

New Developments and Novel Therapeutic Perspectives for Vitamin C^{1,2}

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Abstract

Vitamin C is required for collagen synthesis and biosynthesis of certain hormones and recommended dietary intake levels are largely based these requirements. However, to function effectively as an antioxidant (or a pro-oxidant), relatively high levels of this vitamin must be maintained in the body. The instability of vitamin C combined with its relatively poor intestinal absorption and ready excretion from the body reduce physiological availability of this vitamin. This inability to maintain high serum levels of vitamin C may have serious health implications and is particularly relevant in the onset and progression of degenerative disease, such as cancer and cardiovascular disease (CVD), which have a strong contributing oxidative damage factor. In this review, we examine recent studies on the regulation of transport mechanisms for vitamin C, related clinical ramifications, and potential implications in high-dose vitamin C therapy. We also evaluate recent clinical and scientific evidence on the effects of this vitamin on cancer and CVD, with focus on the key mechanisms of action that may contribute to the therapeutic potential of this vitamin in these diseases. Several animal models that could be utilized to address unresolved questions regarding the feasibility of vitamin C therapy are also discussed. *J. Nutr.* 137: 2171–2184, 2007.

Introduction

“The medical profession itself took a very narrow and very wrong view. Lack of ascorbic acid caused scurvy, so if there was no scurvy there was no lack of ascorbic acid. Nothing could be clearer than this. The only trouble was that scurvy is not a first symptom of a lack but a final collapse, a premortal syndrome and there is a very wide gap between scurvy and full health.”

—Albert Szent-Gyorgyi

The above quotation, taken from Szent-Gyorgyi's Nobel Prize acceptance speech, was remarkably prescient. Few nutritional issues have received as much attention or been as hotly debated as the dietary requirement for vitamin C since the discovery of this vitamin in 1932. The recognition that vitamin C may also be important in cancer and heart disease has spurred renewed interest in dietary vitamin C requirements with the view that

amounts consumed should account for a potential therapeutic role in ameliorating chronic disease.

Vitamin C is an essential nutrient for the biosynthesis of collagen, L-carnitine, and the conversion of dopamine to norepinephrine (1). Under physiological conditions, it functions as a potent reducing agent that efficiently quenches potentially damaging free radicals produced by normal metabolic respiration of the body (2). Though most animals are able to synthesize large quantities of vitamin C endogenously, humans lost this capability as a result of a series of inactivating mutations of the gene encoding gulonolactone oxidase (GULO)³, a key enzyme in the vitamin C biosynthetic pathway (3,4). These mutational events were estimated to have occurred about 40 million years ago, rendering all descending species, including humans, ascorbic acid deficient (4). Acute lack of vitamin C leads to scurvy, manifest by blood vessel fragility, connective tissue damage, fatigue, and, ultimately, death.

Humans normally acquire vitamin C from a large variety of dietary sources through a substrate-saturable transport mechanism involving the ascorbate-specific transporters. Due to saturation and low expression of the transporter, combined with substrate-induced downregulation (5), the effective serum vitamin C concentrations attainable by oral ingestion are controlled at low levels (6). This inability to maintain serum ascorbic acid and the consequent reduction in antioxidant capacity may result in an increased flux of harmful reactive oxygen species (ROS) (7). The impact of low-level serum vitamin C and the consequent accumulation of ROS may have a profound effect on aging populations and may in part contribute to the high incidence of degenerative diseases, such as cancer and

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³ Abbreviations used: BH4, (6R)-5,6,7,8-tetrahydro-L-biopterin; CVD, cardiovascular disease; DHA, dehydroascorbic acid; ECM, extracellular matrix; eNOS, endothelial nitric oxide synthase; GLUT, glucose transporter; GULO, gulonolactone oxidase; HIF-1 α , hypoxia-inducible factor-1 α ; ICAM, intercellular adhesion molecule; NO, nitric oxide; NOS, nitric oxide synthase; oxLDL, oxidized LDL; RNS, reactive nitrogen species; ROS, reactive oxygen species; SVCT, sodium vitamin C cotransporter; VEGF, vascular endothelial growth factor.

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heart disease (8). Therefore, high-dose vitamin C treatment may ameliorate age-related degenerative diseases (8).

Our growing understanding of the mechanisms of vitamin C transport, newly-described physiological roles, and the potential involvement of vitamin C in cancer and heart disease have led to calls for reappraisals of the dietary requirements for this vitamin (8–10). In this review, we will examine the function and regulation of vitamin C transporters and potential implications in vitamin C treatment at both experimental and clinical stages. We will focus on recent evidence supporting a potential role for vitamin C in degenerative disease, including cancer and cardiovascular disease (CVD), and will review the new developments in animal models that will be critical tools in resolving outstanding questions.

Vitamin C Transport

As a polar compound with a relatively large molecular weight, vitamin C cannot readily cross the cell membrane by simple diffusion. The flux of vitamin C in and out of the cell is controlled by specific mechanisms, including facilitated diffusion and active transport, which are mediated by distinct classes of membrane proteins such as facilitative glucose transporters (GLUT) and sodium vitamin C cotransporters (SVCT), respectively.

