

Mitochondria, Energy and Cancer: The Relationship with Ascorbic Acid

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This paper is dedicated to the memory of Dr. Brian Leibovitz and Dr. Hugh D. Riordan.

Abstract *Ascorbic Acid (AA) has been used in the prevention and treatment of cancer with reported effectiveness. Mitochondria may be one of the principal targets of ascorbate's cellular activity and it may play an important role in the development and progression of cancer. Mitochondria, besides generating adenosine triphosphate (ATP), has a role in apoptosis regulation and in the production of regulatory oxidative species that may be relevant in gene expression. At higher concentrations AA may increase ATP production by increasing mitochondrial electron flux, also may induce apoptotic cell death in tumor cell lines, probably via its pro-oxidant action. In contrast, at lower concentrations AA displays antioxidant properties that may prevent the activation of oxidant-induced apoptosis. These concentration dependent activities of ascorbate may explain in part the seemingly contradictory results that have been reported previously.*

Ascorbic Acid

Ascorbic acid (ascorbate, vitamin C, AA) is a water-soluble vitamin needed for the growth and repair of tissues in the body. It is necessary for the formation of collagen, an important protein of skin, scar tissue, tendons, ligaments and blood vessels. AA is essential for the healing of wounds and for the repair and maintenance of cartilage, bones and teeth. AA is one of many molecules with antioxidant and pro-oxidant capacity.

Proposed mechanisms of AA activity in the prevention and treatment of cancer include: Enhancement of the immune system

by increased lymphocyte production and activity;¹ stimulation of collagen formation, necessary for "walling off" tumors; inhibition of hyaluronidase by keeping the ground substance around the tumor intact and preventing metastasis;¹ inhibition of oncogenic viruses; correction of an ascorbate deficiency commonly seen in cancer patients; expedition of wound healing after cancer surgery;² enhancement of the anticarcinogenic effect of certain chemotherapy drugs;³⁻⁵ reduction of the toxicity of chemotherapeutic agents;⁶ prevention of cellular free radical damage;⁷ production of hydrogen peroxide;^{8,9} and neu-

tralization of carcinogenic substances.^{10,11}

The use of AA supplementation in large doses for the prevention and treatment of cancer has been reported by various groups. Cameron and Pauling performed experiments that utilized high doses of AA for the treatment of cancer.¹² These experiments proved that AA in high doses appears to be safe for the majority of individuals. Extensive epidemiological evidence points to the capacity of AA to prevent cancer at a number of sites. Some of the few studies which have been conducted on the use of high dose ascorbate in the treatment of cancer have yielded promising results.^{8,9} While AA alone may not be enough of an intervention in the treatment of most active cancers, it appears to improve quality of life and extend survival time in most cases and it should be considered as part of the treatment protocol for all patients with cancer.¹¹

The main function of AA in small quantities is as a hydrophilic antioxidant, but in high doses in malignant cells it may act as pro-oxidant. Experiments in rodents suggest that ascorbate administration increases host survival times and inhibit tumor growth.^{13,14} Clinical trials with AA have yield mixed results. In two Scottish studies, terminal cancer patients given intravenous AA (10g/day) had longer survival times than historical controls.^{15,16} A Japanese study yielded similar results,¹⁷ but two double-blind studies at the Mayo clinic using oral AA (10g/day) showed no benefit.^{18,19} We should mention that oral AA supplementation is unlikely to produce plasma ascorbate levels sufficient to kill tumor cells directly.²⁰ Intravenous AA at higher doses has been effective in individual cases.^{8,21-23}

The energy producing process of malignant cells is mainly anaerobic and the transformation of normal cells to malignant may be due to defects in aerobic respiratory pathways.²⁴⁻²⁶ Oxygen, the final electron acceptor in the electron transport system is of great importance in the ascorbate-induced inhibitory action, because of the production of hydrogen peroxide (H_2O_2) from a radical out of the AA molecule. Oxygen by itself has

an inhibitory action on malignant cell proliferation²⁷ by directly interfering with anaerobic respiration (fermentation and lactic acid production), a common energy mechanism utilized by malignant cells. It would be worth investigating the status of the mitochondria of malignant cells because González and colleagues²⁸ believe this may be relevant in the origin of malignancy. A problem in electron transfer in the malignant cells might well be coupled to a defective mitochondrial membrane and AA may help correct this electron transfer problem by balancing mitochondrial membrane potential of malignant tissue. The inhibitory action on cancer cells by ascorbic acid has been described since 1952.²⁹ AA not only has antioxidant properties but also pro-oxidant activity capable of selective cytotoxic effects on malignant cells when provided in high concentrations.^{8,9,28}

