

The Potential Role of Ascorbic Acid and its Salts in the Management
of Malignant Diseases

by

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A dissertation submitted in partial satisfaction of the
requirements for the degree of

Doctor of Philosophy

2005

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Abstract

The effects of ascorbic acid on the prevention and treatment of malignant disease have been controversial for many years. This manuscript reviews the history of ascorbic acid in the prevention and treatment of malignancies and presents original research data that support the use of ascorbic acid and its salts in the management of people with malignancies. In particular, the research data demonstrate that host-nontoxic concentrations of ascorbic acid that are toxic to tumor cells can be achieved in human beings with cancer. Other mechanisms by which ascorbic acid can be utilized as a nontoxic cancer treatment are discussed. Clinical protocols which have been used successfully by clinicians for several years are also described. The objective of this manuscript is to provide an updated scientific basis for the use of ascorbic acid, especially intravenous salts of ascorbic acid, as an adjuvant treatment in pharmacological nutritional oncology.

Acknowledgments

Research conducted by this candidate and described herein was supported in part by a grant from the Flossie West Charitable Trust and the Olive White Garvey Trust, and by the BioCommunications Research Institute. I wish to thank my father, Hugh D. Riordan, M.D., who gave me the opportunity and inspiration to research this subject—a visionary doctor who helped so many people by thinking of people more than thinking inside the box. I thank my great friend, Michael Gonzalez, Ph.D, D.Sc. my mentor, friend and colleague, who has spent endless hours of his valuable time contemplating and commiserating about this subject that is so important to both of us. I thank Xiaolong Meng, M.D. for being a great friend and intellectual fencing opponent, and Joe Casciari, a fantastic colleague and linear thinker. And, finally, and most importantly, I want to thank my wife, Shirley, and our wonderful children, Chloe, Michaela, Brinn, and Tierney, who have unselfishly sacrificed pieces of their lives to allow me to pursue my dreams.

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Chapter 1: Introduction

1.1. The Research Questions

This candidate addresses the following questions:

- Is there evidence that ascorbic acid (AA) at high concentrations can act as a pro-oxidant agent by generating hydrogen peroxide in the presence of oxygen?
- Is there evidence that vitamin C at high concentrations is preferentially toxic to cancer cells *in vitro* and *in vivo*?
- Can tumor cytotoxic concentrations of AA be clinically achieved by intravenous administration?
- Have AA oxidation products demonstrated antitumor activity?
- Does AA exhibit secondary anticancer mechanisms, perhaps by increasing the intracellular matrix, by suppressing angiogenesis, by increasing immunocompetence, or by acting as a mitochondrial energy intermediate?
- Are there certain nutrients, such as alpha lipoic acid, and vitamin K3, which potentiate the efficacy of ascorbic acid in its tumor preferential cytotoxicity, and which augment the potency of AA as a tumor cytotoxic chemotherapeutic agent?
- In the field of pharmacological nutritional oncology, what clinical possibilities might AA offer?

1.2 Goals of This Work

In addressing the questions listed above, this dissertation presents original laboratory and clinical research conducted and/or participated in by this candidate, as well as a literature review and analysis of the field and related research conducted by others.

The objective of this manuscript is to provide an updated scientific basis for the use of ascorbic acid, especially intravenous salts of ascorbic acid, as an adjuvant treatment in pharmacological nutritional oncology.

1.3 Organization

Beginning with descriptions of the biochemical properties and the biological functions of ascorbic acid, this manuscript progresses through an explanation of the mechanisms of ascorbic acid *in vitro*, *in vivo*, and in the management of malignant diseases. Specific data are presented in each section, with a discussion of this material preceding the conclusions.

Chapter 2: Biochemistry of Ascorbic Acid

History and Background

Ascorbic Acid (vitamin C) is a ketolactone with a molecular weight of 176.13 g/mol and molecular formula $C_6H_8O_6$. Although knowledge of its properties has advanced considerably since its discovery, a full scientific understanding of its numerous and complex functions is still evolving.

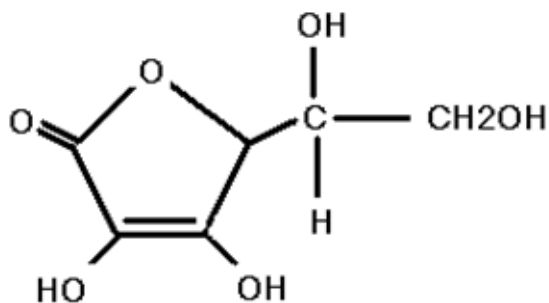
In 1913, Dr. E.V. McCollum, et al., at the University of Wisconsin, reported two types of “necessary food factors”, with one type being described as soluble in fats, and the other type as soluble in water (Pauling, 1986). In 1915, he named the vitamin that prevents scurvy “water soluble C”, as opposed to, for example, the one that prevents rickets, which he named “fat soluble D”. A number of efforts were made during subsequent years to isolate “water soluble C” from lemon juice and from other food, mostly without success. The term “vitamin” became the popular label for these “necessary food factors”, and in 1928, Albert-Szent Gyorgyi, M.D., Ph.D., the Hungarian physiologist and biochemist, became the first to successfully isolate vitamin C. His achievement occurred while he

was working on another problem, however, and he did not initially identify the substance that he had discovered. (Szent-Gyorgyi, 1980)

Dr. Szent-Gyorgyi had begun, in 1922, to study the oxidation reactions involved in the formation of brown pigmentation that appears in certain decaying fruits. He found that cabbages contain a reducing agent which prevents the formation of this brown pigment, and, coincidentally, he also found that the adrenal glands of animals contain the same, or a similar, reducing agent. While working in the Netherlands, he began his efforts to isolate this unidentified reducing agent from plant tissues and from adrenal glands. In 1928, while studying in Cambridge, England, on a fellowship from the Rockefeller Foundation, he succeeded in isolating the substance. He then spent a year at the Mayo Clinic, in Rochester, Minnesota, where he isolated 25 grams of the substance, and although he also discovered its chemical formula, $C_6H_8O_6$, no one had yet been able to identify it. At the suggestion of the editor of the journal to whom he submitted his paper on this discovery, Szent-Gyorgyi named this unknown compound "hexuronic acid". (Moss RW, 1988). He received his Ph.D. for this research, and in 1930 he then returned to Hungary where he found that Hungarian paprika also contains large amounts of this still unknown substance. In 1932, Szent-Gyorgyi and his collaborators in Hungary, along with the American investigators Waugh and King, were finally able to show that this substance is vitamin C. Szent-Gyorgyi then gave some of the crystalline material to the British sugar chemist, W.M. Haworth, who determined its structural formula, establishing the intermolecular connections between its atoms. Szent-Gyorgyi and Haworth together then changed its name to "ascorbic" acid, which translates literally as "without scurvy", to indicate the properties of this acidic substance in preventing and curing scurvy (Szent-Gyorgyi, 1960). In 1937, Albert Szent-Gyorgyi was awarded the Nobel Prize in Medicine or Physiology for this work, for his discoveries concerning the processes of biological oxidation, with special recognition of his achievements in isolating and describing vitamin C and in elucidating the role of fumaric acid in these processes.

Glucose: $C_6H_{12}O_6$

Ascorbic Acid: $C_6H_8O_6$



Ascorbic Acid molecule

The ascorbic acid molecule is strikingly similar to a glucose molecule. The molecular structure of dehydroascorbic acid, the oxidized form of ascorbic acid, bears the same resemblance. With the enzyme L-gulonolactone oxidase, glucose is converted into ascorbic acid by surrendering 4 hydrogen atoms, thereby producing 2 water molecules as a byproduct. Most mammals synthesize ascorbate in their livers in this manner, but humans and other primates (as well as fruit bats and guinea pigs) lack this enzyme and must therefore obtain ascorbic acid by external means only (Shimura et al., 1998). Whether occurring within the living cells of animals that produce their own vitamin C, or in the chemical plants that manufacture “synthetic” vitamin C, the chemical process is the same. As Linus Pauling stated, “The very simplicity of the molecule and its manufacture from glucose, the principal fuel sustaining life in tissue cells, suggest the importance of vitamin C and explain its ubiquity in the tissues of the body.” (Pauling, 1986)

In body fluids, ascorbic acid is dissociated into an ascorbate ion and a hydrogen ion. The hydrogen ion combines with basic groups of proteins or with a bicarbonate (HCO_3) ion, while it is this ascorbate ion that participates in the numerous physiological reactions that require vitamin C. This ascorbate ion is especially important in the synthesis of the protein collagen. The salts of ascorbic acid, in particular sodium ascorbate and calcium ascorbate, also dissolve in body fluids to produce ascorbate ions with the same properties. Although vitamin C may be taken by mouth, in solution or in tablet form, as ascorbic

acid, as sodium ascorbate, or as calcium ascorbate, only the latter two, the salts, may be taken intravenously, since the acid solution damages veins and tissues.

Ascorbic acid is able to engage on both sides of the universal oxidation reduction reaction, by either subtracting or adding hydrogen atoms to a molecule, and this property accounts for its vital role in fundamental cellular processes. Ascorbic acid is readily and reversibly oxidized to dehydroascorbic acid by the surrender of two hydrogen atoms to oxidizing agents, and dehydroascorbic acid, in turn, is easily reduced to ascorbic acid by picking up two hydrogen atoms. The reducing power of ascorbic acid and the oxidizing power of dehydroascorbic acid are responsible for many of the physiological roles in which these acids are engaged.

Among other functions, vitamin C is essential to the synthesis of collagen. Collagen, a protein consisting of strong white fibers (stronger than steel wire of the same weight), yellow elastin networks, and macropolysaccharides, constitutes the connective tissue that holds our bodies together. When people die of scurvy, they have stopped manufacturing collagen to the point that the cellular architecture of the body can no longer hold itself together; joints, cartilage and tendons, vessels and veins, gums and organs break open and lose their structural integrity as well as their functional ability, without adequate amounts of ascorbic acid, and death results. The role that ascorbic acid plays in the synthesis of collagen is of key importance in the extracellular matrix, which will be described in more detail in section 4.1.4.

A commonly acknowledged biochemical property of ascorbic acid is its role in the acceleration of hydroxylation reactions throughout a number of biosynthetic pathways. In many of these reactions, ascorbate either directly or indirectly provides electrons to enzymes that require prosthetic metal ions in a reduced form to achieve full enzymatic activity. Ascorbate thus plays a vast and seemingly unique role in the electron transport chain, and in its ability to scavenge free radicals (Cathcart, 1985). Additionally, one of its most widely recognized biochemical roles is that of cofactor for prolyl and lysyl hydroxylase enzymes in the biosynthesis of collagen (Levine, 1986).

The three dimensional architecture of vitamin C plays an important role in determining its biochemical functions. As a chiral substance, vitamin C exhibits “handedness”. Ascorbic acid is more correctly known as “L-ascorbic acid”, to identify its molecule as left-handed rather than right-handed. Throughout this thesis, “AA” and “LAA” are both used interchangeably to refer to the same substance: that which is known as ascorbic acid, as L-ascorbic acid, as ascorbate or as vitamin C.

Some inorganic substances, such as mineral quartz, form both left- and right-handed crystals, and this handedness determines their properties. (One rotates the plane of polarized light in a clockwise direction, and the other rotates it in a counterclockwise direction, though at exactly the same angle). Likewise, chirality is also a defining feature of the function of organic substances. The chirality of organic molecules, built around the carbon atom, is derived from a property of carbon and its four bonds, to which either left- or right-handed molecules may be attached. All carbohydrates are chiral. Glucose, also known as dextrose, is, as its name implies, right-handed; fructose, also known as levulose, is, as its name implies, left-handed. L-ascorbic acid is synthesized from D-glucose. Enzymes are chiral, as are all of the more than twenty amino acids that make up the proteins in human beings, in other animals, and in plants. Chains of amino acid residues are folded into stable proteins in such a way that their structures are only stable when they constitute exclusively one type of handedness or the other, but they cannot exhibit both simultaneously. It is an interesting occurrence in nature that all amino acids are left-handed, L-amino acids, except for glycine, which is identical with its mirror image.

Ascorbic acid has 3 isomers, yielding 4 ascorbic acid molecules with identical atomic constituents linked to one another in the same order, but arrayed differently in three-dimensional space. The right-handed isomer, D-ascorbic acid, also known as D-xyloascorbic acid, possesses the same melting point, water solubility and other chemical properties as L-ascorbic acid, yet it does not share the same biochemical properties and is incapable of protecting against scurvy. D-ascorbic acid exhibits no vitamin C activity, and neither do the other 2 polymers; however, all of these stereoisomers exhibit the same

activity as reduction and oxidation agents. Nevertheless, the 3 isomers of L-ascorbic acid offer no protection against scurvy, nor could they be utilized in any of the laboratory or clinical research described in this paper (unless otherwise indicated), for which only L-ascorbic acid is suitable. (A few notable exceptions to this exist, in which some of the isomers have been found to exhibit greater preferential tumor cytotoxicity than L-ascorbic acid). The unique properties of L-ascorbic acid therefore depend not merely upon its reducing and oxidizing abilities, but upon the specific three-dimensional configuration of its molecules. Unlike its 3 isomers, only L-ascorbic acid is able to fit into a complementary cavity in the hydroxylation enzymes, thereby forming a reactive complex, by which it is involved in collagen synthesis and a host of other processes.

This hydroxylation reaction also plays a major role in many other physiological processes, and L-ascorbic acid is required in each one. Carnitine is synthesized from the amino acid lysine in order to fuel the contraction of muscle fiber, for example, and this synthesis transpires through five successive reactions, each catalyzed by a specific enzyme. The second and fifth steps involve hydroxylation, for which L-ascorbic acid is needed. Hydroxylation reactions mediated by large amounts of L-ascorbic acid in the adrenal glands also convert the amino acid tyrosine first to dopa, then to dopamine and finally to noradrenaline in the manufacture of the adrenaline hormone, and for this reason L-ascorbic acid is needed in especially large quantities in the adrenals. L-ascorbic acid is not destroyed in this cycle but is reconstituted from semidehydroascorbate via the electron transport mechanism, another fundamental process for which L-ascorbic acid is essential.

Without the hydroxylation of proline, collagen is soluble at body temperature. Clearly, the role of ascorbic acid in such hydroxylation processes is crucial to the normal functioning of healthy cells.

Chapter 3: Biological Functions of Ascorbic Acid

As described by Casciari, et al. (2000), cancer patients exhibit extra vitamin C consumption. The unusually high need for ascorbic acid as found in cancer patients is no doubt a result of the multitude of biological functions in which this vitamin plays a key role. When the body is burdened with the molecular and cellular demands of cancer, the physiological importance of vitamin C becomes magnified.

Ascorbate, as it exists in most biological settings ($pK=4.2$), is an essential vitamin for humans (Food and Nutrition Board, 1989). As mentioned, scurvy, the deficiency disease arising from a lack of ascorbate, can reach life threatening levels and even death (Guthrie, 1989). Ascorbate is considered to be the most important antioxidant in extracellular fluid (Sies, Stahl, and Sundquist, 1992). Its water solubility allows it to be distributed throughout the body, with high concentrations found in a number of tissues including the eye lens, white blood cells, adrenals and pituitary glands (Levine, 1986). Ascorbate is also required in the synthesis of carnitine from lysine (Leibovitz and Mueller, 1993), neurotransmitter synthesis (Levine, 1986), cytochrome P-450 activity, cholesterol metabolism and detoxification of exogenous compounds (Shils, 1994; Block et al., 1991), and as an antioxidant (Sies et al., 1992). In addition, when given in large doses (mainly intravenously), ascorbate may function as an ergogenic aid. There is also evidence of ascorbate increasing cell respiration and ATP production in osteoblasts (Komarova, Ataulakhanov, and Globus, 2000). These particular functions of ascorbate may be of great relevance to patients suffering chronic degenerative diseases, especially those with chronic fatigue syndrome, AIDS and cancer.

This ergogenic activity reported for large doses of ascorbate is probably due to the oxidation reduction potential of ascorbate, as it is capable of providing necessary electrons to the electron transport system in the mitochondria for increased energy production. This participation of ascorbate in electron transport reactions was first postulated over 70 years ago by Albert Szent-Gyorgyi (Szent-Gyorgyi, 1960).

Chapter 4: Ascorbic Acid in the Management of Cancer

4.1 Mechanisms

Ascorbic acid has a variety of identified functions related to cancer management. Among other roles, ascorbate is required by the mixed function oxidases for the hydroxylation of amino acids (Cameron et al., 1979). The mixed function oxidases are a group of closely related microsomal enzymes that metabolize many classes of compounds and are particularly important in the inactivation of chemical carcinogens. Microsomal metabolism of carcinogens yields products generally of a water soluble nature which greatly increases their rate of excretion. In addition, ascorbate has been shown to protect against nitrate induced carcinogenesis (Mirvish et al., 1972). Another important anticancer function of ascorbate when provided in large quantities is that it enhances the removal of sodium via the urine thereby reducing the level of sodium ions in the serum. In cancer, there is a disturbed sodium/potassium ratio. It has been suggested that vitamin C may also have a role inhibiting prostaglandins of the 2-series in carcinoma cells (Beetens and Hermen, 1983; El Attar and Lin, 1992). In the process of prostaglandin biosynthesis, the release of arachidonic acid from cell membrane phospholipids is implicated as one of the synergistic signals leading to cell proliferation. Recently, ascorbate has been shown to stabilize p53, a protein involved in cell proliferation control (Reddy, Khanna, and Singh, 2001).

Ascorbate is considered one of the strongest reductants and radical scavengers. Ascorbate reduces unstable oxygen, nitrogen and sulphur centered radicals; in addition, it acts as the primary defense against aqueous radicals in blood (Niki, 1991). In studies with human plasma, ascorbate has been found to protect plasma lipids against detectable peroxidative damage induced by aqueous peroxy radicals (Fritsch, 1997). Thus, by efficiently trapping peroxy radicals in the aqueous phase before they can reach the lipid rich membranes and initiate lipid peroxidation, ascorbate protects biomembranes against primary peroxidative damage. Ascorbate may also protect membranes against peroxidation due to its synergistic antioxidant function with vitamin E. Ascorbate may enhance or reinstate the activity of tocopherol (vitamin E), the principal lipid soluble

antioxidant (Niki, 1991). When both vitamins are present in environments suffering enhanced oxidative stress, ascorbate reacts with the tocopheroxyl (chromanoxyl) radical that arises in cell membranes as a result of vitamin E antioxidant activity, and the ascorbate regenerates tocopherol while transferring the oxidative challenge to the aqueous phase (Vandenberg, Kuypers, Roelofsen, and Op den Kamp, 1990). At this point, the less reactive ascorbate radical can be enzymatically reduced back to ascorbic acid by an NADH-dependent system (Chan, 1993; Levine et al., 1991; Packer, Slater, and Wilson, 1979; Burton, Wronska, and Stone, 1990). This is also believed to be the mechanism by which ascorbate reduces nitrates and prevents the formation of carcinogenic nitrosamines (Mirvish, 1974).

The literature on ascorbic acid and cancer is vast. Since 1952, ascorbate has been proposed as a chemotherapeutic agent (McCormick, 1952), and even as early as 1949 its cytotoxicity to cancer was suggested (Klenner, 1949). Hundreds of articles, including an array of *in vitro*, *in vivo*, cell, animal and human studies have been published on this topic (see Padayatty et al., 2003, for a general review of vitamin C; see Tamayo and Richardson, 2003, for a general review of ascorbate and cancer). Worthy of particular mention is the first comprehensive review of the field, published in 1979 in *Cancer Research* (Cameron, Pauling, and Leibovitz, 1979). In the intervening years since this seminal publication on nutritional oncology by Drs. Cameron, Pauling and Leibovits first appeared, a new body of and data has subsequently arisen, with new evidence for the chemotherapeutic potential of ascorbic acid.

In the following sections of this chapter, this doctoral candidate shall describe a literature review of such new data on the anticancer mechanisms of ascorbic acid.

4.1.1 Cytotoxicity

Ascorbic acid and its salts (AA) have been found to be preferentially toxic to tumor cells both *in vitro* and *in vivo*. This phenomenon first came to the attention of this doctoral candidate through the work of Benade et al., described in 1969 (Benade et al., 1969). They theorized that the preferential toxicity was due to the relative deficiency of catalase

in tumor cells. This theory has since been validated by others (Maramag, Menon, Balaji, Reddy and Laxmanan, 1997). Given in high enough doses to maintain plasma concentrations above levels that have been shown to be toxic to tumor cells *in vitro*, AA has the potential to selectively kill tumor cells in a manner similar to other tumor cytotoxic chemotherapeutic agents. However, most studies of AA and cancer to date have not utilized high enough doses of AA to maintain tumor cytotoxic plasma concentrations of AA. Data are presented herein which demonstrate the ability to sustain plasma levels of AA in humans above levels which are toxic to tumor cells *in vitro*, suggesting the feasibility of using AA as a tumor cytotoxic chemotherapeutic agent.

Cytotoxic drugs began to be considered consistently successful for the therapy of some cancers around 1950 (Kennedy, 1991). A large jump in the cure rate for several cancer types – especially childhood, acute lymphoblastic leukemia, Hodgkin's disease, and testicular tumors – was seen between 1950 and 1990 (from 0% for all in 1950 to 75%, 80% and 90% respectively) (Krakoff, 1991). Other, relatively common, types of cancer (Boring, Squires, Tong and Montgomery, 1994), including head and neck, large bowel, stomach, pancreatic, liver, cervical cancers, and melanoma, for the most part remain refractory to cytotoxic chemotherapy, with and without adjuvant chemotherapy, with no demonstrable prolongation of life (Krakoff, 1991).

Even though the term “chemotherapy” generally includes hormonal and cytotoxic agents, the discussion herein is limited to cytotoxic agents. Whether they are alkylating agents, antimetabolites, or antibiotics, the rationale for using chemotherapeutic agents in the treatment of malignancy is to preferentially induce cytotoxicity of malignant cells. Because of the similarities between normal and malignant cells – both being born of the same host – a chemotherapeutic dose which is cytotoxic to cancer cells can also be toxic to normal cells. Oncologists must often push the limits of acceptable toxic side effects in order to effect a remission. Ideally, there should be a large gap between the lower dose required for efficacy and the higher dose of toxicity to the patient. Adverse effects of chemotherapy include hair loss, nausea and vomiting, cardiac toxicity, and secondary cancers (DeVita, Hellman and Rosenberg, 1982). One of the most common toxic

manifestations of many cytotoxic agents is bone marrow suppression (Krakoff, 1991), which can lead to immune suppression and hematopoietic dysfunctions. Because infectious complications are one of the major causes of death in cancer patients (DeVita et al., 1982), more host-nontoxic compounds – particularly compounds without immune suppressive qualities – should be investigated for their chemotherapeutic value.

