BASIC SCIENCES RESEARCH

Effects of High Dose Ascorbate Administration on L-10 Tumor Growth in Guinea Pigs

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Sodium ascorbate is preferentially toxic to tumor cells at high concentrations. It has not been established, however, whether sufficient intra-tumor ascorbate concentrations are safely achievable *in vivo*. We administered sodium ascorbate subcutaneously or orally for eighteen days to Sewall-Wright strain-2 guinea pigs bearing intradermal L-10 hepatocarcinoma tumors. Tumor masses and intra-tumor ascorbate concentrations were determined at necropsy. L-10 cells formed tumors that metastasized to the lymph nodes, with tumor burdens reaching nearly 50 grams in untreated animals. Subcutaneous injections of ascorbate (500 mg/kg/day) inhibited tumor growth by as much as sixty-five percent, with oral supplementation reducing it by roughly fifty percent. Tumor growth correlated inversely with intra-tumor ascorbate concentration, the latter exceeding 2 mM in some cases. Ascorbate concentrations sufficient to kill tumor cells can be safely achieved in solid tumors in vivo, suggesting a possible role for high dose intravenous ascorbate in treating cancer.

Key Words: Ascorbic acid, Vitamin C, Neoplasm, Guinea pig

Most tumors are commonly treated with chemotherapy and radiation, interventions that can compromise the immune system and cause harsh side effects. Nutrient-based strategies that support overall patient health while reducing tumor burden would, if available, provide an appealing alternative to the current standard of care. One such strategy, first proposed by Cameron and Pauling in the 1970's, calls for the administration of vitamin C (ascorbic acid, ascorbate) to cancer patients at doses two to three orders of magnitude above the United States government's recommended daily allowance of the vitamin (1). While a pair of clinical trials conducted at the Mayo clinic suggested that oral administration of vitamin C (10 g/day) did not benefit terminal cancer patients (2, 3), other clinical studies indicate that ascorbate can be effective against tumors when it is administered intravenously (4-7).

The pharmacokinetics of vitamin C are such that intravenous administration may elevate plasma ascorbate levels one or two orders of magnitude above those attainable with oral dosing. This may be important in light of the fact that, at millimolar concentrations, sodium ascorbate can generate cytotoxic levels of hydrogen peroxide (8, 9). Since tumor cells are often catalase deficient, they are more sensitive to hydrogen peroxide than normal cells. Published reports indicate that intravenous ascorbate protocols can be safely used to achieve plasma ascorbate concentrations equivalent to those necessary to kill tumor cells in culture (10–12). It is not known yet what ascorbate concentrations can be achieved in solid tumors, though it has been observed that vitamin C accumulates in solid tumors at concentrations higher than those in surrounding normal tissue (13, 14). If millimolar ascorbate concentrations can be attained in tumors, vitamin C may prove useful as an anti-cancer agent.

The purpose of the research described in the present report was to simultaneously measure tumor growth rates and intra-tumor ascorbate concentrations in tumor bearing guinea pigs treated with injected or orally administered sodium ascorbate. We chose a guinea pig model because guinea pigs share with primates the inability to produce vitamin C. A transplantable hepatocarcinoma model, the L-10 cell line, is available for use with inbred Sewall-Wright strain 2 guinea pigs. L-10 cells can be injected intradermally to form metastatic solid tumors that are fatal in ninety

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days (15, 16). We were able to measure tumor mass and tumor ascorbate concentrations in L-10 tumors after seventeen days of therapy and to assess the correlation between these two variables.

