

Pharmacologic doses of ascorbate act as a prooxidant and decrease growth of aggressive tumor xenografts in mice

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Ascorbic acid is an essential nutrient commonly regarded as an antioxidant. In this study, we showed that ascorbate at pharmacologic concentrations was a prooxidant, generating hydrogen-peroxide-dependent cytotoxicity toward a variety of cancer cells *in vitro* without adversely affecting normal cells. To test this action *in vivo*, normal oral tight control was bypassed by parenteral ascorbate administration. Real-time microdialysis sampling in mice bearing glioblastoma xenografts showed that a single pharmacologic dose of ascorbate produced sustained ascorbate radical and hydrogen peroxide formation selectively within interstitial fluids of tumors but not in blood. Moreover, a regimen of daily pharmacologic ascorbate treatment significantly decreased growth rates of ovarian ($P < 0.005$), pancreatic ($P < 0.05$), and glioblastoma ($P < 0.001$) tumors established in mice. Similar pharmacologic concentrations were readily achieved in humans given ascorbate intravenously. These data suggest that ascorbate as a prodrug may have benefits in cancers with poor prognosis and limited therapeutic options.

cancer | hydrogen peroxide | oxidation | free radical | vitamin C

Vitamin C (ascorbate) is an essential micronutrient used as a co-factor by numerous biosynthetic enzymes. An additional viewpoint is that ascorbate serves as an antioxidant and increased intake from either foods or dietary supplements might promote good health (1). Cancer chemoprevention studies have used this antioxidant rationale to examine a putative inverse association between tumor incidence and ascorbate ingestion (2, 3). In contrast to this line of investigation, we have tested the hypothesis that pharmacologic concentrations of ascorbate may engender a prooxidant cytotoxic state within tumors. In our initial *in vitro* experiments, we observed hydrogen peroxide (H_2O_2)-dependent cytotoxicity after ascorbate exposure ($EC_{50} < 4$ mM) in five cancer cell lines, whereas normal cells were resistant (4). The *in vivo* pharmacokinetics of ascorbate treatment was subsequently determined in rats (5). These dosing and biodistribution data in rodents showed that oral ascorbate administration produced concentrations that cannot exceed 0.2 mM in plasma and extracellular fluids because of physiologic tight control, similar to mechanisms that exist in humans (6–8). Pharmacologic concentrations of ascorbate (>0.2 mM) in body fluids could be attained only when oral tight control mechanisms were bypassed by parenteral (i.v., i.p.) ascorbate administration routes. Pharmacologic ascorbate concentrations in plasma resulted in the formation of both ascorbate radical and H_2O_2 in extracellular fluid of the tissue parenchyma (5). On the basis of these data, the efficacy of parenteral ascorbate administration on tumor growth *in vivo* was examined by using the dose-toxicity relationships of ascorbate in numerous types of cancer cells *in vitro*.

Results

Range of Cancer Cell Sensitivity to Ascorbate-Derived Hydrogen Peroxide. An extensive panel of 43 tumor and 5 normal cell lines were exposed to ascorbate *in vitro* for ≤ 2 h to mimic clinical pharmacokinetics, and the effective concentration that decreased survival 50% (EC_{50}) was determined. EC_{50} was <10 mM for 75% of tumor cells tested, whereas cytotoxicity was not evident in normal cells with >20 mM ascorbate (Fig. 1A). The addition of catalase to the medium ameliorated death of ovarian carcinoma (Ovar5), pancreatic carcinoma (Pan02), and glioblastoma (9L) cells exposed to 10 mM ascorbate (1 h), indicating cytotoxicity was mediated by H_2O_2 (Fig. 1B), which is consistent with previous work on a more limited sampling of cancer cell types (4, 9).

Pharmacological Ascorbate Treatment Decreases Tumor Growth.

Given their relative sensitivity, the efficacy of pharmacologic ascorbate administration on the growth of Ovar5, Pan02, and 9L tumors was examined in nude mice. The acidity of ascorbate solutions was neutralized to pH 7 with sodium hydroxide. A maximum tolerated dose for ascorbate was limited by potential stress from osmotic imbalance after injection into the peritoneal cavity. A treatment dose of 4 g ascorbate/kg body weight either once or twice daily did not produce any discernible adverse effects. Treatment commenced after tumors reached a palpable size of 5–7 mm in diameter.

