Pharmacological Ascorbic Acid Suppresses Syngeneic Tumor Growth and Metastases in Hormone-refractory Prostate Cancer

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Abstract. *Aim: The aim of this study was to test for the influence of ascorbic acid on tumorigenicity and metastases of implanted PAIII prostate cancer adenocarcinoma cells in syngeneic LW rats. Materials and Methods: Hormone-refractory prostate cancer PAIII cells were implanted subcutaneously into immunologically intact, Lobund-Wistar (LW) rats. Intraperitoneal pharmacological doses of ascorbic acid were administered each day for the ensuing 30 days. On the 40th day, animals were sacrificed. Local tumor weights were measured, and metastases were counted. Results: At the end of the 40 day experimental period, the primary tumors were found to be significantly reduced in weight (p=0.026). In addition, sub-pleural lung metastases were even more profoundly reduced in number and size (p=0.009). Grossly enlarged ipsilateral lymph node metastases declined from 7 of 15 rats to 1 of 15 rats. Conclusion: Pharmacological doses of ascorbic acid suppress tumor growth and metastases in hormone-refractory prostate cancer.*

Pharmacological doses of ascorbic acid have recently been shown to reduce the growth and weight of human, rat and murine tumor xenografts in athymic, nude mice (1). Specific tumor cell types tested included ovary (Ovcar5), pancreas (Pan02) and glioblastoma (9L). In experiments with the gliobastoma cell line, metastases were entirely absent from the treated cohort, but present in 30% of the controls. The process by which ascorbic acid suppresses tumor xenografts in these model systems appears to depend on the generation of hydrogen peroxide (H_2O_2) in extracellular fluid (2, 3).

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Hydrogen peroxide induces necrosis in tumor cells, but not in normal cells (2). A presently unknown metalloprotein mediator is believed to link the formation of pharmacological ascorbate concentrations to H_2O_2 (3). Similar results have been reported for the TLT mouse hepatoma cell line implanted in the irradiated NMRI/nude mouse (4). However, all of these models have compromised immune systems, which may contribute to the apparent efficacy of ascorbic acid. If the same result could be obtained in an animal model with an intact immune system, the use of ascorbic acid to treat cancer would have important translational consequences (5).

One of the most challenging human tumors to treat is hormone-resistant prostate cancer (HRPC) (6-8). Patients can develop irreversible, aggressive, therapy-resistant metastatic disease if untreated, or if relapse occurs following failed therapy (9). Therefore, to study a human-like rodent model for HRPC, we have turned our attention to the implanted PAIII hormone-resistant prostate cancer cell in the syngeneic Lobund-Wistar (LW) rat (10). The PAIII cell line is derived from a germfree LW rat with spontaneous advanced stage II HRPC (11), and has been routinely shown to form large primary tumors when injected subcutaneously (12). In 99% of cases, these tumors also develop metastases to local lymph nodes and to lung (9). This pattern of disease has remained constant over time (9, 13, 14). In addition to its faithful metastatic character, the PAIII cell is androgen-receptor-negative (15). As expected, it is also insensitive to testosterone deprivation (9). However, PAIII cells are positive for the estrogen receptors, ERα and ERβ (16), and are suppressed by the selective ER modulator trioxifene (LY133314) (16). Finally, the tumors formed by PAIII cells appear histologically as human-like, poorly differentiated anaplastic lesions (14, 18). Considering all of these properties together, the PAIII/LW rat system appears to be a very compelling small animal model for hormone insensitive prostate cancer in the normal, immunocompetent human population.
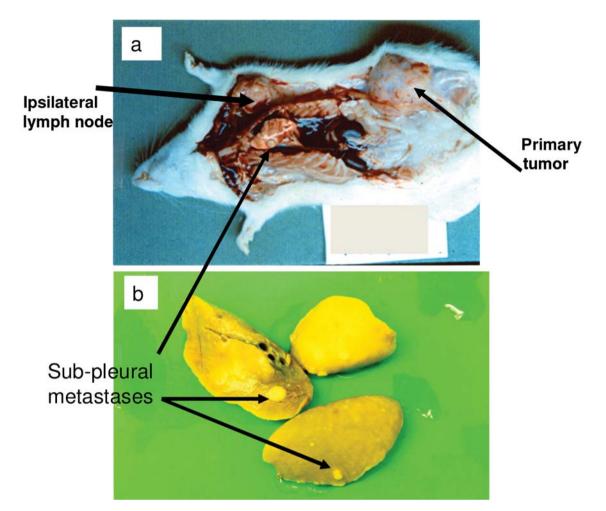
Figure 1. *Images of primary PAIII tumor, axillary lymph node, and lung metastases in LW rat. a: Gross image of LW rat implanted with PAIII tumor cells. Primary PAIII tumor (labeled arrow) is shown for a control rat inoculated with 10⁶ tumor cells. An enlarged, ipsilateral axillary lymph node is shown (see labeled arrow). Sub-pleural metastases are shown (see labeled thin arrow). b: Sub-pleural lung metastases. Lung metastases are grossly shown on rat lung surface. Large and small metastases are seen. The data in this paper are based on total sub-pleural metastases observed using ×5 magnification.*