There is a 10- to 100-fold greater content of catalase in normal cells than in tumor cells (Benade et al., 1969). This potentially creates a large gap between the toxic dose for normal cells and for tumor cells of agents which induce hydrogen peroxide generation. Ascorbic acid and its salts (AA) have been shown to be preferentially toxic to tumor cells *in vitro* (Benade et al., 1969; Bram et al., 1980; Noto et al., 1989; Helgestad et al., 1990; Park, Aniare, Savin and Hoogstraten, 1980; Yamafuji et al., 1971; Yagashita et al., 1976; Koch et al., 1978) and *in vivo* (Cohen and Krasnow, 1987; Lupulesco, 1991; Varga and Airoidi, 1983; Pierson and Meadows, 1983; Chakrabarti and Dasgupta, 1984). This preferential cytotoxicity has been demonstrated to be related to intracellular hydrogen peroxide generation (Benade et al., 1969; Noto et al., 1989; Cohen and Krasnow, 1987). AA thus belongs in a class of substances which, given at the correct dosage, can preferentially induce cytotoxicity of tumor cells with negligible toxic effects to the host.

The extent of the preferential cytotoxicity to tumor cells exhibited by ascorbic acid may be influenced by a variety of factors. In an effort to understand these factors in more detail, numerous studies have been conducted in which various combinations of other nutrients with vitamin C, and even the derivatives and isomers of vitamin C, are tested on different types of tumor cells.

In one such experiment, in 2001, researchers in the Department of Oral and Maxillofacial Surgery at the Peking University School of Stomatology in Beijing investigated the combined effect of sodium ascorbate and menadione (vitamin K3) on the viability of various cultured cells (Zhang et al., 2001). Zhang et al. found that human oral squamous cell carcinoma (HSC-2, HSC-3) and human promyelocytic leukemia (HL-60) cells are more sensitive to this particular vitamin combination than are normal cells (human

gingival fibroblast HGF, human periodontal ligament fibroblast HPLF, and human pulp cell HPC). The combination of vitamin C and vitamin K3 produces synergistic cytotoxicity against all 6 cell lines. Treatment with vitamin C or vitamin K3, or their combination, induces internucleosomal DNA fragmentation only in HL-60 cells, but not in the oral tumor cell lines (HSC-2, HSC-3, HSG). ESR spectroscopy shows that vitamins C and K3 produce radicals under alkaline conditions and that the combination of these two vitamins synergistically enhanced their respective radical intensities.

In recent years, the combined mechanisms of vitamins C and K3 have been further studied by others. Gilloteaux et al. (1998; 1995) reported utilizing an MTT/formazan assay to evaluate the antitumor activity of vitamin C and vitamin K3, singly and in combination, against a human prostatic carcinoma cell line (DU145). Both vitamins C and K3, when given alone, exhibited antitumor activity, but only at elevated doses. When vitamins C and K3 were combined, however, at a C:K3 ratio of 100:1, and administered to the carcinoma cells, the 50% cytotoxic concentrations (CD50) of the vitamins decreased 10- to 60-fold. Subsequently, the DU145 cells were examined with transmission and scanning electron microscopy (TEM and SEM) following a 1 hour treatment with vitamins C and K3 administered separately, and combined at their 50% cytotoxic dose. Morphological data suggest that treatment with individual vitamins affects the cytoskeleton, the mitochondria, and other membranous components of the cell. In this study, scanning electron microscopy and transmission electron microscopy revealed the synergistic antitumor activity of vitamin C and vitamin K3 when combined, specifically against human prostatic carcinoma cells. The investigators observed that treatment with this particular vitamin combination appears to potentiate the effects of the individual vitamin treatment, specifically inducing abundant necrotic cells, with surviving cells displaying morphological defects that are characteristic of cell injury.

Along these lines, researchers in Belgium investigated the role of vitamins C and K3 in inducing autschizis in cancer cells, when this combination was used as a coadjuvant in cancer therapy (Verrax et al., 2003). Deficiency of alkaline and acid DNase has been found to be a hallmark of all non-necrotic cancer cells in animals and humans. These

enzymes are reactivated at early stages of cancer cell death by vitamin C (acid DNase) and vitamin K3 (alkaline DNase). Moreover, the coadministration of these vitamins (in a ratio of 100:1, for vitamins C and K3, respectively) produced selective cancer cell death. Detailed morphological studies indicated that cell death is produced mainly by autoschizis, a new type of cancer cell death. Several mechanisms are involved in such a cell death induced by vitamins C and K3, which include the formation of H₂O₂ during vitamin redox cycling, oxidative stress, DNA fragmentation, no caspase-3 activation, and cell membrane injury with progressive loss of organelle-free cytoplasm. Changes in the phosphorylation level of some critical proteins leading to inactivation of NF-kappaB appear as main intracellular signal transduction pathways. The increase in knowledge of the mechanisms underlying cancer cell death by combined vitamins C and K3 may ameliorate the techniques of their *in vivo* administration. The aim of these investigators was to prepare the introduction of the association of vitamins C and K3 into human clinics as a new, non-toxic adjuvant cancer therapy.

Calderon et al. (2002) further explored the synergistic actions of vitamins C and K3 as an adjuvant cancer treatment. The decision of stressed cells to die or to survive is made by integrated signals at different levels through multiple check-points. However, initiation and continued progression toward cell death by “apoptosis” (to be described in further detail in sections 4.1.3 and 5.1.3) in cancer cells may be blocked by mutation of the tumor suppressor p53, or by overexpression of members of the bcl-2 family of proteins. The existence of such mechanisms indicates that cancer cells lose the controls regulating their cell cycle. Therefore, the activation of their programmed cell death appears as a major therapeutic target. Oxidative stress can stimulate growth, trigger apoptosis, or cause necrosis depending upon the dose and the exposure time of the oxidizing agent. A large body of evidence supports the idea that oxidative stress induced by redox cycling of vitamins C and K3 in association surpasses cancer cellular defense systems and results in cell death. Several types of cell death may be produced, namely autoschizis, apoptosis and necrosis. Combined vitamin C and K3 administration *in vitro* and *in vivo* have been found to produce tumor growth inhibition and increase the life-span of tumor-bearing mice. CK3-treatment (combining vitamins C and K3 in the appropriate proportions)

selectively potentiates tumor chemotherapy, produces sensitization of tumors resistant to some drugs, potentiates cancer radiotherapy and causes inhibition of the development of cancer metastases without inducing toxicity in the host. Calderon et al. therefore propose the association of vitamins C and K3 as an adjuvant cancer therapy which may be introduced into human cancer treatment, such as radiation therapy, without any change in the classical anticancer protocols, and without any supplementary risk for patients.

Besides vitamin K3, there are other factors which have been found to potentiate the preferential tumor cytotoxicity of ascorbic acid. These nutrients include alpha lipoic acid, bioflavonoids, quercetin, selenium, niacinamide, and biotin (which enhances vitamin K production in the gut). Such factors have been found to augment the ability of ascorbic acid to induce apoptosis in the tumor cells.

In order to understand and describe the cytotoxicity of ascorbic acid in more depth, researchers at the Linus Pauling Institute of Science and Medicine studied the growth suppression of a malignant leukemia cell line *in vitro* with L-ascorbic (LAA) acid and its derivatives (Roomi, House, Eckert-Maksic, Maksic, and Tsao, 1998). AA is a gamma-crotonolactone derivative with reactive hydroxyl groups at the 2- and 3-positions and an ethylene glycol substitution at the 4-position. This particular study addressed the underlying chemical structural features for the toxicity of AA. The researchers tested *in vivo*, using malignant leukemia cell line P388D1, (i) LAA and its isomers, (ii) substitution at the 2-position: -PO₄, -SO₄, O-Me, O-octadecyl, (iii) substitution at the 6-position: -PO₄, -SO₄, -palmitate, -stearate, (iv) substitution at the 2,6-position: dipalmitate, (v) 6-deoxy derivative: -Cl, -Br, -NH₂ and (vi) dihydroxy gamma-crotonolactone with substitutions at the 4-position: -H, -CH₃, -CH₂-CH₃ and -CH=CH₂. LAA and its isomers were found to be very cytotoxic even at very low concentrations. All 6-substituted and 6-deoxy derivatives were found to be as toxic as AA. However, 2-substituted and 2,6-disubstituted AA derivatives were nontoxic. Interestingly, dihydroxy gamma-crotonolactone, with or without substitution at the 5-position, also exhibited toxicity. These results suggest that the underlying criterion for AA toxicity resides in dihydroxy gamma-crotonolactone moiety. The investigators found that either

substitution in the hydroxy groups or saturation of the double bond will render the molecule inactive.

Researchers in Japan performed a comparative study of the antitumor action between sodium 5,6-benzylidene-L-ascorbate (SBA) and sodium ascorbate (Sakagami, Satoh, and Kochi, 1997). Sakagami et al. found SBA and ascorbate to produce ascorbate radicals during decomposition, as well as elevated oxidation potential and oxidized methionine to methionine sulfoxide, in the regular culture medium. These compounds were found to induce apoptotic cell death (characterized by internucleosomal DNA fragmentation) in human myelogenous leukemic cell lines, although they killed most of the other tumor cell lines by necrosis without induction of internucleosomal DNA fragmentation. The cytotoxic activity of SBA and ascorbate was significantly enhanced in the presence of copper, and the stimulation effect of copper was reduced by a heavy metal antagonist. However, the cytotoxic activity of SBA was only slightly modified by iron, cysteine analog or catalase, in contrast to ascorbate, which was highly sensitive to all these agents. Additionally, intravenous administration of SBA induced degeneration in chemically-induced hepatocellular carcinoma whereas ascorbate was inactive. These data suggest the differential mode of antitumor action between these two compounds.

In Germany, researchers explored the uptake and cytotoxicity of ascorbic acid and dehydroascorbic acid in neuroblastoma (SK-N-SH) and neuroectodermal (SK-N-LO) cells (Baader, Bruchelt, Trautner, Boschert, and Niethammer, 1994). Baader et al. found ascorbic acid (AA) to be cytotoxic to neuroblastoma cells both *in vitro* and *in vivo*. They investigated whether the reduced ascorbic acid or the oxidized form (dehydroascorbic acid, DhAA) and its rapidly formed metabolites were the main cytotoxic agents. In neuroblastoma SK-N-SH cells, AA was found to be more cytotoxic than DhAA, although considerably higher amounts of [14C]DhAA than of [14C]AA were incorporated. By contrast, SK-N-LO cells derived from neuroectodermal tissue showed a similar uptake, but were much less injured by both substances. The researchers observed that uptake of [14C]AA and [14C]DhAA was impaired in the presence of dithiothreitol and glutathione. Once inside the cell, [14C]DhAA was partially reduced to [14C]AA. From these data

they concluded that at least part of AA is oxidized prior to its uptake, and that the reduced form of AA, as well as perhaps ascorbyl radicals (but not DhAA or its metabolites), are the most important forms in mediating cytotoxic reactions in neuroblastoma cells. Furthermore, the results were found to strengthen the previous assumption that AA acts as a pro-oxidant in neuroblastoma cells. The investigators concluded by endorsing the use of AA in the treatment of neuroblastoma, especially in combination with existing chemotherapeutics.

Ascorbic acid has been found to enhance the cytotoxicity of other chemotherapy drugs in cell culture. Kurbacher et al. (1996) utilized a microplate ATP bioluminescence assay to test the sensitivity of two human breast carcinoma cell lines, MCF-7 and MDA-MB-231, to doxorubicin (DOX), cisplatin (DDP), and paclitaxel (Tx), alone and in combination with ascorbic acid (300). In both cell lines, vitamin C exhibited cytotoxic activity at high concentrations (10⁽²⁾-10⁽³⁾ microM). Both cell lines were also resistant to DOX, with MCF-7 exhibiting DDP-resistance, while MDA-MB-231 exhibited moderate sensitivity to DDP. Both cell lines were strongly sensitive to Tx. Vitamin C, both at non-cytotoxic (1 microM) and moderately cytotoxic concentrations (10⁽²⁾ microM) improved the cytotoxicity of DOX, DDP, and Tx significantly. Combination effects between vitamin C and DDP or Tx were partly synergistic and partly additive or subadditive whereas a consistent synergism was found between vitamin C and DOX. Although ascorbic acid was found to improve the antineoplastic activity of these cell lines, the mechanisms by which vitamin C potentiates the particular cytostatics studied herein remain unclear, and the authors recommended further studies.

Other researchers in Germany explored the lipid peroxidation that is induced by ascorbic acid in neuroectodermal SK-N-LO cells with high endogenous ferritin content and loaded with MAb-ferritin immunoconjugates (Lode et al., 1994). Neuroblasma-and other malignant cells often contain elevated amounts of iron-rich ferritin and H₂O₂ and may therefore be a potential target for pro-oxidative effects of ascorbic acid (AA), generating cytotoxic products, e.g., by lipid peroxidation (LPO). Lode et al. investigated the influence of H₂O₂ and iron, either in its free form or bound to ferritin, on AA-induced

LPO using erythrocyte ghosts as a model system. Results of these experiments showed that AA could induce LPO not only in the presence of free available iron but also in the presence of ferritin. Similarly, AA induced significant LPO in neuroectodermal SK-N-LO cells with elevated intracellular ferritin levels. These LPO-promoting effects of ferritin in the presence of AA on SK-N-LO cells were also observed using ferritin-immunoconjugates. For this purpose, ferritin was bound to human monoclonal antibodies (MAb-ferritin) capable of recognizing ganglioside GD2, which is present in large quantities on cell surfaces of SK-N-LO and many neuroblastoma cells. The investigators therefore concluded that the pro-oxidative effects of AA could be exploited in the treatment of ferritin rich neuroblastoma in combination with chemotherapy or with MAb-ferritin immunoconjugates.

It has similarly been demonstrated that ascorbic acid and glutathione (GSH) have actions in common and can act complementarily, and that an important function of GSH is the elimination of reactive oxygen species (ROS) as well as the reduction of DHA (Meister, 1994). The majority of ascorbic acid is oxidized extracellularly to DHA (Agus et al., 1999), transported into the cell by glucose transporters, and then rapidly reduced by a GSH-dependent mechanism (Vera et al., 1993; Wang et al., 1997). Ascorbic acid may also oxidize spontaneously or in the presence of transition metals to form DHA and H₂O₂ (Chiou, 1983). Co-treatment of cells with ascorbic acid and DHA results in enhanced cytotoxicity, as increasing levels of H₂O₂ cause the oxidation of cellular macromolecules and DHA.

In 1969, researchers at the NCI (National Cancer Institute) reported that ascorbic acid was highly toxic to Ehrlich ascites cells *in vitro*. The goal of the study was to exploit the 10- to 100-fold lower catalase activity in tumor cells compared to normal cells. The proposed cytotoxic mechanism was the generation of toxic hydrogen peroxide. The toxicity was greatly enhanced by concomitant administration of 3-amino-1,2,4-triazole (ATA), a catalase inhibitor. Catalase and glucose added to the culture medium and a low oxygen tension reduced the toxic effects of AA and ATA. The addition of vitamin K3

(menadione sodium bisulfite) to the medium overcame the protective effects of low oxygen tension and glucose (Benade et al., 1969).

In 1977, Bram et al. reported preferential ascorbic acid cytotoxicity for several malignant melanoma cell lines, including four human-derived lines (Bram et al., 1980). They found that catalytic concentrations of Cu^{2+} greatly increased the preferential toxicity for melanoma cells. Another French group also found that AA and Cu^{2+} were toxic to mouse melanoma cells *in vitro* (Noto and Taper, 1989). Noto and Taper reported that AA plus vitamin K3 had growth inhibiting action against three human tumor cell lines at nontoxic levels. Helgestad et al. recently reported that a new malignant T-cell line, isolated from a boy with malignant lymphoma, was very sensitive to AA in culture at concentrations achievable in human plasma (Helgestad, Pettersen, Storm-Mathisen, 1990). In 1980, Park et al. reported that several leukemic cell cultures were sensitive to AA at concentrations attainable *in vivo*, while normal hemopoietic cells were not suppressed (Park, Aniare, Savin, and Hoogstraten, 1980). Metabolites of AA have also shown antitumor activity *in vitro* (Yamafuji et al., 1971; Yagashita et al., 1976).

From studies such as those described above, there is also increasing evidence that ascorbic acid is selectively toxic to some types of tumors in its capacity as a pro-oxidant rather than as an antioxidant (Bram et al., 1980; Bruchelt et al., 1993). The following section shall address the pro-oxidant properties of ascorbic acid in more detail.

4.1.2 Pro-oxidation

The primary oxidative, oxidant and pro-oxidant properties of ascorbate are described herein.

Ascorbic acid not only possesses antioxidant activity at low concentrations, but it also exhibits prooxidation activity at higher concentrations (Gonzalez, Mora, Riordan NH, Riordan HD, and Mojica, 1998; Yamamoto, Takahashi, and Niki, 1987; Rowly and Halliwell, 1983). It has been suggested that ascorbate promotes oxidative metabolism by

inhibiting utilization of pyruvate for anaerobic glycolysis (Ramp and Thorton, 1968). Ascorbate in high doses inhibits prostaglandins of the 2-series (arachidonic acid derived), which have been correlated with increased cell proliferation (Beetens and Hermen, 1983). Also, a growth inhibitory effect has been produced by ascorbate or its derivatives in at least seven types of tumor cells (Mikino, Sakagami, and Takeda, 1999; Nakamura and Yamafuji, 1968; Yamafuji, Nakamura, Omura, Soeda, and Gyotoku, 1971; Omura, Tomita, and Yasuhiko, 1974; Tomita, Eto, and Lio, 1974; Poydock, Reikert, Rice, and Aleandri, 1982; Leung, Miyashita, Young, and Tsao, 1993). Although this inhibitory action was not observed in normal fibroblasts by some research groups (Mikino et al., 1999; Nakamura and Yamafuji, 1968; Yamafuji et al., 1971; Omura et al., 1974; Tomita et al., 1974; Poydock et al., 1982), other researchers have nevertheless reported this action in fibroblast inhibition (Leung et al., 1993; Avakawa, Nemoto, Suzuki, and Otsuka, 1994; Peterkofsky and Prather, 1971; Yve, Niedra, and Baum, 1980; Jampel, 1990).

Such cytotoxic effects produced by ascorbate in an array of cell lines (mostly malignant) have been attributed to its pro-oxidant activity (Gonzalez et al, 1993). Furthermore, ascorbate and its radicals potentiate the activation of transcription factor NF- κ B, which has been associated with the inhibition of cell growth (Muñoz, Blazquez, Ortiz, Gómez-Díaz and Navas, 1997). The role of hydrogen peroxide, other peroxides and metabolites shall be addressed in more detail below.

Ascorbate can generate hydrogen peroxide (a reactive oxygen species) upon oxidation (with oxygen) in biological systems (Halliwell, 1996; Alcain and Buron, 1996; Asano et al., 1999). This action can be enhanced by divalent cations such as iron and copper (Rowly and Halliwell, 1983; Tsao, Dunhan and Leung, 1988; Jonas, Riley, and Wilson, 1989). Hydrogen peroxide may further generate additional reactive species such as the hydroxyl radical and secondary products of oxidation such as aldehydes, which can compromise cell viability mainly by damaging biomembranes (González and Riordan NH, 1996). However, these oxidative reactions may only form in minute quantities *in vivo*, in healthy organisms. This is mainly because most transition metal ions are

attached to binding proteins in serum, which makes them unavailable to participate in biochemical reactions (Gutteridge, Richmond, and Halliwell, 1980).

Nevertheless, these oxidation reactions may take place in pathological states such as malignancy, in which cohesive forces that inhibit the liberation of the metal ion from the protein as well as the control of the cell's replication mechanisms are drastically reduced (Gutteridge, Richmond, and Halliwell, 1980). These reactive species are capable of inducing multiple negative cellular effects such as DNA strand breakage, disruption of membrane function via lipid peroxidation, and depletion of cellular ATP (Gonzalez, 1992). The failure to maintain high ATP production (cell energy level) may be a consequence of oxidative inactivation of key enzymes, especially those related to the Krebs cycle and the electron transport system. A distorted mitochondrial function (transmembrane potential) may result. This aspect could suggest an important mitochondrial role in the carcinogenic process. In this respect, ascorbate may serve yet another metabolic and physiological function by providing reductive energy, the necessary electrons with which to direct energy pathways in the mitochondria (Szent-Gyorgyi, 1980; Schwartz, 1996; Sigal and King, 1936; Landauver and Sopher, 1970; Cathcart, 1991). Interestingly, ascorbate has been detected in the mitochondria and also found to be regenerated internally there (Li, Cobb, Hill, Burk, and May, 2001).

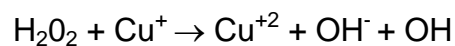
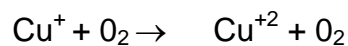
In general, the cytotoxicity induced by ascorbate seems to be primarily mediated by hydrogen peroxide (Mikino, Sakagami and Takeda, 1999; Nakamura and Yamafuji, 1968; Yamafuji, Nakamura, Omura, Soeda, and Gyotoku, 1971; Peterkofsky and Prather, 1971; Clement, Ramalingam, Long and Halliwell, 2001; Gonzalez, Schemmel, Dugan, Gray and Welsch, 1993; Sakagami, Satoh, Hakeda, and Kumegawa, 2000; Iwasaka et al., 1998; Davies, 1999). Of interest, this phenomenon has been observed in proliferating cells in which very low levels of hydrogen peroxide (3-15 μ M) stimulate cell division, whereas greater concentrations induce cell growth arrest, apoptosis and/or necrosis (Davies, 1999). It has also been shown that the amount of hydrogen peroxide generated was proportionally dependent on the ascorbate concentration while inhibited by serum (Avakawa, Nemoto, Suzuki and Otsuka, 1994; Dasgupta and Zdunek, 1992; Sakagami et

al., 1997; Sakagame et al., 1996). Human serum, as part of its normal contents, has certain proteins such as albumin and glutathione with antioxidant capacity that may stabilize ascorbate, directly or indirectly, by chelating available transition metals. In addition, serum contains antioxidant enzymes such as catalase, which decomposes hydrogen peroxide. Other antioxidant enzymes are glutathione peroxidase and superoxide dismutase that complement the catalase function.