Xenograft experiments showed that parenteral ascorbate as the only treatment significantly decreased both tumor growth and weight by 41–53% ($P = 0.04$ – 0.001) for Ovar5, Pan02, and 9L tumors (Fig. 2 A–F). Metastases, present in $\approx 30\%$ of 9L glioblastoma controls, were absent in ascorbate-treated animals (data not shown).

In Situ Analysis Shows Prooxidant Metabolism of Ascorbate at Pharmacological Concentrations Is Achievable in Human Subjects.

To explore potential mechanisms underlying ascorbate action *in vivo*, blood samples and interstitial fluids from s.c. and 9L tumor sites were obtained by microdialysis in athymic mice. Parenteral administration of a single ascorbate dose (4 g per kilogram of

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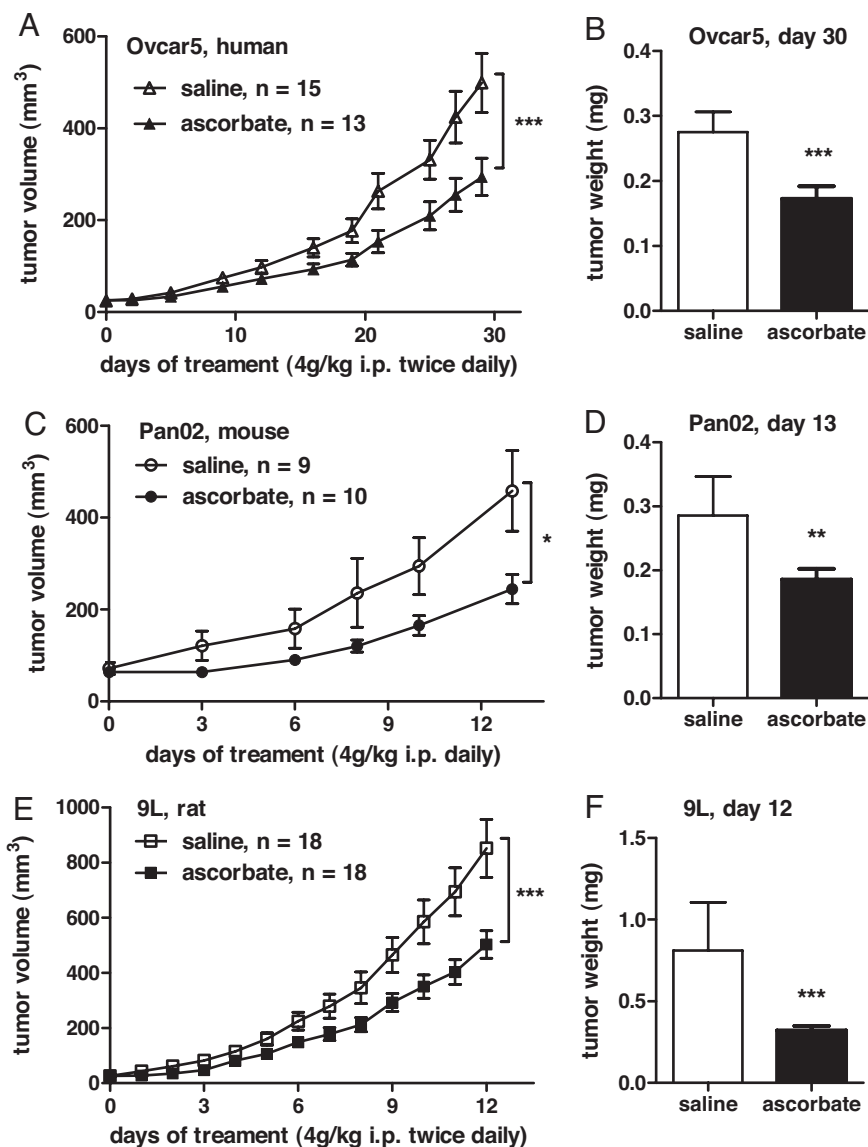


