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Mega-doses of L-ascorbic acid alter the antineoplastic effects of ionizing radiation in EMT6 cells *in vitro*

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by

Karina Ann Lund

2006

MEGA-DOSES OF L-ASCORBIC ACID ALTER THE ANTINEOPLASTIC EFFECTS OF IONIZING RADIATION IN EMT6 CELLS *IN VITRO*. Karina Ann Lund (Sponsored by Dr. Sara Rockwell). Department of Therapeutic Radiology, Yale University School of Medicine, New Haven, CT.

ABSTRACT

Despite the common usage of high-dose vitamin C among breast cancer patients, the published medical literature is not in agreement as to how mega-dose vitamin C may interact with conventional therapy to affect clinical outcomes. The purpose of this study was to investigate the interaction of mega-dose vitamin C with radiation therapy and with doxorubicin in the treatment of breast cancer. Cultures of EMT6 mouse mammary tumor cells were treated concurrently with varying dose of vitamin C and either radiation or doxorubicin. A clonogenic assay was then performed to determine the surviving fraction of the cells. The surviving fractions of cells in cultures receiving different doses of vitamin C were compared among themselves as well as with controls and dose response curves were generated. Results show that ascorbic acid administered in concentrations of 1 mM or 10 mM 4 hours before x-irradiation protected the cells from radiation-induced cytotoxicity. The dose-modifying factors for 1 mM and 10 mM ascorbic acid as compared to controls were 1.23 and 1.37 respectively. These results support the hypothesis that mega-dose vitamin C, when taken concurrently with radiation therapy, protects cancer cells from the cytotoxic effects of ionizing radiation. No evidence was found to suggest that mega-dose vitamin C alters the antineoplastic effects of doxorubicin.

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INTRODUCTION

Prevalence of Complementary and Alternative Medicine Use among Cancer Patients

The National Center for Complementary and Alternative Medicine (http://nccam.nih.gov/), a component of the National Institutes of Health, defines complementary and alternative medicine (CAM) as "a group of diverse medical and health care systems, practices, and products that are not presently considered to be part of conventional medicine." Complementary medicines are used concomitantly with conventional medicine, whereas alternative medicines are used to substitute for conventional medicine.

It is well documented that CAM is widely used by cancer patients. In 1998, Ernst and Cassileth published a review of the literature documenting the prevalence of CAM use among cancer patients. They reviewed 26 surveys from 13 countries which found a prevalence of CAM use among adult cancer patients ranging from 7 to 64 percent with an average prevalence of 31.4 percent (1). The study authors attribute this wide variability to a lack of specificity in the definition of CAM within studies and inconsistency in its definition between studies. Nevertheless, they conclude that the use of CAM by cancer patients is both common and widespread. That same year, a national survey conducted by Eisenberg *et al.* found that the prevalence of CAM use among the general population increased from 33.8 percent in 1990 to 42.1 percent in 1997 ($p \leq 0.001$) (2). According

to the survey results, one of the therapies that increased the most during that time was megavitamins, defined by Dictionary.com as "a dose of a vitamin greatly exceeding the amount required to maintain health."

Since 1998, multiple new studies have been published which further support the high prevalence of CAM use among cancer patients. In a study of CAM use by breast cancer survivors in Ontario, Canada, 66.7 percent of the survey respondents reported using CAM (3). In a cohort study of 480 patients in Massachusetts with newly diagnosed early-stage breast cancer, 10.6 percent of the study participants had used CAM prior to being diagnosed with breast cancer, and 28.1 percent initiated new use of CAM after surgery for their cancer (4). A 2004 survey of 500 women with breast or gynecologic cancers at the M.D. Anderson Cancer Center found that 48 percent of the women used CAM (5). In a study of patients with advanced-stage breast cancer, 73 percent of patients reported using CAM (6). Given the high prevalence of CAM use among cancer patients, an understanding of the interactions of CAM with conventional cancer therapies is clinically important.

Vitamin C

Vitamin C (ascorbic acid) is an important water-soluble dietary vitamin. It is absorbed in the distal small intestine via a sodium-dependent cotransport mechanism (7) and excreted renally. The recommended daily allowance of vitamin C for females is 75 mg and for males is 90 mg; daily intake should not exceed 2000 mg (8). Nearly all of

the vitamin C ingested is absorbed into the circulation up to a daily intake of 100 mg (9). However, as ingestion increases above this dose, a progressively smaller percentage of dietary vitamin C is absorbed (10). Scurvy is a clinical syndrome that develops with vitamin C deficiency. Symptoms of scurvy include: petechiae, ecchymoses, bleeding gums, hyperkeratosis, arthralgias, impaired wound healing, neuropathy, weakness, malaise and depression (11). Symptoms of vitamin C overdose include renal calculi, nausea, gastritis and diarrhea (8).

Although the mechanism of action of vitamin C is not yet fully understood, much has been learned about the biochemical functions it performs, which are many and varied. Vitamin C plays an important role in collagen synthesis and tissue repair (12). Many of the symptoms observed in scurvy, for example, are due to impaired collagen synthesis secondary to vitamin C deficiency. In the gut, vitamin C enhances iron absorption (13). Once absorbed, vitamin C acts as a reversible reducing agent in a variety of biochemical reactions involving iron and copper, in some cases resulting in the alteration of enzyme activity (13). The antioxidant properties of vitamin C make it capable of stabilizing molecular oxygen and scavenging free radicals (12).

Nobel laureate Linus Pauling's 1979 book, Cancer and Vitamin C, opened the public debate over the effectiveness of vitamin C in the prevention and treatment of cancer. In this book, Pauling and his co-author, Ewan Cameron, argued that mega-dose vitamin C improves clinical outcomes in cancer patients (14). In support of their argument, Cameron and Pauling presented the results of a study of 100 patients with

terminal cancer. These patients were given 10 g of vitamin C daily and their survival was compared with that of 370 case controls. Results showed a remarkable increase in survival time among the patients treated with high-dose vitamin C. The study was criticized for exhibiting selection bias as the study cases were all Dr. Cameron's patients whereas case controls were taken from historical chart reviews of patients treated at a different hospital. A group at the Mayo Clinic subsequently attempted to confirm Pauling's findings by conducting a prospective, randomized, double-blind trial comparing high-dose vitamin C with placebo in terminal cancer patients who had been previously treated with conventional therapy (15). The Mayo Clinic group found instead that the patients in the vitamin C arm of the study deteriorated more quickly than those in the placebo arm. After receiving criticism from Dr. Pauling for including in their study patients who had a prior history of chemotherapy (16), the Mayo Clinic group conducted a second study with identical treatment arms but in patients who had not previously been exposed to conventional cytotoxic chemotherapy (17, 18). Again, no benefit of vitamin C was observed.

A significant amount of research has recently sought to answer the question of whether vitamin C may have a role as an adjuvant therapy in the treatment of cancer either to protect normal tissues or to potentiate antineoplastic activity. Current literature will be reviewed separately for studies investigating the interaction of vitamin C with radiation and with doxorubicin.

Radiation

Radiation therapy is widely used in the treatment of human cancers and certain other hyperproliferative diseases. Greater than 60 percent of cancer patients undergo radiation therapy at some point during the course of their illness. Radiation therapy can be used with curative intent, for palliation of symptoms or as adjuvant therapy (19). Adjuvant radiation therapy may be used to shrink a tumor prior to surgery as well as to kill remaining malignant cells following surgery. Chemotherapy given prior to or concomitantly with adjuvant radiotherapy may make tumors more sensitive to the effects of radiation. Two of the most common methods of delivering radiation to patients are external beam radiation, which uses a linear accelerator, cobalt-60 irradiator or other machine to deliver radiation from outside the body to the tumor, and brachytherapy in which γ-radiation is delivered directly to the tumor from within the body (20). Brachytherapy implants can be intracavitary, interstitial or intravenous. Of course, the goal in devising any radiation treatment plan is to kill cancer cells with the greatest possible efficacy while minimizing damage to normal tissue.