It has been suggested that ascorbate promotes oxidative metabolism by inhibiting the utilization of pyruvate for aerobic metabolism.³⁰ Also, an inhibitory effect on growth of several types of tumor cells has been produced by ascorbate and/or its derivatives. This inhibitory action was not observed in normal fibroblasts.³¹ This cytotoxic activity produced by ascorbate in an array of malignant cell lines has been associated to its pro-oxidant activity.³¹⁻³⁸ Ascorbate can generate H_2O_2 (a reactive species) upon oxidation with oxygen in biological systems.³⁹ H_2O_2 may further generate additional reactive species such as the hydroxyl radical and aldehydes which can compromise cell viability.⁴⁰ These reactive species may induce strand breaks in DNA, disrupt membrane function via lipid peroxidation or deplete cellular ATP.³⁹ The failure to maintain ATP production may be a consequence of oxidative inactivation of key enzymes of the aerobic pathway on the ATP uses. The cytotoxicity induced by ascorbate seems to be primarily mediated by H_2O_2 generated intracellularly by ascorbate's metabolic oxidation to dehydroascorbate.^{9,14,41-44} In addition this antiproliferative action of ascorbate in cultured malignant cells, animal and human tumor has been increased by the addition of the cupric ion, a catalyst for the

oxidation of ascorbate.¹⁴ It has also been suggested that the selective toxicity of ascorbate in malignant cells may be due to a reduced level of catalase in these cells, leading to cellular damage through the accumulation of H₂O₂.^{9,28,41-45} There is a 10 to 100 fold greater content of catalase in normal cells than in tumor cells.²⁰ For this reason the combination of megadoses of ascorbate together with oxygen and copper seems logical as part of a non-toxic treatment protocol for cancer patients.⁴⁶ Moreover, a lack of superoxide dismutase (SOD) has been detected in the mitochondria of cancer cells.⁴⁷ This deficiency will impair the function of the Krebs cycle forcing anaerobic metabolism and the concomitant production of lactic acid.⁴⁶

This information suggests that the mitochondria may be one of the principal targets of ascorbate activity and play an important role in the development and progression of cancer. Since the Warburg research in the 1930s,²⁵ few scientists have worked with the mechanism of mitochondrial respiratory alterations in cancer. Also, in relation to this it is relevant to understand the relationship between of AA, mitochondrial alterations and the apoptosis process pathway.

Mitochondria and Apoptosis

Mitochondria play a central role in oxidative metabolism in eukaryotes.⁴⁷ The primary purpose of mitochondria is to manufacture adenosine triphosphate (ATP), which is used as the primary source of energy by the cell. Mitochondria have several important functions besides the production of ATP. Mitochondria are also essential in the processing of important metabolic intermediates for various pathways involved in the metabolism of carbohydrates, amino acids, and fatty acids. In addition to oxidative phosphorylation, mitochondria are involved in other critical metabolic processes.⁴⁸ These variety of functions corresponds to the variety of mitochondrial diseases including diabetes, cardiomyopathy, infertility, migraine, blindness, deafness, kidney and liver diseases, stroke, age-related neurodegenerative disorders such as Parkinson's, Alzheimer's

and Huntington's disease, and cancer.⁴⁹

Mitochondria also play a role in the regulation of apoptosis. This organelle can trigger cell death in a number of ways: by disrupting electron transport and energy metabolism, by releasing and/or activating proteins that mediate apoptosis and by altering cellular redox potential.

Apoptosis, a physiological process for killing cells, is critical for the normal development and function of multicellular organisms. Abnormalities in cell death control contribute to a variety of diseases, including cancer, autoimmunity and degenerative disorders.³⁰ Electron microscopic analysis has identified the morphological changes that occur during apoptosis, which include chromatin condensation, cytoplasmic shrinkage, and plasma membrane blebbing.^{1,51} These studies have also noted that during the early stages of apoptosis, no visible changes occur in mitochondria, the endoplasmic reticulum, or the Golgi apparatus. However, others have more recently reported swelling of the outer mitochondrial membrane^{52,53} and release of cytochrome c^{54,55} and apoptosis inducing factor, an oxidoreductase-related flavoprotein,⁵⁶ from the mitochondrial intermembrane space.⁵⁰ Molecular changes induced during apoptosis include internucleosomal DNA cleavage⁵² and randomization of the distribution of phosphatidyl serine between the inner and outer leaflets of the plasma membrane.⁵⁷ These morphological and molecular changes are elicited by a broad range of physiological or experimentally applied death stimuli and are observed in cells from diverse tissue types and species.⁵⁰