Results obtained with tumor xenografts in athymic nude mice demonstrate ascorbate-dependent reduction in both the primary tumor mass and the incidence of metastases. However, all of these models have compromised immune systems, which may contribute either to an enhanced or diminished efficacy of ascorbic acid. We therefore hypothesized that if carcinotoxic actions of ascorbate were independent of a compromised immune system then ascorbic acid should similarly suppress both the PAIII-dependent primary tumor mass and the incidence of PAIII lung metastases in a normal, syngeneic LW rats. We report here that ascorbic acid treatment does lead to both the suppression of the primary tumor mass, and also to the reduction in the incidence of lung metastases. Additionally, we found that ascorbic acid treatment changes the quantitative relationship

between primary tumor weight and the number of lung metastases from random to essentially linear. This is the first report of ascorbic acid effects on tumor biology in a syngeneic, immune-competent rodent system.

Materials and Methods

Cells and tumor induction/treatment protocol. PAIII tumor cells were passaged in LW rats, as previously described (11). To initiate the experiment, 10⁶ cells were implanted subcutaneously into the flanks of 30 adult LW rats, aged 3 months, (12). For the present experiment, 15 of these rats were also treated pharmacologically with ascorbic acid, as described in the protocol below. Two complete experiments were performed. The pioneer experiment, with fewer rats, had very similar results to the second, large-scale experiment, as described in this paper.
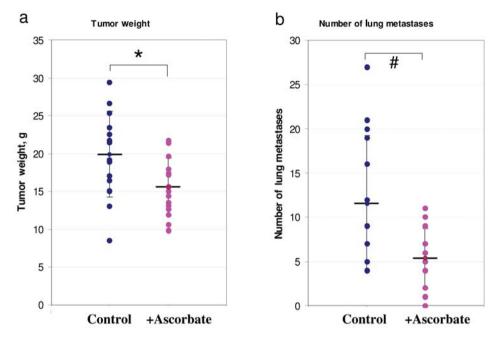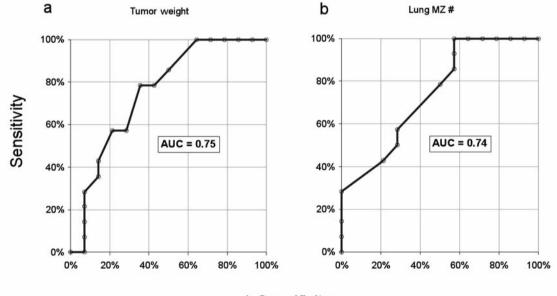
Figure 2. *Influence of ascorbate on primary tumor weight and number of lung metastases. a: Influence of ascorbate treatment on primary tumor weight. Fifteen LW rats were treated with 10⁶ PAIII cells, as described. Fifteen other rats were inoculated with the same number of PAIII cells, but also treated with ascorbate (4 g/kg), as described. After 40 days, rats were sacrificed and primary tumors dissected out for measurement of weight. The difference in weight between treated and untreated rats is significant (*p=0.026). b: Influence of ascorbate treatment on number of lung metastases. Lungs from the animals analyzed in Part a were fixed in Bouin's Fixative, and the total number of metastases counted. The difference in numbers of lung metastases in the two conditions is significant (#p=0.009).*