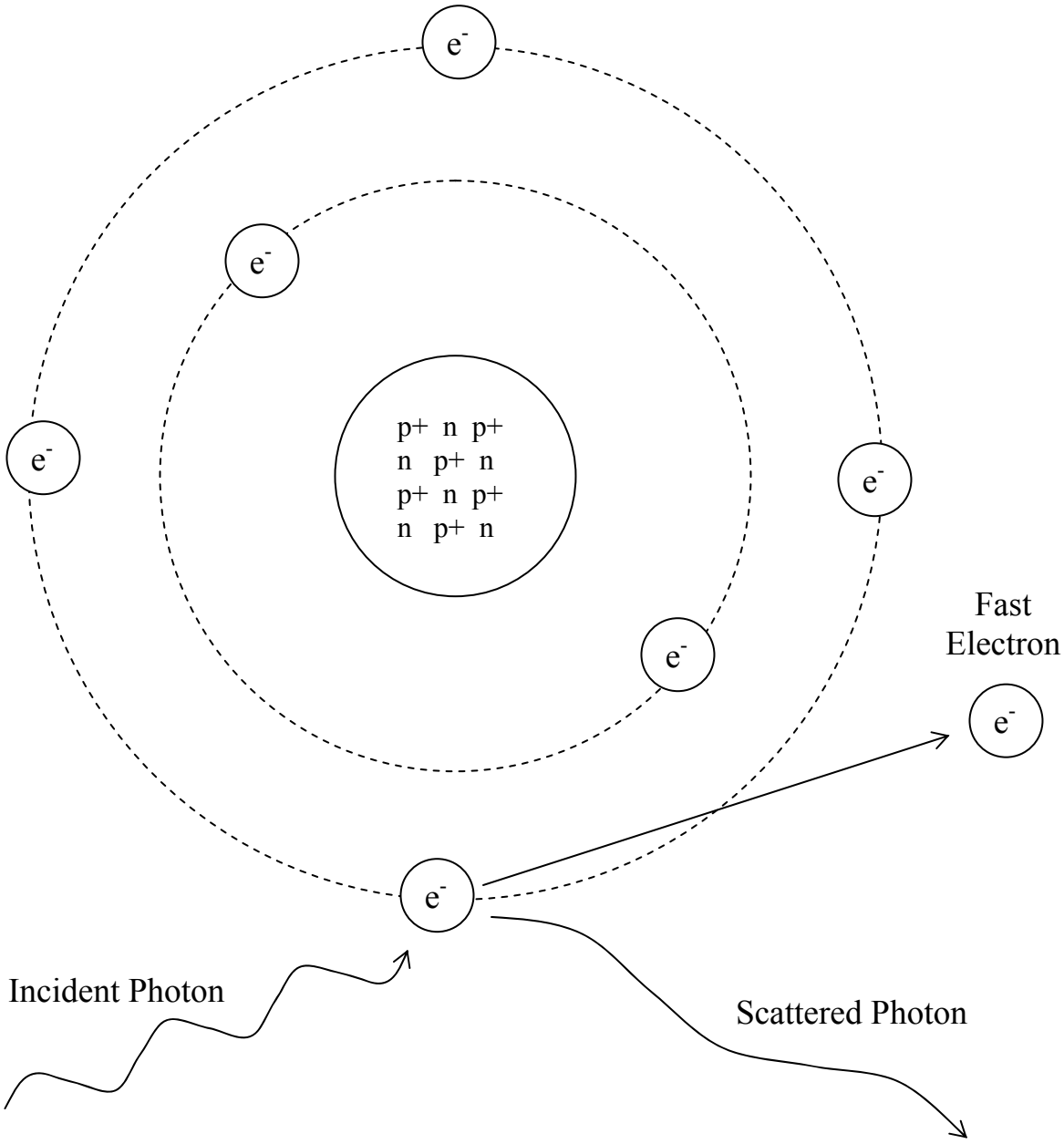
When radiation is absorbed by living tissue, energy may be transferred to an electron causing excitation of that electron. Radiation that has sufficient energy to eject an orbital electron from the atom is referred to as ionizing radiation. There are several different types of ionizing radiation that are used clinically in therapeutic radiology. X-rays and γ-rays are both electromagnetic radiations with similar spectrums of frequency and energy, which produce ionization (19, 20). When orbital electrons change

between high-energy and low-energy states, x-rays are emitted that contain the energy released in these transitions. Gamma rays are emitted from the nuclei of certain radioactive elements as they decay. Particulate radiations (i.e. electrons, protons, α-particles, neutrons or other particles accelerated to high velocities) can also produce ionization (19, 20). Both electromagnetic and particulate radiations are used in cancer therapy.

X-ray photons are typically absorbed by tissue in one of two ways: by Compton scattering or by photoelectric absorption (20, 21). Pair production, a third way in which a photon can be absorbed, results in the production of an electron-positron pair. This occurs only when the photon energy is greater than 1.02 MeV, a condition that is not met in the experiments conducted for this thesis. In Compton scattering, which dominates at high energies, the incident photon transfers a portion of its energy to a loosely bound electron of an atom of the absorbing tissue causing that electron to be ejected from the atom as a fast-moving particle (see Figure 1). The photon, deflected from its original path, continues with the remaining energy. In contrast, when radiation is absorbed by the photoelectric process, which dominates at low energies, the incident photon interacts with a tightly bound electron, transferring all of its energy to that electron (see Figure 2). The resultant fast-moving electron, having been ejected from the atom, has a kinetic energy equal to the energy of the incident photon minus the electron's binding energy. The vacancy left by the ejected electron is filled either by an outer orbital electron from within the same atom or by an electron from outside the atom. This filling of the vacancy causes the emission of characteristic x-rays. Regardless of whether x-rays are absorbed

FIGURE 1. Absorption of an x-ray photon by Compton scattering. The incident photon transfers a portion of its energy to a loosely bound electron of an atom of the absorbing tissue causing that electron to be ejected from the atom as a fast-moving particle. The photon, deflected from its original path, proceeds with reduced energy. Key: e^- represents an electron; p+ represents a proton; n represents a neutron. This figure was adapted from Hall (20).

FIGURE 1.


e-
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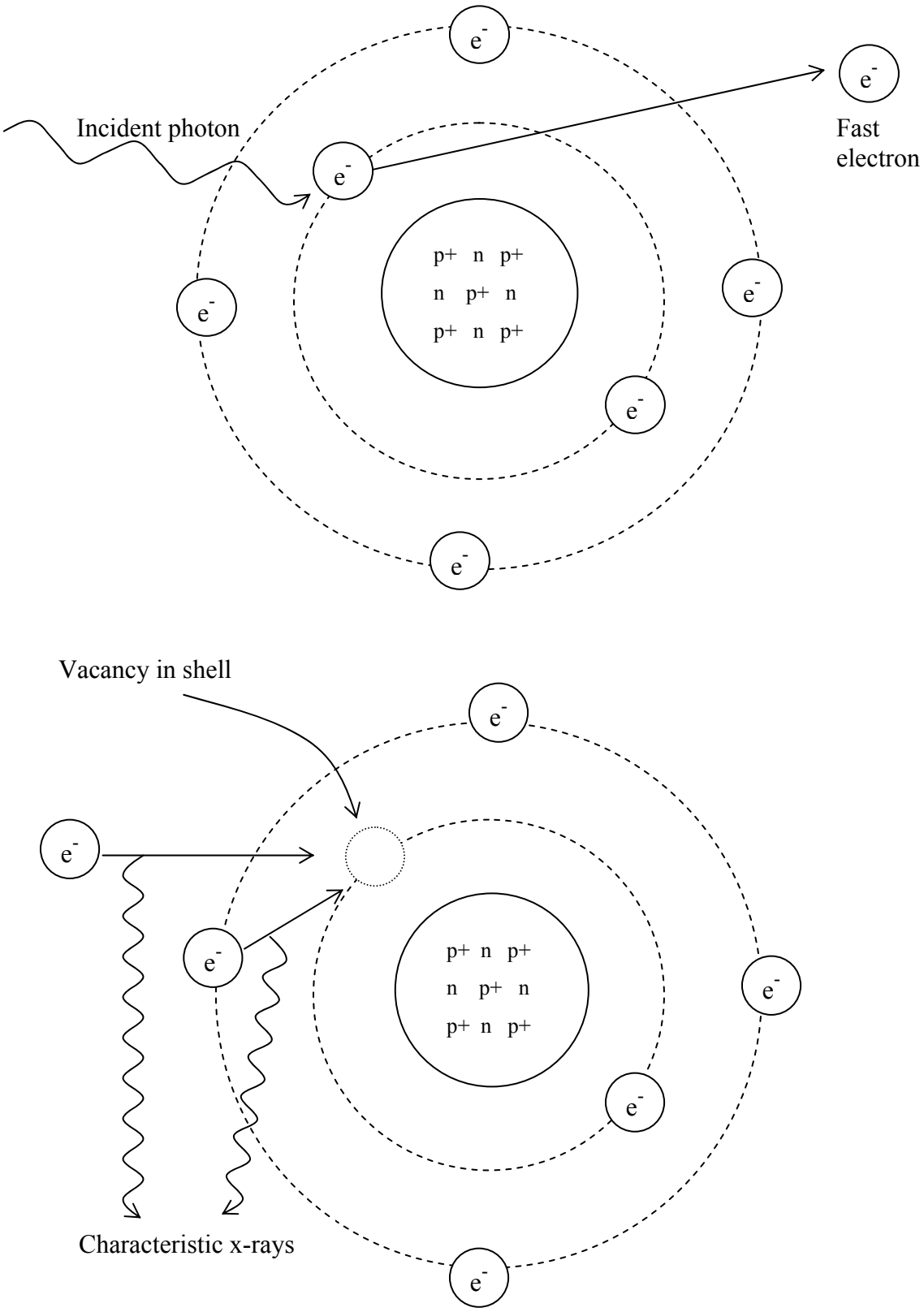
n p+ n
Fast
Electron
e-
e-
Incident Photon
Scattered Photon

FIGURE 2. Absorption of an x-ray photon by the photoelectric process. The incident photon interacts with a tightly bound electron, transferring all of its energy to that electron and ejecting the electron from the atom. The vacancy left by the ejected electron is filled either by an outer orbital electron from within the same atom or by an electron from outside the atom. This filling of the vacancy causes the emission of characteristic x-rays. Key: e^- represents an electron; p+ represents a proton; n represents a neutron. This figure was adapted from Hall (20).

