Effects Of High Dose Ascorbate On Tumor Growth In Guinea Pigs

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EFFECTS OF HIGH DOSE ASCORBATE ON
TUMOR GROWTH IN GUINEA PIGS

Submitted in Partial Fulfillment of the Requirements
for Graduation with Honors to the Department of Biology
at Carroll College, Helena, Montana

Brian O'Hollaren

March 30, 1979
This thesis for honors recognition has been approved for the Department of Biology by:

Dr. James J. Mahlon, Advisor

Joseph Harrington

Mr. Guido Bugni, Reader

March 30, 1979
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INTRODUCTION

Recent studies indicate that a host's susceptibility to cancer is determined to a large extent by the natural resistance of the host to the disease (1). Consequently, there has developed a wave of interest concerning the theory that measures designed to enhance host resistance will result in an inhibition of malignant invasive growth.

One important factor in host resistance is the availability of ascorbic acid (2-4). It has been shown that ascorbate can be used to inhibit in-vivo formation of highly carcinogenic N-nitroso compounds (5, 11). In tumorigenesis experiments, ascorbate inhibited lung adenoma induction in A strain mice by nitrite and amines (6, 7), and transplacental induction of neurogenic tumors in rats and hamsters by ethylurea and nitrite (8, 9).

Studies on tumor production in rats showed that massive doses of ascorbate protected rats against liver tumor production by aminopyrine and sodium nitrite (10).

Besides being useful in the blockade of the nitrosation reaction, high dose ascorbate treatment can be useful in the inhibition of tumor induction and growth in that it serves to cause a significant increase in the collagen content of the animal's tissues.
The following study was performed for the purpose of investigating the effects of high dose sodium ascorbate on the rate of neoplastic induction and growth in guinea pigs.
MATERIALS AND METHODS

Tumor Induction and Treatment

Twelve 2-month old male Strain Two guinea pigs were obtained from Rocky Mountain Research Lab. The animals, whose weights varied from 400 to 429 g, were injected intradermally 2 cm behind the left clavicle with 1 ml of Line 10 hepato-carcinoma tumor cell suspension at a concentration of $1.1 \times 10^6$ cells/ml.

The animals were then divided into three groups of four. Each group was marked with a characteristic dye. Group One was marked with blue dye and used as a control group which received no ascorbate except for the trace amount present in their food. Group Two was marked with purple dye and started ascorbate treatment 6 days after injection of tumor cells. Group Three was marked with orange dye and started ascorbate treatment immediately following injection of tumor cells.

After the sudden death of all controls, 2 animals from each experimental group were used to establish a new control group.

Ascorbate administration was accomplished by dissolving sodium ascorbate in water and force feeding the mixture to the animals daily after a water deprivation period of 6 to 7 hrs. Each treated animal received approximately 3.5 g of ascorbate daily.
Melanoma Tumor Measurements

External ulcerous tumor growth was monitored by means of a small metal micrometer. The total surface area of each tumor was measured daily to the nearest .01 cm². Growth of the tumors was recorded and comparisons were made between the groups. (See Figs. 1, 2, and 3.)

Determination of Serum Ascorbate Levels

A modification of the method of Strohecker and Henning was used to determine serum ascorbate levels (12).

One g Merck soluble starch was added to 10 ml distilled water and this mixture was added to 90 ml boiling distilled water. This solution was used as the indicator. One ml of the ascorbate-containing liquid was added to the starch solution and this mixture was titrated with a .01N iodide solution until endpoint was reached.

Standard solutions of .01, .05, .10, .25, .5, and 1 mg ascorbate per ml of water were titrated many times in order to obtain invariable results from which the concentration of ascorbate in the serum could be determined. (See Figs. 4, 5, and 6.)

Tumor Removal and Weight Determination

All animals were opened surgically on day 24 and inspected for tumor growth. Tumors were removed with a fine point scalpel. All foreign tissue was removed from the surface of the tumors;
the tumors were then washed in saline followed by a washing in distilled water.

Each tumor was blotted dry with tissue paper and weighed to the nearest .01 g with a Mettler balance. The results were then recorded. (See Figs. 7 and 8.)

A special effort was made to detect any sign of metastasis. Any tumors resulting from metastasis were removed, washed, and weighed, using the same method mentioned above. All tumors were then placed in formalin.

Collagen Staining and Preparation of Specimens for Mounting

Specimens measuring 2.5 x 1.5 cm were cut from two of the tumors which seemed to be the most uniform. The tumor specimens were transferred from 100% formalin to a 10% formalin solution and allowed to remain for 24 hrs.

The specimens were then imbedded in paraffin and the paraffin was cut longitudinally into sections 6 μm thick with a microtome.

The sections were deparaffinized and hydrated with distilled water.

The specimens were then mordanted in Bouin's solution for 1 hr at 56°C, cooled to room temperature, then immediately washed in running water until the yellow color disappeared. A distilled water washing was then performed to remove any Bouin's solution.
Biebrich acid fuschin stain was then applied for 15 min prior to a 5 min soaking in Aniline Blue solution. A distilled water washing was then repeated.

Twenty-five percent glacial acetic acid was then applied for 5 min by soaking.