Figure 3. *Receiver operating condition (ROC) curves for ascorbate effects on primary tumor weight and number of lung metastases. a: ROC curve for ascorbate effects on primary tumor weight. Area under the curve (AUC) is 0.75. A perfect discriminating cut-off point would have a value of 1.0. b: ROC curve for ascorbate effects on the number of lung metastases. Area under the curve (AUC) is 0.74. A perfect discriminating cut-off point would have a value of 1.0.*
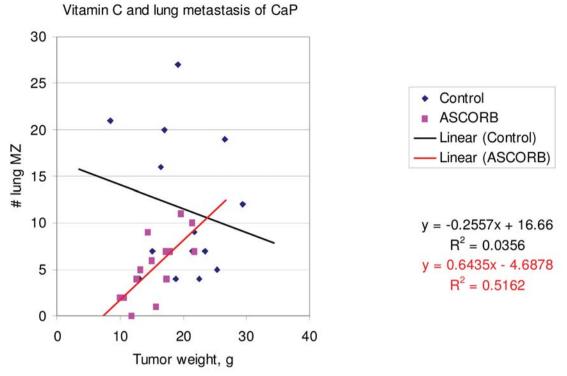
Vitamin C and lung metastasis of CaP

Figure 4. *Ascorbate effects on the relationship between primary tumor weight and number of lung metastases. Data relating tumor weight to metastatic number in the absence of ascorbate treatment are shown in solid diamonds. The R^2 value is only ~0.04, indicating essentially random distribution. Data relating tumor weight to metastatic number in the presence of ascorbate treatment are shown in solid squares. The R2 value is ca. 0.5, indicating a somewhat linear relationship. The vertical intercept is ca. –4.7, indicating a discrete lag between primary tumor weight and metastatic number.*

Following a ten-day period for primary tumor growth, neutralized ascorbic acid (4 g/kg body weight; ca. 600 mg/day/rat, depending on initial weight; neutralized to pH 7 with NaOH) was injected intraperitoneally (IP) every day for the next 30 days. Control rats received equivalent volumes of sterile distilled water. At the end of the experiment, animals were sacrificed to obtain the primary tumor weight, to record ipsilateral axillary lymph node size, and to analyze lungs for sub-pleural metastases. Animals were cared for in accord with University of Notre Dame guidelines

Measurement of tumor progression. This measurement was performed exactly as previously described (12). Briefly, rats were weighed, and then sacrificed by deep anesthesia with inhaled isofluorene, and exsanguinated from the exposed heart. This improved the visibility of metastatic foci. The primary tumor was dissected out and weighed. The lungs were removed and inflated through the trachea with Bouin's fixative. The whole lung was then placed in Bouin's fixative for 24 hours, and the fixative then replaced with 70% ethanol. Lungs were examined for sub-pleural metastases at ×5 magnification. Total numbers of metastases for each lung were then recorded. Sub-pleural tumors were observed here, as in the past, to expand into the parenchyma of the lung tissue.

Statistics. Statistics were calculated using Student's *t*-test. A *p*-value of <0.05 was taken as indicative of a significant difference between compared data. Receiver operating charactic (ROC) curves were calculated by standard methods (25).