Materials and Methods

L-10 tumor growth and therapy. The L-10 cell line is a transplantable hepatocarcinoma that grows poorly in culture but can be maintained in the ascites fluid of Sewall-Wright strain-2 guinea pigs by intraperitoneal (IP) implantation. L-10 cells collected from ascites fluid can be implanted intradermally (ID) at the guinea pig's left flank to form solid tumors that metastasize to the regional axillary lymph nodes and are fatal if left untreated. Our protocols for raising L-10 cells in ascites and for producing intradermal tumors were similar to previously reported methods (15–17). Briefly, 10^7 L-10 cells (courtesy of Dr. Ronald Neumann, National Institutes of Health, Bethesda, MD) were thawed from liquid nitrogen, rinsed in PBS, and injected into the intraperitoneal cavity of two six-to-eight week old Sewall-Wright guinea pigs. At the first signs of abdominal distension (typically two to three weeks after implantation), the guinea pigs were euthanized by carbon dioxide inhalation and the ascites fluid was removed. The L-10 tumor cells from ascites were washed and re-suspended in saline at a concentration of five million cells per milliliter. A 200 mL cell suspension volume of this L-10 cell solution was injected intradermally into the left flanks of six-to-eight week old Sewall-Wright guinea pigs using a 30-gauge needle. Ten days after implantation, we began ascorbate treatments. Sodium ascorbate was injected subcutaneously or administered orally as follows. For subcutaneous injections, a 100 mg/mL solution of vitamin C was prepared by diluting a 500 mg/mL buffered ascorbate injection solution (Merit Pharmaceutical) five-fold with distilled sterile water. Injections were administered under the loose skin at the nape of the neck using a 22-gauge needle. To administer the vitamin orally, we supplemented each guinea pig's daily allotment of water (150 ml) with the appropriate ascorbate dose. All treatments were given daily five days per week.

Necropsy and ascorbate measurements. The animals were euthanized by carbon dioxide inhalation four weeks after tumor implantation. Immediately after euthanasia, blood samples were obtained by cardiac puncture. The blood was transferred to heparin tubes and centrifuged at high speed for obtain plasma. A 3 ml aliquot of plasma was then stabilized by mixing to 4.5 ml cold aqueous 3% metaphosphoric acid (MPA) solution. This plasma/MPA mixture was then frozen in liquid nitrogen. At a later date, these samples were thawed and cold-filtered so that vitamin C concentrations could be measured. The tumor (both primary and metastases was excised and massed; then a roughly 2 cc sample of tumor was added to 3 ml MPA and quickly minced with scissors. The tumor/MPA solution was then frozen in liquid nitrogen. In some cases, the adrenal glands were also excised, massed, minced, stabilized in MPA, and frozen in liquid nitrogen. At a later date, these tissue samples were homogenized and cold-filtered so that vitamin C concentrations could be measured using High Pressure Liquid Chromatography (HPLC), as described elsewhere (18).

Results

The L-10 intradermal tumor model performed as expected. We obtained solid tumors with diameters of roughly 14 mm ten days after implantation, consisted with reports in the literature (15 – 17, 19, 20). At the time of necropsy, untreated guinea pigs had large tumor burdens (primary tumor or palpable metastases reaching diameters of 4 cm or more) and were just beginning to show signs of lethargy. Small primary tumors were present at the injection site accompanied by a larger metastasis between the injection site and the rear leg. In some cases, a second metastasis formed near the front leg. We found quite a bit of variability in tumor size, with the tumor burden in untreated guinea pigs ranging from 29 grams to 79 grams. The overall average tumor burden in untreated guinea pigs was 49 ± 18 (SD) grams.

Tumor sizes at time of necropsy, as well as ascorbate concentrations in tumors, adrenal glands, and blood plasma, are shown in Table 1. In the first experiment, untreated guinea pigs were compared to guinea pigs given 500 mg/kg/day ascorbate orally or subcutaneously. As expected, plasma ascorbate levels were increased by supplementation, with injections having a particularly dramatic effect. Since injections were given roughly ½ hour prior to necropsy, this likely affects early post-injection pharmacokinetics. Tissue ascorbate concentrations in adrenal glands and tumors were much higher than those seen in plasma, indicating that these tissues accumulate and perhaps store the vitamin. Supplementation increased these tissue ascorbate levels substantially. Ascorbate administration was associated with decreased tumor mass; moreover, the mean tumor mass in guinea pigs given subcutaneous vitamin C injections was significantly lower than that for guinea pigs given the vitamin orally ($p = 0.01$).