Fig. 2. Impact of pharmacological ascorbate on tumor growth. Tumors were grown in the flanks of athymic mice to a volume of $\approx 50 \pm 10$ mm³, and treatment commenced with either ascorbate (4 g per kilogram of body weight) or osmotically equivalent saline by i.p. injection as indicated. Data (\pm SEM) show growth curves and final tumor weight with either saline (\square) or ascorbate (\blacksquare) treatments in mice bearing Ovar5 (A and B), Pan02 (C and D) and 9L (E and F) tumors. *P* values were calculated by unpaired *t*-test: *, *P* < 0.01; **, *P* < 0.005; ***, *P* < 0.001.

data support that pharmacologic ascorbate concentrations, which can readily be achieved in humans (Fig. 3E), diminished growth of several aggressive cancer types in mice (Fig. 2) without causing apparent adverse effects.

We observed that ascorbate radical was an essential intermediate in H₂O₂ generation from pharmacologic ascorbate (Fig. 4). Ascorbate radical concentrations in extracellular fluids of both mice and rats were evident over a wide dose range of ascorbate, reaching a steady-state plateau of >500 nM at tissue ascorbate concentrations of >20 mM. Despite corresponding ascorbate concentrations in blood, minimal ascorbate radical and no H₂O₂ were evident (5) (Fig. 3). These data suggest that the lifetimes of ascorbate radical and H₂O₂ in blood are limited to below the detection limit, likely because of the predominance of erythrocyte peroxidase capacity (18). Data generated using microdialysis technique show that the putative metallocalcatalyst(s) for the generation of ascorbate radical and H₂O₂ was present within extracellular fluids, including tumor interstitial space (Fig. 3) (5).

Our previous work suggested that catalytic activity in serum was mediated by a protein (or proteins), because activity was heat-labile and between 10 and 30 kDa in size (4). Ascorbate is a reducing cofactor for a select small group of metal-centered enzymes (19, 20). Pharmacologic concentrations of ascorbate may react with a larger set of metallocalcatalysts with higher *K_M*s for ascorbate that otherwise are not engaged in normal biological conditions. This degeneration toward increased nonspecific reactions with pharmacologic ascorbate, with the subsequent formation of H₂O₂, may underlie the physiologic basis of tight control in ascorbate homeostasis.

It was notable that the tumor parenchyma experienced an early and sustained increase in H₂O₂ after ascorbate treatment relative to s.c. sites (Fig. 3D). This finding may be because of either an enhanced formation or decreased destruction of H₂O₂ within tumor interstitium relative to normal extracellular fluid. These intratumoral H₂O₂ concentrations of >125 μ M persisted for >3 h after ascorbate administration, similar to endogenous

that otherwise have poor outcomes, such as pancreatic and ovarian carcinomas and glioblastoma.

Materials and Methods

Cells and Cytotoxicity Assessment. Cell lines were either purchased from American Type Culture Collection or donated by colleagues: Chuxia Deng (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health), William DeGraff (National Cancer Institute, National Institutes of Health), Peter Eck (National Cancer Institute, National Institutes of Health), Corinne Griguer (University of Alabama, Birmingham, AL), Lucia Martiniova (National Institute of Child Health and Human Development, National Institutes of Health), Marsha Merrill (National Institute of Neurological Disorders and Stroke, National Institutes of Health), James Mitchell (National Cancer Institute, National Institutes of Health), Ana Robles (National Cancer Institute, National Institutes of Health), Anthony Sandler (Children's National Medical Center, Washington, DC), Emily Shacter (Center for Biologics Evaluation and Research, United States Food and Drug Administration), Lyuba Varticovski (National Cancer Institute, National Institutes of Health), and Lalage Wakefield (National Cancer Institute, National Institutes of Health). Cells (1×10^4) in logarithmic growth phase were cultured at 37°C in 5% CO₂/95% air in recommended growth media containing 10% FCS and exposed to serial dilutions of ascorbate (0–20 mM, pH 7) for 2 h and washed and cultured for an additional 24–48 h in growth medium in the absence of ascorbate. Ascorbic acid was buffered to pH 7.0 with sodium hydroxide and prepared immediately before use. EC₅₀ values indicate the concentration of ascorbate that reduced survival by 50% determined by viability assays as previously described (4, 25). Human lymphocyte and monocytes were freshly elutriated from peripheral blood donors. EC₅₀ values for 13 of 43 cells in Fig. 1A were previously shown (4). Catalase (600 units/ml; Sigma) was prepared immediately before use.