FIGURE 2.


Incident photon
Fast electron
p+ n p+
n p+ n
p+ n p+
Vacancy in shell
Characteristic x-rays

by Compton scattering or by the photoelectric process, the production of fast-moving free electrons is essential to cause damage to critical molecules such as DNA.

Ionizing radiation can damage DNA either directly or indirectly (20). If an incident photon happens to be absorbed by a critical molecule such as DNA, that photon can cause direct biological damage. The vast majority of radiation-induced damage, however, is produced indirectly. In this case, free electrons produced by photon absorption interact with nearby molecules, commonly with water, to produce free radicals (22). Free radicals are highly reactive atoms or molecules that carry an unpaired orbital electron in an outer shell. These free radicals initiate a chain of reactions with other nearby molecules to produce a large number of ions, free radicals and other chemically reactive species that then go on to damage critical cellular molecules, most importantly DNA (22).

The damaging biologic effects of indirectly acting radiation can be modified by chemical agents that act as either radiosensitizers or radioprotectors (20, 22, 23). This is in contrast to the direct action of radiation that cannot be modified by these agents. A radiosensitizer is a chemical agent that increases the damage induced by a given dose of radiation, whereas a radioprotector prevents or diminishes the damage produced by the radiation. In other words, radiosensitizers cause decreased cell survival following radiotherapy, steepening the slope of the radiation dose-response curve; while radioprotectors cause increased cell survival, decreasing the slope of the curve (see the Materials and Methods section for more information on dose-response curves).

Molecular oxygen is an example of a radiosensitizer. Oxygen is a frequent participant in chemical reactions that produce the ions and free radicals which go on to cause biologic damage. On the other hand, chemical compounds that scavenge free radicals can protect cells and tissues from the damaging effects of radiation. Antioxidants, because of their properties as free radical scavengers, have been studied as potential clinically important radioprotectors (23).

In order to study the effects of agents that might alter the cellular response to radiation, it is important to have a precise definition of what we mean by "cell survival." When discussing the survival of cancer cells, the clinically relevant question to ask is whether the cells have the capacity to grow and form a tumor, to locally invade surrounding tissues, or to metastasize to distant sites. After being irradiated, a given tumor cell might continue to live and carry on with its metabolic functions; but if it is unable to divide indefinitely to produce a large number of daughter cells, it has become clinically inconsequential and can be considered dead. Hall refers to this phenomenon as "reproductive death" (20). In order to be considered a survivor, a cell must maintain the capacity to produce a colony of new cells. In other words, cell survival is defined in terms of clonogenicity.

Current *in vitro* data available from the scientific literature is inconclusive in respect to the possible interaction between high-dose vitamin C and radiotherapy. *In vitro* experiments conducted by Witenberg *et al*. demonstrated that preincubation with an ascorbic acid derivative protects HL60 myeloid leukemia cells against x-irradiation-

induced apoptosis (24). In a study of normal human lymphocytes treated with radiation *in vitro*, Konopacka and Rzeszowska-Wolny found evidence for a radioprotective effect of vitamin C at all tested concentrations when the vitamin was administered after irradiation (25). When given prior to irradiation, vitamin C was found to have a significant protective effect at low concentration (1 μg/mL) and a sensitizing effect at the highest concentration tested (20 μg/mL), though the sensitizing effect was not significant. Ortmann *et al*. studied the effect of vitamin C on radiation-induced apoptosis in normal human lymphoblastic cells *in vitro* (26). When given prior to irradiation, vitamin C had a slight but statistically significant radiosensitizing effect at 100 μM, the highest concentration tested. When given immediately after irradiation, vitamin C had a radioprotective effect at 0.01 μM, the lowest concentration tested.

The cumulative data obtained from animal models have been similarly inconclusive. In their study of Ehrlich ascites tumor cells *in vivo*, Koch and Biaglow found that dehydroascorbate, a metabolite of vitamin C, sensitized the tumor cells to radiation (27). Tewkif *et al*. administered ascorbic acid to mice *in vivo* and studied its effects on solid tumor growth and on tumor control by radiation (28). They found that tumor growth was significantly faster in control mice compared to mice receiving vitamin C. Additionally, tumor control by radiation was better in the ascorbic acid group than in controls. Okunieff performed *in vivo* experiments in mice investigating the effect of high-dose ascorbic acid on the radiation response of both normal tissues and transplanted tumor (29). Results showed that vitamin C protected normal bone marrow and skin from radiation damage without affecting the dose of radiation required to control 50 percent of

tumors or to achieve remission. Sarma and Kesavan found vitamin C to have a protective effect against γ-ray-induced chromosomal damage in mice as evidenced by a reduction in the frequency of micronuclei and chromosomal aberrations in the bone marrow cells of vitamin C-treated mice compared to controls after whole body γ-irradiation (30). Konopacka *et al*. conducted *in vivo* experiments in mice investigating the modifying effect of vitamin C on the clastogenic activity of γ-rays as measured by the number of micronuclei in bone marrow polychromatic erythrocytes and exfoliated bladder cells (31). When given before irradiation, vitamin C was found to be a radioprotector at low doses (50-100 mg/kg/day) and a radiosensitizer at the highest dose tested (400 mg/kg/day). When given after irradiation at the same high dose, however, vitamin C acted as a radioprotector.

Given the high prevalence of CAM use among cancer patients and the inconclusive results of the experimental data to date, the question of whether high-dose vitamin C alters the efficacy and/or toxicity of radiotherapy is an important clinical question to answer so that clinicians may better advise their patients.

Doxorubicin

Doxorubicin is an intravenously administered chemotherapeutic agent used commonly in the treatment of many cancers including cancers of the breast, endometrium and thyroid as well as both acute lymphocytic and acute myelogenous leukemias, Hodgkin's and non-Hodgkin's lymphomas, osteosarcoma, Kaposi's sarcoma, and soft-

tissue sarcomas. Acute and reversible adverse effects of doxorubicin include myelosuppression, nausea, vomiting and cardiac arrhythmias. The most important adverse effect of the drug, however, which may develop after weeks or months of repeated treatments with doxorubicin is irreversible chronic cardiomyopathy. The development of cardiomyopathy secondary to doxorubicin is related to the total cumulative dose of drug given (32). For this reason, it has been recommended that patients receive a total cumulative dose no greater than 550 mg/m^2 of body surface area (32) and that cardiac performance be assessed prior to the initiation of, during, and after completion of therapy with doxorubicin (33).

Doxorubicin is classified as an anthracycline antibiotic. Although its mechanism of action is not fully understood, doxorubicin is believed to cause its antineoplastic effects through a multifactorial, complex process. Multiple mechanisms of action have been proposed. For example, it has been postulated that doxorubicin alters DNA in a number of ways including: inducing DNA strand breaks, inducing DNA cross-links, interfering with DNA strand separation and helicase activity, or inhibiting DNA synthesis by intercalating with the sugar phosphate backbone or inhibiting DNA polymerase (34). A second category of proposed mechanisms has to do with disruption of membranes. Doxorubicin has been found to cause a dose-related decrease in membrane fluidity (35) as well as altering other membrane properties (36). A third major class of hypothesized mechanisms involves the generation of free radicals and other reactive oxygen species (34). This is one of the main mechanisms believed to be responsible for the cardiotoxicity of the drug.

In theory, free radical scavengers (such as antioxidants, which bind to free radicals thereby preventing oxidative damage) could protect cardiac and other normal tissues from the free radical-mediated damage caused by doxorubicin. The resultant reduction in adverse effects could allow for the use of increased drug doses leading to a possible improvement in tumor control. However, consideration must be given to the possibility that any protection provided to normal tissues may also be afforded to the tumor, essentially protecting cancer cells from the very treatment intended to destroy them.