In a second experiment, we compared controls to guinea pigs given oral ascorbate along with various doses (0, 83, 200, or 500 mg/kg/day) of subcutaneously injected ascorbate. These data confirmed the effects of ascorbate

Table I. Tumor Mass and Plasma Ascorbate Concentration Data for L-10 Tumor-bearing Guinea Pigs.

EXPERIMENT 1: Comparison of oral and injected ascorbate treatments

	Ascorbate (mg/kg)		Tumor	Ascorbate Concentration (mM)		
	Oral	Injected	Mass (g)	Tumor	Adrenal	Plasma
Control	0	0	35 ± 3	0.45 ± 0.28	1.42 ± 0.80	0.08 ± 0.01
Oral	500	0	26 ± 2†	0.91 ± 0.45	4.09 ± 0.17†	0.23 ± 0.09†
Injected D	0	500	19 ± 2†	1.42 ± 0.51†	4.60 ± 0.97†	2.84 ± 0.45†

EXPERIMENT 2: Treatments with various doses of injected (with oral) ascorbate

	Ascorbate (mg/kg)		Tumor	Ascorbate Concentration (mM)		
	Oral	Injected	Mass (g)	Tumor	Adrenal	Plasma
Control	0	0	50 ± 20	0.91 ± 0.34	2.90 ± 0.68	0.16 ± 0.03
Oral	500	0	32 ± 19†	1.31 ± 0.40	3.69 ± 0.40	0.13 ± 0.01
Injected A	500	83	24 ± 3†	1.59 ± 0.91†	3.64 ± 0.80	1.14 ± 0.74†
Injected B	500	200	30 ± 15	1.25 ± 0.68	3.47 ± 0.74	0.97 ± 0.11†
Injected C	500	500	17 ± 4†	2.61 ± 0.51†	4.77 ± 1.02†	2.16 ± 0.63†

EXPERIMENT 3: Injected ascorbate treatments with or without 10 mg/kg/day lipoic acid.

	Ascorbate (mg/kg)		Tumor	Ascorbate Concentration (mM)		
	Oral	Injected	Mass (g)	Tumor	Adrenal	Plasma
Control	0	0	61 ± 18	0.11 ± 0.11	NO DATA	NO DATA
Ascorbate	500	500	32 ± 3†	0.85 ± 0.63	NO DATA	NO DATA
Lipoic	500	500	22 ± 5	0.85 ± 0.40†	NO DATA	NO DATA

†Errors given as standard deviations. Significantly different ($p < 0.05$) from control values.

treatment on tumor growth and tissue ascorbate concentrations that were observed in the first experiment. As indicated in Figure 1, a general dose-response affect could be observed in most cases, with tumor masses being cut to less than half the control values in guinea pigs treated at the highest doses. It is interesting to note that combining ascorbate injections with orally administered ascorbate did not seem to enhance these effects over those seen with injections alone. In our third experiment, we tested ascorbate injections alone or in combination with lipoic acid injections. We initially administered lipoic acid subcutaneously at a dose of 100 mg/kg/day; however, this proved toxic to some of the guinea pigs, forcing us to reduce the does to 10 mg/kg/day. These data confirmed the value of ascorbate therapy, while suggesting that the addition of lipoic acid reduced tumor burdens further over those seen with ascorbate alone ($p = 0.07$).

Since intra-tumor ascorbate concentrations in the millimolar range were attained with treatment, we were interested in determining if tumor size correlated with tumor ascorbate levels. The correlation between these two variables is shown in Figure 2. The correlation coefficient