Hydrogen peroxide is most likely generated intracellularly by ascorbate's metabolic oxidation to dehydroascorbate, with the subsequent formation of the superoxide anion which, in the presence of oxygen, is further reduced to hydrogen peroxide. Hydrogen peroxide reduces cellular levels of thiols and can initiate membrane lipid peroxidation (Mikino et al., 1999; Nakamura and Yamafuji, 1968; Yamafuji et al., 1971; Omura et al., 1974; Tomita et al., 1974; Povdock et al., 1982; Leung et al., 1993; Jonas et al., 1989; Clement et al., 2001; Sakagami et al., 1997; Gonzalez et al., 1993; Gonzalez, 1995; Sestili et al., 1996; Iyanagi et al., 1985; Venugopal, Jamison, Gilloteaux, 1996). As mentioned previously, this antiproliferative action of ascorbate in malignant cultured cells, animal and human tumor xenografts, has been augmented by the addition of the cupric ion, a catalyst for the oxidation of ascorbate (Leung et al., 1993; Tsao et al., 1988; Tsao et al., 1989; Poydock, 1982; Satoh, Kadofuku, and Sakagami, 1997; Gonzalez et al., 2002). Additionally, the combination of ascorbate and copper has been shown to inactivate lactate dehydrogenase (Nelson, Pazdernik, and Samson, 1992), the enzyme responsible for the reduction of pyruvate to lactate (a metabolic dead end product prevalent in anaerobic environments such as in cancer). Copper in the form of copper sulfate may also inhibit tyrosinase activity (Powers, Gibson, Bates, Primhak and Beresford, 1994; Palumbo, Misuraca, D'Ischia, and Prota, 1985). It has also been suggested that the selective toxicity of ascorbate in malignant cells may be due to reduced levels of antioxidant enzymes, catalase, superoxide dismutase and glutathione peroxidase in these cells, leading to cellular damage through the accumulation of hydrogen peroxide (Riordan NH, Riordan HD, Meng, Li, and Jackson, 1995; Satoh et al., 1997; Benade, Howard, and Burk, 1969; Punnonen et al., 1994; Jaruga and Olinste, 1994; Sun, Colburn, and Oberley, 1993; Bozzi, Mavelli, Mondovi, Strom, and Rotilio, 1979). As mentioned,

there is a 10- to 100-fold greater content of catalase in normal cells than in tumor cells (Riordan NH, et al., 1995; Benade, Howard, and Burk, 1969). Furthermore, the addition of vitamin K3 (menadione) to ascorbic acid produces a synergistic antitumor activity (Venugopal, 1996; Noto et al., 1989; Gilloteaux et al., 1998 and 2001). Since menadione is reduced intracellularly via one or two electron transfer actions (probably by ascorbic acid), this may lead to the formation of hydrogen peroxide and other reactive oxygen species, concomitant with the depletion of glutathione. Decreases of glutathione have also been associated with ascorbate metabolism (Grad et al., 2001). Interestingly, a new form of cell death, “autoshizis”, has been described for this synergistic vitamin phenomenon (with vitamins C and K) in which tumor cells undergo profound perturbations that ultimately kill the cells in a manner that is distinct from apoptosis, oncosis or necrosis (Gilloteaux et al., 1998, and 2001; Grad et al., 2001; Jamison et al., 2002). For this reason, the combination of megadoses of intravenous ascorbate together with oxygen, vitamin K, lipoic acid, coenzyme Q10 and small doses of copper may seem logical as part of a nontoxic treatment protocol for cancer. Melanoma cells have been found to contain higher levels of copper than other lines for melanogenesis; the presence of copper sulfate induces H₂O₂ production and increases ascorbate-induced tumor cytotoxicity within the cells. Intravenous administration of ascorbate can yield plasma levels which are high enough to achieve this desired toxic effect by the ascorbate on malignant cells (Riordan NH, and Riordan HD, 1995; Riordan NH, Riordan HD, and Casciari, 2000; Casciari, Riordan NH, Schmidt, Meng, Jackson, and Riordan HD, 2001).

Ascorbate - Copper Interaction:



Researchers in Japan have investigated this phenomenon of hydrogen peroxide production in cancerous tissue, by intravenous administration of sodium 5,6-benzylidene-

L-ascorbate (Asano et al., 1999). Investigating whether or not the antitumor action of sodium 5,6-benzylidene-L-ascorbate (SBA) is mediated via an oxidation-involved mechanism, they used three different systems: 3'-methyl-4-dimethylaminoazobenzene (DAB)-induced rat hepatocellular carcinoma (*in vivo*), its homogenate (semi *in vivo*), and cultured cells (*in vitro*). They found that an oral intake of DAB irreversibly produced hepatocellular carcinoma in rats, with a maximum incidence of carcinogenesis after 4 months. Intravenous administration of SBA induced vacuolar, eosinophilic degeneration and nuclear debris, producing greater amounts of ESR signal of ascorbate radical and hydrogen peroxide (H₂O₂)-derived chemiluminescence (CL) (H₂O₂-CL) in the cancerous tissue than in the normal tissue. When SBA was directly added to the homogenates, higher amounts of the ascorbate radical and H₂O₂-CL were generated in cancerous tissues. When SBA was added to the RPMI1640 medium supplemented with 10% fetal bovine serum, methionine was oxidized to methionine sulfoxide and H₂O₂ was produced in amounts that sufficiently induce apoptotic cell death in human promyelocytic leukemic HL-60 cells. Cytotoxic activity of SBA was significantly reduced by catalase. The researchers concluded that antitumor activity of SBA *in vivo* might at least in part be due to H₂O₂, produced from SBA.

Ascorbic Acid Oxidation Products:

Ascorbic acid oxidation products, such as dehydroascorbic acid, 2,3-diketogulonic acid and 5-methyl 1-3, 4-dehydroxytetrone, all degradation products of ascorbic acid, have demonstrated antitumor activity (Leung et al., 1993; Tsao et al., 1988; Tsao et al., 1989; Poydock, 1982; Edgar, 1970). In addition, other compounds arising from the oxidation or degradation of ascorbate can inhibit tumor growth. The most effective ones are gamma-cronolactone and 3-hydroxy-2-pyrone. The available evidence suggests that these vitamin C oxidation products and/or metabolic byproducts have a function controlling mitotic activity. All active compounds consist of an unsaturated lactose ring with a double bond conjugated with a carbonyl group. This might suggest that the particular structural feature of the lactose ring may be relevant to the antitumor activity (Leung et al., 1993). This antitumor activity shown by these compounds could be due to

their ability to produce active molecular species that inhibit tumor growth, such as hydrogen peroxide and certain aldehydes. Most of these compounds are very unstable and their growth inhibitory activities could be attributed to their chemical instability. These antiproliferative mechanisms of ascorbic acid and/or its oxidation products upon tumor cells are expected to be of a very complex nature, since the mechanisms seem to involve a series of pleiotropic chain reactions. Large amounts of ascorbic acid intake can change the levels of certain amino acids in body fluids (Tsao and Miyashita, 1984; Tsao and Miyashita, 1985; Basu, 1979; Bensch et al., 1985) and may deplete the bioavailability of lysine and cysteine, two amino acids which are required for rapidly growing tumors (Garland et al., 1986). Experiments using tissue homogenate show that the interactions between ascorbate, metal ions and oxygen are capable of inducing structural changes in protein (Basu, 1979; Bensch et al., 1985; Garland et al., 1986). These electron-induced or charge transfer changes need a conductor in order to proceed; and proteins can serve as electronic conductors for these reactions. Metal ions such as copper are good electrical conductors because their valence bonds are partially filled and there is plenty of space for interactions. The resulting molecules containing one or more uncoupled electrons are very reactive free radicals. We should remember that cells need to reduce cohesiveness and dismount part of their structure to be able to divide, in other words, to de-differentiate. This proposed unstable state of cellular organization facilitates free radical damage in the malignant cell. This same principle is used in chemotherapy treatment, such as with the drug doxorubicin (adriamycin). The difference is that the free radicals formed by the drug are of a much more potent nature, thus much more toxic, to such an extent that they can damage normal tissue and be systemically detrimental to the cancer patient. Doxorubicin causes cardiac toxicity, nausea, vomiting and hair loss (see Moss, 1995, for a review). Dehydroascorbic acid (the oxidized, nonionic and more lipid soluble form of ascorbate) and the semidehydroascorbic acid radical have been shown to promote lipid peroxidation (Edgar, 1970).

One of the investigators (MJG) in the research contributed by this doctoral candidate has demonstrated that secondary products of lipid peroxidation have an inhibitory action on human malignant cell proliferation (Gonzalez et al., 1991; Gonzalez and Riordan NH,

1996; Gonzalez et al., 1993; Gonzalez, 1995). There is also evidence to suggest that dehydroascorbic acid may work as a mitotic inhibitor *in vivo* (Riordan HD, 1990). Dehydroascorbate may prevent cell division by inhibiting protein synthesis at the ribosomal level. Interestingly, prolonged exposure to high concentrations of dehydroascorbic acid may cause irreparable damage resulting ultimately in complete lysis of the cells. It should be noted that dehydroascorbic acid is unstable and must be constantly produced in order to be maintained in high concentrations. Under the appropriate conditions, it may revert back to ascorbic acid. This recycling occurs when extracellular ascorbate is oxidized, transported as dehydroascorbic acid and reduced intracellularly to ascorbate (Wang et al., 1997). Actually, many cell types transport ascorbate solely in its oxidized form, through facilitated glucose transporters. These cells accumulate large intracellular concentrations of ascorbate by reducing dehydroascorbate to ascorbate, a form that is trapped intracellularly. Other cells can transport ascorbate in its reduced form through a sodium-dependent co-transporter (Spielholz et al., 1997). To ascorbate's advantage, tumor cells have an increased requirement for glucose (Warburg, 1956). To compensate for this increased need for glucose, tumor cells increase their quantity of glucose transporters (Younes, Lechago, Somoano, Mosharaf, and Lechago, 1996). This action greatly enhances the entrance of either ascorbate or its oxidized form, dehydroascorbate into the cancer cell, thus facilitating the action of ascorbate as a selective, nontoxic chemotherapeutic agent. These issues are very relevant in the clinical use of ascorbic acid and dehydroascorbic acid. Dehydroascorbic acid may be further metabolized to 2,3-diketogulonic acid or, as mentioned above, reduced back to ascorbic acid. It is conceivable that ascorbate may have a preferential cytotoxicity against tumor cells and this can be associated to its selective uptake by the cancer cell and the intracellular generation of hydrogen peroxide via redox reactions with no toxic effects on normal tissue (Riordan NH, et al., 1995; Noto et al., 1989; Jackson et al., 1995; Garland et al., 1986). The most likely reason for this can be a quantitative difference in the content of the enzyme catalase mentioned earlier (Benade et al., 1969). It is important to recognize that ascorbate's antioxidant or pro-oxidant characteristics depend on the redox potential of the surrounding environment at a specific point in time, and on the quantity of ascorbate. It is conceivable that nutrients that have chemopreventative properties may

be capable of inhibiting the continual growth of transitory clones of cells through their antagonistic pro-oxidant activity. In contrast, uncontrolled pro-oxidant activity can generate excess free radicals (reactive oxygen species) that can be deleterious to cellular membranes and DNA (Schwarz, 1996; Alcaín et al., 1990; Arnold et al., 2001; Djuric et al., 1993; Gonzalez et al., 1996; Gonzalez et al., 1997). This paradoxical role of antioxidants and pro-oxidants has been analyzed previously (Gonzalez et al., 1996; Gonzalez et al., 1997; Allen and Venkatrai, 1992). Interestingly, during differentiation there is an increased cellular production of oxidants that appear to provide one type of physiological stimulation for changes in gene expression that lead to a terminal differentiated state (Allen and Venkatrai, 1992).

Oxygen, the final electron acceptor, is of great importance to the ascorbate induced cytotoxic action on cancer cell proliferation by interfering with anaerobic respiration (fermentation), a commonly used energy mechanism of malignant cells. It would be worth investigating the status of the mitochondria of malignant cells since there is reason to believe that this may be relevant to the origin of malignancy (John, 2000). A problem in electron transfer activity might involve coupling to defective mitochondria, in which case vitamin C may help correct this electron transfer problem (Ingebretsen and Normann, 1982).

4.1.3 Apoptosis

As described, it has been well documented that vitamin C is one of the major water-soluble antioxidants present in cells and in plasma (Stich et al., 1976; Speit et al., 1980), and a number of studies have demonstrated that, under certain conditions and at high enough concentrations, ascorbic acid functions as a pro-oxidant and increases DNA damage. The process by which these specific properties of ascorbic acid culminate in cell death is known as apoptosis.

Apoptosis is an active process of cell death characterized by cellular shrinkage, membrane blebbing, chromatin condensation, and DNA fragmentation. Impairment of

apoptosis has been implicated in many human diseases including malignancies (Chinnaiyan and Dixit, 1996). Several mitochondrial specific events have been known to precede apoptosis, including alteration of the ratio of Bcl-2 /Bax and cytosolic translocation of cytochrome C (Jayshree et al., 2000), which in turn can activate a downstream apoptotic cascade of activation of caspases and cleavage of PARP (poly [ADP-ribose] polymerase) (Kluck et al., 1997). It has subsequently been confirmed that clinical response is associated with incomplete cytodifferentiation and the induction of apoptosis via caspase activation in leukemic cells (Soignet et al., 1998).

It has been found that 10 nM-1 mM of vitamin C induces apoptosis in neuroblastoma cells, and in melanoma cells, and in human myelogenous leukemic cell lines within 24 hours by oxidative stress (De Laurenzi et al., 1995; Fujinaga et al., 1994). Vitamin C was also shown to be an important modulator for the growth of mouse myeloma cells in an *in vitro* colony assay (Park et al., 1971). Subsequent studies also established that the growth of leukemic progenitor cells from patients with acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS) may be profoundly modulated by ascorbic acid (Park, 1985; Park et al., 1992). According to recent *in vivo* studies, intravenous administration of sodium 5,6-benzylidene-L-ascorbate (SBA) to inoperable cancer patients induces a significant reduction in tumor volume, without any adverse side effects (Sakagami et al., 1991). Furthermore, recent clinical studies indicate that manipulation of ascorbic acid levels *in vivo* can result in clinical benefit for patients with AML as well as solid tumors (Kim et al., 2001; Park et al., 2001). The results of Park et al. indicate that ascorbic acid induces growth arrest and apoptosis of HL-60 cells in a concentration-dependent manner. Such results indicate that ascorbic acid causes apoptosis through inducing the decrease of Bcl-2/Bax ratio, the release of cytochrome C from mitochondria to cytosol, activation of caspase-9 and caspase-3, and cleavage of PARP.

Additionally, the oxidation and metabolism of DHA results in the increased formation of metabolites that cause cellular damage (Gautam et al., 1999).

Despite the complexity of the mechanisms involved, apoptosis has been successfully replicated in numerous studies such as these cited herein. Selectively induced apoptosis in tumor cells by vitamin C may constitute a unique, host non-toxic strategy in the management of malignant disease. Original research conducted by this doctoral candidate on the mechanisms of apoptosis shall be further elucidated in section 5.1.3.

4.1.4 The Extracellular Matrix and Collagen Production

Ascorbic acid metabolism is associated with a number of mechanisms known to be involved in host resistance to malignant disease. Cancer patients are significantly depleted of ascorbate. This could indicate an increased requirement and utilization of this substance to potentiate these various resistance mechanisms.

One of the most basic functions of vitamin C is the prevention of scurvy, as previously described. Scurvy results from the severe dietary lack of ascorbate, and is a syndrome of generalized tissue disintegration at all levels. It involves the dissolution of the intercellular ground substance, the disruption of collagen bundles, and the disruption of the lysis of the inter-epithelial and inter-endothelial cements, resulting in ulceration with secondary bacterial colonization as well as vascular disorganization with edema and interstitial hemorrhage. An additional result involves generalized undifferentiated cellular proliferation with specialized cells throughout the tissue reverting to a primitive form (Cameron et al., 1979). The generalized stromal changes of scurvy are identical to the local stromal changes observed in the immediate vicinity of invading neoplastic cells (McCormick, 1959). Thus, stromal resistance may be a physical line of defense against cancer by encapsulating neoplastic cells with a dense fibrous tissue. This feature can be enhanced by high doses of ascorbate. Resistance of the intercellular ground substance to local infiltration, and the degree of lymphocytic response are among other stromal factors involved in resistance which are enhanced by vitamin C. A brisk lymphocytic response is a systemic factor indicative of enhanced host resistance, and is associated with a more favorable prognosis of the disease. In order to proliferate, cells must escape the restraint imposed by highly viscous intercellular glycosaminoglycans, and they must do this by the

release of the enzyme hyaluronidase (Dresden, Heilman and Schmidt, 1972). There is evidence that a physiological hyaluronidase inhibitor is a oligoglycosaminoglycan that requires ascorbic acid for its synthesis (Cameron and Pauling, 1973; McCormick, 1959). Changes in hyaluronic acid have been shown to be conducive to cell proliferation (Yoneda et al., 1998). Additionally, the role of ascorbate in the synthesis of collagen is vitally important in the extracellular matrix. The collagen-rich extracellular matrix, including the basement membrane, is a major barrier to the metastatic and invasive spread of cancer cells (Cameron et al., 1979). The intercellular matrix is reinforced by a tri-dimensional network of interlacing collagen fibers, and the amount of collagen present determines the strength of the tissue as well as its resistance to malignant infiltration. Lack of ascorbate sharply reduces hydroxylation of prolyl and lysyl residues into hydroxyproline and hydroxylysine, leading to instability of the triple helix of collagen (Kennedy, 1976), which is a common feature of both scurvy and cancer. The function of vitamin C in the synthesis of collagen is also of central importance in wound healing, including in decubital ulcers, in recovery from surgery and in other accidental traumatic injuries (Ringsdorf and Cheraskin, 1982).

Composed of only two amino acids, glycine and hydroxyproline, collagen consists of long polypeptide chains containing about one thousand amino acid residues, or about 16,000 atoms. Three-dimensionally, the polypeptide chains of collagen's two amino acids are coiled in a left-handed helix, with three of these helical strands twisted about each other in a right-handed superhelix. In the multisteped synthesis of collagen, the three-stranded precursor to collagen, "procollagen", is assembled first, with the amino acids glycine and proline as its principal components. Procollagen is then converted to collagen through a reaction that substitutes a hydroxyl group, OH, for hydrogen, H, at certain points in the proline residues, converting these residues to hydroxyproline and securing the chains within the triple helix. The further hydroxylation of lysine residues yields hydroxylysine, which permits the cross-linking of the triple helices with the networks of fiber within tissue throughout the body.

Two enzymes, prolyl-4-hydroxylase and lysyl-hydroxylase, catalyze these hydroxylation reactions, and vitamin C works with each in inducing the reactions. For each hydrogen atom that is replaced by a hydroxyl group, OH, one molecule of vitamin C is destroyed. Vitamin C is therefore much more than merely a catalyst in the production of collagen, and its destruction during the reaction is the reason for the continual necessity of replenishing vitamin C in the body.

Prolonged exposure of cultures of human connective tissue cells to ascorbate *in vitro* has been found to induce an eightfold increase in the synthesis of other proteins. To explore the roll of vitamin C in cell attachment and detachment, researchers in France studied the inhibition of cell proliferation and fibronectin biosynthesis by sodium ascorbate (Peterszegi, Dagonet, Labat-Robert, and Robert, 2002). Recognizing that the importance of ascorbate on the production of extracellular matrix proteins, such as elastin and collagens, has already been well documented, these researchers were among the first to publish studies concerning the effects of ascorbate on fibronectin biosynthesis. Fibronectin is important for cell attachment and for proliferation, and they investigated the effects of sodium ascorbate on these processes as well as on cell viability and fibronectin biosynthesis by human skin fibroblasts *in vitro*. They then followed cell proliferation by monitoring the incorporation of [(3)H]-thymidine, viability by the MTT-test, cell adherence by counting adherent and nonadherent cells, and fibronectin biosynthesis by immunoprecipitation of biosynthetically labelled fibronectin. Their results indicated that in the presence of ascorbate, the fibroblasts showed a biphasic growth pattern. At 500 microM ascorbate, [(3)H]-thymidine incorporation was stimulated by 15% when compared to the controls, and higher concentrations gradually decreased proliferation up to 36% of the control value at 5 mM. These effects of ascorbate on DNA synthesis were followed to > 1.25 mM by a strong inhibition, cytotoxic effect and cell death. The non-adherent cell count increased to 10% of the total population at 2.5 mM and to 31% at 5.0 mM ascorbate. Increasing concentrations of ascorbate resulted in a dose-dependent decrease of fibronectin biosynthesis, both in the culture supernates and cell extracts. Superoxide-dismutase or catalase could inhibit sodium ascorbate-induced cytotoxicity and partially re-establish fibronectin biosynthesis,

whereas desferrioxamine, ergothionein and vitamin E were found to be inefficient. The results indicate that ascorbate decreases fibronectin biosynthesis of cultured human skin fibroblasts, thereby producing cell detachment and decreased proliferation, and this effect is mediated primarily by the reactive oxygen species and can be inhibited by superoxide-dismutase and catalase.