Table I. *Statistical analysis of ascorbate effect on transplanted PAIII prostate cancer.*

		Body weight	Tumor weight	Lung MZ, #
Control	Avg	366.6	19.9	11.6
	SD	26.1	5.6	7.6
ASCORB	Avg	344.6	15.6	5.4
	SD	23.7	3.8	3.4
t-Test		0.02781	0.02566	0.00987

Results

Implanted PAIII cells develop large primary tumors, and metastatic lesions in lymph nodes and lung. Figure 1a shows the location of a typical subcutaneous primary tumor in the flank of one of the control rats used for this study. Also shown is an ipsilateral, axial lymph node, which is frequently found to be grossly enlarged due to tumor cells. In this experiment, 7 out of 15 control rats typically showed this gross lesion, compared with 1 of 15 rats in those treated with

Table II. *Dataset for PAIII-implanted hormone-resistant prostate cancer cells in LW rats.*

	Control				ASCORB		
Rat#	Body weight	Tumor weight	Lung MZ, #	Rat#	Body weight	Tumor weight	Lung MZ, #
1	399	16.47	16	1	356	19.66	11
2	379	21.7	9	2	385	15.04	6
3	376	22.56	4	3	335	21.38	10
4	358	13.03	4	4	330	14.42	9
5	357	23.45	7	5	374	13.14	5
6	382	17.04	20	6	303	9.87	2
7	342	19.18	27	7	303	11.86	0
8	328	26.63	19	8	330	17.23	7
9	405	25.3	5	9	337	10.64	2
10	385	8.49	21	10	345	17.9	7
11	377	29.41	12	11	355	21.7	7
12	385	15.08	7	12	357	17.39	4
13	323	21.46	7	13	350	12.67	4
14	336	18.84	4	14	364	15.65	1
15	270	15.02	Lost	15	350	13.51	Lost
Average	366.6	19.9	11.6		344.6	15.6	5.4
SD	26.1	5.6	7.6		23.7	3.8	3.4

ascorbic acid. Sub-pleural metastases, occurring typically in all PAIII-implanted rats, are also shown. Figure 1b shows subpleural metastases in lungs from a control implanted rat. These tumors initiate as subpleural metastases, and penetrate the parenchyma of the lung. Both large and small metastatic lesions are found.

Ascorbate treatment reduces primary tumor weight and number of lung metastases. As shown in Figure 2a, treatment of LW rats with implanted PAIII cells resulted in all rats developing primary tumors. The tumor weights averaged 19.9±5.6 (S.D.) g. However, when the parallel cohort of implanted rats were also treated with ascorbic acid, the average tumor weight declined to 15.6±3.8 (S.D.) g. This was a significant difference (*t*-test: *p*=0.026). As anticipated, all PAIII-implanted rats developed sub-pleural lung metastases (see Figure 2b). However, when these implanted animals were treated with ascorbate, there was a substantial reduction in numbers of lung metastases. The reduction was approximately 50%, from 11.6±7.6 (S.D.) to 5.4±3.4 (S.D.). The *t*-test indicates that the difference is very significant (*p*=0.009). These data are also summarized in Tables I and II. Classical ROC curves are shown for the effect of ascorbate treatment on tumor weight (Figure 3a) and cognate number of lung metastases (Figure 3b). The values for the area under the curve (AUC) for both tumor weight and number of metastases are 0.75 and 0.74, respectively. Perfect separation of data would correspond to an AUC of 1.0. Thus the ROC curves are consistent with the observed degree of data overlap between control and treated animals for both measures.

Ascorbate treatment changes the quantitative relationship between primary tumor weight and number of lung metastases. Figure 4 shows the relationship between weight of the primary tumor for each rat, and the number of lung metastases in each of the rats. In the case of the control rats (untreated with ascorbate), the distribution of tumor weights and cognate metastatic numbers appears to be somewhat random. In an attempt to determine a relationship, we tested whether a linear relationship could be determined. Based on the slope of –0.26 and the R^2 value of 0.036, we conclude that a linear relationship is not present (see solid blue diamonds). However, for those rats treated with ascorbate, there is a proportional, and objectively linear relationship, based on the slope of +0.64 and an R^2 value of 0.52 (see solid red squares). Furthermore, based on the negative intercept on the x-axis, ascorbate treatment appears to create a mathematically defined lag between primary tumor development and initiation of observable metastases. Such a lag cannot be defined in untreated animals. Thus ascorbate treatment appears to change the fundamental relationship between primary tumor mass and dependent metastases.