Cell culture and animal model data examining the interaction of vitamin C and doxorubicin is inconclusive. Wells *et al.* studied the effect of L-ascorbic acid 2-phosphate, a less active and more stable ascorbate derivative, on doxorubicin toxicity in two strains of human breast cancer cells, one that is doxorubicin-sensitive and one that is doxorubicin-resistant. Whereas L-ascorbic acid 2-phosphate had no effect on the survival of cells of the doxorubicin-sensitive strain, it caused enhanced resistance in cells of the doxorubicin-resistant strain (37). A separate study of human breast cancer cells treated *in vitro* with doxorubicin found that vitamin C potentiated the cytotoxicity of doxorubicin (38). Shimpo and Fujita *et al.* studied the effects of ascorbic acid and two of its derivatives, CV-3611 and ascorbyl palmitate, on both the antitumor activity and the cardiotoxicity of doxorubicin in mice and guinea pigs (39, 40). They found that the incidence of doxorubicin-induced cardiomyopathy was significantly decreased in the animals receiving ascorbic acid while antitumor activity was not reduced. Furthermore,

mice and guinea pigs treated with any of the three forms of vitamin C together with toxic doses of doxorubicin lived longer than those treated with doxorubicin alone. Atunes and Takahashi investigated the effect of vitamin C on normal bone marrow cells of the Wistar rat treated with doxorubicin *in vivo* (41). They found that only the lowest studied dose of vitamin C demonstrated a protective effect as measured by total number of chromosome aberrations. The highest doses of vitamin tested produced no change in outcome compared to control.

Given the high prevalence of CAM use among cancer patients and the contradictory nature of the data available to date, the question of whether high-dose vitamin C alters the efficacy and/or toxicity of doxorubicin is an important clinical question to answer so that clinicians may better advise their patients.

EMT6 In Vivo – In Vitro Tumor System

Given the technical and ethical limitations of studying tumor response to therapy in humans, experimental oncology has been faced with the challenge of developing animal and cell culture models for human cancers that lend themselves to experimentation. Spontaneous tumors, while valuable experimental models, present a high level of variability, offer only small numbers of homogeneous tumors for experimentation, and are difficult to use in the measurement of therapeutic efficacy of different treatments. Inbred rodent strains make available large numbers of genetically identical animals, which have the benefit of greatly reducing variability in *in vivo*

experiments. These animals can be used to study transplanted tumor lines or to induce and study autochthonous tumors, tumors studied in the host of origin. *In vitro* models allow for even greater control over the experimental environment and interventions as well as more precise assays of cell survival. Despite having ideal characteristics for experimental manipulation, however, cells studied *in vitro* are not a perfect model for tumors existing *in vivo*. Unlike cells grown in culture, cells within a single tumor exist in different states of proliferation and in a variety of different environments in terms of oxygen concentrations, pH, nutrient availability and other parameters. *In vivo-in vitro* cell lines have been developed to address this problem.

In vivo-in vitro cell lines are transplanted tumor cell lines that have been selected or adapted for growth in cell culture. Prior to the development of an *in vivo-in vitro* tumor system, data from cell culture was compared with that from animal experiments using different cell lines. The great advantage of an *in vivo-in vitro* tumor system is that it allows for study of a single cell line both in culture and in animals, thereby benefiting from the advantages offered by both models. For example, cells can be injected into research animals and the subsequent tumors studied and treated in an environment that closely resembles that of spontaneous human tumors. These same solid tumor cells, having been treated *in vivo*, can then be suspended, plated and assayed *in vitro* for cell survival.

The EMT6 cell line, used in all experiments of the present study, is an *in vivo-in vitro* tumor system first isolated and characterized by Rockwell *et al.* (42). The original

tumor was the primary mammary tumor KHJJ-1 that arose in a BALB/c mouse. This tumor was serially transplanted in BALB/c mice and then put into culture. After multiple *in vitro* passages, the cell line was cloned and several of the clones characterized. The sixth clone, EMT6, was selected for having desirable characteristics for a model system to be used in experimental oncology. The EMT6 cell line grows rapidly in culture with a doubling time of about 12 to 16 hours, has a high plating efficiency of about 80 percent, and produces tumors when injected into BALB/c mice (43). Because of its lack of differentiation, the EMT6 tumor cell line, like all *in vivo-in vitro* tumor systems, should be considered a model for aggressive, rapidly growing tumors in general as opposed to being a model for a narrower subset of human tumors which share the same histology as the EMT6 cell line.

STATEMENT OF PURPOSE

To investigate the potential clinical significance of the concomitant use of mega-dose vitamin C with conventional cancer therapies, I asked whether ascorbic acid alters the response of breast cancer cells *in vitro* either to radiation or to doxorubicin, a commonly used antineoplastic agent. The interactions of varying doses of L-ascorbic acid with ionizing radiation and doxorubicin were analyzed using cultures of EMT6 mouse mammary carcinoma cells, the specific aim being to elucidate any modifying effect of the vitamin on the antineoplastic effects of treatment.

MATERIALS AND METHODS

Cell Culture

EMT6 mouse mammary carcinoma cells (sub-line EMT6-Rw) were used for all experiments. Experimental cell cultures were initiated using exponentially growing cells from already established cell lines. These cells were incubated at 37ºC in a humidified atmosphere of 95 percent air and 5 percent CO_2. Stock cultures were routinely passed by laboratory personnel using Waymouth's medium supplemented with 15 percent serum (made up of fetal clone and fetal bovine serum at a 1:1 ratio), and antibiotics (100 U/mL penicillin-G, 100 µg/mL streptomycin sulfate, 5 µg/mL gentamycin and 0.125 µg/mL fungizone). To ensure tight control of L-ascorbic acid levels, experimental cultures were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 percent dialyzed fetal bovine serum (dFBS), neither of which contain ascorbic acid or other redox-active small molecules. The experimental culture medium was additionally supplemented with the same antibiotics as the Waymouth's medium described above. All cell cultures were grown in 60-mm Petri dishes.

Drugs and Radiation

Powdered L-ascorbic acid, obtained from Sigma-Aldrich, was stored at room temperature and protected from light to prevent degradation. Immediately prior to each

experiment, ascorbic acid powder was dissolved in sterile phosphate-buffered saline (PBS) at a pH of 7.1 and sterilized by filtration through a 0.22-μm filter. The solution was then protected from light until it was added to the cell culture medium. Petri dishes were immediately returned to the incubator after addition of ascorbic acid to minimize light exposure.

Cells were irradiated with graded doses of radiation using 250 kVp x-rays (15 mA, 2 mm A1 equivalent filtration) from a Siemen's Stabilipan x-ray therapy machine at a dose rate of 1.1 Gy/min.

Doxorubicin was obtained from American Pharmaceutical Partners, Inc.

Growth Experiments

Preliminary growth studies were performed to determine the toxicity of L-ascorbic acid in the absence of radiation or antineoplastic agents. EMT6 mouse mammary tumor cells were harvested from exponentially growing cultures and plated into 60-mm dishes in a total of 5 mL DMEM and at an initial concentration of 2×10^5 cells per dish. Prior to cell plating, the culture medium was allowed to equilibrate in the incubator to attain the proper pH and temperature. Four hours after cell culture initiation, the plated cells were treated with solutions of high purity L-ascorbic acid powder dissolved in PBS to produce concentrations of 100 μM, 1 mM or 10 mM in the culture medium. A fourth group, not treated with ascorbic acid, served as the control. Each of

the four groups was then incubated and allowed to grow. Each day, two Petri dishes were selected from each treatment group. The cells in these dishes were suspended and counted; and the number of cells per dish was calculated. Cells from one dish per group were plated and a colony formation assay was performed to assess viability (see below for details). Although this experiment was repeated twice, the first experiment followed the growth of the cells in cultures for only 2 days. When the experiment was repeated, cell growth was studied daily for 4 days. The duration of the study was chosen so that the cultures were followed throughout the period when the control cultures were expected to be in exponential growth. Only data from the second experiment is shown.

Radiation Experiments

To determine the effect of L-ascorbic acid on cellular radiosensitivity, exponentially growing stock EMT6 tumor cells were trypsinized, suspended in DMEM, counted and diluted to a concentration of 2×10^5 cells/mL. One mL of cells was then plated into each of 18 Petri dishes containing 4 mL of DMEM that had been previously equilibrated to the proper pH and temperature. Cultures were incubated for 3 days under the conditions described above, allowing the cells to reach the middle of the exponential growth phase prior to treatment. After 3 days of growth, the medium was removed from all dishes and replaced with fresh DMEM. Two treatment groups were then treated with solutions of high purity L-ascorbic acid powder dissolved in PBS to produce concentrations of either 1 mM or 10 mM in the culture medium. Cultures grown and treated under identical conditions but in the absence of L-ascorbic acid were used as

controls. After 4 hours of exposure to ascorbic acid, cells were irradiated with doses of 0.0, 2.5, 5.0, 7.5 or 10.0 Gray. Immediately following irradiation, the medium was removed from the cultures, and the cells were washed, detached from the dish using 0.05 percent trypsin, suspended in Waymouth's medium and assayed for colony formation (see below for details). This experiment was repeated three times.