Other research has shown that vitamin C regulates keratinocyte viability, the epidermal barrier, and the basement membrane *in vitro*, while also reducing wound contraction after the grafting of cultured skin substitutes (Boyce, Supp, Swope, and Warden, 2002). Cultured skin substitutes have become useful as adjunctive treatments for excised, full-thickness burns, but skin substitutes lack the identical anatomy and physiology of native skin, and deficiencies of structure and function may, it is postulated, result from nutritional deficiencies in culture media. To address this hypothesis, Boyce et al. titrated vitamin C at 0.0, 0.01, 0.1, and 1.0 mM in a cultured skin substitute model on filter inserts, and cultured skin substitute inserts were then evaluated at 2 and 5 weeks for viability by incorporation of 5-bromo-2'-deoxyuridine (BrdU) and by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) conversion. Subsequently, cultured skin substitute grafts consisting of cultured human keratinocytes and fibroblasts attached to collagen-glycosaminoglycan substrates were incubated for 5 weeks in media containing 0.0 mM or 0.1 mM vitamin C, and then grafted to athymic mice. The cultured skin substitutes (n = 3 per group) were evaluated *in vitro* at 2 weeks of incubation for collagen IV, collagen VII, and laminin 5, and through 5 weeks for epidermal barrier by surface electrical capacitance. The cultured skin substitutes were grafted to full-thickness wounds in athymic mice (n = 8 per group), evaluated for surface electrical capacitance through 6 weeks, and scored for percentage of original wound area through 8 weeks, and for HLA-ABC-positive wounds at 8 weeks after grafting. The data indicate that incubation of cultured skin substitutes in media containing vitamin C results in greater viability (higher BrdU and MTT), more complete basement membrane development at 2 weeks, and better epidermal barrier (with lower surface electrical capacitance) at 5 weeks *in vitro*. After grafting, cultured skin substitutes with vitamin C

developed functional epidermal barrier earlier, had less wound contraction, and had more HLA-positive wounds at 8 weeks than without vitamin C.

These results suggest that incubation of cultured skin substitutes in media containing vitamin C extends cellular viability, promotes formation of epidermal barrier *in vitro*, and promotes engraftment. Improved anatomy and physiology of cultured skin substitutes that result from nutritional factors in culture media may be expected to improve efficacy in the treatment of full-thickness skin wounds. Such data also demonstrate the key importance of vitamin C in wound healing in general, not merely in skin.

In 2003, scientists in India studied the role of ascorbic acid on wound healing in mice exposed to different doses of gamma radiation (Jagetia, Rajanikant, and Rao, 2003). Jagetia et al. examined the alteration of radiation-induced changes in wound contraction, collagen synthesis and wound histology by ascorbic acid in mice exposed to 10, 16 and 20 Gy of fractionated (2 Gy/fraction) gamma radiation. The animals were given double-distilled water or ascorbic acid daily before exposure to 2 Gy/day of fractionated irradiation. A full-thickness skin wound was then created on the dorsum of the irradiated mice, and the progression of wound contraction and collagen synthesis were examined as histological evaluations were carried out at various times after wounding. Irradiation caused a dose-dependent delay in wound contraction, and pretreatment with ascorbic acid resulted in a significant increase in wound contraction. The greatest increase in wound contraction was observed 6 and 9 days after wounding in both groups. Pretreatment with ascorbic acid augmented the synthesis of collagen significantly as revealed by an increase in hydroxyproline content. The collagen deposition, as well as fibroblast and vasculature densities, declined in a dose-dependent manner in groups receiving radiation alone as indicated by histological evaluation. Pretreatment with ascorbic acid ameliorated the observed effect significantly. The investigators demonstrated that pretreatment with ascorbic acid resulted in a significant reduction of radiation-induced delay in wound healing, as shown by earlier wound closure and by increased collagen content, as well as in fibroblast and vascular densities.

As important as vitamin C is in such processes, however, the role of vitamin C throughout the body extends far beyond its known activity in collagen synthesis. The following section addresses another major area in which vitamin C is involved, namely, immunology.

4.1.5 Immune Function

Secondary Anticancer Mechanisms of Ascorbic Acid

Ascorbate is essential for the efficient working of the immune system. The immunocompetence mechanisms are a combination of humoral and cell mediated defensive reactions, with ascorbate involved in a number of ways. In terms of humoral immunocompetence, ascorbate is essential for immunoglobulin synthesis (Lewin, 1976). In cell mediated immunity, immunocompetence is exercised overwhelmingly by lymphocytes which contain high concentrations of ascorbate relative to other cells. In addition, ascorbate is required for active phagocytosis (Goetzl, Wasserman, Gigli, and Austen, 1974), and it has also been shown to enhance interferon production (Lewin, 1976; Siegel, 1975; Dahl and Degre, 1976).

In order to learn how the immune system changes with age, researchers in Spain studied the effects of several antioxidants on the natural killer cell function of aging mice (Ferrandez, Correa, Del Rio, De la Fuente, 1999). Ferrandez et al. studied the changes with aging in the effect *in vitro* of the antioxidants thiazolidine-4-carboxylic acid (or thioproline), N-acetylcysteine (NAC), ascorbic acid (AA), and alpha-tocopherol (vitamin E, or VE) on the natural killer (NK) activity in mononuclear cells from axillary nodes, spleen, thymus and peritoneal leukocytes from BALB/c male mice. They studied young (8+/-2 weeks), adult (24+/-2 weeks), mature (48+/-2 weeks), and old (72+/-2 weeks) animals, using a nonradioactive cytotoxic assay with cells from the murine lymphoma YAC-1 as target cells, and a relation effector of cells/target cells of 10/1. The concentrations of the different antioxidants were: 1 mM for thioproline and N-acetylcysteine, and 5 microM for ascorbic acid and alpha-tocopherol, which had induced

a maximum effect in previous dose-response experiments. The results demonstrated that, in general, the antioxidants mentioned above cause an enhancement of the NK activity at all ages studied, with this stimulation being higher with thioproline and N-acetylcysteine than with ascorbic acid and alpha-tocopherol. The effects were similar for the three lymphoid organs and the peritoneum. This stimulation of the NK activity by antioxidants is an important favorable response, especially in old mice, in which age results in a decrease in NK function and, therefore, in a higher incidence of neoplasia.

Continuing with the same idea as Ferrandez et al., further studies have examined the activities of ascorbic acid on natural killer cell activity, *in vivo* (Heuser and Vojdani, 1997). After exposure to many toxic chemicals, NK function can be decreased significantly. Weeks or months later, natural killer (NK) function can rebound to normal levels in some people, while being suppressed for prolonged periods of time in others. In view of this, Heuser et al. decided to study the effect of buffered vitamin C on NK, T and B cell function in patients who had been exposed to toxic chemicals. After the first blood draw, 55 patients immediately ingested granulated buffered vitamin C in water at a dosage of 60 mg/Kg of body weight. 24 hours later, blood was again drawn for a follow-up study of NK, T and B cell function. Vitamin C in high oral dose was found to be capable of enhancing NK activity up to ten-fold in 78% of patients. Lymphocyte blastogenic responses to T and B cell mitogens were restored to the normal level after vitamin C usage. The signal transduction enzyme protein kinase C (PKC) appeared to be involved in the mechanism of induction of NK activity by vitamin C. The results of this study indicate that immune functional abnormalities can be restored after toxic chemical exposure by oral usage of vitamin C.

4.1.6 Activities Related to Stress and Cortisol Secretion

Since adrenal glands contain an unusually high level of vitamin C in comparison to other body organs, a measure of their function is often indicative of the total health of the individual. Dr. William Wells at Michigan State University (Wells, personal

communication, 2001) conducted research with mice in which the arteries of the adrenal cortex were catheterized in order to measure their output. When these mice were subjected to stress, Dr. Wells found that, after their adrenals had been depleted of their vitamin C reserves, the adrenal cortices then began producing cortisol. Injecting laboratory mice with cortisol has been found to induce thymic involution, so the production of cortisol by the adrenal cortex is understood to have negative immunological consequences. This is just one of many examples of the detrimental effects of not having adequate amounts vitamin C in the body.

Because of their high vitamin C content, which is the highest of any organ in the body, adrenal glands have played a central role in the evolution of our understanding of vitamin C. As described in chapter 2, it was from the adrenal glands of animals that vitamin C was first isolated, and its molecular structure identified, by Albert Szent-Gyorgyi, M.D., Ph.D., who later won the Nobel Prize for this work.

Investigating further the relationship between ascorbic acid deficiency and cortisol, Enwonwu et al. studied the effect of marginal ascorbic acid deficiency on saliva levels of cortisol in the guinea pig (Enwonwu, Sawiris, and Chanaud, 1995). These researchers found that male guinea pigs who were subjected to prolonged marginal ascorbic acid deficiency developed features of functional adrenal hypercorticism. Compared with age- and sex-matched controls who were fed an adequate diet for a similar period, the ascorbate deficient guinea pigs exhibited no change in submandibular gland weight, but they did elicit a significant ($p < 0.005$) reduction in stimulated whole-saliva flow rate. Perhaps most importantly, plasma cortisol concentration (nmol/L) was found to be significantly increased ($p < 0.005$) in the ascorbate deficient animals (998.21 57.19 as compared to 254.66 15.62 for the controls). Associated with marked hypercortisolaemia in the deficient animals was a significant ($p < 0.01$) but less prominent increase in the whole-saliva cortisol level, resulting in a mean saliva/plasma cortisol ratio of 46% for this group compared to 72% for the controls. Increased corticosteroid levels have been shown to suppress immunological and inflammatory responses, particularly neutrophil function, in addition to impairing the production of some cytokines, inhibiting collagen

synthesis, and impairing wound healing as well as bone matrix formation. In humans, tissue ascorbate levels may be depleted by numerous conditions such as aging, stress, smoking, ionizing radiation, ingestion of drugs, protein malnutrition, diabetes, and other pathological states which are among the risk factors for xerostomia and periodontal/oral mucosal lesions. From this particular study, the authors suggest that increased salivary and blood levels of glucocorticoids in such conditions may indicate a reduced ability of the host to mount an effective immune response against oral pathogens.

4.2 Further *In Vitro* Observations

As an essential nutrient in humans, primates, the guinea pig, and any collagenous species lacking the L-gulonolactone oxidase enzyme, ascorbate is involved in many cellular functions, including the modulation of cell growth and differentiation.

Concluding the literature review, this section addresses a particular *in vitro* study in which researchers in Spain explored the ability of ascorbate to either reduce or stimulate the growth of tumor cells, depending on the cell type. Alcaín and Buron (1994) found that an inhibitory effect is not limited exclusively to the biologically active isomer L-ascorbate, but that isoascorbate and D-ascorbate are actually more effective in reducing cell growth than is L-ascorbate. These results confirm that ascorbate has a cytotoxic effect, rather than a cytostatic effect, by killing cells directly. However, the investigators found that only L-ascorbate is able to stimulate cell growth, even though the precise mechanisms of this stimulation remain unknown. L-Ascorbate was found to stimulate the *in vitro* differentiation of several mesenchyme-derived cell types by altering the expression of multiple genes as the cell progresses through specific differentiation programs. Stimulation of collagen matrix at gene transcription, mRNA stabilization, hydroxylation, and secretion are key functions of L-ascorbate, which was also found to prevent cell transformation by stabilization of the differentiated state, and through cooperation with other agents to induce differentiation in the particular cell line (leukemia) under investigation in this study. Meanwhile, the exact mechanisms of activity utilized by the isomers of ascorbic acid remain topics of further study.

Thus, although numerous cellular mechanisms of ascorbic acid and its isomers have been clearly elucidated, other mechanisms still remain unknown. Although ascorbic acid certainly plays a key role in a myriad of physiological processes, the full extent of its properties, and the full implications thereof, promise to remain topics of fertile research in the future.

4.3 Animal Studies

A logical extension of *in vitro* studies, animal studies such as that described in this section further explore the mechanisms by which ascorbic acid is responsible for its selective cytotoxicity.

Subcutaneous injections of ascorbic acid (AA) were found to significantly potentiate the curative effects of chemotherapy on advanced Lewis lung carcinoma in mice (Cohen and Krasnow, 1987). Orally administered AA was seen to inhibit DNA, RNA, and protein synthesis in epithelial neoplastic cells in mice and in rats (Lupulesco, 1991), as well as inhibit transplantable melanoma tumor development in mice (Varga and Airoidi, 1983). AA also enhanced carbidopalevodopa methyl ester antitumor activity against pigmented B16 melanoma in mice (Pierson and Meadows, 1983). Administered to the drinking water of Swiss mice, AA (0.1%) inhibited the growth of solid sarcoma 180 and increased the survival time in comparison to the controls (Chakrabarti and Dasgupta, 1984). Tsao et al., (1988; 1989) reported that AA in the drinking water of mice significantly inhibited the growth of human mammary tumor fragment xenografts implanted in immunocompetent mice. AA was also effective as a tumor inhibitor in this model when given in the diet along with cupric sulfate (Tsao et al., 1988). As a dietary additive alone, AA was not effective. This finding supports the theory that the inhibitory action of AA is due to hydrogen peroxide (and hydroxyl radical) production because of the known ability of Cu^{2+} to catalyze the production of these substances in the presence of AA (Halliwell and Gutteridge, 1989).

Chapter 5: Original Research on Ascorbic Acid in the Management of Cancer

5.1 Mechanisms

Data presented herein demonstrate that plasma AA concentrations exceeding those required to kill 100% of tumor cells *in vitro* can be sustained in humans, and that those levels can generally only be obtained by intravenous administration of AA. Research indicates that intravenous AA, administered in sufficient doses to achieve plasma concentrations that have demonstrable cytotoxic effects on tumor cells, constitutes an effective chemotherapeutic agent. Additionally, AA exhibits the following positive characteristics:

- It preferentially kills neoplastic cells.
- It is virtually non-toxic at any dosage (Cameron et al., 1979).
- It does not suppress the immune system, unlike most chemotherapy agents.
- It increases animal and human resistance to infectious agents by enhancing lymphocyte blastogenesis, enhancing cellular immunity, strengthening the extracellular matrix, and enhancing bactericidal activity of neutrophils and modulation of complement protein (Anderson, 1981; Henson et al., 1991; Kennes, 1983; Klenner, 1949; Klenner 1951; Klenner 1971; McCormick 1952; Panush et al., 1982; Siegel and Leibovitz, 1982; Siegel and Morton, 1977; Yonemoto et al., 1976).
- It strengthens the structural integrity of the extracellular matrix which is responsible for stromal resistance to malignant invasiveness (Cameron et al., 1979).

In a study reported in the British Journal of Cancer (Casciari, Riordan NH, et al., 2001), this doctoral candidate investigated the cytotoxicity of ascorbate, lipoic acid, and other antioxidants in hollow fibre *in vitro* tumors (Casciari et al., 2001). Vitamin C (ascorbate) is toxic to tumor cells, and has been suggested as an adjuvant cancer treatment. In this research, our goal was to determine if ascorbate, in combination with other antioxidants, could kill cells in the SW620 hollow fibre *in vitro* solid tumor model at clinically achievable concentrations. Ascorbate anti-cancer efficacy, alone or in combination with

lipoic acid, vitamin K3, phenyl ascorbate, or doxorubicin, was assessed using annexin V staining and standard survival assays. A two-day treatment with 10 mM ascorbate was found to increase the percentage of apoptotic cells in SW620 hollow fibre tumors. Additionally, lipoic acid synergistically enhanced ascorbate cytotoxicity, reducing the 2 day LC(50) in hollow fibre tumors from 34 mM to 4 mM. Lipoic acid, unlike ascorbate, was equally effective against proliferating and non-proliferating cells. Ascorbate levels in human blood plasma were measured during and after intravenous ascorbate infusions. Infusions of 60 g produced peak plasma concentrations exceeding 20 mM with an area under the curve (24 h) of 76 mM h. Thus, we found that tumoricidal concentrations may be achievable *in vivo*. Ascorbate efficacy was enhanced in an additive fashion by phenyl ascorbate or vitamin K3. The effect of ascorbate on doxorubicin efficacy was concentration-dependent; low doses were protective while high doses increased cell killing.

Such a study is representative of the chemotherapeutic properties of ascorbic acid as elucidated throughout this chapter. In the following sections, further tumor cytotoxicity mechanisms investigated by this doctoral candidate shall be described.

5.1.1 *in Vitro* Preferential Toxicity

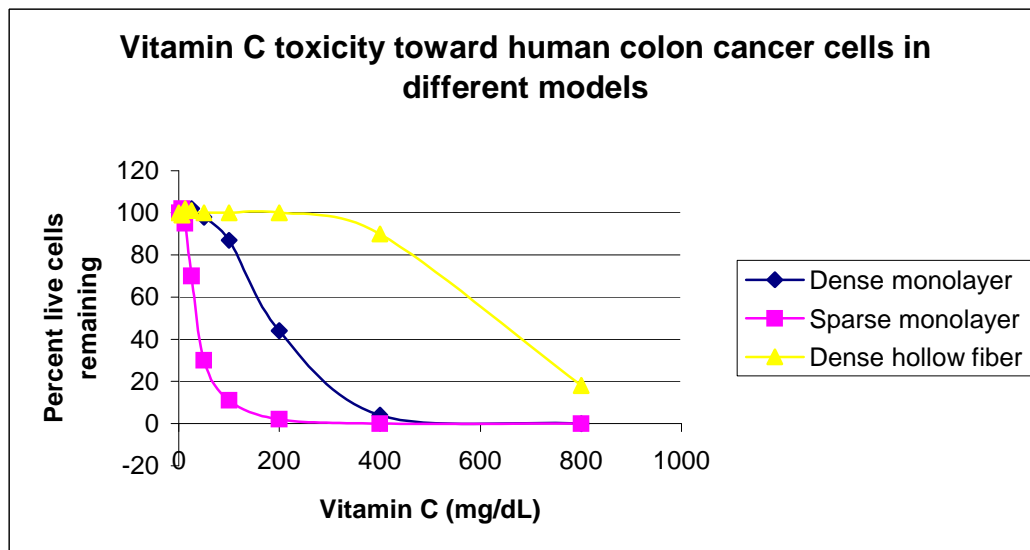
Vitamin C has advantageous characteristics as a chemotherapeutic agent. Rather than possessing adverse side effects as most chemotherapeutic drugs do, vitamin C has side benefits, such as increasing collagen production, and enhancing immune function.

In support of such findings, original research by this candidate has found that AA is toxic to several types of human tumor cells at concentrations which are nontoxic to normal cells. Figure 1 shows the AA percent dose-response of a newly established human colon tumor cell line and a normal human colon fibroblast cell line (ATCC CCD18-Co). The AA begins to reduce cell proliferation in the tumor cell line at the lowest concentration, 1.76mg/dl, and is completely cytotoxic to the cells at 7.04mg/dl, while significant inhibition of the normal cells is demonstrated only at a dose of 28.18 mg/dl and 100%

cell death is realized only at a dose of 56.36 mg/dl (an 8-fold higher dose than the tumor cells). In addition, the normal cells grew at an enhanced rate at the low dosages (1.76 and 3.52 mg/dl). The graph also displays preferential toxicity of AA for tumor cells. Greater than 95% toxicity to human endometrial adenocarcinoma and pancreatic tumor cells (ATCC AN3-CA and MIA PaCa-2) occurred at 20 and 30 mg/dl, respectively. No toxicity or inhibition was demonstrated in the normal, human skin fibroblasts (ATCC CCD 25SK), even at the highest concentration of 50 mg/dl.

At the medical Center where I conducted these studies, we began to investigate the effects of vitamin C on cultured tumor cells in 1991. We found that vitamin C was preferentially toxic to tumor cells, meaning that it killed tumor cells before killing normal cells. Our early findings on preferential vitamin C toxicity were published in 1994 (Riordan NH et al., 1994). In that paper we also described a so called “serum effect,” wherein the toxicity of vitamin C was reduced by the presence of human serum. The inhibitory effects of serum led us to the conclusion that the concentrations of vitamin C which were toxic to tumor cells in our early studies (5 to 50 mg/dL) would not necessarily be toxic *in vivo*.

Figure 1



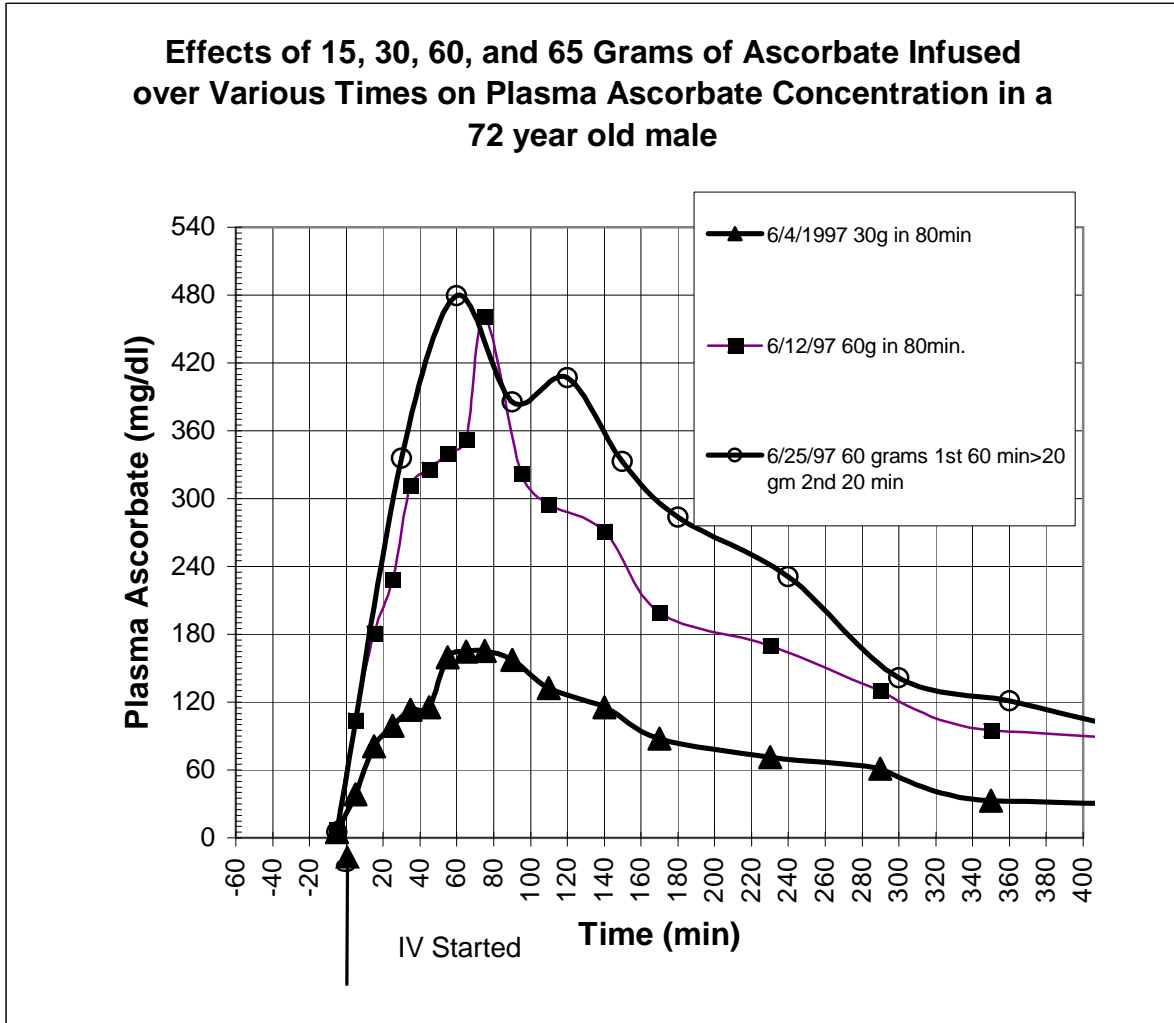
We therefore began a series of experiments in which we tried more closely to mimic the *in vivo* tumor micro-environment. In particular, we began testing for toxicity of vitamin C toward cultured tumor cell lines using dense monolayers and hollow fiber tumor models to mimic the three-dimensionality of tumors. We used human sera as culture media to include the serum inhibitory activity seen in previous assays.

