Sodium Ascorbate Enhancement of Carbidopa-Levodopa Methyl Ester Antitumor Activity against Pigmented B16 Melanoma

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ABSTRACT

We report here the single and combined antitumor activity on B16 melanoma in female C57BL/6 × DBA/2F1, mice bearing s.c. tumors of sodium ascorbate, carbidopa-levodopa methyl ester, and dietary phenylalanine and tyrosine deficiency. Groups of 15 mice were fed continuously one of three test diets both with and without sodium ascorbate (30 mg/ml) in the drinking water beginning 2 weeks before inoculation of 10⁶ melanoma cells. The test diets included the following amounts of tyrosine-phenylalanine: commercial, 1.09 and 0.64%; purified, 0.6 and 0.3%; and deficient, 0.08 and 0.04%. Drug-treated groups received daily injections of carbidopa (100 mg/kg) and levodopa methyl ester (1000 mg/kg) i.p. for 15 days beginning 1 day after tumor transplant. Tumor growth curves and median survival time were determined.

Ascorbate stimulated tumor growth in the commercial diet group. In mice fed the purified diet, ascorbate inhibited growth in some tumors, while it had no effect on others. Ascorbate inhibited tumor growth in mice fed the deficient diet, which itself severely inhibited tumor growth, and in this group increased survival by 82%. Drug treatment had little effect on tumor growth and survival of mice fed the commercial diet, but it significantly decreased growth and moderately increased survival of mice fed the purified diet. The deficient diet enhanced drug activity and increased survival of tumor-bearing mice by 73%.

Combined therapy had little effect in mice fed the commercial diet; however, mice fed the purified diet and receiving drug and ascorbate had smaller tumors and lived 55% longer. In mice fed the deficient diet, the combination retarded tumor growth and increased survival dramatically by 123%. These data indicate that adding ascorbate and restricting tyrosine and phenylalanine in combination with levodopa methyl ester therapy may become an important strategy for treating malignant melanoma.

INTRODUCTION

Levodopa and other related catechol compounds possess significant antitumor activity against a variety of animal tumor models (43–45, 49, 51, 52). In addition, levodopa and dopamine may become useful in the treatment of human melanoma (46, 48, 50). We reported recently that the efficacy of levodopa methyl ester in B16 melanoma is diet dependent and that the antimelanoma activity of this drug can be enhanced greatly by restricting the level of tyrosine and phenylalanine in the diet (27). Other dietary constituents probably influence this activity. One of these, ascorbate, has been shown to be cytotoxic to melanoma cells in culture (6, 32); however, it also prevents the oxidation of levodopa to dopaquinone (42) which Wick (47) has shown to be important to the tumoricidal activity of this drug. Therefore, ascorbate may have either an additive effect or an inhibitory effect on the antitumor activity of levodopa.

Our research investigates the in vivo interaction between levodopa methyl ester and ascorbate in B16 melanoma in conjunction with dietary tyrosine and phenylalanine restriction. We report here that supplemental ascorbate enhances the antimelanoma effect of this drug and that the magnitude of the response is dependent upon the dietary level of tyrosine and phenylalanine.

MATERIALS AND METHODS

Chemicals. All chemicals and drugs used in this study were reagent grade. Levodopa methyl ester and ascorbate were purchased from Sigma Chemical Co., St. Louis, Mo. Carbidopa was a gift from Merck Sharp and Dohme, West Point, Pa.

Mice. Specific-pathogen-free female C57BL/6 × DBA2F1 mice were purchased from Harlan/Sprague-Dawley, Madison, Wis., at 4 to 6 weeks of age. Adult mice at 14 to 16 weeks of age and averaging 22.1 ± 1.2 (S.D.) g were used in all experiments. The mice were maintained ad libitum on a locally manufactured natural product diet containing 17.8% crude protein, 2.6% dietary crude fat, and 6.7% crude fiber until they were distributed into experimental dietary groups. Three test diets were used in this study: (a) a commercial diet (LabBlox sterilizable animal diet) obtained from Allied Mills, Chicago, Ill., containing 1.09% phenylalanine and 0.64% tyrosine; (b) a purified diet containing 0.6% phenylalanine and 0.3% tyrosine; and (c) a deficient diet containing 0.08% phenylalanine and 0.04% tyrosine. The latter 2 diets were purchased from BioServ, Inc., Frenchtown, N. J. The composition of the test diets was reported previously (27). Mice were accustomed to the diets for 2 weeks before the antimelanoma experiments.