Doxorubicin Experiments

To determine the effect of L-ascorbic acid on clonogenic cell survival after treatment with doxorubicin, exponentially growing stock EMT6 tumor cells were trypsinized, suspended in DMEM, counted and diluted to a concentration of 2×10^5 cells/mL. One mL of cells was then plated into each of 15 Petri dishes containing 4 mL of DMEM that had been previously equilibrated to the proper pH and temperature. Cultures were incubated for 3 days allowing the cells to reach the middle of the exponential growth phase prior to treatment. After 3 days of growth, the medium was removed from all dishes and replaced with fresh DMEM. Two treatment groups were then treated with solutions of high purity L-ascorbic acid powder dissolved in PBS to produce concentrations of either 1 mM or 10 mM in the culture medium. Cultures grown and treated under identical conditions but in the absence of L-ascorbic acid were used as controls. After 2 hours of exposure to ascorbic acid, cells were treated with doxorubicin at concentrations of 0.0, 0.1, 0.4, or 1.6 μg/mL. The doses of doxorubicin were selected based on prior studies (44). After a total exposure time of 4 hours for ascorbic acid and 2 hours for doxorubicin, the medium was removed from the cultures, and the cells were

washed, trypsinized, suspended in Waymouth's medium and assayed for colony formation as described below. This experiment was repeated three times.

Colony Formation Assay

To determine the survival of cells following treatment, colony formation assays were performed for all experiments described above. After trypsinizatioin, the single-cell suspensions were counted using a Coulter Counter model ZBI. Cell volume distributions were also determined to ensure that the cell counts were accurate and uncompromised by the clumping together of multiple cells. Cells were then diluted and plated at low densities in Waymouth's medium. Each treatment group was plated at multiple different densities to ensure that the resultant colonies would grow at such a density that individual colonies could be distinguished and counted. Cells were then incubated and allowed to grow for 2 weeks at which time the colonies were washed, fixed with methanol and stained with crystal violet. Each culture was projected on the wall for magnification and colonies of 50 cells or more were counted to determine the survival of cells with clonogenic capacity. To ensure an objective count, colony counting was performed by lab workers other than this student investigator who were blinded to the treatments received by the cells in the different Petri dishes. All the cultures from each experiment were counted by a single observer to prevent error from inter-observer variability.

Data Analysis

After all the cultures from an experiment were counted, the plating efficiency of each group was calculated. Plating efficiency (PE) is the percentage of single cells plated that gives rise to colonies and is calculated by the following equation:

$$\mathrm{PE} = \frac{\text{number of colonies formed}}{\text{number of cells plated}} x100$$

In these experiments the mean plating efficiency of untreated control cells was 94.0% with a standard deviation of 15.1.

A surviving fraction (SF) for each treatment group was determined by taking the ratio of the plating efficiency of the treated culture to that of the untreated control culture (i.e. cells treated with neither ascorbic acid nor radiation) plated in the same experiment on the same day:

$$\mathrm{SF} = \frac{\text{treatment group plating efficiency}}{\text{control group plating efficiency}}$$

These surviving fractions were used to generate a radiation dose-response curve. This was accomplished by conducting a non-linear regression analysis using the StatMost Statistical Package to obtain the best fit of the following equation to the experimental data (20, 21):

$$SF = 1 - \left(1 - e^{-D/D_0}\right)^n$$

This equation defines the surviving fraction (SF) at a given radiation dose (D). The other variables in the equation define different parts of the dose-response curve. D_0 describes the slope of the linear segment of the curve. Specifically, D_0 is the dose required to reduce the surviving fraction by $1/e$ on the linear portion of the curve. The extrapolation number, n, is a description of the curve's shoulder. More precisely, n is the calculated surviving fraction at which the linear section of the survival curve, extrapolated back to zero dose, hits the y-axis. Because vitamin C alone was found to produce a small toxic effect in the radiation studies, the SF's of the groups treated with vitamin C plus radiation were normalized using the PE's of the vitamin C-treated cultures before these curves were fitted.

To quantify the extent to which vitamin C alters the radiation response of EMT6 cells, data from the dose-response curve was used to calculate the dose-modifying factor (DMF) for ascorbic acid. DMF for radioprotectors is defined as the ratio of the dose of radiation with the modifying agent (i.e. vitamin C) to the dose of radiation without the agent that results in the same level of cell survival (20):

$$DMF = \frac{\text{radiation dose with modifying agent resulting in an SF of } 0.1}{\text{radiation dose without modifying agent resulting in an SF of } 0.1}$$

DMF's were calculated by extrapolating the computer-fitted curves to a surviving fraction of 0.1. A DMF greater than 1.0 indicates protection, whereas a DMF less than 1.0 indicates sensitization. The Wilcoxon test was performed using the Statmost Statistical Package to determine whether the differences between the curves were statistically significant. Statistical significance was defined as a p-value of less than 0.05.

RESULTS

Growth Experiments

Preliminary growth studies examined the growth curves for EMT6 cells to determine the effects of different doses of ascorbic acid on the growth of these cells *in vitro*. The three different concentrations of L-ascorbic acid used in the experiment were 100 μM, 1 mM and 10 mM. Cells grown under identical conditions but in the absence of ascorbic acid were used as controls. Figure 3 shows the growth curve of each of the four groups of cells studied. No error bars are shown because this graph represents data from one single experiment. When EMT6 cultures were grown in the presence of 1 mM or 10 mM concentrations of vitamin C, there was a significant reduction in cell growth compared to control cultures. The cells grown in the presence of the smallest concentration of vitamin C tested, 100 μM, showed a slight decrease in growth. This slight reduction in cell growth, however, cannot be tested for statistical significance based on data from only one experiment.

After counting the cells in each of the four groups to determine cell growth, the cells were then assayed for clonogenic capacity. On each of the 4 days of the experiment, cells from one dish per treatment group were plated in fresh media not containing any ascorbic acid. The cells were incubated for 2 weeks and were then washed, fixed and stained. Colonies of 50 cells or more were counted. In this way, the clonogenic capacity was determined for cells after exposure to ascorbic acid for 1, 2, 3 or 4 days for each of the 3 concentrations of vitamin C tested. Surviving fractions were then calculated by

FIGURE 3. Growth study of EMT6 cells grown in the presence of different concentrations of L-ascorbic acid. Points are arithmetic means of cell numbers from 2 dishes, obtained in a single experiment. Key: λ represents control cells; ❍ represents cells treated with 100 μM L-ascorbic acid; ▼ represents cells treated with 1 mM L-ascorbic acid; ∇ represents cells treated with 10 mM L-ascorbic acid.

FIGURE 3.