Using these new culture conditions, we found that the cytotoxic concentration of vitamin C for most human tumor cell lines was indeed much higher than previously described, as indicated in Figure 1.

5.1.2 Pharmacokinetics

Given this information, that higher concentration of vitamin C were required to become cytotoxic to tumor cells, we needed to learn more about the human pharmacokinetics of vitamin C. There were no data on the concentrations of vitamin C that were achievable in human beings after high-dose intravenous vitamin C. We therefore began a series of experiments to yield data for modeling pharmacokinetics of high doses of vitamin C.

Figure 2



We gave a series of vitamin C infusions to a 72 year old male who was in excellent physical condition except for slowly progressing, non-metastatic carcinoma of the prostate. Before the infusions, and at intervals thereafter, blood was drawn from a heparin lock (not the site of infusion). The plasma was separated and analyzed for plasma vitamin C concentration using a microplate-based 2,6-dichlorophenol-indophenol assay.

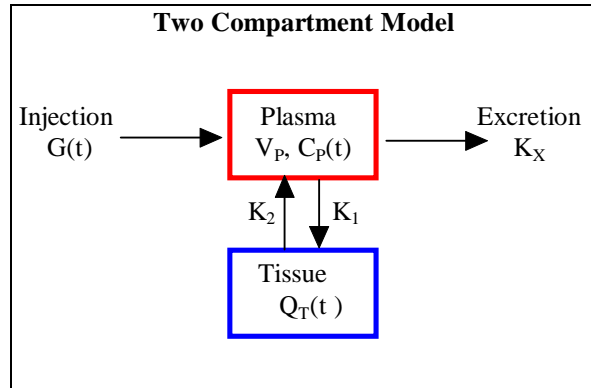
Some of the results are given in Figure 2. From this experiment, we observed that a 30 gram infusion was not adequate to raise plasma levels of vitamin C to a level that was toxic to tumor cells (>200 mg/dL for dense monolayers and >400 mg/dL for hollow fiber

models). Infusion of 60 grams resulted in a brief (30 min) elevation of plasma levels of vitamin C above 400 mg/dL, while 60 grams infused over 60 minutes immediately followed by 20 grams infused over the next 60 minutes resulted in a 240 minute period in which the vitamin C plasma concentration was near or above 400 mg/dL.

Ascorbate Pharmacokinetics

Using data from the above experiments, we designed a two-compartment model with four adjustable parameters (V_P , K_X , K_1 , K_2) to fit the data. The model schematic and the data fit are shown in the accompanying Figure 3.

Figure 3



The parameter K_X represents the rate of excretion of ascorbate out of the blood (renal excretion), while parameter K_1 represents the rate of diffusion of ascorbate from the blood into tissue, and K_2 represents the diffusion rate for return of ascorbate to the blood stream from the tissue compartment.

Table 1

Date:	5/29/09	6/4/97	6/5/97	6/11/97	6/12/97	6/18/97	6/19/97	6/25/97	6/26/97
Dose (g):	15	30	60	30	60	65	65	65 =>20	65
Infusion (min)	45	80	160	40	80	60	60	60 =>60	60
K_X (min^{-1}) (excretion)	0.026	0.027	0.024	0.025	0.022	0.025	0.022	0.023	0.027
K_1 (min^{-1}) (tissue absorb)	0.195	0.214	0.653	0.307	0.124	0.447	0.200	0.150	0.884
K_2 (min^{-1}) (tissue efflux)	0.066	0.060	0.165	0.091	0.038	0.141	0.062	0.040	0.235
K_2 / K_1 (efflux/absorb)	0.337	0.279	0.253	0.296	0.302	0.317	0.310	0.265	0.266
Max. C_p (mg/dl)	125.7	186.1	285.3	233.3	427.8	458.4	495.0	472.8	397.2
Ave. (1-8hr) C_p (mg/dl)	37.3	70.6	150.1	76.0	165.4	167.6	185.4	221.5	151.9

We used this two compartment model to obtain kinetic parameters from each of the *in vivo* vitamin C experiments where sufficient time-concentration data were obtained. We first fitted all four parameters, then fixed the blood plasma volume at 30 dl (near the average value for all experiments) and floated the three K values. Results are given in Table 1, above. The average parameter values for all experiments are shown in Table 2.

Table 2

Parameter	Mean	SD
K_x	0.025	0.002
K_1	0.353	0.261
K_2	0.100	0.067
K_2/K_1	0.292	0.028

To see if there was any systematic variation in parameter values over time, the parameter K_x , and the ratio K_2/K_1 for each experiment were plotted against time. The excretion constant, K_x , was remarkably uniform, as was the ratio K_2/K_1 (see Figure 4). The tissue uptake rate constant, K_1 , did vary, but not in a systematic way. We use the ratio K_2/K_1 to point out that while K_2 also varies, its variation merely compensates for variations in K_1 . We conclude from this analysis that the pharmacokinetic parameters of the subject did not change over a one month period of regular ascorbate infusions.

Figure 4

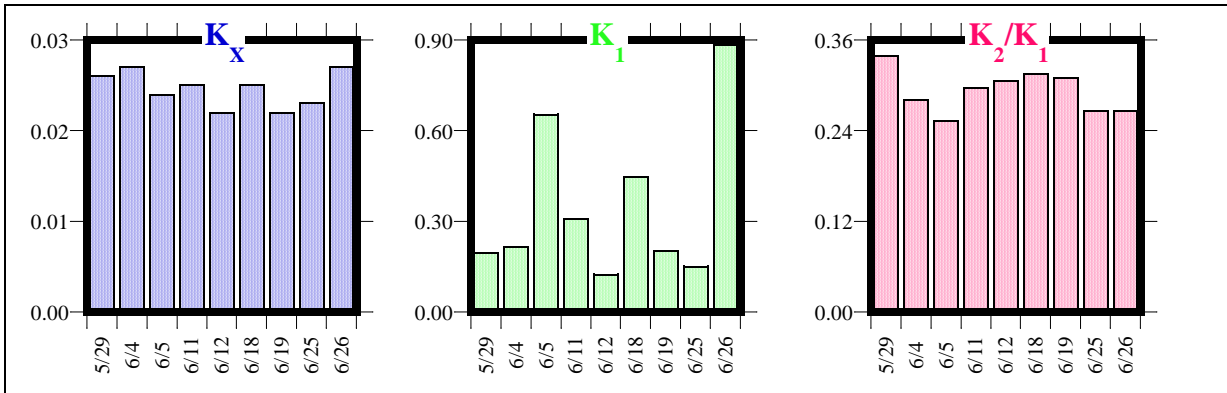
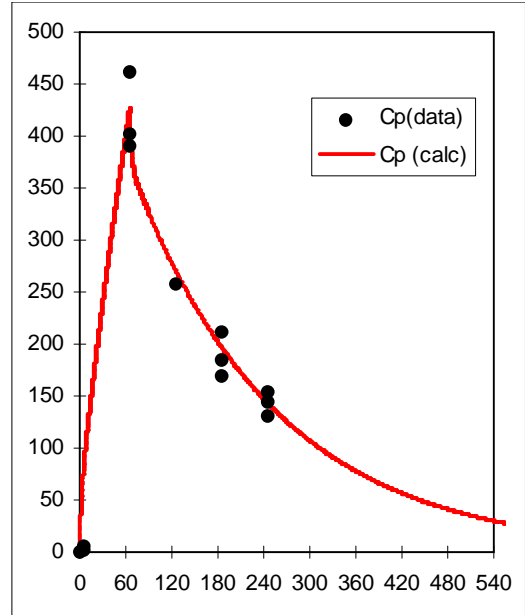


Figure 5

Other Pharmacokinetic Studies

We plotted data points from three recent studies (over a three month period) against the theoretical curve obtained using the average parameter values given above. The results are shown in Figure 5.

Clearly, there was excellent agreement between the theoretical curve and the recent data, suggesting the ascorbate pharmacokinetics for this subject were the same over the three month treatment period. This further supports the conclusion that the ascorbate transport parameters for a given subject remain relatively constant during the time of treatment.



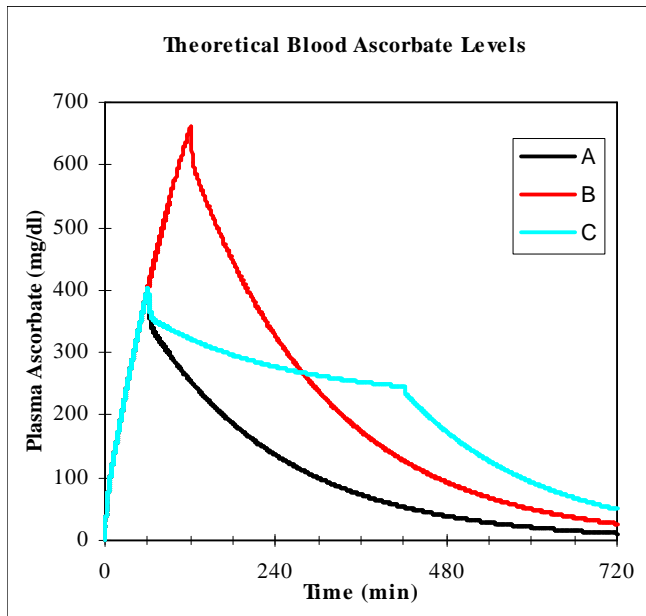
Computer Protocol Simulations

A program was then constructed to predict the plasma ascorbate levels in various protocols based on the pharmacokinetic parameters obtained above. Three protocols were simulated:

- A) 1 hour at 60 g/hr
- B) 2 hour at 60 g/hr
- C) 1 hour at 60 g/hr followed by 6 hour at 10 g/hr

Predicted plasma ascorbate levels for these protocols are shown in Figure 6. Prolonged infusions at higher doses obviously give the highest peak values, while a “trickle” infusion can be used to maintain plasma levels at a desired level.

Figure 6

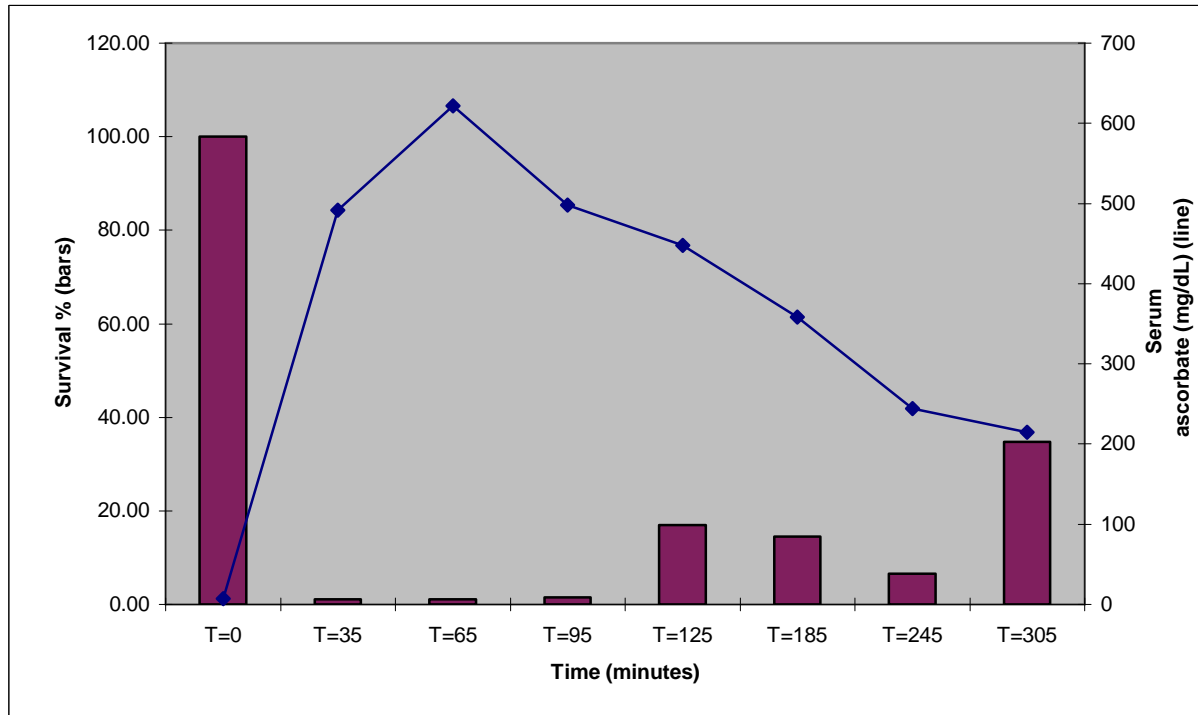


This computer simulation can be a useful tool for examining what plasma levels we can expect from various protocols, once the pharmacokinetics for a given subject are known from an initial experiment.

EFFECTS OF HUMAN SERUM OBTAINED FROM SUBJECT RECEIVING INTRAVENOUS VITAMIN C ON TUMOR CELL GROWTH

To more closely study the probability of *in vivo* cytotoxic effects of intravenous vitamin C, we planned and performed the following experiment. Dense monolayers of human prostate tumor cells (PC-3 from ATCC) were created in microplates. A patient with carcinoma of the prostate was given 65 grams of intravenous vitamin C (in 500 cc sterile water for injection) over 65 minutes. Serum separator blood tubes were drawn before the infusion and at intervals thereafter from a heparin lock separate from the infusion site. The final blood draw was 5 hours after the infusion began. Some of the collected serum was then tested for vitamin C concentration, and the rest was heat-inactivated and used as culture media for the prostate tumor cells. The cells were incubated for five days when the numbers of viable tumor cells were determined using a previously described carboxy-fluorescein diacetate/microplate fluorometer viable cell determination method. The results of the experiment are graphed in Figure 7. Greater than 97% cytotoxicity was observed for the serum samples taken at 35, 65, and 95 minutes after the beginning of the IV vitamin C. During those periods, the serum concentration was greater than 400 mg/dL. After that time, the toxicity decreased. The last sample taken (vitamin C concentration 213 mg/dL) resulted in 64% inhibition compared to the controls.

Figure 7. Effects of human serum removed from patient before and at intervals after intravenous infusion of vitamin C (60 grams) on cultured human prostate tumor cells.

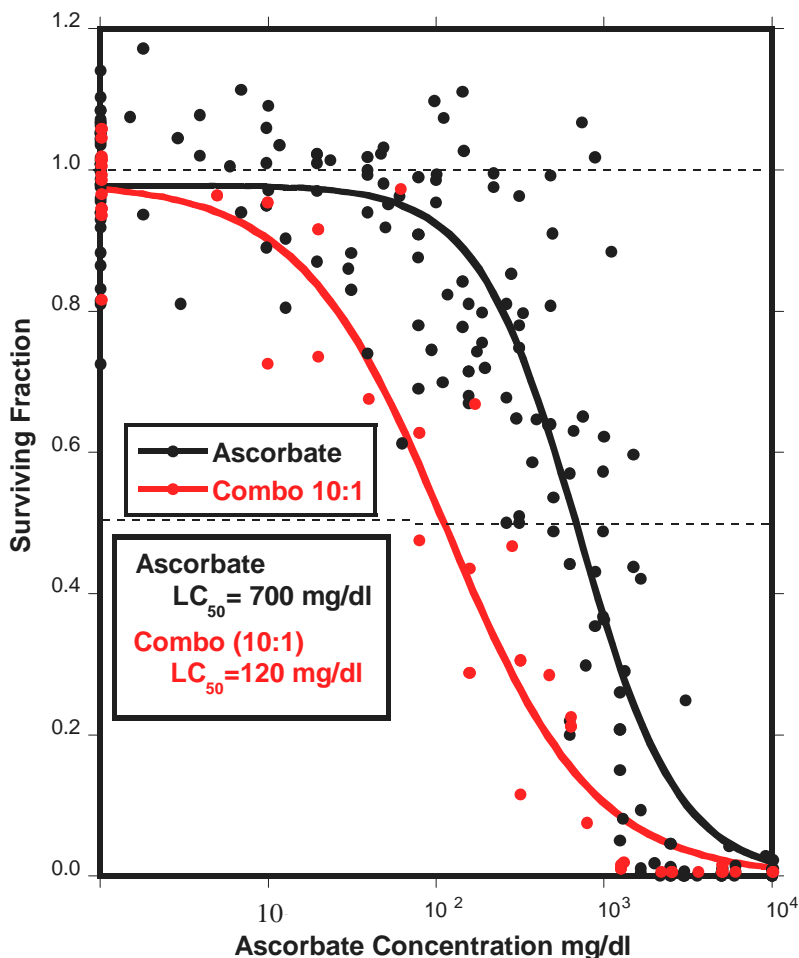


POTENTIATION OF PREFERENTIAL TOXICITY OF VITAMIN C

Because plasma concentrations of vitamin C of greater than 200 mg/dL are problematic to maintain, we began looking for ways to increase the sensitivity of tumor cells to vitamin C. During experimentation we found that lipoic acid (a water and lipid soluble antioxidant that recycles vitamin C) can enhance the tumor toxic effects of vitamin C. Figure 8 illustrates dose response of tumor cells in a hollow fiber tumor model exposed to vitamin C with and without lipoic acid. Lipoic acid decreased the dose of vitamin C required to kill 50% of the tumor cells from 700 mg/dl to 120 mg/dl.

Figure 8
Effect of Lipoic Acid on Ascorbate Efficacy

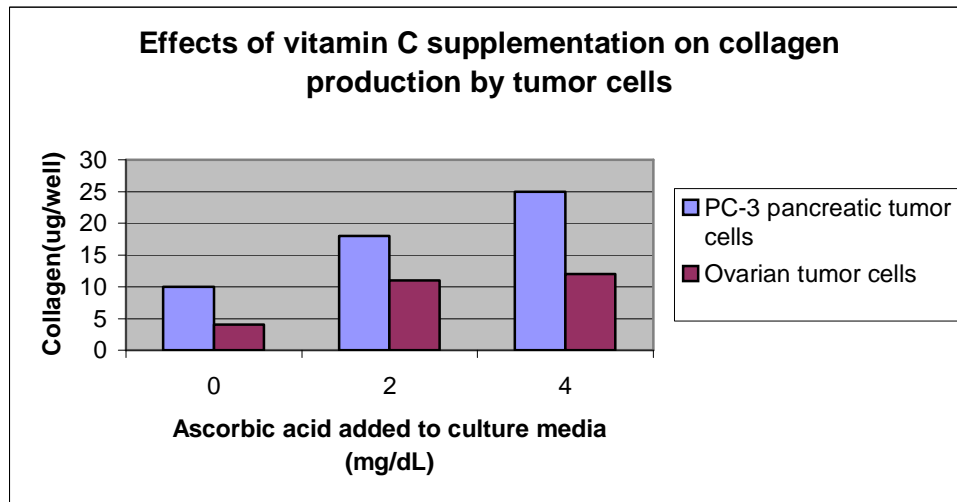
SW620 human colon carcinoma cells grown as hollow fiber solid in vitro tumors exposed for 48 hours to Sodium Ascorbate alone or combined with Lipoic Acid Sodium (Combo 10:1).



EFFECTS OF HIGH-DOSE VITAMIN C ON TUMOR CELL COLLAGEN PRODUCTION

It is well known that vitamin C is required for the hydroxylation of proline, and that low levels of vitamin C can be a limiting factor in the production of collagen. Because many tumor cells produce collagenase and other proteolytic enzymes, we wanted to determine if vitamin C supplementation would increase collagen production by tumor cells, thereby having a balancing effect on collagenase. In an experiment, we supplemented cultured tumor cells with vitamin C concentrations that are achievable with oral supplementation (2 and 4 mg/dL) and measured the collagen produced using a well-known method (Riordan HD et al., 1998). We found that indeed, these concentrations of vitamin C greatly increased the production of collagen. Data are summarized in Figure 9.

Figure 9



5.1.3 Apoptosis

Intravenous Vitamin C and Apoptosis in Cancer Cells

As described in chapter 2, the ascorbic acid molecule closely resembles a glucose molecule. Cancer cells have an affinity to glucose, and consequently they also have an affinity to ascorbic acid. Once inside a tumor, ascorbic acid is cytotoxic to tumor cells through the induction of “apoptosis”.

In normal cells, apoptosis is a form of cell death. When a cell's DNA is damaged, the cell will “commit suicide” in an attempt to keep the damage from reproducing and creating a cancer cell. The immune system is constantly working to maintain health, and apoptosis is a natural, automatic mechanism for the removal of damaged cells. Cells are programmed to replace themselves when the DNA becomes damaged. The program is found in the gene p53, the tumor-suppressing gene. Before rapid, uncontrolled replication may occur, as in cancer, the p53 gene instructs the cell to undergo programmed cell death, or apoptosis.

In cancer cells, however, the “programming” goes awry. The damage mutates the p53 gene, which in turn instructs the cells to multiply even though the DNA is damaged. Apoptosis in cancer cells makes the cancer visible to the immune system. Apoptosis causes the inside of the cell to invert and become the outside of the cell. Consequently,

phosphatidylserene, which ordinarily is found on the inside of the cell, is instead found on the outside and this triggers the immune system. Phosphatidylserene is not only found on the outside of cancer cells, but also on the outside of viruses and aged blood cells that are ready to be replaced.