Xenograft and Treatment Procedures. Tumor cells (Ovcar5, 5×10^6 ; Pan02, 1×10^6 ; 9L, 2×10^6) suspended in normal saline solution were injected s.c. into the flanks of female athymic mice (Ncr-nu/nu aged 5–8 weeks). When tumor volume reached 25–50 mm³, treatment commenced with ascorbate (4 g per kilogram of body weight) by i.p. injection. Ascorbate was prepared as 1 M stock solution in sterile water adjusted to pH 7 with NaOH. Control mice received an identical regimen of osmotically equivalent saline solution. Longitudinal tumor volume was calculated from caliper measurements using

volume = (length) × (width)² × 0.5. At the end of the experiments, mice were killed with final tumor weight and metastases assessed by gross necropsy.

In Situ Sample Acquisition. Mice were anesthetized and maintained for microdialysis as previously described (5) with the following modifications: separate probes (CMA/20 4 × 0.5 mm, 20 kDa cutoff) were implanted into tumor tissue (right flank) and s.c. spaces (left flank) and perfused (1 μl/min) with sterile 0.9% saline solution. After a 30-min baseline period, a single dose of ascorbate (4 g per kilogram of body weight, pH 7) was given by i.p. injection at 0 min and probe eluates were collected simultaneously from each site in 30-min intervals. Relative recovery of analytes through the probe membrane was: ascorbate 12%, ascorbate radical 65%, and H₂O₂ 20%. Blood was collected from the tail vein into heparinized hematocrit tubes, and analytes were determined as single point measures every 30 min.

Analytical Chemistry. Ascorbate and ascorbate radical concentrations in plasma and microdialysates were determined by HPLC separation with electrochemical detection and electron paramagnetic resonance, respectively, as previously described (5). Formation of H₂O₂ was determined by simultaneous collection of dialysate into tubes containing peroxyxanthone (20 μM) either with or without catalase (600 units/ml), followed by fluorescence spectroscopy as previously described (5). Nonspecific background signal and low probe relative recovery restricted the lower limit of sensitivity to 20 μM.

Human Studies. Plasma ascorbic acid concentrations were measured in participants enrolled in two clinical trials using i.v. ascorbic acid as cancer therapy at the University of Kansas (ClinicalTrials.gov registration numbers: NCT00284427 and NCT0022831). The trials were approved by the University of Kansas Human Subjects Committee, and written informed consent was obtained from each participant. A single starting ascorbate dose of 15 g over 30 min at 0.5 g/min was infused with subsequent dose escalation of 25 g over 50 min, 50 g over 100 min, 75 g over 150 min, and 100 g over 200 min. Venous blood samples ($n = 8, 4$ at 100 g) were drawn at the completion of each infusion, and plasma was immediately prepared and frozen to –80°C until analyzed.