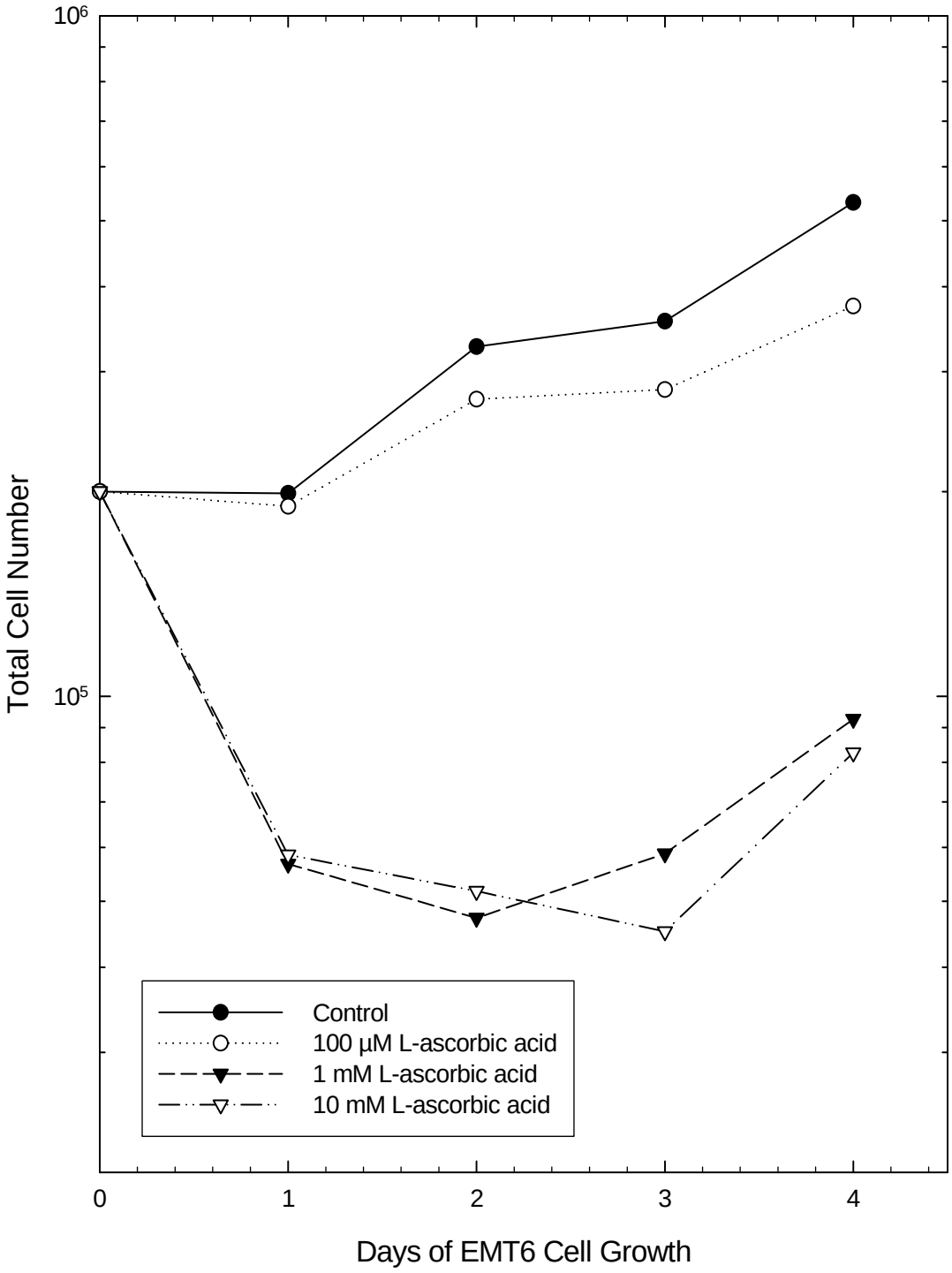
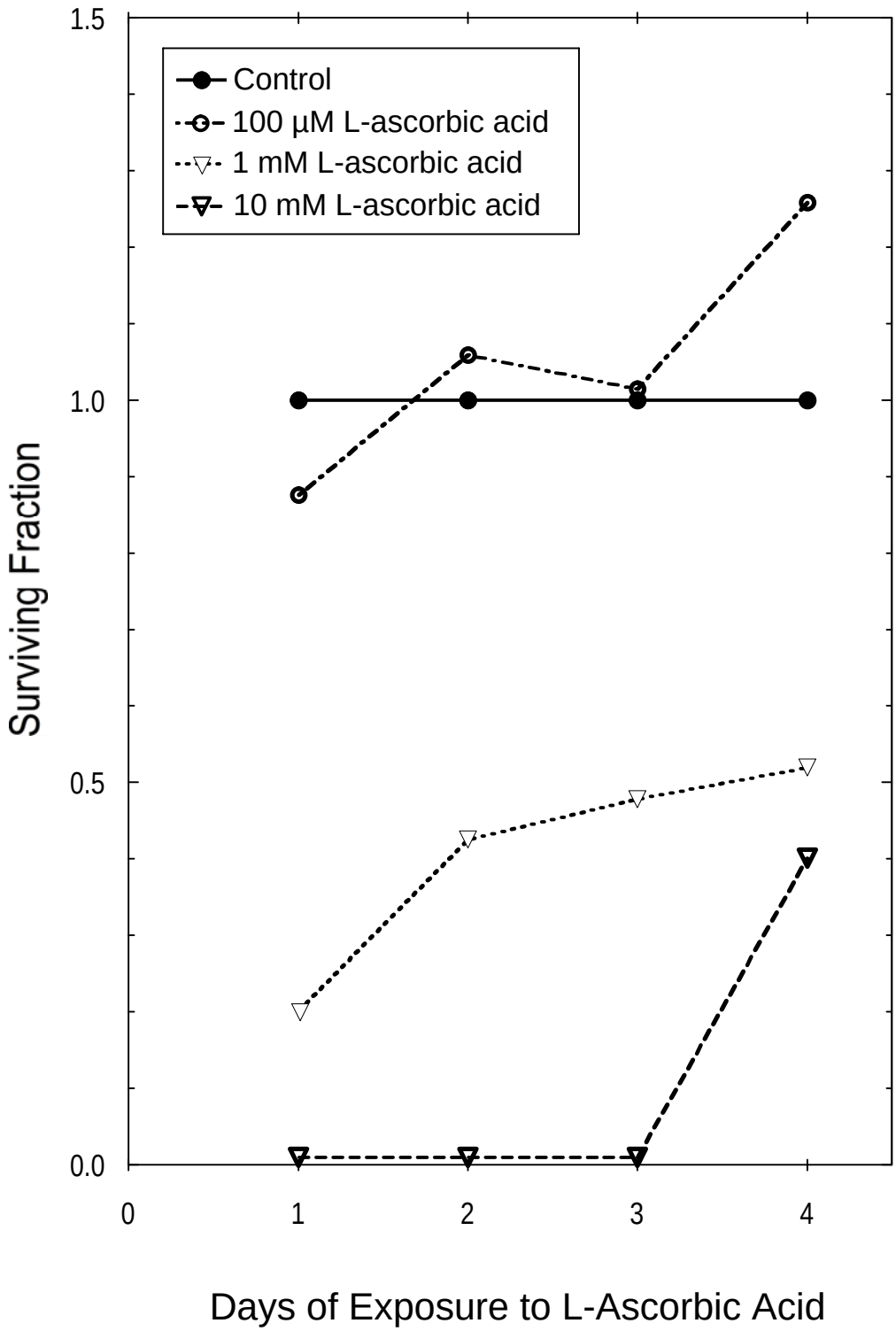

10^6
10^5
Total Cell Number
0
1
2
3
4
Days of EMT6 Cell Growth
Control
100 µM L-ascorbic acid
1 mM L-ascorbic acid
10 mM L-ascorbic acid

FIGURE 4. Survival of EMT6 cells grown in the presence of different concentrations of L-ascorbic acid. Each point is the surviving fraction of cells from one dish, obtained in a single experiment. Key: λ represents control cells; ❍ represents cells treated with 100 μM L-ascorbic acid; ▼ represents cells treated with 1 mM L-ascorbic acid; ∇ represents cells treated with 10 mM L-ascorbic acid.

FIGURE 4.


Control
100 µM L-ascorbic acid
1 mM L-ascorbic acid
10 mM L-ascorbic acid
Surviving Fraction
1.5
1.0
0.5
0.0
0
1
2
3
4
Days of Exposure to L-Ascorbic Acid

comparing the plating efficiencies of the treatment groups to that of the control group. Figure 4 illustrates these surviving fractions. The cells exposed to 1 mM or 10 mM vitamin C show a significant reduction in clonogenic capacity compared to cells in the control group. This means that the presence of high concentrations of vitamin C in this study not only caused a reduction in cell proliferation (see Figure 3), but also reduced the capacity of existing cells to produce new colonies even after the vitamin was removed from the culture medium (see Figure 4). Clonogenicity was seen to improve, however, with increasing exposure time to the vitamin as illustrated by the positive slopes of the lines graphed in Figure 4.

In summary, results of a preliminary growth study suggest a slightly toxic effect of 100 μM vitamin C in comparison to control, and a significant toxicity of 1 mM and 10 mM L-ascorbic acid causing a reduction in both cell proliferation and clonogenic capacity. The growth study results presented above were considered when choosing the concentrations of ascorbic acid to be used in subsequent studies of the effect of vitamin C on the outcomes of treatment with radiation and doxorubicin. Concentrations of 1 mM and 10 mM L-ascorbic acid were selected for these subsequent experiments despite the demonstrated toxicity of the vitamin at these levels. It is important to note that in the preliminary growth study, cells were exposed to ascorbic acid for durations of 1, 2, 3 or 4 day, whereas the cells in subsequent experiments were exposed to the vitamin for only 4 hours.