In laboratory studies which have shown that vitamin C is cytotoxic to cancer cells, there are three types of tumor models: the sparse monolayer, the dense monolayer, and the hollow fiber tumor model. Most of the solid tumors in the body are of the hollow fiber tumor model, with the sparse and dense monolayer models being found on the outer layers of the tumor. Studies show that plasma levels of just 200 mgs/deciliter (one tenth of a liter) will kill all the cells in the dense and sparse models, but not in the hollow fiber tumor model. Plasma levels of 700 mgs/deciliter yield between 50% to 65% cell survival in the hollow fiber tumor model.

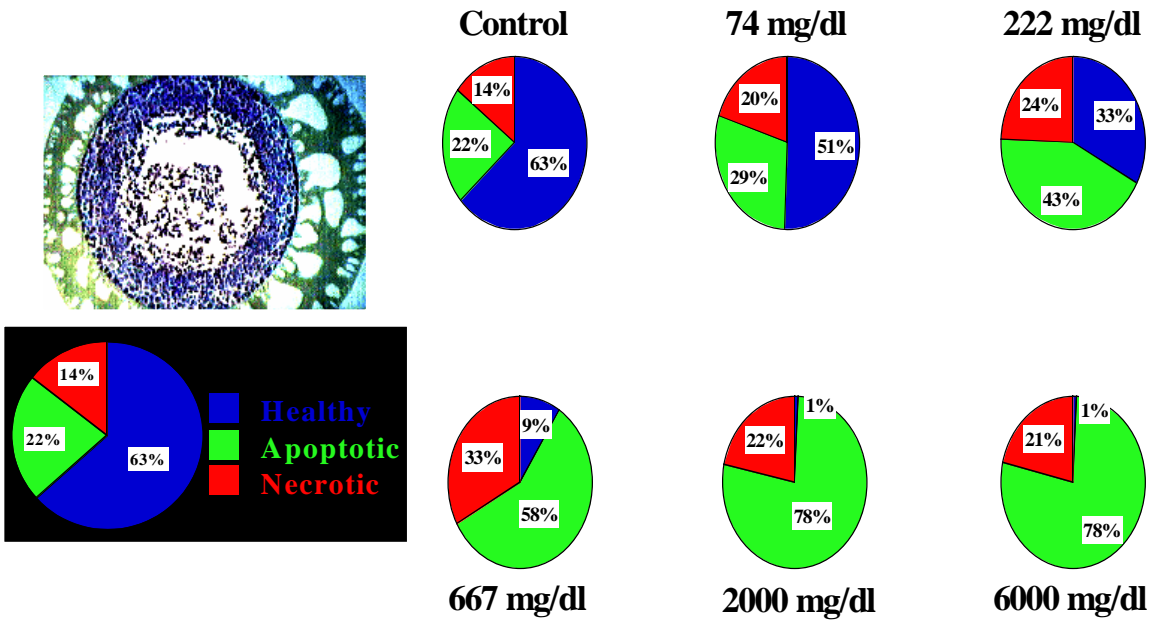
Maintaining this high plasma level of vitamin C has been difficult, and the level sought in IV C treatment is 400 mgs/deciliter. Oral supplementation of vitamin C yields plasma levels only around 10 mgs/deciliter. To attain the higher levels, vitamin C must be administered intravenously.

Cancer cells need glucose for their metabolism, but because of the structural similarity of vitamin C to glucose, cancer cells readily intake vitamin C. At plasma levels of 400 mgs/deciliter, vitamin C is no longer an antioxidant, but instead acts as a pro-oxidant. Vitamin C at these levels is peroxidative, causing oxidation. The cancer cells lack the catalase enzyme needed to convert the peroxide, so as a result of this peroxidation, an aldehyde is formed that is toxic to the cancer cells. With repeated treatments over time, the peroxidation causes the cancer cells to become apoptotic.

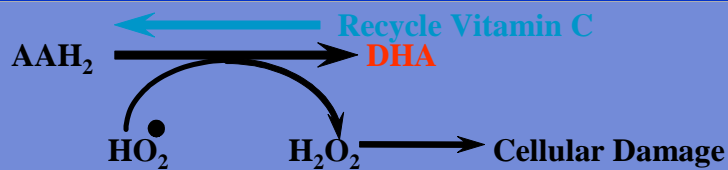
Apoptosis assays

From a study reported in the British Journal of Cancer (Casciari, Riordan NH, et al., 2001), this author and doctoral candidate examined the extent of apoptosis in ascorbate-treated and -untreated HFST, by dual staining with FITC conjugated annexin V (AV) and propidium iodide (PI). Annexin V has a natural affinity for phospholipid

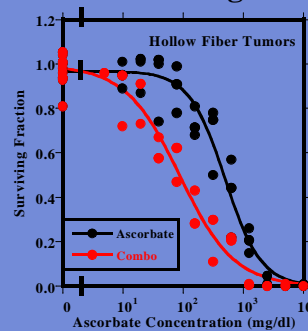
phosphatidylserine, a membrane molecule that trans-locates from the intracellular side of the membrane to the extracellular side soon after the initiation of apoptosis. HFST were treated for 2 days with various concentrations of ascorbate. Cells were then collected from the hollow fibres. Some were seeded in 96-well plates to measure surviving fraction with the SRB assay, while the remainder were stained using the ApoAlert™ Annexin V FITC Apoptosis Kit (Clonotech Laboratories, Palo Alto, CA) for analysis using an EPICS XLMCL flow cytometer (Coulter Corporation, Miami, FL). The excitation wavelength was 488 nm and the detection wavelengths were 525 nm and 620 nm for AV and PI, respectively. Cells staining negative for both markers were considered viable, since AV and PI are membrane impermeable, while cells staining positive for annexin V only were considered apoptotic and cells staining positive for both markers were considered necrotic. Percentages of viable, apoptotic, and necrotic cells were determined as a function of ascorbate concentration. Our results showed that apoptosis in SW620 HFST was confirmed by dual staining with propidium iodide and annexin V. Cells grouped into 3 clusters; low PI and annexin V staining cells with high forward scatter and low granularity; low PI and high annexin V staining cells with low forward scatter and high granularity; and cells with high levels of both PI and annexin V. The viable cell fractions decreased while the apoptotic and necrotic cell fractions increased with increasing ascorbate concentration. This trend was consistent with the effects of ascorbate on surviving fraction. Ascorbate concentrations on the order of 10 mM (~200 mg dl⁻¹) were required to kill a significant percentage of the tumour cells. We conducted a dose-response experiment with sodium chloride to determine what osmotic and sodium levels SW620 HFST could tolerate. Sodium chloride concentrations of 180 mM were necessary to reduce HFST cell survival over a 2-day period. Thus, ascorbate toxicity at 10 mM was not due to hyper-osmotic effects. The following pie charts show relative percentages when ascorbate, lipoic acid and other antioxidants were combined in hollow fibre *in vitro* tumors.



Combination Therapy



- Additive to Recycle Vitamin C
 - Toxic to non-proliferating tumor cells
 - Enhances Vitamin C Toxicity
- Required Vitamin C Dose Reduced Roughly 4-Fold**



5.2 Human Studies

A logical extension of animal studies (cited in chapter 4), the human studies described in this section, as conducted by this author and doctoral candidate, further explore the cytotoxicity mechanisms of ascorbic acid.

Although the use of very high-dose intravenous AA for the treatment of cancer was proposed as early as 1971 (Klenner, 1971), and Cameron published a protocol for the use of AA in the treatment for cancer (Cameron, 1991) which included initial intravenous AA administration, there was, prior to the investigations of this doctoral candidate, no large study of intravenous AA at levels high enough to maintain plasma levels above a level known to inhibit or kill tumor cells. Two of the investigators (HDR and JAJ) involved in the research conducted by this candidate reported apparent positive effects of intravenous AA on metastatic kidney adenocarcinoma (Riordan HD et al., 1990). In light of our own studies, it is relevant to note that Cameron and Pauling published extensive suggestive evidence for prolonged life in terminal cancer patients who were orally supplemented (both with and without initial intravenous AA therapy) with 10 g/day of AA (Cameron and Pauling, 1974; Cameron and Campbell, 1974; Cameron, Campbell and Jack, 1975; Cameron and Pauling, 1976; Cameron and Campbell, 1991; Cameron and Pauling, 1978; Cameron et al., 1979; Pauling, 1980). Although intravenous AA was administered during both of these studies by Cameron and Pauling, plasma levels during infusion were not monitored, and therefore it was not possible to determine if cytotoxic plasma levels of AA were achieved. By contrast, plasma levels of AA were monitored in the studies conducted by this doctoral candidate.

It is also relevant to note, for purposes of comparison, that Morishige and Murata (1979), and Murata et al. (1982), reported evidence for increased survival and prolongation of life in terminal cancer patients with oral AA supplementation. By contrast, however, Creagan et al. (1979) and Moertal et al. (1985) reported that oral AA had no effect on the survival of patients with advanced cancer, with Moertel et al. (1985) similarly reporting that oral AA did “not increase the survival” of another group of advanced cancer patients who had no prior chemotherapy. These negative results from controlled trials have been

the subject of much debate centered mostly on protocol. These arguments will not be rehashed here because the long-term, oral dosage used in those experiments (10 g/day), while substantial and capable of producing immunostimulatory and extracellular matrix modulation effects, was not high enough to achieve plasma concentrations that are generally cytotoxic to tumor cells in culture. Indeed, it is important to note two significant points from these controlled trials: firstly, that such relatively low dosages (10 g/day) of vitamin C were administered, and secondly, that the vitamin C was administered orally. Both of these facts will contrast sharply with the protocol employed by this doctoral candidate.

An average of high and low normal plasma AA limits from five studies of adults yields a normal range (95% range) of 0.39-1.13 mg/dl (Lentner C. Geigy Scientific Tables, 1984). The highest plasma level of AA that we (Riordan NH, et al.) have seen achievable in humans via oral supplementation is 4.5 mg/dl. The lowest cytotoxic level of AA *in vitro* for any of the cellular studies mentioned above was 0.88 mg/dl for a malignant lymphoma (Helgestad et al., 1990). This low cytotoxic level of AA is exceedingly rare. In our experience, 50-400 mg/dl of AA is required *in vitro* to kill 100% of tumor cells within 3 days. A 100% kill level of 300 mg/dl for endometrial carcinoma cells and 400 mg/dl for pancreatic carcinoma cells are typical.

For a chemotherapeutic agent to be effective, its plasma levels must reach the tumor cell toxicity range. The longer that the plasma level can be kept in that range, the more effective the agent will be. For this reason, plasma pharmacokinetic studies are sometimes performed to assess the plasma level of chemotherapeutic agents over time.

Because AA is so readily cleared from the body, we decided to measure plasma levels of AA during extended intravenous infusions of AA in a few cancer patients (Riordan NH et al., 2000; 1996; 1995). The AA determination method was that of Henry (1964). A representative example is a pancreatic cancer patient, a male aged 69, weighing 70 kg. After initial screening for a toxic reaction to intravenous AA with small doses given

intravenously during a 1 hour infusion, he was given large doses of AA in 1000 cc Ringer's Lactate infused over an 8-hour period.

One hour after beginning his first 8-hour infusion of 115 g AA (Merit Pharmaceuticals, Los Angeles, CA), the plasma AA was measured and found to be 3.7 mg/dl, and at 5 hours it was 19 mg/dl. During his fourth 8-hour infusion, 8 days later, the 1 hour plasma level was 158 mg/dl, and the 5 hour level was 185 mg/dl. Both values in the fourth 8-hour infusion were well above the concentration required to kill 100% of human pancreatic tumor cells in our laboratory.

Plasma levels during the first infusion were much lower than during the fourth infusion; this indicates an enormous capacity for destruction of ascorbate by this individual and highlights the need for measurements to ensure that adequate plasma levels of AA are achieved during therapy.

Plasma levels of over 100 mg/dl have been maintained in 3 patients for more than 5 hours using continuous intravenous infusion. The patient cited above has, to date, received 39 of the 8-hour infusions of AA, ranging in dose from 57.5 to 115 g, over a 13-week period. A recent CT scan revealed that there had been no progression of tumor growth during the treatment period.

Altogether, six patients were infused intravenously with similar doses of AA over 8-hour periods with no reported side-effects. In all cases, the patients had either been given no further therapeutic options by their oncologists, had refused further conventional treatment, or in one case, requested the use of AA in conjunction with standard chemotherapy. Intravenous AA administration in these cases was approved by our Institutional Review Board. Further studies with this method of administration are detailed in subsequent sections.

5.2.1 Achievement of Cytotoxic Concentrations of AA in Human Sera After Intravenous Infusions of Ascorbic Acid

As previously described, ascorbic acid and its salts have been found to be preferentially toxic to tumor cells *in vitro* and *in vivo*. Given in high enough doses to maintain plasma concentrations above levels that have been shown to be toxic to tumor cells *in vitro*, AA has the potential to selectively kill tumor cells in a manner similar to other tumor cytotoxic chemotherapeutic agents. Most studies of AA and cancer to date have not utilized high enough doses of AA to maintain tumor cytotoxic plasma concentrations of AA. Herein, data are presented which demonstrate the ability to sustain plasma levels of AA in humans above levels which are toxic to tumor cells *in vitro*, and suggest the feasibility of using AA as a tumor cytotoxic chemotherapeutic agent.

The concentrations of ascorbate toxic to cancer cells *in vitro* can be achieved clinically by intravenous administration. In studies conducted by the RECNAC Project of the Bio-Communications Research Institute (directed by this candidate), we observed that a seemingly large dose of a 30 gram infusion of AA given to a cancer patient was not adequate to raise the plasma level to a level that was toxic to tumor cells as reported *in vitro* (> 200 mg/dL for dense monolayers and > 400 mg/dL for hollow fiber models) (Riordan NH et al, 2001; 2000; 1999; 1998; 1997; 1996, 1995). Infusion of 60 grams resulted in a brief (30 minute) elevation of plasma levels of vitamin C above 400 mg/dL, while 60 grams infused over 60 minutes immediately followed by 20 grams infused over the next 60 minutes resulted in a 240 minute period in which the vitamin C plasma concentration was near or above 400 mg/dL, a concentration proven cytotoxic. Lipoic acid (Thiolic acid), an aqueous and lipid soluble antioxidant that recycles vitamin C, decreased the dose of vitamin C required to kill 50% of tumor cells from 700 mg/dL to 120 mg/dL. Lipoic acid can mediate the reduction of dehydroascorbic acid and improves mitochondrial function. It is conceivable that other energy intermediates such as acetyl-L-carnitine, coenzyme Q10, B-complex vitamins, magnesium, and alpha-ketoglutarate-aspartate, among others, will prove to be of benefit against cancer either by interacting directly with ascorbate (redox) or by stimulating/ improving and/or correcting aerobic

metabolism in the mitochondria. This information supports the hypothesis that certain oxidation intermediates and/or aerobic metabolism cofactors originating from nutrients or from their interaction can act as active antineoplastic agents. It seems that the cytotoxic effects of ascorbate and its derivatives are ascribed to the chemical properties related to their molecular structural characteristics and not to vitamin activity. In general, we have proposed that the pro-oxidant activity exhibited by ascorbic acid is the main mechanism by which it inhibits cancerous growth and metastasis, and that this constitutes its proposed role as energy intermediate and as a possible secondary or accessory anticancer mechanism.

As previously described, one of the investigators (MJG) in the research directed by this doctoral candidate has demonstrated that secondary products of lipid peroxidation have an inhibitory action on human malignant cell proliferation (Gonzalez et al., 1991; Gonzalez and Riordan NH, 1996; Gonzalez et al., 1993; Gonzalez, 1995). Furthermore, there is evidence to suggest that dehydroascorbic acid may work as a mitotic inhibitor *in vivo* (Riordan HD, 1990). Dehydroascorbate may prevent cell division by inhibiting protein synthesis at the ribosomal level. Interestingly, prolonged exposure to high concentrations of dehydroascorbic acid may cause irreparable damage resulting ultimately in complete lysis of the cells. It should be noted that dehydroascorbic acid is unstable and must be constantly produced in order to be maintained in high concentrations. Under the appropriate conditions, it may revert back to ascorbic acid. This recycling occurs when extracellular ascorbate is oxidized, transported as dehydroascorbic acid and reduced intracellularly to ascorbate. (The reader is referred to section 4.1.2).

As also previously described, the preferential cytotoxicity exhibited by ascorbic acid against tumor cells may be associated with the intracellular generation of hydrogen peroxide via redox reactions, and the selective uptake of this by cancer cells with no toxic effects on normal tissue (Riordan NH, et al., 1995; Noto et al., 1989; Jackson et al., 1995; Garland et al., 1986). Specific examples of the clinical applications of such reactions are described in the succeeding sections.

5.2.2 Case Studies of Long-Term Survivors Who Received AA Infusions

This section continues a description of original laboratory research and clinical studies conducted by this doctoral candidate.

5.2.2.1 Non-Hodgkins Lymphoma

RESOLUTION OF NON-HODGKIN'S LYMPHOMA WITH INTRAVENOUS VITAMIN C: 2 CASES

MH, a 66 year old white female, was diagnosed with a large peri-spinal (L4-5) malignant, non-Hodgkin's lymphoma (diffuse large cleaved cell of B-cell lineage) in January of 1995. Her oncologist recommended localized radiation therapy and Adriamycin-based chemotherapy. She began localized radiation therapy 5 days per week for 5 weeks on January 17th, 1995, but refused chemotherapy. On January 13th, 1995 she was started on intravenous vitamin C, 15 grams in 250 cc Ringer's Lactate 2 times per week, which she continued after completing the radiation therapy. She also began taking several oral supplements to replace those found to be deficient by laboratory testing, and empirical co-enzyme Q-10, 200 mg BID. She was also successfully treated at that time for an intestinal parasite.

On May 6th, 1995, MH returned to her oncologist with swelling and painful supraclavicular lymph nodes. One lymph node was removed and found to contain malignant lymphoma cells. In spite of recommendations for chemotherapy and more radiation, MH refused and continued with her intravenous vitamin C and oral regimen. Within 6 weeks, the supraclavicular nodes were barely noticeable. She continued intravenous vitamin C infusions until December 24th, 1996. She has been followed with regular physical exams and has had no recurrence. During a telephone follow-up on March 23rd, 1999, she was well without recurrence.

Comment: This case is rare in that the patient refused chemotherapy, which in all likelihood would have been curative. She also had a so-called recurrence of her

lymphoma during intravenous vitamin C therapy months after her radiation therapy had ended. The possibility exists that the lymphoma cells in her lymph nodes were there at the initial diagnosis and the adenopathy occurred during immune recognition of those cells. Also of note is the fact that this patient received only 15 grams of vitamin C per infusion. According to our model, this is not a high enough dose to achieve cytotoxic concentrations of vitamin C in the blood. Therefore, any effect of vitamin C could only be attributed to its biological response modification characteristics.

In the fall of 1994, a 73 year-old white male farmer from Western Kansas was diagnosed with wide-spread non-Hodgkin's lymphoma. Biopsies and CT-scan revealed bilateral tumor involvement in his anterior and posterior cervical, inguinal, axillary and mediastinal lymph node beds. Bone marrow aspirate was negative for malignant cells. He was treated with chemotherapy for 8 months, which resulted in remission. In July of 1997 he began losing weight (30 lbs), at which time he returned to his oncologist and a CT-scan at that time showed recurrence. He was placed on chemotherapy in September of 1997, and in December of 1997 he developed leukopenia followed by extensive left sided Herpes Zoster. As a result, the chemotherapy was stopped. In March of 1998 he became a patient at our medical Center and began receiving intravenous vitamin C along with oral nutrient supplementation that included lipoic acid. His vitamin C dose was escalated until he was receiving 50 grams in 500 cc sterile water two times per week. He continued on that dose for 11 months. Three months after beginning vitamin C therapy, a CT-scan showed no evidence of malignancy. Another CT-scan, in February of 1999, was also clear and he was declared to be in complete remission by his oncologist. Also of note is that this patient was addicted to sleeping pills when first seen at our Center, but after 3 months of intravenous vitamin C therapy he replaced the sleeping pills with Kava tea.