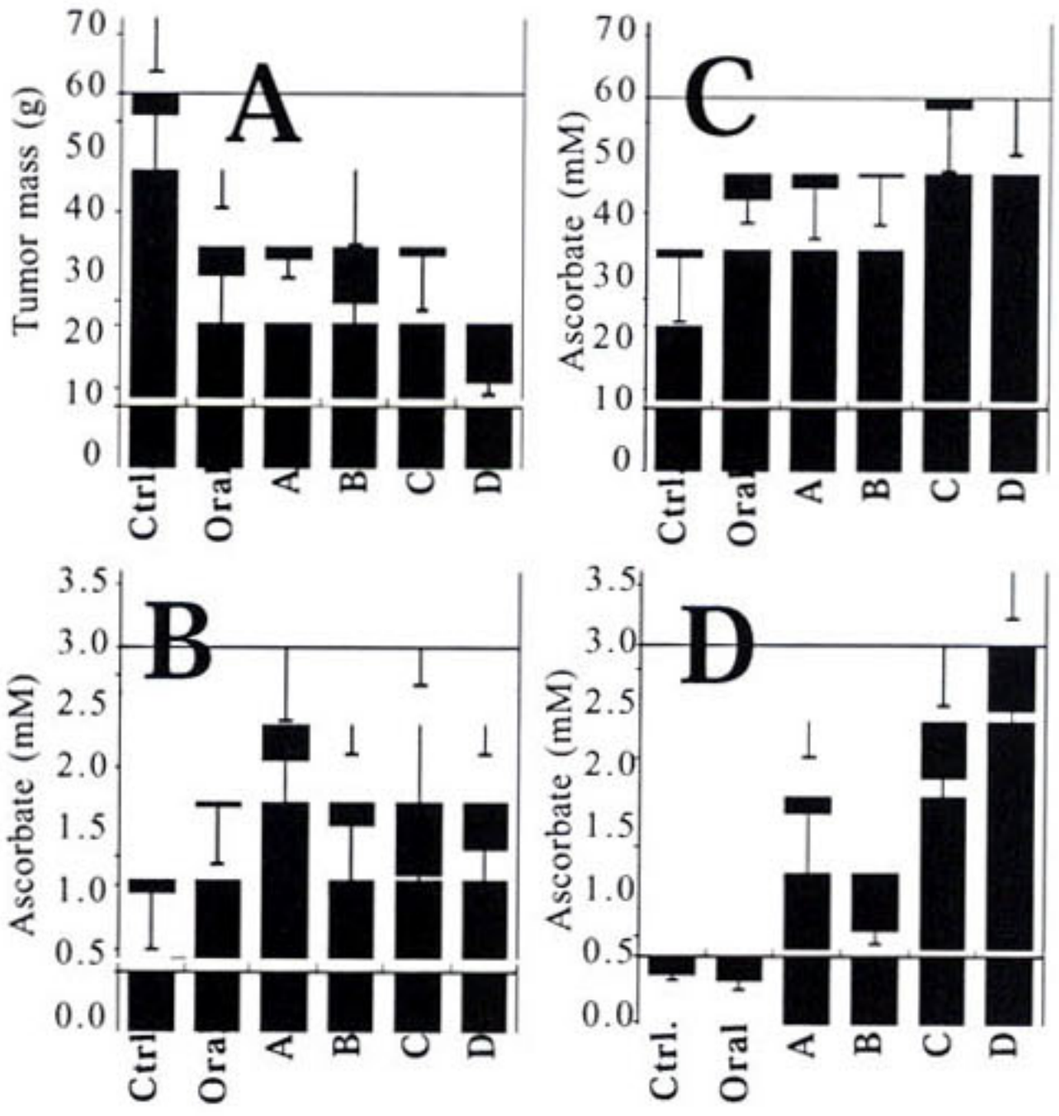

Figure 1. Averages of tumor burdens. A) Tumor ascorbate concentrations; B) adrenal gland ascorbate concentrations C) and plasma ascorbate concentrations D) from L-10 tumor bearing guinea pigs.