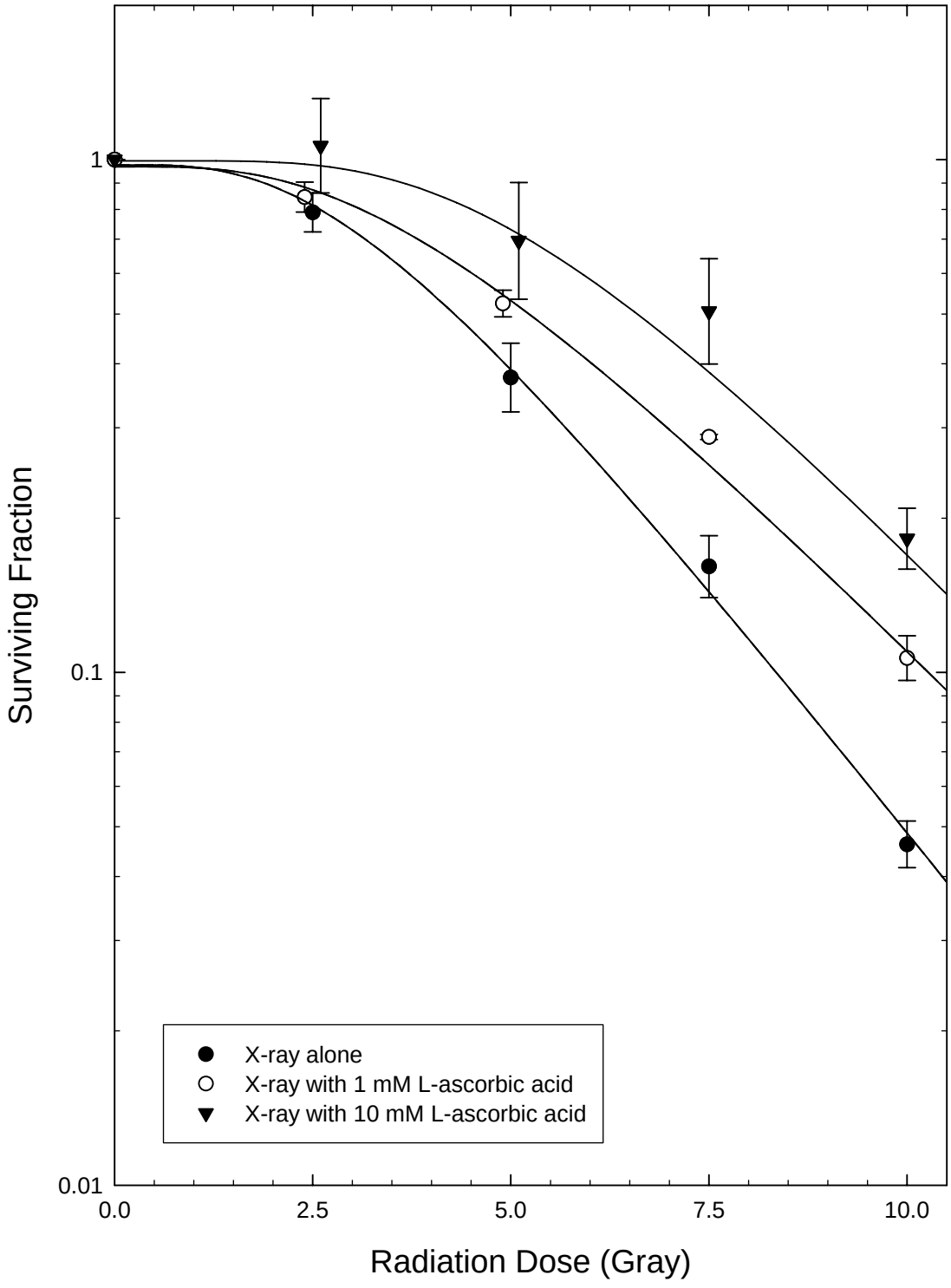
Radiation Experiments

Figure 5 shows data from experiments examining the effects of two different high concentrations of L-ascorbic acid on the cellular radiosensitivity of EMT6 tumor cells. Data points on the graph represent the geometric means ± the standard errors of the mean (S.E.M.s) of surviving fractions determined in three independent experiments. Following convention, radiation dose is plotted along the x-axis on a linear scale while surviving fraction is plotted along the y-axis on a logarithmic scale. The curves representing cells treated with 1 mM and 10 mM vitamin C have been corrected for ascorbic acid toxicity. The surviving fractions for cells treated with vitamin C alone were 0.86 and 0.56 for cells treated with 1 mM and 10 mM vitamin C, respectively. As expected, cell survival decreases with increasing dose of ionizing radiation as illustrated by the down-sloping curves in all three groups.

The figure shows a significant change in the radiosensitivity of cultures treated with radiation plus vitamin C as compared to those treated with radiation alone. The survival curve for cultures treated with 1 mM ascorbic acid lies above the control curve on the graph. The difference between these two curves was found to be statistically significant ($p = 0.012$). The survival curve for cultures treated with 10 mM ascorbic acid lies even further above the control curve ($p = 0.003$) and well above the curve representing survival of cells treated with the lower dose of vitamin C ($p = 0.011$). This higher rate of cell survival among those cells exposed to high-dose ascorbic acid

FIGURE 5. Effects of L-ascorbic acid on the radiation dose-response curve of exponentially growing EMT6 cells. Points are geometric means ± standard errors of the mean (S.E.M.s) of survivals determined in 3 independent experiments. Curves were fitted as described in the Material and Methods section. Key: λ represents control cells; ❍ represents cells exposed to 1 mM L-ascorbic acid for 4 hours immediately prior to irradiation; ▼ represents cells exposed to 10 mM L-ascorbic acid for 4 hours immediately prior to irradiation.

FIGURE 5.


Surviving Fraction
1
0.1
0.01
0.0
2.5
5.0
7.5
10.0
Radiation Dose (Gray)
X-ray alone
X-ray with 1 mM L-ascorbic acid
X-ray with 10 mM L-ascorbic acid

indicates a protective effect of the vitamin. Furthermore, this protective effect was greatest at the highest concentration of vitamin C tested.

The dose-modifying factor (DMF), the ratio of the dose of radiation with the modifying agent to the dose of radiation without the agent (in this case, vitamin C) that results in the same level of cell survival (in this case, a surviving fraction of 0.10), was calculated from these survival curves. A DMF greater than 1.0 indicates protection, whereas a DMF less than 1.0 indicates sensitization. The DMF for 1 mM ascorbic acid was calculated to be 1.23. This means that a concentration of 1 mM vitamin C alters the antineoplastic effects of radiation such that in order to achieve the same level of cell damage, a dose of radiation 23 percent higher would need to be given. The calculated DMF for 10 mM ascorbic acid was 1.37, indicating that at this higher concentration of vitamin C the radiation dose would need to be 37 percent higher in order to result in the same level of radiation-induced damage. In summary, the results of radiation experiments show that mega-doses of vitamin C protect EMT6 mouse mammary tumor cells from the cytotoxic effects of ionizing radiation.