5.2.2.2 Pancreatic Cancer

A 70 year old Caucasian male who had been diagnosed with pancreatic cancer in December of 1996 was seen at our medical Center. After exploratory surgery, he had

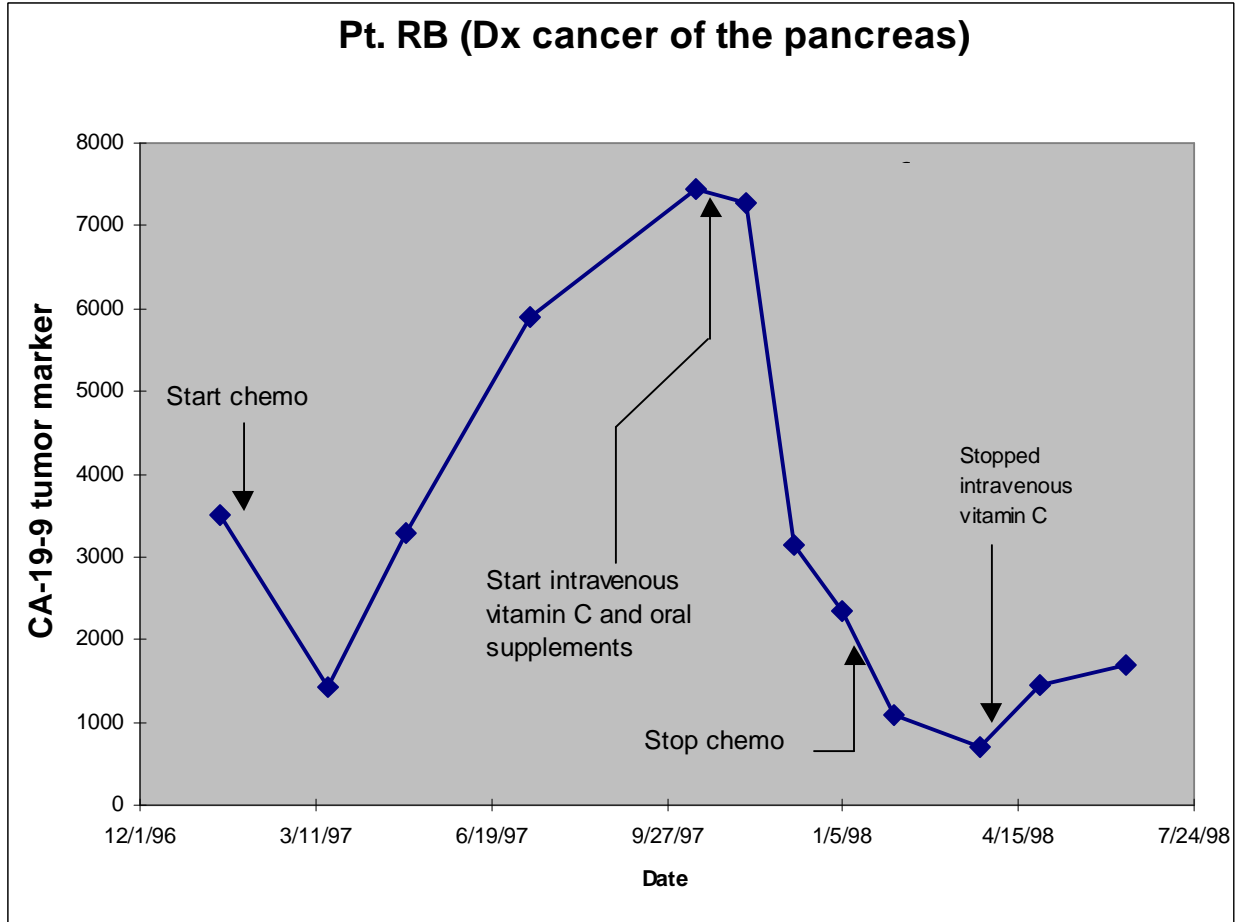
been diagnosed with a low-grade mucinous carcinoma of the pancreas. During surgery there was found to be widely metastatic disease affecting all intra-abdominal organs. In January of 1997 he was started on Gemzar, to which he had an allergic reaction so he was placed on weekly 5-FU for 9 weeks. He was then placed back on Gemzar in June of 1997 along with Decadron to counteract his allergy. One tumor marker for pancreatic cancer is a carbohydrate antigen known as CA-19-9, and in spite of chemotherapy his CA-19-9 continued to elevate until he was seen at our Center. At that time his CA 19-9 was 7,400 U/mL (normal <33). He was then placed on a regimen of 15 grams of intravenous vitamin C, given two times weekly, and 300 mg of alpha lipoic acid, taken twice per day orally, as well as other nutrient supplements in which the patient was determined by laboratory blood analysis to be deficient. One month later, his dose of vitamin C was increased to 25 grams twice per week. Within two months, the patient's antigen level had dropped to 3,200 units/mL of serum. At that time, his dosage of vitamin C was increased incrementally to 30, 50 and finally 75 grams. In January of 1998, the patient opted to stop chemotherapy while continuing lipoic acid and ascorbate treatments. The levels of CA-19-9 continued to fall. In March of 1998, the patient's CA-19-9 was 700 units/ml serum. The patient's antigen levels during the course of treatment with ascorbate and lipoic acid were analyzed over time and continued to decrease. The patient improved and decided to discontinue the ascorbate and lipoic acid therapy in March of 1998, after six months of treatment. He died four months later.

An analysis of the pharmacokinetics at work in this particular case study is useful. His first vitamin C infusion had contained 15 grams administered over one hour, which yielded a plasma concentration of 34 mg/dL of vitamin C immediately following that infusion. By contrast, the plasma vitamin C level of a healthy person receiving such an infusion may be expected to reach 120-200 mg/dL. On his first visit to our Center he was also placed on a broad-spectrum nutritional program, and his intravenous dosage of vitamin C was increased to 75 gram infusions bi-weekly.

The patient's CA-19-9 serum concentration had declined during this treatment until March of 1998, when he received the results of a CT-Scan of the abdomen/pelvis which

showed no change compared to a CT in January. He related that he felt as if he was wasting his money at that time, and stopped his bi-weekly intravenous vitamin C. The reader is referred to the following graph.

Figure 10



A graph of the patient's serial serum CA-19-9 is given in Figure 10, above. The evidence in this case suggests that the intravenous vitamin C was acting as a cytostatic and not a cytotoxic agent. When the patient went off the protocol, the tumors became active again. The evidence also suggests that intravenous vitamin C was working independently of the chemotherapy, given that the CA-19-9 level continued to decrease after chemotherapy was discontinued. This patient died at home on July 4th.

In a different case study, a 68-year old white male was a self-referral to our medical Center in December of 1993 (Riordan HD, Jackson, Hunninghake, and Riordan NH,

1995). Two months previously, he was seen at another medical facility for painless jaundice (bilirubin was 14 mg/dL), "black urine," pain in the stomach and a rapid weight loss of 21 pounds. A CT scan and abdominal angiogram suggested a blocked bile duct and a pancreatic mass. An operation was performed and because of its location, all of the tumor could not be removed. An area of the tumor 4 cm x 2 cm x 4 cm in size was removed. The gallbladder, head of the pancreas, distal stomach, and duodenum were also removed and a complete "Whipple" procedure performed. The pathology report showed a grade 1 adenocarcinoma of the pancreas with metastasis to 1 of 7 regional lymph nodes (T3, N1, Mo). A month after the operation the patient developed hyperglycemia. He was placed on the ADA diet with blood glucose monitoring twice a day. After a short period, the blood glucose returned to, and remained, normal. Three months prior to the Whipple procedure, he had a transurethral resection for an enlarged prostate which proved to be benign.

After discussing treatment options with an oncologist, the patient decided not to take conventional chemotherapy and radiation. At our medical Center, a complete physical, psychological and biochemical examination was done on the patient. He was an alert, pleasant, 68-year old male who weighed 140 pounds and was 70 inches tall. Significant laboratory data included blood DHEA at 39.7 ng/dL (normal: 200 to 335), beta carotene at 2.4 ug/dL (normal: 10 to 85), and vitamins A, C and E in the non-supplementing normal range. Urine vitamin C was 10 mg/dL (our normal is 20 to 40), and the RBC essential fatty acid profile showed low gamma linolenic, low palmitoleic fatty acids and a low stearic/oleicratio ratio. His fructosamine was 313 umol/L (normal: 175 to 272) and blood glucose was 326 mg/dL. Hair tissue analysis showed calcium, magnesium and sodium to be low.

A blood analysis for G6PD, a BUN, creatinine and urinalysis was done before I.V. vitamin C was started. All were normal. Appropriate supplements were started for those identified as low or sub-optimal by the laboratory results.

The patient initially received a small dose of vitamin C in Ringer's Lactate during a one hour infusion to screen for toxic reactions. The next infusion of 115 g was given in 1,000

mL of Ringer's Lactate over an 8 hour period. One hour into the infusion, the plasma C level was 3.7 mg/dL and at 5 hours it was 19 mg/dL. During the fourth 8-hour infusion (8 days later), the 1 hour plasma C level was 158 mg/dL and at 5 hours it was 185 mg/dL. Both values are well above the concentration required to kill 100% of human pancreatic tumor cells as found in our research laboratory. The low plasma levels of C in this patient during the first infusion compared to the fourth infusion shows the value of measuring the plasma level to see that adequate levels are achieved during therapy. The patient received 39 of the 8-hour infusions in doses ranging from 57.5 to 115 g over a 13-week period, the length of the treatment protocol with high dose I.V. vitamin C.

A CT scan of the abdomen six months after the surgery failed to detect any progression of the tumor. A recurrence of the tumor occurred after the amount and frequency of I.V. vitamin C was significantly reduced so the patient could travel in his motor-home (for family reunions, etc). The patient lived for 12 months after the initial diagnosis of cancer of the head of the pancreas. He received no chemotherapy or radiation treatment and enjoyed a good quality of life until the time of his death.

As previously described, altogether, six patients have been infused intravenously with similar doses of vitamin C over 8-hour periods with no reported side-effects. In all cases, the patients had either been given no further therapeutic options by their oncologists, had refused conventional treatment, or had requested I.V. vitamin C in conjunction with standard chemotherapy.

5.2.2.3 Colorectal Cancer

COMBINED INTRAVENOUS VITAMIN C AND CHEMOTHERAPY IN A PATIENT WITH STAGE IV COLORECTAL CARCINOMA

In April of 1997, RL, a 51-year-old white male, was first seen at our medical Center. He was well other than having type II diabetes until the previous fall when he developed painless bright red rectal bleeding. A work-up demonstrated the presence of a distal colon lesion. On December 31 of 1997 he underwent an anterior colon resection and appendectomy at a local hospital. The colon tumor penetrated through the bowel wall

and into the surrounding pericolonic adipose tissue. Two large hepatic metastases were discovered at the time of surgery; one was biopsied. Pathology revealed that the colon lesion was a moderately differentiated adenocarcinoma, and the liver biopsy was metastatic adenocarcinoma. Following surgery he received chemotherapy with weekly 5-FU and leucovorin for twelve cycles with a decrease in his CEA from 90.2 to 67.7. The patient and his wife, who is an R.N., asked the chemotherapist about getting intravenous vitamin C along with the chemotherapy. The oncologist informed them that vitamin C would not be of any value.

RL was then seen at Pittsburg University hospital on May 13th of 1997 where he underwent liver resection to segments three and five. During surgery, the stomach was mobilized off the inferior surface of the liver and a frozen section of this area was taken and metastatic adenocarcinoma confirmed. The pathology report showed metastatic carcinoma consistent with colon primary within the desmoplastic tissue and adjacent hepatic parenchyma from the stomach wall and liver. Segments three and five both contained multiple nodules. His CEA was 9.8 post surgery. The Pittsburg University oncologist informed him his prognosis was very poor and that he should go home and begin chemotherapy again. RL and his wife asked this oncologist if he should use intravenous vitamin C. He responded, "I know of no studies which showed that this [vitamin C] would eradicate or delay progression of cancer."

In spite of the two no-confidence recommendations for the use of intravenous vitamin C, RL returned to our Center for infusions after recovering from surgery in June of 1997. He also began receiving weekly 5-FU (1,100 mg) and Leucovorin (1,300 mg) treatments administered by his local oncologist. His first vitamin C infusion was 15 grams over one hour. The dose was gradually increased during bi-weekly infusions. On September 9th, 1997, a post-intravenous vitamin C (100 gram in 1000 cc sterile water infused over 2 hours) plasma concentration of vitamin C was measured and found to be 355 mg/dL. He was then started on intravenous vitamin C, 100 grams, twice weekly. His wife gave most of these infusions at home. In addition to the vitamin C, he was given recommendations

for oral vitamin and mineral supplementation to increase levels of nutrients in which he was found to be low.

He kept up his vitamin C infusions until February of 1998 when he traveled to Florida for a vacation. While on vacation he continued the 5-FU/Leucovorin injections. After a two weeks hiatus from the vitamin C infusions he began to experience nausea, diarrhea, stomach pain, and stomatitis; common side effects of 5-FU. The side effects stopped when he restarted intravenous vitamin C.

He continued on chemotherapy and 100 gm Bi-weekly intravenous vitamin C until April 1st of 1998. Other than the brief period of side-effects mentioned above, RL had no other side effects during the year of chemotherapy. He never experienced leukopenia, thrombocytopenia, or anemia, as many patients do.

During April of 1998 we began to taper his intravenous vitamin C. The doses were: 75 grams, one time per week for 2 months; then 75 grams, one time every other week for 2 months; then 75 grams, one time every month for two months; and then 50 grams, one time per month for 6 months.

RL's CEA dropped into the normal range on July 31, 1997, and has remained normal (<3.0 ng/mL) to the time of his last follow-up visit (March 20, 1999). A CT-scan in October 1998 showed no evidence of metastatic disease. During an interview in 1999, he described himself as "perfectly healthy."

Comment: This report demonstrates four things about intravenous vitamin C in this patient's case: 1) Intravenous vitamin C was not encouraged by his oncologists; 2) He did not take the advice of his oncologists on the issue of intravenous vitamin C usage; 3) His only side effects of chemotherapy occurred during a hiatus from intravenous vitamin C therapy and disappeared upon reinstatement of vitamin C infusions; and 4) For this patient intravenous vitamin C did not work against the chemotherapy, as demonstrated by his complete remission.

Another patient, a 29 year old male with metastatic colon carcinoma, was also seen at our medical Center. His cancer had been discovered during exploratory surgery, and multiple metastases were found on the omentum, transverse and sigmoid colon, and the peritoneum. Biopsy samples from these metastases were obtained during surgery and most of the tumor mass was not removed. A cell culture line was established from the tissue biopsies, and the patient's tumor cells were tested to determine the sensitivity of these particular tumor cells to ascorbic acid. An ascorbate concentration of 30 mg/dl was found to be toxic to 100 percent of the patient's tumor cells, so a target level of plasma concentration of ascorbic acid was selected at three times this level which had been found to be toxic to the patient's tumor cells. Intravenous ascorbate was begun and the infusion quantity and rate required to achieve and maintain the targeted plasma ascorbic acid concentrations of 90 mg/dl was determined. Twenty hour infusions of ascorbate were given three times per week, and after four months of therapy, a surgical ileostomy was performed due to chronic diarrhea. During the surgery, the multiple metastases were observed to have decreased significantly in size and to have a flaccid consistency.

Another patient, a 53 year old Caucasian male who had been diagnosed with colorectal cancer and liver metastases, had undergone surgery to remove the largest of the liver metastases. When first seen at our medical Center, we started him on low dose 5-fluorouracil chemotherapy, intravenous vitamin C, and oral alpha lipoic acid. He was also given various nutritional supplements to combat diagnosed deficiencies. The vitamin C dose was gradually increased to 100 grams, administered twice weekly by intravenous infusion. He continued therapy until March of 1998, when he was declared to be cancer-free. Follow-up monitoring continued to verify that he remained cancer-free.

5.2.2.4 Breast Cancer

INTRAVENOUS VITAMIN C IN A PATIENT WITH END-STAGE METASTATIC BREAST CARCINOMA

In 1995, a hospitalized 68 year old lady with widely metastatic end-stage breast cancer was seen. Her most recent bone scan had shown metastases to "nearly every bone in her skeleton." She was experiencing bone pain which was not controlled with narcotics. At the time of her first consultation she had blood clots in both subclavian veins, and shortly thereafter contracted cellulitis in her left arm and hand secondary to an errant arterial blood draw. After the blood clots were treated with Activase R, she was placed on intravenous vitamin C, at 30 grams per day initially, increasing to 100 grams per day over 5 hours. Within one week, the once bed-bound patient began walking the halls of the hospital. Several hospital staff reported that she looked like a new person. Her cellulitis cleared, and she was discharged from the hospital. At home she received 100 grams of intravenous vitamin C 3 times per week. Three months after starting the vitamin C therapy, a bone scan revealed resolution of several skull metastases. Six months after starting the vitamin C, she fell while shopping at a mall, and subsequently died of complications from pathological fractures.

5.2.2.5 Prostate Cancer

A 73 year old male with metastatic prostate cancer was seen at our medical Center. A baseline blood sample was drawn from the patient and the serum was separated. An intravenous infusion of 105 grams of ascorbate was given over 5 hours. At the end of the 5 hours, another blood sample was taken and again the serum was separated. The pre-infusion serum sample was assayed and found to contain an ascorbic acid concentration of 3.4 mg/dl, and the post-infusion serum samples were assayed and found to contain an ascorbic acid concentration of 165 mg/dl. The cytotoxic effectiveness of both serum samples was tested against a line of human prostate tumor cells called "PC-3", from the American Type Culture Collection. The PC-3 cells were plated at 3,000 cells per well in plastic 96 well tissue culture plates 24 hours before the blood was taken from the patient. 100% and 50% (diluted with complete culture medium, DMEM) serum solutions from both samples taken from the patient were added to 16 wells each of the PC-3 cells which had been plated earlier. After a five day incubation, the number of surviving tumor cells in the wells containing the 100% and 50% solutions of the post-infusion serum sample was compared to the number of surviving tumor cells in the wells containing the 100%

and 50% solutions of the pre-infusion serum sample. The percent survival calculations yielded 100% tumor cell survival in both pre-infusion serum solutions, whereas in the post-infusion serum solutions a 15% survival of tumor cells was seen in the 50% serum solution, and a 19% survival of tumor cells was seen in the 100% serum solution. These results demonstrate that a beneficial level of tumor cytotoxic effectiveness can be achieved by the elevation of ascorbic acid concentration in the patient's tumor serum by means of intravenous infusion of ascorbic acid. The patient continued to receive ascorbic acid infusions accordingly, and his prostate metastases were controlled without the addition of chemotherapy or radiation.

An interesting phenomenon also occurs when culturing prostate tumor cells in the presence of high concentrations of ascorbic acid. The prostate tumor cells begin producing large quantities of collagen (data not shown). In some cases the candidate would need to literally "chip off" prostate tumor cells from the plastic substrate when they had been cultured with high concentrations of ascorbate. The candidate also observed a group of prostate cancer patients who received periodic intravenous vitamin C infusions (roughly 1 time per month) for a period of years. In the candidate's experience, these men, with biopsy-proven prostate cancer, did not progress to metastatic disease. One case in particular, a 70 year old patient started treatment with a PSA of 8 in 1996. During the next 5 years he received intravenous infusions of 50 grams one time approximately every month. During the five year period, his PSA rose steadily until in 2001 when it was measured at 110. At that time the patient had a Prostatecint test which is a nuclear medicine test that entails injection of a radio-labeled antibody to prostate-specific membrane antigen (PSMA) followed some time later by a scan. The Prostatecint test is thought to be much more sensitive at picking up prostate cancer spread outside of the prostate. At 75 years old, 5 years after original diagnosis, and with a PSA of 110, the man was sexually active, had a rock-hard prostate and no prostate cancer outside of the prostate capsule according to the Prostatecint test. The candidate hypothesizes that in the case of prostate cancer, high doses of vitamin C may allow the prostate cancer cells the ability to produce large amounts of collagen. This collagen then may "glue" the cancer cells in place, preventing metastatic spread. It is also of interest to note that prostate

tumor cells are less sensitive to the cytotoxic effects of ascorbate than most other tumor cell types. The reduced sensitivity may be related to the utilization of the ascorbate by the tumor cells in the hydroxylation of proline reaction required to produce structural collagen.

(The reader is also referred to the study involving a patient with non-metastatic prostate cancer, described in “Pharmacokinetics,” section 5.1.2 and to the “Discussion regarding prostate cancer cells preferential accumulation of ascorbate).

5.2.2.6 Renal Cell Carcinoma

PATIENTS WITH RENAL CELL CARCINOMA TREATED WITH INRAVENOUS VITAMIN C

One of the investigators of our research (HDR) reported positive effects of vitamin C therapy in a patient with adenocarcinoma of the kidney in 1990 (Riordan HD et al., 1990). This report described a 70-year-old white male, GW, diagnosed with adenocarcinoma of his right kidney. Shortly after a right nephrectomy, he developed metastatic lesions in the liver and lung. The patient elected not to proceed with standard methods of treatment. Upon his request, he began intravenous vitamin C treatment, starting at 30 grams twice per week. Six weeks after initiation of therapy, reports indicated that the patient was feeling well, his exam was normal, and his metastases were shrinking. Fifteen months after initial therapy, the patient’s oncologist reported the patient was feeling well with absolutely no signs of progressive cancer. The patient remained cancer-free for 14 years. He died of congestive heart failure at the age of 84.

A second case study, published in 1998, described another complete remission in a patient with metastatic renal cell carcinoma. The patient, BR, was a 52-year-old white female from Wisconsin diagnosed with non-metastatic disease in September of 1995. In October of 1996, eight metastatic lung lesions were found: seven in the right lung and one in the left (measuring between 1 and 3 cm). The patient chose not to undergo chemotherapy or radiation treatments. The patient was started on intravenous vitamin C and specific oral nutrient supplements to correct diagnosed deficiencies, and a broad-spectrum oral

nutritional supplement in October of 1996. The initial dose of intravenous vitamin C was 15 grams, subsequently increased to 65 grams after two weeks. The patient was given two infusions per week. Intravenous vitamin C treatments were continued until June 6th of 1997. An x-ray taken at that time revealed resolution of all lung metastases but one. The patient discontinued intravenous vitamin C infusions at that time and continued taking the broad-spectrum oral nutritional supplement. A radiology report on a chest x-ray taken January 15th of 1998 stated that no significant infiltrate was evident, and there was resolution of the left upper lobe lung metastasis. In February of 1999, a chest x-ray showed no lung masses, and the patient reported being well at that time.

5.2.2.7 Lung Cancer

A 47 year old male with metastatic fibrous histiocytoma was seen at our medical Center. Three metastatic lesions to the lungs were initially presented, as revealed by chest x-ray, with two masses in the left lung and one mass in the right lung. All masses measured approximately 2 cm in diameter. Plasma levels of 110 mg/dl of ascorbate were achieved with intravenous infusions of ascorbate. After seven months of therapy, a chest x-ray revealed that the right lung mass had disappeared and one of the left lung masses had decreased in size (to about 1 cm) with decreased radio-opacity. After twelve months of therapy, the left lung mass which had regressed was completely resolved. Although no change was observed in the size of the remaining left lung metastasis after twelve months of therapy, the patient's condition nevertheless improved without the use of chemotherapy or radiation.

5.2.2.8 Ovarian Cancer

A 60 year old female with metastatic ovarian cancer was seen at our medical Center. After six months of standard chemotherapy, a second exploratory surgery was performed, at which time metastases throughout the abdominal cavity were found. The patient was started on intravenous ascorbate infusions at the quantity and rate of flow required to maintain a plasma concentration of 80 mg/dl. The patient's level of cancer antigen CA-

125, which had been elevated from the time of diagnosis and throughout most of the chemotherapy regimen, returned to normal and remained within normal limits throughout her intravenous ascorbate therapy for the next seven months.

5.2.3 Patents

This doctoral candidate has received 2 U.S. and international patents for inventions related to the research and work described herein. Specifically for the treatment methods described above, in the clinical cases listed in the preceding sections, this doctoral candidate has received the following U.S. patents:

In 1997, U.S. Patent # 5,639,787, for the "*Therapeutic Method for the Treatment of Cancer,*" shared jointly with HD Riordan, M.D.