The animal facility is accredited by the American Association for Accreditation of Laboratory Animal Care and conforms to their standards for the care and use of laboratory animals. Experimental mice were housed in polycarbonate cages (15 x 30 cm) containing sterilized wood chip bedding in groups of 5 mice/cage. Food intake was determined daily to an accuracy of ±0.5 g which allowed for slight inefficiencies in food retrieval. Fluid intake was measured from inverted conical glass tubes calibrated to 1 ml. Some mice received ascorbate solutions (30 mg/ml). Fresh food and fluids were offered ad libitum daily.

Tumor. A highly pigmented B16 melanoma, originally obtained from the Mason Research Institute, Worcester, Mass., was used in all experiments. The tumor was maintained in vivo as a s.c. transplant. All tumor suspensions, prepared according to the method of Fidler et al. (13), were >95% viable by trypan blue exclusion tests. Mice received 10⁶ viable cells s.c. in a fixed volume of 0.1 ml. Tumor volume was calculated from the mean of 3 individual caliper measurements of tumor diameter using
the formula for a hemiellipsoid \(\frac{1}{6} \times \text{mean diameter}^3\). Tumor diameters were measured every other day once the tumors were palpable.

**Antimelanoma Experiments.** Mice were divided into experimental groups each containing 15 mice, and some groups were given ascorbate solution for 2 weeks before tumor inoculation. The test diets and ascorbate were continued throughout the experiment. Mice were given injections of tumor on Day 0 and given drug from Days 1 to 15. Drug groups received daily injections of carbodopa (100 mg/kg) 90 min before injecting levodopa methyl ester (1000 mg/kg). Both drugs were prepared in 0.9% NaCl solution and injected i.p. in a volume equal to 1% of mouse body weight. All other groups were given equivalent injections of 0.9% NaCl solution. The antitumor response was determined by changes in mean tumor volume and by increase in median survival time.

**Statistical Analysis.** The data were analyzed by multivariate analysis. The differences between 2 means were determined using Duncan's multiple range test (38).

### RESULTS

As shown in Table 1, drug treatment had little effect on median survival time of s.c. tumor-bearing mice fed the commercial diet, a modest effect in mice fed the purified diet, and enhanced activity in mice fed the deficient diet. These data confirm our results reported previously for mice bearing i.p. tumors (27).

Ascorbate supplementation increased survival in all dietary groups, and in all groups, the antitumor activity was greater than the activity of levodopa methyl ester. Combination therapy with the drug and ascorbate in mice fed the commercial diet was no more effective at increasing survival than were the treatments alone. Ascorbate and drug treatment more than doubled the increase in survival of mice fed the purified diet compared to those given drug treatment alone, but the combination was not synergistic. The chemotherapeutic response to combination therapy was enhanced greatly in mice maintained on the deficient diet, and this resulted in a 123% increase in survival.

| Diet group | Range  | Median | Increase in survival (%)
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<tr>
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<tbody>
<tr>
<td>Commercial</td>
<td>Untreated</td>
<td>18-31</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>Drug</td>
<td>20-35</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Ascorbate</td>
<td>22-37</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Drug + ascorbate</td>
<td>20-50</td>
<td>27</td>
</tr>
<tr>
<td>Purified</td>
<td>Untreated</td>
<td>17-32</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td>Drug</td>
<td>17-46</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Ascorbate</td>
<td>22-82</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Drug + ascorbate</td>
<td>22-82</td>
<td>34</td>
</tr>
<tr>
<td>Deficient</td>
<td>Untreated</td>
<td>24-58</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td>Drug</td>
<td>22-56</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Ascorbate</td>
<td>32-72</td>
<td>40</td>
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<tr>
<td></td>
<td>Drug + ascorbate</td>
<td>33-70</td>
<td>49</td>
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a) % of increase = \(\frac{\text{Median survival of treated mice within dietary group}}{\text{Median survival of untreated mice within dietary group}}\) - 1 \times 100.