Discussion

These data show that pharmacological ascorbic acid inhibits both the growth and the metastatic potential of hormone-refractory PAIII cells, when these cells are implanted in the syngenic, immune-competent LW rat. This is the first report of pharmacological ascorbic acid effects on tumor biology in a syngeneic, immune-competent rodent system. Thus the

observations made by Chen *et al.* (2008) (1), with tumor xenografts in athymic nude mice, appear to be reproducible in a small animal system with significant, documented clinical similarities to HRPC in man. Pharmacological concentrations of ascorbate only partially suppresses tumor growth and metastases mediated by PAIII cells. These data are thus consistent with the reports for xenotransplants into athymic nude mice (1), or into the NMRI/ heterozygous nude mouse (4). The suppressive effect is qualitatively significant for the incidence of ipsilateral lymph node metastases, and quantitatively significant for sub-pleural lung metastases. The ROC curves (Figure 3) indicate that the effects are quantitatively similar for both the growth of the primary tumor and the occurrence of metastases. Inasmuch as the PAIII system is among the most robust and aggressive of the transplanted prostate cancer systems, we suggest that the pharmacological ascorbate effects documented here would most likely be even more powerful if tested with less aggressive systems. The attractive aspects of ascorbate as a therapeutic agent for humans include that a phase I trial has already been completed to test the safety of pharmacological doses of ascorbate, and there are few documented side-effects (18). Known limitations do exist for the patients with renal insufficiency or G6PD deficiency (18, 19).

The PAIII tumor system is sensitive to other drugs. For example, pretreatment of LW rats with *N*-(4-hydroxyphenyl)retinamide suppresses tumorigenesis and subpleural lung metastases induced by *in vivo*-passaged PAIII cells, as used in this paper (20). In addition, for a modified system of cultured PAIII cells injected into the tail vein of LW rats, tumor suppression is observed with the selective estrogen receptor modulators trioxifene (LY133314; (16)) and raloxifene (LY156758; (21)); and the anticoagulants warfarin (22) and hirudin (23). However, in the present study, tumors were allowed to develop for ten days *prior to* the ascorbic acid treatment. The consequence is that the metastatic program for the PAIII tumor seems to be significantly altered by the ascorbate treatment protocol. For example, as shown in Figure 3, in the presence of continuous dosing with ascorbic acid, the relationship between primary tumor weight and number of metastases becomes essentially linear. By contrast, without ascorbate, the relationship between tumor weight and number of lung metastases is essentially random. Furthermore, in the presence of ascorbate, there is a discrete, mathematically defined lag between the growth of the primary tumor and the eventual occurrence of detectable metastases in the lung. By contrast, no such lag is apparent in untreated control LW rats.

The most likely interpretation of the surprising observation of linearity is the possibility that in the presence of ascorbate there is a critical mass of the primary tumor that must be reached before metastases can occur. The lag mass seemed to be the same for both ascorbate and control tumors,

the difference for ascorbate being the reduction in number of metastases and the linearity with tumor mass. Thus the use of pharmacological ascorbate may open a hitherto unknown, limited window of therapeutic opportunity into the metastatic process. Clearly this relationship offers an opportunity for further study of ascorbic acid action on the biology of the PAIII cell system. The recent report that dehydroascorbic acid (oxidized ascorbate) lacks antitumor properties shows that tumor suppression depends on the specific chemical structure of ascorbic acid (24) Dehydroascorbate also opposes the action of some anti-cancer drugs, a property opposite to that reported for pharmacological ascorbic acid in the NMRI/nude mouse system (4). We therefore conclude that pharmacological ascorbate needs to be considered for addition to the limited therapeutic armamentarium that can currently be deployed against HRPC.

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