In 2001, U.S. Patent # 6,284,786, and in 2002, U.S. Patent # 6,448,287, both for the "*Treatment of Cancer Using Lipoic Acid in Combination with Ascorbic Acid,*" both shared with JJ Casciari, Ph.D.

5.3 Safety and Toxicity Concerns for High-Dose Intravenous AA Therapy

Ascorbic acid is remarkably nontoxic at high levels (10 to 100 times the RDA when taken po). Nevertheless, some minor toxic effects have been reported. These side effects include: acidosis, oxaluria, renal stones, glycosuria, renal tubular disease, gastrointestinal disturbances, sensitivity reactions, conditioned need, prothrombin and cholesterol disturbances, vitamin B₁₂ destruction, fatigue and sterility (Barness, 1974). Of all of these, gastrointestinal disturbances are perhaps the most consistent and prevalent problem following the ingestion of large quantities of oral ascorbic acid, since nausea, abdominal cramps and diarrhea are frequently mentioned as negative side effects. These effects are lessened or eliminated by taking ascorbic acid as a buffered salt or immediately after meals. The amount of oral ascorbic acid tolerated by a patient without producing diarrhea increases proportionately to the stress or toxicity of the ailment (Cathcart, 1981).

Bowel tolerance doses of ascorbic acid ameliorate the acute symptoms of many diseases. Lesser doses often have little effect on acute symptoms but assist the body in handling the stress of disease and may reduce the morbidity of the disease (Cathcart, 198). Many of the toxic effects reported for taking large amounts of vitamin C in reality are insignificant, rare and of minor consequence. Nevertheless, a word of caution should be given for patients with glucose-6-phosphate deficiency. These patients, when given high doses of ascorbic acid, may be at risk of developing hemolysis (Rees, Kelsey and Richards, 1993). Before applying ascorbic acid therapy, patients should be screened for this deficiency. Also, while on ascorbic acid therapy, intake of inorganic selenium (Na selenite) should be avoided. A possibility exists that ascorbic acid may reduce selenite and render it unavailable for tissue uptake (Gonzalez, 1990). In regard to kidney stones, these are formed mostly in alkaline urine (calcium oxalate stones). High doses of ascorbate make the urine acidic, thus preventing stone formation. There are various studies that have addressed this issue (Sutton, Basu and Dickerson, 1983; Erden, Hacisalihoglu, Kocer, Simsek and Nebioglu, 1985; Tsao and Leung, 1988; Gerster, 1997; Wandzilak et al. [undated]), and found no evidence of ascorbate increasing the risk of kidney stone formation.

In relation to ascorbic acid given intravenously, no ill effects have been reported with doses as high as 150 to 200 grams over a 24 hour period (Riordan NH et al., 1995; Riordan HD et al., 1990; Jackson et al., 1995; Riordan NH et al., 2000; Casciari et al., 2001; Klenner, 1971; Cathcart, 1985). Ascorbate is more efficient when administered intravenously than when given orally because it bypasses the gut, and higher circulating levels are achieved for longer periods of time. Another valid concern when applying ascorbate intravenously is rapid tumor hemorrhage and necrosis (Campbell and Jack, 1979). Patients may become very ill because their bodies cannot cope with the sudden task of getting rid of such a large mass of dead tissue. This concern applies mainly to patients suffering from advanced disease with a very considerable tumor load, especially of highly aggressive, rapidly dividing tumors. This might be the main reason not to overload the body's detoxification systems (skin, kidneys, colon and liver) while on ascorbate therapy. Ascorbic acid has a unique advantage relative to other currently

utilized remedies for cancer, in that it is generally harmless and safe even at sustained high doses for prolonged periods of time. Evidence supports the concept of using high dose intravenous ascorbic acid for extended periods, in doses high enough to achieve and maintain plasma levels above those which have been found to be preferentially cytotoxic to tumor cells *in vitro*. Ascorbic acid is one of the safest and most valuable substances available to the physician.

Although very rare, tumor necrosis, hemorrhage, and subsequent death should be of the highest priority and concern in the safety of intravenous AA for cancer patients. While there is little data on the safety of the high doses of AA that we are proposing here other than the reports of Klenner (1979; 1971; 1953; 1951; 1949; 1948), who reported no ill effects of dosages as high as 150 g intravenously over a 24-h period, many of the publications cited earlier describe the relative lack of toxicity of high dose oral AA. Cathcart (1985; 1981; 1975) describes no ill effects with doses of up to 200 g/d in patients with various pathological conditions. According to Rivers (1987), high dose AA is generally safe, with contraindications in the following circumstances: renal insufficiency, chronic hemodialysis patients, unusual forms of iron overload, and oxalate stone formers. Care should be taken to screen potential patients for the above-mentioned conditions prior to beginning any high-dose AA therapy. Screening for red cell glucose-6-phosphate dehydrogenase deficiency, which can give rise to hemolysis of red blood cells under oxidative stress, should also be performed. After screening, any cancer therapy should be started at a low dosage to ensure that tumor hemorrhage does not occur.

Cameron (1991) described a rebound effect that can occur in response to high circulating levels of AA. He proposed that abnormally low levels of AA can occur between intravenous infusions of AA and that this effect is caused by increased levels of hepatic enzymes responsible for degradation and metabolism of AA (52-65). We (Riordan NH, et al.) have not found that to be the case, at least when the patient is orally supplementing between infusions. All of our patients have supplemented between infusions, therefore we have no data that allows us to directly compare our results to Cameron's. Plasma

levels of two patients who were receiving 15 and 22.5g infusions of AA 3 times weekly and orally supplementing to bowel tolerance with <10 g/d of AA were 2.4 and 2.8 mg/dl immediately prior to infusions. Another patient who was also supplementing with oral AA and receiving infusions 2 times weekly recorded plasma AA levels between 2.6 and 4.5 mg/dl on the 6 occasions prior to infusion. These examples indicate that a scorbutic rebound effect can be avoided with oral supplementation.

Because of the possibility of a rebound effect, measurement of plasma levels during the periods between infusions should be performed to ensure that no such effect takes place. Every effort should be made to monitor plasma AA levels when a patient discontinues intravenous AA therapy.

CLINICAL EXPERIENCES & SAFETY

We (Riordan NH, et al.) recently completed a Phase I clinical trial of high-dose intravenous vitamin C at the University of Nebraska Medical Center. Subjects with end-stage metastatic gastrointestinal cancer were infused with intravenous vitamin C continuously for 24 hours using a drug pump. Five dose levels were used: 150, 300, 430, 570, and 710 mg/kg/day. Those doses were approximately equal to doses of 10, 20, 30, 40, and 50 grams per day for a 70 kg person. No toxicities attributable to vitamin C were seen. There were no significant changes in complete blood counts in the subjects. Given that these doses were given over 24 hours, the highest plasma concentration of vitamin C seen in any of the subjects was 66.5 mg/dL.

5.4 Combination of AA with Chemotherapy and Radiation Therapy

In a study described in the British Journal of Cancer (Casciari, Riordan NH, et al.), this doctoral candidate describes our work with vitamin C and doxorubicin. Because of concerns that vitamin C would protect tumour cells from the effects of chemotherapeutic agents, we conducted experiments in SW620 HFST combining sodium ascorbate with doxorubicin. Results suggest that the effect of ascorbate on doxorubicin efficacy is concentration dependent. Ascorbate concentrations of 5 mg dl⁻¹ (0.25 mM), an amount

that might be relevant during oral supplementation, protected SW620 cells from doxorubicin to a small degree, while cytotoxic ascorbate concentrations (500 mg dl⁻¹, or 25 mM) increased cell killing. When ascorbate and doxorubicin were combined at a 50,000:1 mass ratio, they had an additive but not synergistic effect, reducing the doxorubicin LC₅₀ by nearly 50%. For example, a doxorubicin concentration of 0.02 mM combined with 3 mM vitamin C would kill roughly 25% of SW620 HFST cells in 2 days, while 0.02 mM doxorubicin alone would only kill 5% of the cells.

Prasad et al. (1999) has found that multiple dietary antioxidants enhance the efficacy of standard and experimental cancer therapies and decrease their toxicity. Prasad (2004) describes cancer patients as classifiable into 3 groups: those receiving standard or experimental therapy, those who have become unresponsive to these therapies, and those in remission who are at risk for recurrence or a second new cancer. While impressive progress in standard cancer therapy has been made, the value of such therapy in the management of solid tumors may have reached a plateau. Presently, as Prasad describes, there is no conventional strategy to reduce the risk of recurrence of the primary tumors or of a second cancer among survivors. Patients unresponsive to standard or experimental therapies have few options except for poor quality of life for the remainder of the time of their survival. Therefore, Prasad (2004) argues in favor of additional approaches being developed to improve the efficacy of current cancer management. In particular, he proposes that an active nutritional protocol which includes high doses of multiple dietary antioxidants and their derivatives (vitamin C, alpha-tocopheryl succinate, and natural beta-carotene), but not endogenously made antioxidants (glutathione- and antioxidant enzyme-elevating agents), when administered as an adjunct to radiation therapy, chemotherapy, or experimental therapy, may improve the efficacy of the standard treatment by increasing tumor response and decreasing toxicity. This nutritional protocol may also be used when patients become unresponsive to standard therapy or to experimental therapy, in order to improve the quality of life and possibly increase survival time. Prasad et al. (1999) also propose that after completion of standard therapy and/or experimental therapy, a maintenance nutritional protocol that contains lower doses of antioxidants and their derivatives, together with modification in diet and lifestyle, may

reduce the risk of recurrence of the original tumor and development of a second cancer among survivors. Experimental data and limited though promising human studies suggest that use of these nutritional approaches may improve oncologic outcomes and decrease toxicity.

As the data indicate, ascorbic acid and its salts offer a safe and nontoxic method of cancer treatment, whether utilized exclusively or as an adjuvant therapy with standard medical chemotherapy and radiation.

Chapter 6: Discussion

Many oncologists believe that the issue of AA and cancer is closed mainly due to the Mayo Clinic studies of Creagan et al. (1979). Even those directly involved in cancer research with AA may be skeptical of its therapeutic value due to the high levels required for cytotoxic effects – even if those effects are preferential. One aspect of AA administration remains paramount, namely, the means by which plasma levels of AA may be maintained above the levels that have a direct cytotoxic action on tumor cells. Such matters have been addressed by the examples provided herein. As described, awareness of rare contraindications in patients with widely disseminated and rapidly proliferating tumors is also of prime importance in the safe intravenous administration of ascorbic acid, as widespread tumor hemorrhage and necrosis, resulting in death, may result. Although the outcomes in such cases are disastrous, they are similar to the description of tumor-necrosis-factor-induced hemorrhage and necrosis in mice and still, nevertheless, seem to demonstrate the ability of AA to kill tumor cells *in vivo*.

Another matter of central interest and concern is the precise extent to which *in vitro* AA cytotoxicity may be extrapolated to *in vivo* conditions, beyond those already measured. *In vitro* cultures contain “free” iron or copper ions, and these ions, which are capable of catalyzing the oxidation of AA, could be responsible, at least in part, for the cytotoxicity of AA. The *in vitro* levels of these ions, particularly for copper, are probably unachievable in plasma. However, the work of Tsao et al. suggests that some ionic copper is available *in vivo* (at least in mice) as a catalyst for ascorbate oxidation. Tsao

reported that dietarily supplemented ionic copper was found to potentiate the inhibitory effect of ascorbate on human mammary xenografts in mice. In the case of the cell culture data, it is unlikely that ionic copper was responsible for catalytic oxidation of ascorbate, as no copper was added to, or was contained in the original formulation of, the medium. The above-mentioned necrosis and hemorrhage case reports, and the work of Tsao, suggest that the limitation of ionic catalysts is not always critically limiting, but this issue requires further clarification and study. It may be possible, for example, to find ways to adjust *in vivo* conditions to ensure adequate vulnerability of tumor cells to high levels of AA.

Certainly the extrapolation of *in vitro* data to *in vivo* therapeutic value is limited in multiple ways, one of which is the effect of serum concentration on toxic effects. 20% human serum added to the culture medium of human prostate tumor cells (ATCC PC-3) has been shown to partially protect the cells from the inhibitory effects of AA. Begin et al. reported a similar, dose dependent, protective effect of fetal calf serum on the toxicity of polyunsaturated fatty acids toward human breast cancer cells. This leaves us not knowing the exact toxic dose of AA for either normal or tumor cells. The combination of the data in which toxic effects of AA on one normal cell line were observed at 58.36 mg/dl and the lack of side effects in patients maintaining >100 mg/dl plasma levels suggests that there is at least some negative shift of AA toxicity when moving from *in vitro* to *in vivo*. The precise preferential *in vivo* tumor toxic levels of AA have yet to be determined.

One other factor which could influence how *in vitro* effects are extrapolated to *in vivo* effects is active transport of AA into certain tissues. Several tissues have been identified as containing greater than plasma concentrations of AA (wt of AA/unit of wet tissue), thus indicating an active transport of AA. In descending order, tissue levels of AA in humans rank as follows: adrenals, leukocytes, pituitary, brain, eye lens, pancreas, kidney, liver, spleen, heart-muscle, and plasma. Plasma concentrations of AA required for toxicity of both normal and tumor cells in these tissues could potentially be lower.

Major contributing factors to the beneficial effects of ascorbate may lie outside of its preferential cytotoxicity activity.

Tumor cells may become resistant to the cytotoxic effects of ascorbate. Tumor cells, or at least subsets of tumor cells, can and do become resistant to the cytotoxic effects of other cytotoxic agents. Why would we expect vitamin C to be any different? Multi-drug resistance is a common finding after chemotherapeutic regimens. This doctoral candidate has clinically observed what he believes to be cases of drug resistance of tumor cells to high dose intravenous vitamin C therapy. The cases that are the most dramatic and stand out in his memory are cases of pancreatic cancer. Three cases of advanced metastatic carcinoma of the head of the pancreas were treated in this candidate's office (unpublished). The cases were all male aged 55, 62, and 72. In all three cases, tumor markers (CA-19-9) decreased and the patients had a good quality of life without increase in tumor burden by radiology for a period of between 11 and 18 months. Then, almost as if a switch were turned off, the CA-19-9 levels began rising in spite of the same intravenous vitamin C regimen.

Another topic of widespread relevance in the field of cancer treatment involves the mechanisms of interaction at work between vitamin C and standard methods of radiation or chemotherapy. A report issued by Memorial Sloan Kettering (Golde et al., 1999) generated widespread publicity on the theoretical proposal that vitamin C might decrease the effectiveness of standard medical treatments of cancer. Golde et al. found that cancer tumors consume large amounts of vitamin C, and from this observation they concluded that vitamin C may interfere with chemotherapy and radiation treatment. "This study is the first to demonstrate exactly how cancer cells acquire large quantities of vitamin C," reported Dr. David Golde, senior author of the study and Physician-in-Chief of Memorial Hospital. "It's possible that taking large amounts of vitamin C could interfere with the effects of chemotherapy or even radiation therapy, since these therapies often kill cells in part by using oxidative mechanisms. It's conceivable then, that vitamin C might make cancer treatment less effective and therefore, it is reasonable that cancer patients undergoing chemotherapy should avoid taking large amounts of this vitamin," said Dr.

Golde.

This doctoral candidate disagrees. Vast and strong scientific evidence exists to disprove the conclusions of Golde et al. Among other reasons, numerous studies have demonstrated the following:

- Vitamin C is preferentially toxic to tumor cells.
- Vitamin C is nontoxic, at any dose, to normal, healthy cells.
- Vitamin C is crucial for maintaining an effective immune system.
- People with cancer have a high requirement for vitamin C.
- Vitamin C is vital for the production of collagen, which is one way the body can slow or stop the spread of cancer.
- Original clinical experiences of this candidate and other researchers with high dose vitamin C demonstrate that it does not decrease the effectiveness of chemotherapy or radiation therapy.
- When IL-2 and IFN-gamma are used, vitamin C blood levels drop to zero.
- The news that vitamin C is “hoarded” by tumor cells should, in the proper prospective, argue in favor of vitamin C use by cancer patients rather than against it.

As has been shown by Benade, Howard, and Burk (1969), Bram et al. (1980), Baader et al. (1994), Lode et al. (1994), and Sakagami et al. (2000; 1997; 1996; 1995; 1991), among others, vitamin C has been found in numerous studies to be preferentially toxic to tumor cells. Furthermore, standard medical chemotherapy per se is ineffective, as less than 5% of all metastatic cancers respond to standard chemotherapy. The reasons for using vitamin C in conjunction with standard medical treatment, as opposed to relying solely upon standard medical treatment alone, should not be ignored.

Lamson and Brignall (2000; 1999) have demonstrated that antioxidants such as ascorbate provide beneficial effects in various types of cancers and no reduction in the efficacy of chemotherapy, nor was the radiation detectable when antioxidants were provided. In

addition, the data show increased effectiveness of conventional cancer therapeutic agents when given with antioxidants, as well as a decrease in adverse effects when both therapies are given concurrently. Moreover, studies by Prasad et al. (2004; 1999) show similar positive results of the combination of antioxidants and conventional treatment. The misconception that vitamin C may reduce the efficacy of standard chemotherapeutic treatments should not prevent clinicians from utilizing ascorbate as adjuvant therapy for cancer. Supporting literature and data are presented herein to corroborate the safety, and indeed the necessity, of ascorbic acid when taken in combination with standard radiation and chemotherapeutic methods of treatment.

To patients (n = 15) with metastatic malignant melanoma, hypernephroma, and colon carcinoma, Marcus et al. (1991) administered a three-phase adoptive immunotherapy protocol: phase 1, 10(5) units (high-dose) interleukin-2 (IL-2) I.V. every 8 hours or 1 mg/m² continuous intravenous infusion; phase 2, 6.5 d rest + leukapheresis; phase 3, 4 d of high-dose IL-2 plus three infusions of autologous lymphokine-activated killer cells. Toxicities of treatment included fever, chills, tachycardia, hypotension, vomiting, diarrhea, and fluid retention. Patients entering the trial were not malnourished, and mean plasma ascorbic acid concentrations before therapy were normal (36.3 +/- 14.2 mumol/L). Mean concentrations dropped by 80% after the first phase of treatment with high-dose IL-2 alone (to 7.4 +/- 4.5 mumol/L). Mean plasma ascorbic acid concentrations remained severely depleted (between 4.5 and 7.4 mumol/L) throughout the remainder of the 15-d treatment. Ascorbic acid concentrations became undetectable (less than 2.8 mumol/L) in 12/15 patients during this time. Blood pantothenate and plasma vitamin E concentrations remained within normal limits in all patients tested throughout the phases of therapy, yet the fact that hypovitaminosis C was induced by the treatment indicates the acute need that cancer patients have for large amounts of vitamin C.

The mounting evidence in favor of vitamin C as a host-nontoxic, preferentially cytotoxic chemotherapeutic agent in the management of cancer is too substantial to disregard.

Chapter 7: Conclusions

From section 1.1, this candidate began this thesis by posing the following questions:

- Is there evidence that ascorbic acid (AA) at high concentrations can act as a pro-oxidant agent by generating hydrogen peroxide in the presence of oxygen?
- Is there evidence that vitamin C at high concentrations is preferentially toxic to cancer cells *in vitro* and *in vivo*?
- Can tumor cytotoxic concentrations of AA be clinically achieved by intravenous administration?
- Have AA oxidation products demonstrated antitumor activity?
- Does AA exhibit secondary anticancer mechanisms, perhaps by increasing the intracellular matrix, by suppressing angiogenesis, by increasing immunocompetence, or by acting as a mitochondrial energy intermediate?
- Are there certain nutrients, such as alpha lipoic acid, and vitamin K3, which potentiate the efficacy of ascorbic acid in its tumor preferential cytotoxicity, and which augment the potency of AA as a tumor cytotoxic chemotherapeutic agent?
- In the field of pharmacological nutritional oncology, what clinical possibilities might AA offer?

Herein, this candidate has provided evidence, which has included original research conducted by this candidate, supporting the following answers to these questions:

- Yes, compelling evidence exists to indicate that ascorbic acid (AA) at high concentrations can act as a pro-oxidant agent by generating hydrogen peroxide in the presence of oxygen.
- Yes, compelling evidence exists that vitamin C at high concentrations is preferentially toxic to cancer cells *in vitro* and *in vivo*.
- Yes, tumor cytotoxic concentrations of AA can indeed be clinically achieved by intravenous administration.
- Yes, AA oxidation products have indeed demonstrated antitumor activity.
- Yes, AA does indeed exhibit secondary anticancer mechanisms, by increasing the intracellular matrix, by suppressing angiogenesis, by increasing immunocompetence, and by acting as a mitochondrial energy intermediate.
- Yes, there are indeed certain nutrients, such as alpha lipoic acid, and vitamin K3, which potentiate the efficacy of ascorbic acid in its tumor preferential cytotoxicity, and which augment the potency of AA as a tumor cytotoxic chemotherapeutic agent.
- In the field of pharmacological nutritional oncology, AA has a multitude of safe, effective, and medically desirable clinical properties to offer, whether AA is administered singly or in combination with other therapeutic agents.

Herein this candidate has presented evidence that vitamin C is useful in the treatment of cancer. In particular, data have been shown which demonstrate that:

1. Vitamin C is toxic to tumor cells.
2. Vitamin C is nontoxic to normal, healthy cells.

3. Concentrations of vitamin C that kill tumor cells can be achieved in humans using intravenous vitamin C infusions.
4. Infusion of a bolus of vitamin C followed by slow infusion can result in sustained concentrations of vitamin C in human plasma.
5. Modeling of vitamin C pharmacokinetics may accurately predict plasma concentrations of vitamin C using varied infusion protocols.
6. Lipoic acid enhances vitamin C induced tumor cell toxicity.
7. Vitamin C in blood concentrations achievable through oral supplementation is capable of increasing collagen production by tumor cells.
8. Vitamin C does not interfere with standard chemotherapy or radiation treatment.
9. Some cancer patients have had complete remissions after high-dose intravenous vitamin C infusions.
10. Since concentrations of vitamin C that kill most tumor cells are not achieved after infusion of 30 grams of vitamin C, remissions in patients treated with this dosage are likely to have occurred as a result of vitamin C induced biological response modification effects rather than its cytotoxic effects.

In light of the data presented herein, this candidate believes that sufficient, and in fact compelling, evidence exists to support further research into the administration of intravenous ascorbic acid in the management of several types of malignancy.

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