b) Numbers in parentheses, increase in survival relative to untreated group fed the purified diet.

The effects of drug, ascorbate, and combination therapy on tumor volume are presented in Charts 1 to 3. No differences in mean tumor volume were observed between the untreated and treated groups in mice fed the commercial diet during the first 15 days after tumor transplant (Chart 1). Tumor growth was inhibited in the drug-treated group for 7 days after therapy was stopped, but within the next 2 days the mean tumor volume was equal to that in the untreated group. Ascorbate stimulated tumor growth. Combination therapy ultimately slowed tumor growth after 19 days compared to the untreated and drug-treated groups; however, this inhibition did not increase survival.

In mice fed the purified diet (Chart 2), the tumor grew faster relative to mice fed the commercial diet, indicating that the commercial diet may contain constituents that are inhibitory to tumor growth or that the nutrients are more biologically available to the tumor from the purified diet. The drug inhibited tumor growth during the treatment period and for 8 days after treatment was stopped. The tumor then grew rapidly, but in contrast to drug-treated mice fed the commercial diet, the mean tumor volume never approached the level of the untreated group. Ascorbate had variable effects on tumor volume; some tumors were sensitive, and some were resistant to inhibition by ascorbate. Until Day 19, the tumor volumes in the ascorbate-supplemented group increased at the same rate as did those in the untreated group, but then 2 distinct populations were evident. Population 1 was refractory to the effects of ascorbate, and the tumors continued to grow until the mice died of very large tumors. At Day 19, the growth of Population 2 stopped for 12 days. At Day 31, the tumors began to grow again but at a slower rate than did the untreated or Population 1 tumors. The mice ultimately developed tumors larger than those in the group designated as Population 1. The reason for this dissociated response
The contribution of the tumor mass to the final body weight. However, the tumor prevents accretion of body weight even when mice are fed the high-protein commercial diet. The drug, ascorbate, and drug-ascorbate combinations do not alter this finding.

Water intake was the same as reported previously (27) and was unaffected by tumor or drug. Mice drank equivalent amounts of ascorbate solution in all dietary groups. The mean ascorbate consumed per mouse was 129 ± 21 (S.D.) mg.

We observed similar changes in mouse weight in the various dietary groups as reported earlier (27), and ascorbate supplementation did not affect weight gain or loss. Ascorbate did not prevent the transient weight loss due to drug treatment. The weight of mice at death was greater than their weight at the time of tumor inoculation. The data in Table 3 take into consideration the contribution of the tumor mass to the final body weight.

When tumor weight is subtracted from the final weight and the corrected body weight is compared to the weight on the day of tumor inoculation, it is evident that tumor-bearing mice are able to maintain weight. However, the tumor prevents accretion of body weight even when mice are fed the high-protein commercial diet. The drug, ascorbate, and drug-ascorbate combinations do not alter this finding.