Apoptosis research has recently experienced a paradigm change in which the nucleus of the cell no longer determines the apoptotic process. The new paradigm states that caspases and more recently, mitochondria constitute the center of death control.⁵⁸ Several observations are compatible with the hypothesis that mitochondria controls cell death: (i) mitochondrial membrane permeabilization generally precedes the signs of advanced apoptosis or necrosis, irrespective of the cell type or the death inducing stimulus;

(ii) this permeabilization event has a better predictive value for cell death than other parameters including caspase activation; (iii) an increasing number of pro-apoptotic effectors act on mitochondrial membranes to induce their permeabilization; (iv) anti-apoptotic members of the Bcl-2 family physically interact with mitochondrial membrane proteins and inhibit cell death by virtue of their capacity to prevent mitochondrial membrane permeabilization; (v) inhibition of mitochondrial membrane permeabilization by specific pharmacological interventions prevents or retards cell death; (vi) cell-free systems have identified several mitochondrial proteins, which are rate-limiting for the activation of catabolic hydrolases (caspases and nucleases). These observations suggest a three-step model of apoptosis: a pre-mitochondrial phase during which signals transduction cascades or pathways is activated (initiation phase); a mitochondrial phase, during which mitochondrial membranes are permeabilized (decision/effector phase); and a post-mitochondrial phase during which proteins are released from mitochondria and cause the activation of proteases and nucleases (degradation phase).⁵⁸

Inducers of apoptosis are relatively diverse and include death factors such as Fas ligand (FasL), Tumor Necrosis Factor (TNF) and TNF-related apoptosis-inducing ligand (TRAIL), genotoxic agents such as anti-cancer drugs, gamma-irradiation and oxidative stress.⁵⁹⁻⁶²

Mitochondria and Cancer

Mitochondria are involved either directly or indirectly in many aspects of the altered metabolism in cancer cells.⁶³ Cancer cells have altered metabolic characteristics that includes: a higher rate of glycolysis,⁶⁴ an increased rate of glucose transport,⁶⁵ increased gluconeogenesis,⁶⁶ reduced pyruvate oxidation and increased lactic acid production,⁶⁷ increased glutaminolytic activity,⁶⁸ reduced fatty acid oxidation,⁶⁹ increased glycerol and fatty acid turnover,⁷⁰ modified amino acid metabolism⁷¹ and increased pentose phosphate pathway activity.⁷² Various tumor cell

lines exhibit differences in the number, size and shape of their mitochondria relative to normal controls. The mitochondria of rapidly growing tumors tend to be fewer in number, smaller in size and have fewer cristae than mitochondria from slowly growing tumors; the latter are larger and have characteristics more closely resembling those of normal cells.⁴⁹

Chemotherapeutic anti-cancer drugs and gamma-irradiation induce apoptosis in tumor cells.^{51,52} Oncogenes and tumor suppressor genes that regulate cell death influence the sensitivity of tumor cells to anti-cancer therapy. Overexpression of Bcl-2 and its pro-survival homologs or the inactivation of Bax not only provides short term protection against apoptosis, but can significantly increase long-term survival with retention of clonogenicity in certain tumor cells that have been treated with anticancer drugs or gamma-irradiation.^{50,73} Thus, the response of cancer cells to therapy is determined by at least two processes: the propensity to undergo mitotic death and the sensitivity to apoptotic stimuli. Many anti-tumor therapies rely on inducing apoptosis in their target cells. The role of caspases in the response or resistance to such drugs has therefore been under intense scrutiny. Because the various caspases can process each other, most of them eventually become activated in cells undergoing apoptosis and this appears to be the case in drug-treated tumor cells.⁷⁴⁻⁷⁹

The observation that in some cancer patients "spontaneous" tumor regression was dependent on circulating levels of TNF80 was the first indication that cell surface receptors might be viable anti-cancer targets. It is now well established that some members of the TNF receptor superfamily induce apoptosis in cells.⁶⁰ Receptor-mediated induction of apoptosis is preceded by ligand-induced trimerization; recruitment of the death-inducing signaling complex (DISC) and activation of procaspase-8.^{81,82} Activated caspase-8 initiates a proteolytic cascade, resulting in apoptotic cell death. However, the use of TNF and other sequence-related ligands as pro-apoptotic agonists has been limited because of the associated systemic toxicity.⁸³