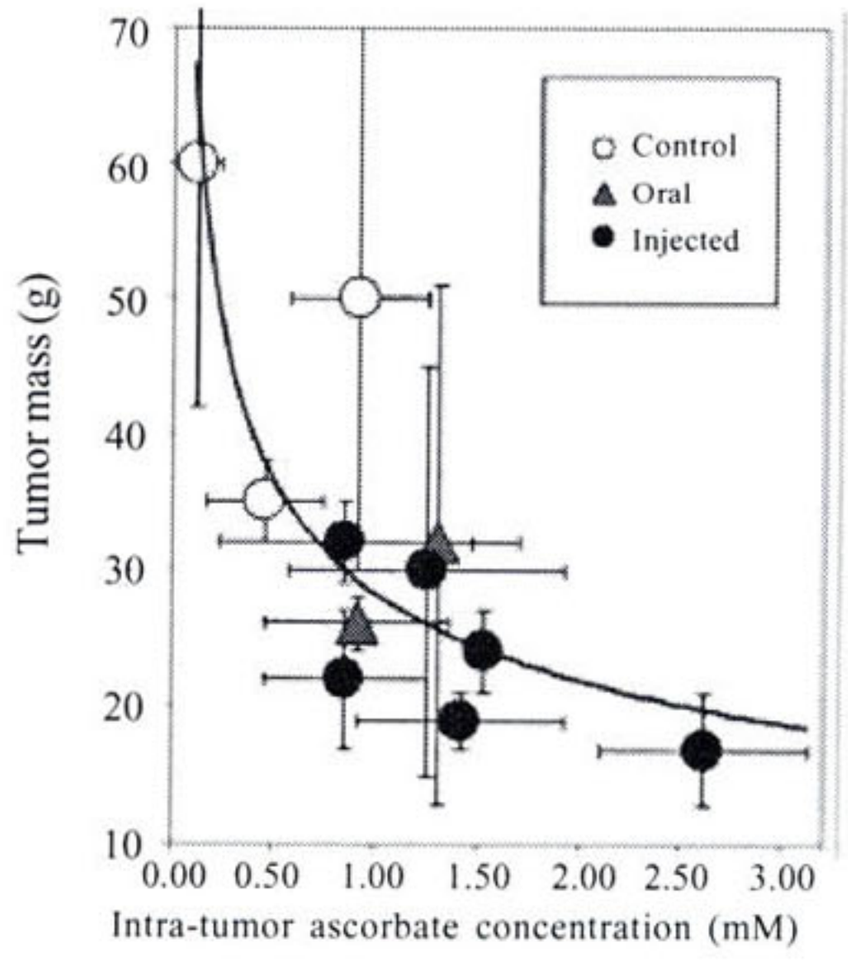

Figure 2. Correlation between intra-tumor ascorbate concentrations and tumor masses in L-10 tumor bearing guinea pigs. (Equation fitting data is: $y = 28.2 \times^{-0.37}$ with $r^2 = 0.61$)

is sufficient to demonstrate a correlation with 95% confidence, and roughly sixty percent the overall variability in tumor mass values can be attributed to variability in intra-tumor ascorbate concentrations. Tumor growth was inhibited by over fifty percent in cases where the intra-tumor ascorbate concentration exceeded 1 mm. Figure 2 seems to indicate, however, that a 'law of diminishing returns' is involved: the effects of tumor ascorbate concentration on tumor mass were most dramatic at low concentrations, with a plateau of sorts being reached at higher ascorbate levels.

Discussion

The use of ascorbate supplementation as an adjuvant treatment for cancer has generated discussion and controversy. Some favor its use based on its roles in extracellular matrix production and immune cell function (21–25), while others fear that antioxidants may protect tumors from the effects of chemotherapy (26). At sufficient concentrations, ascorbate is preferentially toxic to tumor cells *in vitro* (8, 9, 11) and has shown efficacy against tumors in animal models (27 – 29). The potential for exploiting this in the clinic depends on being able to safely deliver ascorbate to tumors at concentrations required (on the order of one millimolar) for this cytotoxic effect. Previous studies suggest that this may in fact be feasible provided that ascorbate is administered intravenously (9, 11, 12). Specifically, peak plasma concentrations of 20 mM, with average concentrations of 3 mM over a twenty-four hour period, were attained with sixty gram infusions in human subjects without ill effects. With the guinea pig model described in the present report, we were able to directly measure ascorbate concentrations in tumors while simultaneously measuring tumor growth rates. Our data indicate that ascorbate concentrations exceeding one millimolar can safely attained in animal tumors and tumor growth rates are inversely related to intra-tumor ascorbate concentration. At the highest ascorbate concentration attained, 2.6 mM, tumor mass at necropsy was roughly 35% of that observed in untreated animals. This leads us to conclude that the use of high dose ascorbate injections to treat cancer, based on ascorbate's toxicity toward tumor cells, deserves further study.