Generally, tumor growth was more invasive in untreated mice fed the commercial and purified diets than in mice fed the deficient diet. Treated mice showed lesser degrees of tumor invasion into the pulmonary cavity. In both purified and deficient dietary groups receiving ascorbate, the primary tumor masses were smaller, more well defined, and less invasive. Secondary tumor masses appeared to be encapsulated. The sizes of the secondary tumors in mice fed the deficient diet were smaller. Ascorbate did not appear to alter the invasion of tumor in mice fed the commercial diet. The combination of ascorbate and drug reduced the size and distribution of secondary tumors with the greatest effect in mice fed the deficient diet. Ascorbate alone or in combination with the drug did not appear to affect melanization of the tumor qualitatively except in the deficient dietary group given drug. Tumors in this group were dark gray in appearance compared to the black tumors observed in the commercial and purified dietary groups. Few lung metastases were present in any of the groups. This finding is probably related to the large tumor inoculum dose (106) used in our study. The mice may have been killed by the primary tumor before evidence of pulmonary metastasis could be detected.
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Table 3

<table>
<thead>
<tr>
<th>Diet group</th>
<th>Wt retention (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>99.8 ± 5.0a</td>
</tr>
<tr>
<td>Drug</td>
<td>99.9 ± 5.1</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>98.9 ± 4.5</td>
</tr>
<tr>
<td>Drug + ascorbate</td>
<td>97.8 ± 8.0</td>
</tr>
<tr>
<td>Purified</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>100.5 ± 6.5</td>
</tr>
<tr>
<td>Drug</td>
<td>101.9 ± 5.6</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>99.0 ± 4.9</td>
</tr>
<tr>
<td>Drug + ascorbate</td>
<td>99.3 ± 8.6</td>
</tr>
<tr>
<td>Deficient</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>91.9 ± 6.6</td>
</tr>
<tr>
<td>Drug</td>
<td>96.3 ± 7.3</td>
</tr>
<tr>
<td>Ascorbate</td>
<td>96.4 ± 6.8</td>
</tr>
<tr>
<td>Drug + ascorbate</td>
<td>96.0 ± 5.4</td>
</tr>
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</table>

a = \frac{\text{Death wt} - \text{tumor wt}}{\text{wt on day of tumor transplant}} \times 100

\text{Mean ± S.D.}

DISCUSSION

In this report, we showed that p.o. ascorbate alone exhibits significant antitumor activity against s.c. B16 melanoma in mice and that it enhances the antitumor activity of levodopa methyl ester, particularly when mice are fed a diet deficient in tyrosine and phenylalanine. The reason for this augmentation in mice fed the deficient diet is unclear, but it may be due to increased incorporation of levodopa (and ascorbate) into melanoma cells as a result of decreasing the competition from tyrosine and phenylalanine as discussed previously (27). Ascorbate also decreases the intestinal absorption of tyrosine in mice and, when given together with a dopa decarboxylase inhibitor, increases the excretion of tyrosine (11). This could starve the tumor for tyrosine and further reduce competition for levodopa uptake. Additional studies will clarify this point. Ascorbate also partially prevented the decrease in food intake associated with the drug administration (Table 2); however, mice still lost weight. This finding supports our previous conclusion that weight loss is due to a direct effect of the drug rather than to drug-induced malnutrition (27). We now show that the weight loss is transient and that mice recover the lost weight after the drug treatment is stopped. In our experiments, ascorbate stimulated tumor growth in mice fed the commercial diet and in some mice fed the purified diet (Charts 1 and 2). Migliozzi (25) reported similar effects in his experiments with methylcholanthrene-induced tumors in guinea pigs. He found that low doses of ascorbate inhibited, while high doses, similar to those used in our experiments, increased tumor size.

Many mechanisms have been proposed to explain the antitumor activity of ascorbate in various tumor systems, and the reader is referred to reviews by Cameron et al. (7) and Prasad (34) for a more complete treatise. We emphasize here only those possibilities that are consistent with the data collected in our experiments, and we have begun additional studies to support our proposals. In our experiments, we observed less invasion in those groups given ascorbate supplements irrespective of the final tumor volume. It is well known that ascorbate promotes collagen synthesis and inhibits proteolytic enzymes involved in its catabolism (7), and that the ability of tumor cells to invade surrounding tissues depends on their ability to degrade basement membrane which contains 40 to 60% collagen (7, 13, 26).

In Ehrlich ascites tumors, ascorbate reduced invasion, and the tumors were characterized by long regions of basement membrane in the connective tissue stroma (18, 40).