Mitochondria, Oxidative Species and differentiation

Cardiolipin is essential for the functionality of several mitochondrial proteins. Its distribution between the inner and outer leaflet of the mitochondrial internal membrane is crucial for ATP synthesis. Garcia-Fernández and colleagues,⁸⁴ investigated alterations in cardiolipin distribution during the early phases of apoptosis. Using two classical models (staurosporine-treated HL-60 cells and TNF- α -treated U937 cells); they found that in apoptotic cells cardiolipin moves to the outer leaflet of the mitochondrial inner membrane in a time-dependent manner. This occurs before the appearance of apoptosis markers such as plasma-membrane exposure of phosphatidylserine, changes in mitochondrial membrane potential, DNA fragmentation, but after the production of reactive oxygen species (ROS). The exposure of phospholipids on the outer surface during apoptosis, thus occurs not only at the plasma membrane level but also in the mitochondria, reinforcing the hypothesis of "mitoptosis" (cell death induce by mitochondria) as a crucial regulating system from programmed cell death, also occurring in cancer cells after treatment with antineoplastic agents.⁸⁴

Several potentially damaging ROS species may arise as by-products of normal metabolism and from chemical accidents.⁸⁵ Superoxide is a one-electron reduction product of molecular oxygen that is formed during normal respiration in mitochondria and by autoxidation reactions. The electron transport chain is not completely secure and electrons may leak onto molecular oxygen prior to their transfer to cytochrome oxidase forming superoxide. H_2O_2 is another ROS that can be formed during normal metabolism.⁸⁶

Not all ROS production is accidental, however, since the body can use these substances for its own benefit. There is some indications that these oxidative products may control cell growth and differentiation.⁸⁷ Nitric oxide finds a multiplicity of uses, for example as a regulator of vascular tone and as a messenger of the central nervous system.⁸⁸ Production of reactive species by activated

neutrophils, macrophages and several other cell types is used in bacterial killing. Indeed ROS may have a variety of functions including regulation of gene expression⁸⁷ and induction of apoptosis;^{89,90} programmed cell death involved in fetal development and tissue remodeling where changes in the type and distribution of tissue occur. An involvement of ROS in the process of cell differentiation has been suggested on the basis of studies with slime mold and *Neurospora crassa*.⁹¹ In both cases differentiation appears to be accompanied by increased production of ROS. Indeed, addition of antioxidants can modulate the process.⁸⁶

We should remember that cells, in order to be able to divide, need to reduce cohesiveness and dismount part of their structure: in other words dedifferentiate. This unstable state of cellular organization facilitates oxidative damage in dividing cells. With this taken into account, AA in high doses in the presence of divalent cations and oxygen may act as a chemotherapeutic pro-oxidant. It should also be pointed out that iron is imbedded in the center of the DNA double helix at certain loci where it seems to function as an oxygen sensor to activate DNA in response to oxidative stress. Because iron is a big molecule, it distorts the outer shape of the DNA helix this distortion depends on the oxidation state of iron. Under non-oxidized (reduced) conditions, iron is present in the ferrous state and the DNA helix is fairly tight around the iron atom; but when the ion is oxidized to the ferric state it opens up the DNA so that transcription into RNA becomes more easy.⁸⁷ This mechanism might as well be a way in which oxidative molecules influence and/or determine differentiation.

AA enters the picture since AA may assure a continuing electron exchange among body tissues and cell mitochondria.⁹² A greater amount of AA in the body enhances the flow of electricity optimizing the ability of the cells to maintain aerobic energy production and production of metabolic intermediaries that arise from this pathway facilitates cell to cell communication and probably enhances cell differentiation. In support of

this theory, it has been documented that osteoblast cells treated with ascorbic acid had a four fold increase in respiration and there-fold increase in ATP production that provided the necessary environment for cell differentiation.⁹³

The antioxidant defenses consist of redox molecules such as AA and vitamin E and enzymes (e.g. superoxide dismutase). Their function is to act as a coordinated and balanced system to protect tissues and body fluids from damage by ROS whether produced physiologically or as a response to inflammation, infection and/or disease.⁸⁶