All specimens were then dehydrated in 95% alcohol, absolute alcohol, and xylene, with 2 changes in each solution. The slides were then mounted with Histoclad\textsubscript{R} (13).
RESULTS

All animals showed positive tumor induction by day 14. Those receiving ascorbate treatment as of day 1 showed 75% positive tumor induction by day 12, and 100% positive tumor induction by day 14. Animals whose treatment was started 6 days after tumor cell inoculation showed 75% positive tumor induction by day 11 and 100% positive tumor induction by day 12. The day of tumor induction for the initial controls could not be determined because of their early death.

All animals in the initial control group contracted severe colds by day 7, lost most anterior limb movement by day 8, and were all dead by day 10. (See Figs. 9, 10, 11, and 12.)

Autopsy of these animals revealed large lymph tumors on the ventral side of the left front leg, coupled with severe edema of all tissues. None of the treated animals developed colds or any sign of edema. It appears that death was caused by a breakdown of the lymph system coupled with an increase in capillary permeability resulting from lack of sufficient ascorbate.

With the establishment of a new control group, tumor growth measurements were made and revealed a rate of growth in the controls that was substantially higher (nearly .4 cm²/day) than that of the treated animals (less than .2 cm²/day).

The tumor growth rate of Group Three (.08 cm²/day) proved to be less than that of Group Two (.16 cm²/day). (See Figs. 1, 2, and 3.)
Iodide titration proved very useful in the determination of serum ascorbate levels. Comparison of ascorbate levels between groups showed that those animals treated with ascorbate had a serum ascorbate level nearly five times higher than that of the controls. (See Figs. 4, 5, and 6.)

Weight determination of the surgically excised melanoma tumors revealed a substantial difference in tumor weight between groups. Those animals treated with ascorbate had a total melanoma tumor weight of 27.47 g. The total melanoma tumor weight of the controls was 37.52 g. This represents a tumor weight which is 27% greater for the untreated animals.

A thorough search for a sign of metastasis showed that in 100% of the controls, tumor cells had metastasized into the lymph nodes, resulting in a lymphoma tumor arising from the lymph gland on the ventral side of the left anterior leg.

One animal from each of Groups Two and Three possessed a lymphoma tumor resulting from metastasis. (See Figs. 7 and 8.)

Determination of collagen content by Masson's trichrome staining procedure indicates that not only on the peripheral regions, but throughout the entire tumors of the treated animals, there was found to be an abnormally large amount of collagen fiber present. In comparison, the tumors of the controls possessed a very low concentration of collagen. (See Fig. 13.)
DISCUSSION

The theory that sodium ascorbate can be effective in the inhibition of tumor induction is supported by the differences seen in the induction times between Groups Two and Three. The reason for this may be due to ascorbate's role in the synthesis of collagen. It has been proposed (1) that when collagen content is substantially increased, thereby "toughening" the visceral organs, the chance for malignant neoplastic invasion is greatly decreased. I propose that this is precisely the mechanism by which the Line 10 hepatocarcinoma malignant tumor growth was inhibited or repressed in the Strain Two guinea pigs used for experimentation.

The occurrence of a slower rate of tumor growth in treated animals, coupled with the fact that the total tumor weight of the treated animals was smaller than that of the controls, tends to substantiate my proposition.

A comparison between trichrome stained slides of tumor sections also reveals that increased ascorbate intake may have a substantial effect on collagen concentration in the animal's tissues.

Arguments used to refute this theory have been based on the assumption that ascorbic acid, being water soluble, is kept in the body in a concentration which is only large enough to fulfill the animal's minimum daily requirement for this
vitamin. Through iodide titration, I have shown this to be false. The ascorbate levels present in serum are directly proportional to the animal's daily ascorbate intake. Since this is true, high dose sodium ascorbate therapy seems to be a simple, safe, and effective means of increasing collagen content in visceral organs, thereby increasing a treated animal's resistance to malignant invasive neoplastic growth.
RATE OF TUMOR GROWTH
Control Group
Figure 1

Ulcerous tumor size (cm²)

Time (Days)
Ulcerous tumor size (cm$^2$)

Figure 2

RATE OF TUMOR GROWTH

Group #2
RATE OF TUMOR GROWTH
Group #3
Figure 3
ml 1% iodide solution needed to reach endpoint
TITRATION FOR SERUM ASCORBATE LEVEL OF CONTROLS

Figure 5

1.5 ml 1% iodide solution needed to reach endpoint

Concentration of Ascorbate:

- Control #1: 0.082 mg/ml serum
- Control #2: 0.061 mg/ml serum
- Control #3: 0.053 mg/ml serum
- Control #4: 0.057 mg/ml serum
TITRATION FOR SERUM ASCORBATE LEVEL OF TREATED ANIMALS
Figure 6

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TUMOR WEIGHT IN CONTROLS

Figure 7

Animal Identification Number
TUMOR WEIGHT IN TREATED ANIMALS

Figure 8
SURVIVAL TIME
INITIAL CONTROL GROUP
Figure 9
SURVIVAL TIME
SECONDARY CONTROL GROUP
Figure 10

Animal Identification Number

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SURVIVAL TIME
GROUP #2
Figure 11

Animal Identification Number

Survival Time (Days)
Survival Time
Group #3
Figure 12

Animal Identification Number

Survival Time (Days)
COLLAGEN CONTENT DETERMINATION BY STAINING

Figure 13

Animal #2, Group 2

Animal #2, Control Group