In addition, it now appears that a peroxidative mechanism is involved in the cytotoxicity of ascorbate against cancer cells. Ascorbate reacts spontaneously with oxygen to yield hydrogen peroxide (8, 10), and peroxides form during ascorbate oxidation to dehydroascorbate (25). This autoxidation of ascorbate is enhanced by cupric ions (2, 20); Bram et al. (6) reported that copper augmented ascorbate toxicity toward melanoma cells. Therefore, autoxidation of ascorbate would probably be enhanced in melanoma patients, since serum copper levels are elevated generally (14). Although peroxides may be generated in situ, cells normally are protected from their toxicity because they contain the detoxifying enzymes, catalase, and glutathione peroxidase (9). Some tumors, however, are deficient in catalase, and they are therefore more susceptible to peroxidative damage (4). Although melanoma cells are not deficient in catalase (32), they still may be susceptible to damage by peroxides because ascorbate inhibits catalase and in other tumor systems, this allows cytotoxic peroxides to accumulate (31, 33, 35). Other tumor systems show a differential sensitivity to generated peroxides, depending on the level of this enzyme and also the levels of reduced glutathione, glutathione reductase, and glucose-6-phosphate dehydrogenase (5). Moreover, peroxide-insensitive tumor cells can be sensitized toward oxidative lysis by inhibiting glutathione reductase (30) or by administering compounds that deplete glutathione (1). Unfortunately, little is known about the levels of these substances in melanoma cells, and further work seems warranted. Although the exact mechanism for ascorbate and peroxide damage to cells is not known fully, most studies indicate that they damage DNA irreversibly (15, 19, 29, 32).

The antitumor activity of levodopa is dependent on selective incorporation of the drug into melanoma cells (43, 45, 52). Once absorbed, this drug may undergo both tyrosinase-dependent and tyrosinase-independent (autoxidation) reactions with ultimate incorporation into melanin (17). Catalysis by tumor tyrosinase generates o-dopaquinone which was shown by Wick (47) to inhibit DNA polymerase through binding to sulfhydryl groups on this enzyme. But levodopa can also undergo autoxidation to form 6-hydroxypopa which subsequently generates 5-hydroxy-p-quinone and peroxides and is cytotoxic to melanoma cells (52). The p-quinone binds only weakly to sulfhydryl groups but is an effective generator of peroxides, superoxide anions, and hydroxyl radicals (17). Autoxidation may explain the activity of levodopa and related catechol compounds in tumor systems lacking tyrosinase (45, 49). Furthermore, the o-quinones of nor-epinephrine and epinephrine, which are even more reactive toward sulfhydryl groups than o-dopaquinone and do not readily autoxidize, are ineffective antitumor agents (12, 16, 17).

Although there have been no previous studies that relate to the combined in vivo synergism between ascorbate and levodopa against melanoma directly, the mechanism of toxicity of 6-hydroxydopamine may serve as a relevant model since levodopa, 6-hydroxydopa, and 6-hydroxydopamine are all selectively toxic to catecholamine-sensitive tumors (17, 33, 41). The toxicity of 6-hydroxydopamine is due to the production of hydrogen peroxide and toxic free radicals (36) and is prevented by catalase (41). In combination with ascorbate, this drug is highly toxic to...
human neuroblastoma cells in culture (36, 37) even at doses of ascorbate that are not themselves toxic. The synergism is thought to be due to an increased production of hydrogen peroxide, since ascorbate is known to augment the production of peroxides by 6-hydroxydopamine (3, 23, 24, 39).

Alternatively, 5-hydroxydopa, a recently discovered metabolite of levodopa formed in the presence of tyrosinase (21), may be involved in the antitumor activity of the ascorbate-levodopa combination, since in vitro ascorbate strikingly increases the production of this compound from levodopa (22). Studies are in progress to evaluate this compound against B16 melanoma and to study its modulation by ascorbate.

REFERENCES

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