Many antioxidant defenses depend on micronutrients or are micronutrients themselves. Examples of the latter are vitamin E (protects against lipid peroxidation) and AA (scavenges some aqueous ROS directly and recycles lipophilic vitamin E). Superoxide dismutase removes superoxide by converting it to H₂O₂ which is then removed by glutathione peroxidase or catalase.⁸⁵

Antioxidants most likely regulate the activation phase of oxidant-induced apoptosis. Oxidants can induce mitochondrial permeability transition and release mitochondrial intermembrane proteins, such as cytochrome c, apoptosis inducing factor (AIF) and mitochondrial caspase.⁹⁴

In health, the balance between ROS and the antioxidant defenses lies slightly in favor of the reactive species so that they are able to fulfill their biological roles and maintain the electrical flow of life. Repair systems take care of damage which occurs at a low level even in healthy individuals.⁹⁵ Oxidative stress occurs when there is a change in this ratio by increasing ROS and this may occur in several circumstances, for instance in disease or malnutrition where there are insufficient redox micronutrients to meet the needs of the antioxidant defenses.⁴⁷ Oxidative stress can be significant especially if the individual is exposed to intra or inter environmental challenges which increase the production of reactive species above "normal" levels, for instance, infection. Oxidative stress may be an important factor in infection if redox micronutrients (e.g. vitamins C and E) are deficient.⁸⁶

Mitochondria and Ascorbic Acid

Different groups are studying the role of AA in mitochondria and apoptosis. Pérez-Cruz and colleagues,⁹⁷ proposed that the AA inhibits FAS-induced apoptosis in monocytes and U937 cells. The FAS receptor-FAS ligand system is a key apoptotic pathway for cells of the immune system. Ligation of the FAS-receptor induces apoptosis by activation of pro-caspase-8 followed by downstream events, including an increase in ROS and the release of pro-apoptotic factors from the mitochondria, leading to caspase-3 activation. They found that AA inhibition of FAS-mediated apoptosis was associated with reduced activity of caspase-3, -8, and -10, as well as diminished levels of ROS and preservation of mitochondrial membrane integrity. Mechanistic studies indicated that the major effect of AA was inhibition of the activation of caspase-8 with no effect on its enzymatic activity. This group suggests that AA can modulate the immune system by inhibiting FAS-induced monocyte death.⁹⁶

AA has been reported to play a role in the treatment and prevention of cancer by Kang and others.⁹⁷ They reported that AA induces the apoptosis of B16 murine melanoma cells via a caspase-8-independent pathway. AA-treated B16F10 melanoma cells showed increased intracellular ROS levels. Also, their results indicated that AA induced apoptosis in these cells by acting as a prooxidant. However, AA-induced apoptosis is not mediated by caspase-8. Taken altogether, it appears that the induction of a pro-oxidant state by AA and a subsequent reduction in mitochondrial membrane potential are involved in the apoptotic pathway on B16F10 murine melanoma cells and that this occurs in a caspase-8-independent manner.⁹⁷

Although a high intake of antioxidant vitamins such as AA and vitamin E may play an important role in cancer prevention, a high level of antioxidants may have quite different effects at different stages of the transformation process. In cancer development, the resistance of cells to apoptosis is one of the most crucial steps. Recently, Wenzel and colleagues,⁹⁸ tested the effects of AA on

apoptosis in HT-29 human colon carcinoma cells when induced by two potent apoptosis inducers, the classical antitumor drug camptothecin or the flavone flavonoid. AA dose-dependently inhibited the apoptotic response of cells to camptothecin and flavone. Also, AA specifically blocked the decrease of bcl-XL by these treatments. An increased generation of mitochondrial O_2 precedes the down regulation of bcl-XL by camptothecin and flavone and AA at a concentration of 1mM prevented the generation of this ROS. In conclusion ascorbic acid at concentrations of 1mM functions as an antioxidant in mitochondria of human colon cancer and thereby blocks drug-mediated apoptosis induction allowing cancer cells to become insensitive to chemotherapeutics. Studies utilizing higher concentrations are necessary to completely understand the mechanism involved. It seems that the different actions of AA in the mitochondria may be dependent on different concentration levels.

Conclusion

AA shows both reducing and oxidizing activities, depending on the environment in which this vitamin is present and its concentration. Higher concentrations of AA induce apoptotic cell death in various tumor cell lines, probably via its pro-oxidant action.⁹ On the other hand, at lower concentrations, ascorbic acid displays antioxidant properties, preventing the activation of oxidant-induced apoptosis.^{9,4} However, the AA specific mechanistic pathways in relation with mitochondria still remain unclear.

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