $1{,}800 \times g$ for 15 min to obtain plasma. Plasma was frozen at -70°C until analyzed for ascorbate.

$Asc^{\bullet -}$ and Ascorbate Detection. $Asc^{\bullet -}$ was measured by X-band EPR (40, 41). Spectrometer (E9 series; Varian) settings were as follows: microwave power, 20 mW; modulation amplitude, 1.0 G; time constant, 0.5 s; and scan rate, 80 G per 8 min. Radical quantitation was performed by using 3-carboxyproxyl as a standard (40). The coefficient of variation was 4%.

Ascorbate was measured by HPLC with coulometric electrochemical detection (12), with a coefficient of variation of 3%. The results for microdialysis were corrected by the recovery rate of the microdialysis membrane and sample dilution from fluid collection.

PX1 Synthesis. For detection of H_2O_2, the boronate fluorophore PX1 was synthesized (19). The precursor 3,6-dihydroxyxanthone (42) was prepared in 95% yield from 2,2′,4,4′-tetrahydroxyacetophenone by thermal cyclization of 250 mg in 15 ml of H_2O at 200°C for 3 h in a Biotage Initiator microwave reactor (Biotage, Uppsala, Sweden). The ^1H NMR (CD_3OD, 300 MHz) parameters were as follows: δ, 8.07 (2H, d, J = 8.73 Hz); 6.85 (4H, m) ppm. MS analysis as calculated for [MH^+] was 229.040 (found, 229.041), which was converted to 3,6-*bis*(trifluoromethanesulfonyl)xanthone by reaction with *N*-phenyl *bis*(trifluoromethanesulfonamide) (19). PX1 [3,6-*bis*(pinacolatoboron)xanthone] was obtained by palladium-catalyzed coupling of the *bis*-triflate with *bis*(pinacolato)diboron (19), using microwave heating with a Biotage Initiator at 160°C for 16 min as a modification. The solution of the crude PX1 in warm toluene was first decolorized with activated carbon before continuing the procedure. Pure PX1 was obtained in a yield of 35%. The ^1H NMR ($CDCl_3$, 300 MHz) parameters were: δ, 8.30 (2H, d, J = 7.82 Hz); 7.94 (2H, s); 7.77 (2H, d, J = 7.82 Hz), 1.39 (24H, s). MS analysis as calculated for [MH^+] was 449.220 (found, 449.230).

H_2O_2 Detection. Microdialysis eluate was collected into tubes containing 20 μM PX1 (initial volume, 60 μl). Eluate was simultaneously collected from the opposite femoral muscle into tubes containing 20 μM PX1 and 600 units/ml catalase (initial volume, 60 μl) to validate the H_2O_2 signal and determine background fluorescence. Samples were collected at 30-min intervals. Spectra were attained on a fluorescent spectrophotometer (PerkinElmer, Shelton, CT) at an excitation wavelength of 350 nm. A peak area between 420 and 500 nm was used for calculating H_2O_2 concentrations, determined from a standard curve comparing signals obtained in the presence and absence of exogenous catalase (Fig. 4*A*). Results were corrected by the throughput (recovery) rate of the microdialysis membrane and a dilution factor from addition of PX1 with or without catalase. The assay coefficient of variation was 7%.

Statistics. Statistical analyses and curve fitting were performed with SigmaPlot 10 (Systat, San Jose, CA). Results were independent of animal sex and age (data not shown). All error bars represent standard deviation. The equation describing H_2O_2 formation in relation to $Asc^{\bullet -}$ concentration in extracellular fluid was the inverse of the equation describing $Asc^{\bullet -}$ formation as a function of ascorbate concentration in either extracellular fluid or plasma, such that H_2O_2 formation was linearly related to ascorbate concentration in extracellular fluid and in plasma.

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