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1. Padayatty SJ, et al. (2003) Vitamin C as an antioxidant: Evaluation of its role in disease prevention. *J Am Coll Nutr* 22:18–35.
2. Zhang S, et al. (1999) Dietary carotenoids and vitamins A, C, and E and risk of breast cancer. *J Natl Cancer Inst* 91:547–556.
3. Hung HC, et al. (2004) Fruit and vegetable intake and risk of major chronic disease. *J Natl Cancer Inst* 96:577–584.
4. Chen Q, et al. (2005) Pharmacologic ascorbic acid concentrations selectively kill cancer cells: Action as a pro-drug to deliver hydrogen peroxide to tissues. *Proc Natl Acad Sci USA* 102:13604–13609.
5. Chen Q, et al. (2007) Ascorbate in pharmacologic concentrations selectively generates ascorbate radical and hydrogen peroxide in extracellular fluid in vivo. *Proc Natl Acad Sci USA* 104:8749–8754.
6. Levine M, et al. (1996) Vitamin C pharmacokinetics in healthy volunteers: Evidence for a recommended dietary allowance. *Proc Natl Acad Sci USA* 93:3704–3709.
7. Levine M, Wang Y, Padayatty SJ, Morrow J (2001) A new recommended dietary allowance of vitamin C for healthy young women. *Proc Natl Acad Sci USA* 98:9842–9846.
8. Padayatty SJ, et al. (2004) Vitamin C pharmacokinetics: Implications for oral and intravenous use. *Ann Intern Med* 140:533–537.
9. Menon M, Maramba C, Malhotra RK, Seethalakshmi L (1998) Effect of vitamin C on androgen independent prostate cancer cells (PC3 and Mat-Ly-Lu) in vitro: Involvement of reactive oxygen species-effect on cell number, viability and DNA synthesis. *Cancer Biochem Biophys* 16:17–30.
10. Cameron E, Pauling L (1976) Supplemental ascorbate in the supportive treatment of cancer: Prolongation of survival times in terminal human cancer. *Proc Natl Acad Sci USA* 73:3685–3689.
11. Cameron E, Pauling L (1978) Supplemental ascorbate in the supportive treatment of cancer: Reevaluation of prolongation of survival times in terminal human cancer. *Proc Natl Acad Sci USA* 75:4538–4542.
12. Creagan ET, et al. (1979) Failure of high-dose vitamin C (ascorbic acid) therapy to benefit patients with advanced cancer. A controlled trial. *N Engl J Med* 301:687–690.
13. Moertel CG, et al. (1985) High-dose vitamin C versus placebo in the treatment of patients with advanced cancer who have had no prior chemotherapy. A randomized double-blind comparison. *N Engl J Med* 312:137–141.
14. Wittes RE (1985) Vitamin C and cancer. *N Engl J Med* 312:178–179.
15. Padayatty SJ, Levine M (2000) Reevaluation of ascorbate in cancer treatment: Emerging evidence, open minds and serendipity. *J Am Coll Nutr* 19:423–425.
16. Hoffer LJ, et al. (2008) Phase I clinical trial of intravenous ascorbate in advanced malignancy. *Ann Oncol*, in press.
17. Padayatty SJ, et al. (2006) Intravenously administered vitamin C as cancer therapy: Three cases. *Can Med Assoc J* 174:937–942.
18. Stone JR, Yang S (2006) Hydrogen peroxide: A signaling messenger. *Antioxid Redox Signal* 8:243–270.
19. Englar S, Seifter S (1986) The biochemical functions of ascorbic acid. *Annu Rev Nutr* 6:365–406.
20. Bruegge K, Jelkmann W, Metzner E (2007) Hydroxylation of hypoxia-inducible transcription factors and chemical compounds targeting the HIF- α hydroxylases. *Curr Med Chem* 14:1853–1862.
21. Tsantes AE, Bonovas S, Travlou A, Sitaras NM (2006) Redox imbalance, macrocytosis, and RBC homeostasis. *Antioxid Redox Signal* 8:1205–1216.
22. Roy S, Khanna S, Nallu K, Hunt TK, Sen CK (2006) Dermal wound healing is subject to redox control. *Mol Ther* 13:211–220.
23. Arbiser JL, et al. (2002) Reactive oxygen generated by Nox1 triggers the angiogenic switch. *Proc Natl Acad Sci USA* 99:715–720.
24. Teoh ML, Sun W, Smith BJ, Oberley LW, Cullen JJ (2007) Modulation of reactive oxygen species in pancreatic cancer. *Clin Cancer Res* 13:7441–7450.
25. Ahmed SA, Gogal RM Jr., Walsh JE (1994) A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: An alternative to [³H]thymidine incorporation assay. *J Immunol Methods* 170:211–224.