Plasma vitamin C concentrations reported in the literature for guinea pigs given cabbage (a good vitamin source) were roughly 0.05 mM, while our mean control plasma concentration was 0.12 mM (30). Reports in the literature also suggest that tissue ascorbate concentrations can be substantially above plasma values; adrenal glands, for instance, typically have ascorbate concentrations of 3 to 6 mM (30–32). Comparing these results with data from Table 1 suggest that tumor bearing guinea pigs (controls) have below normal adrenal ascorbate levels. The marked accumulation of ascorbate in tumors (roughly five times higher than values in plasma for control guinea pigs) may cause other tissues to be ascorbate deficient. Giving ascorbate orally or subcutaneously, however, seems to replenish adrenal ascorbate stores, as shown in Figure 1.

Ascorbate accumulation in tumors may provide a therapeutic advantage, provided concentrations sufficient for cytotoxic effect are reached. Previous in vitro studies with hollow fiber tumors suggest that ascorbate concentrations of 10 mM administered over a two-day period increased the percentage of apoptotic cells (11). Since treatments in tumor bearing guinea pigs occurred over the course of eighteen days, it is not surprising that ascorbate concentration closer to 1 mM affected tumor growth in vivo. Ascorbate injections had a more dramatic effect on tumor growth than oral administration, consistent with higher tumor ascorbate concentrations achieved with this mode of administration. In hollow fiber tumors, lipoic acid enhanced ascorbate efficacy: allowing tumor cell survival to be cut in half by a two-day treatment at 4 mM (11). Our preliminary in vivo data also suggest that lipoic acid can enhance ascorbate efficacy (see Table 1, experiment 3), though more data are needed.

While we were able to successful obtain data demonstrating an inverse correlation between tumor ascorbate concentrations and tumor growth rates, limitations with the L-10 guinea pig tumor model suggest that another model should be chosen, if possible, for future studies. The main disadvantages of the L-10 model were: a) cells cannot be easily maintained in culture, requiring an intraperitoneal inoculation step; b) tumor sizes were quite variable; and c) the lack of tail veins in guinea pigs precluded intravenous ascorbate administration. The difficulty in switching to another rodent model, however, is that mice and rats are able to synthesize their own vitamin C. While the doses we are giving are substantially above normal physiologic levels, animals are capable of cranking up their ascorbate production during times of stress, and there is no way of guaranteeing that this will not affect control data in tumor bearing animals. The ideal choice, if available, would be to conduct future studies in mice or rats that are genetically altered so that they are unable to produce vitamin C metabolically.

Resumen

El ascorbato de sodio es preferencialmente tóxico a las células malignas cuando se encuentra en concentraciones altas. Sin embargo, no se ha establecido en estudios in-vivo si se puede alcanzar en forma segura una

concentración intratumoral suficientemente alta para alcanzar el nivel tumoricida. Administramos ascorbato de sodio por vía subcutánea u oral por 18 días a conejillos de india Sewall-Wright, cepa 2 que tenían hepatocarcinomas L-10 intradermales. Luego de la necropsia se determinó la masa tumoral y la concentración de ascorbato en el tejido tumoral. Las células L-10 formaron tumores que metastizaron a los nódulos linfáticos alcanzando una carga tumoral de casi 50 gramos en los animales no tratados. La inhibición del crecimiento tumoral fue de hasta un 65% con las inyecciones subcutaneas de ascorbato (500 mg/kg/día) y de hasta en un 50% con la suplementación oral. El crecimiento tumoral correlacionó inversamente con las concentraciones intratumorales, excediendo en algunos casos 2 mM. Se puede alcanzar en forma segura concentraciones de ascorbato lo suficientemente altas en el tejido tumoral sólido para matar células malignas en el modelo de conejillos de india. Estos hallazgos sugieren un posible rol para la utilización de altas dosis de vitamina C como terapia para cáncer.

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