Doxorubicin Experiments

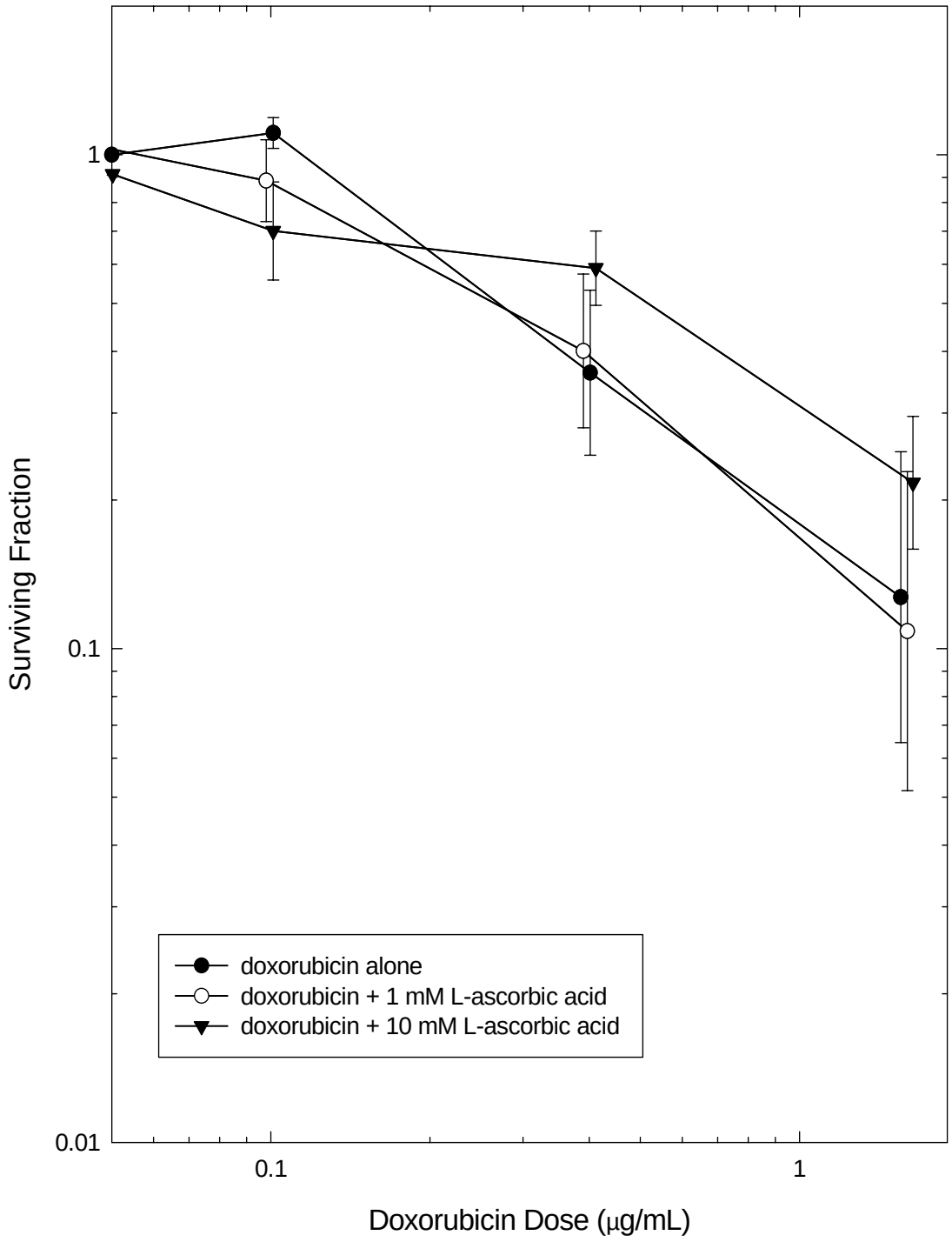
Figure 6 shows data from experiments examining the effects of two different high concentrations of L-ascorbic acid on the survival of EMT6 tumor cells treated with doxorubicin. Data points on the graph represent the geometric means ± S.E.M.s of

surviving fractions determined in three independent experiments. The surviving fractions for cells treated with vitamin C alone were 1.03 and 0.91 for cells treated with 1 mM and 10 mM vitamin C, respectively. Doxorubicin dose is plotted along the x-axis; surviving fraction is plotted along the y-axis. Both doxorubicin dose and surviving fraction are shown on a logarithmic scale. Cell survival decreases with increasing dose of doxorubicin in all three groups, illustrated by the down-sloping curves.

There is no difference in the survival curves of cultures treated with 0, 1, or 10 mM ascorbic acid, as can be seen from the overlapping error bars in the figure. The Wilcoxon test was performed and no statistically significant difference was found between the control group and the cells exposed to 1 mM ascorbic acid ($p = 1.0$), between the control group and the cells exposed to 10 mM ascorbic acid ($p = 0.75$), or between the cells exposed to 1 mM and 10 mM ascorbic acid ($p = 0.75$). This data suggests that L-ascorbic acid has no effect on the sensitivity of EMT6 mouse mammary carcinoma cells to the antineoplastic agent doxorubicin.

FIGURE 6. Effects of L-ascorbic acid on the response of exponentially growing EMT6 cells to doxorubicin. Points are geometric means ± S.E.M.s of survivals determined in 3 independent experiments. Key: λ represents control cells; ❍ represents cells exposed to 1 mM L-ascorbic acid for 4 hours; ▼ represents cells exposed to 10 mM L-ascorbic acid for 4 hours. All cells were exposed to the indicated dose of doxorubicin for 2 hours.

FIGURE 6.


Surviving Fraction
1
0.1
0.01
0.1
1
Doxorubicin Dose (μg/mL)
doxorubicin alone
doxorubicin + 1 mM L-ascorbic acid
doxorubicin + 10 mM L-ascorbic acid

DISCUSSION

The aim of this research was to elucidate any modulatory effect ascorbic acid may have on the cytotoxic effects of radiation or doxorubicin on EMT6 mouse mammary carcinoma cells. The findings from the radiation studies reported here demonstrate that vitamin C decreases the radiosensitivity of EMT6 tumor cells. The dose-modifying factors for 1 mM and 10 mM ascorbic acid as compared to controls were 1.23 and 1.37, respectively. This indicates a radioprotective effect of vitamin C. Furthermore, greater radioprotective effect is seen with the higher concentration of vitamin C. Specifically, the data show that in order to achieve the same level of cell damage in the presence of 1 mM or 10 mM vitamin C, one would have to administer a dose of radiation 1.23 or 1.37 times that actually given, respectively. No chemomodulatory effects of vitamin C were observed in the doxorubicin studies.

It is important to view these results in the context of other research that has been done on vitamin C and its potential role as an adjuvant treatment in cancer therapy. The clinical question is essentially this: given the well-established antioxidant properties of vitamin C, does vitamin C reduce the toxicity of radiotherapy or chemotherapy to normal tissues without compromising their antineoplastic effects? Furthermore, if this question cannot be definitively answered on the basis of current medical research, what recommendation about vitamin C supplementation should be made to cancer patients undergoing conventional treatment?

Because the results of this thesis support the hypothesis that vitamin C alters the antitumor effects of radiation but not of doxorubicin, this discussion will focus on the existing evidence for and debate over whether vitamin C is beneficial to cancer patients undergoing radiotherapy. Overall, *in vitro* and animals studies investigating the interaction between ascorbic acid and radiotherapy have been contradictory and inconclusive (24-31). Particularly concerning for its clinical implications is the fact that some studies not only fail to find a beneficial effect of vitamin C, but rather demonstrate that vitamin C may actually cause harm by reducing the antineoplastic efficacy of conventional therapies (24, 37).

Of course, although cell culture and animal data are important in deciphering the interactions of vitamin C with conventional cancer therapies, a randomized, double-blind, placebo-controlled clinical trial is necessary to definitively answer the question of whether vitamin C supplementation has a role to play in cancer treatment. Such a trial would need to include large numbers of patients to have sufficient power to detect small but important differences between the vitamin C and control groups. To date, no such clinical trial has been done. The closest approximation is an historical cohort study conducted in 2002 by Lesperance *et al.* (45). In this study, survival and recurrence outcomes for 90 women with unilateral non-metastatic breast cancer who had been prescribed mega-doses of vitamin C in addition to other vitamins and minerals while concurrently undergoing conventional therapy were compared to 180 rigorously-matched controls. Patients were not excluded from the study based on the type of conventional treatment received. Sixty-nine percent of study subjects were treated with systemic

therapy, with or without radiation or surgery. Sixty-five percent were treated with radiation, with or without systemic therapy or surgery; the radiation status of 6 percent of the patients is unknown. Results showed shorter disease-free survival and breast cancer-specific survival for the mega-dose vitamin group compared to controls. Although these differences were not statistically significant ($p = 0.07$ for disease-free survival; $p = 0.16$ for breast cancer-specific survival) they none-the-less reinforce the concern that the use of high-dose vitamin C during cancer therapy cannot be assumed to be benign.

As always in the quest to find more efficacious and better-tolerated treatments for cancer, the concept of the therapeutic ratio is both clinically relevant and important. The therapeutic ratio is defined as the maximum tolerated dose of a drug to the effective dose. When assessing the clinical benefit of any potential therapy modulator, such as vitamin C, it is imperative to consider the effect on the response of the tumor as well as the effect on the response of the dose-limiting normal tissues. Treatments that lead to equal changes in tumor response and dose-limiting normal tissue response will have a net effect of zero improvement in outcome. Only those treatments that increase tumor response relative to the dose-limiting normal tissue response will result in therapeutic gain (22). Following this logic, in order to demonstrate the value of vitamin C as an adjuvant therapy to radiation, it is not sufficient to show that vitamin C protects dose-limiting normal tissues from radiation injury. One must also provide evidence that there is *relative* protection of these normal tissues compared to the tumor.

The scientific and medical data currently available do not provide sufficient evidence to support the claim that vitamin C improves the therapeutic ratio of either radiation or doxorubicin. In fact, results of the research conducted for this thesis suggest that high-dose ascorbic acid may actually decrease the efficacy of radiotherapy. Given the high prevalence of complementary and alternative medicine use among cancer patients, it is extremely important that clinicians address the topic of vitamin supplementation with their patients who are undergoing standard therapy. In her recent review article of antioxidants during chemotherapy and radiotherapy, D'Andrea cautions that if antioxidants compromise the efficacy of cancer treatment by even a few percentage points, their use could lead to hundreds or thousands of deaths per year (46). Until proven otherwise, high-dose vitamin C must be considered potentially dangerous when taken by cancer patients concomitantly with radiation, and patients should be advised against it.

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