Facilitated diffusion through GLUT transporters

Gradient-driven transport of the oxidized form of vitamin C, dehydroascorbic acid (DHA), is mediated by a class of facilitative GLUT, which has no detectable affinity for the reduced, biologically-active forms such as ascorbic acid and ascorbate (11). The reduced vitamin C, DHA, can be indirectly imported by a three-step mechanism involving: 1) extracellular oxidation of ascorbate to DHA; 2) transport of DHA by the GLUT transporter; and 3) intracellular reduction of DHA to ascorbate (Fig. 1).

The GLUT transporters mediate the absorption of DHA in an energy-independent manner and their kinetic properties can be robustly modeled by Michaelis-Menten kinetics (11). Based on apparent transport affinities (K_m), GLUT1 and GLUT3 are the major transporters for DHA influx among GLUT isoforms and have kinetic constants similar to those of glucose transport (11). Another DHA transporter, GLUT4, was later identified (12). GLUT1 and GLUT3 are predominantly located in osteoblast (13), muscle (14), and retinal cells (15) and mediate the influx of DHA in these cells. GLUT1 is also expressed on the endothelial cells at the blood brain barrier and may be partially responsible for accumulation of vitamin C in the brain (16) (Table 1). However, this mechanism may not be physiologically relevant, as competitive inhibition of DHA transport by glucose likely reduces vitamin C uptake by GLUT1 to insignificant levels. Therefore, accumulation of vitamin C in the brain is mainly achieved through a sodium-dependent mechanism mediated by the SVCT transporters (17,18).

Sharing the same transporters as glucose, GLUT-mediated transport of DHA is competitively inhibited by glucose (8,11,12,16,19). This raises the possibility that changes in serum glucose levels, especially those occurring during disease, may attenuate the bioavailability of vitamin C leading to secondary pathologies due to the depletion of circulating vitamin C. Indeed, this characteristic type of secondary pathology has been observed under hyperglycemic conditions caused by diabetes (20–22) and may be treated, at least partially, by clinical administration of vitamin C.

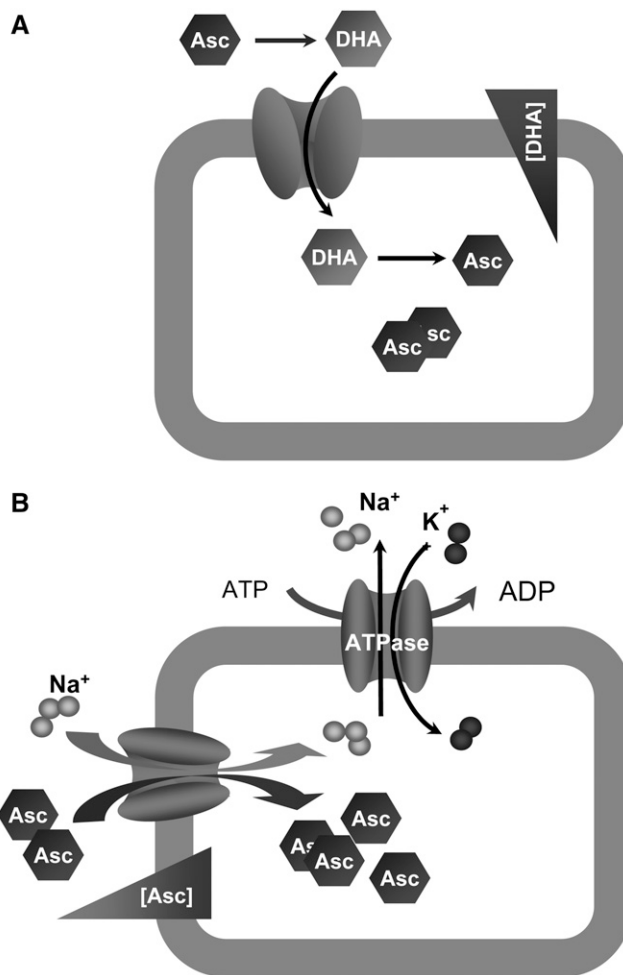


FIGURE 1 Mechanisms of vitamin C transport. Transport via GLUT (A) requires extracellular oxidation of ascorbate to DHA. DHA is imported by GLUT and reduced back to ascorbate in the cell. The concentration gradient of DHA is thus maintained. Ascorbate is coupled to sodium and transported directly by SVCT (B). The excess intracellular sodium is actively exported in exchange for extracellular potassium through a sodium-potassium ATPase.

In addition to glucose inhibition, the GLUT transporters are also subject to hormonal control (23). In the presence of both follicle-stimulating hormone and insulin-like growth factor I, the expression of GLUT 1 is upregulated in granulosa cells (23). Similarly, GLUT4 expression in cells is stimulated by addition of insulin (12).

The impact of serum glucose levels and endocrinal hormone status on vitamin C transport underscores the necessity of examining serum glucose concentrations in conjunction with vitamin C levels to understand how alterations in vitamin C status contribute to various diseases in humans.

The facilitated transport mechanism by GLUT has been implicated in the protection against oxidative damage (24). Administration of DHA has been shown to protect neural cells from experimentally-induced ischemic stroke by increasing antioxidant levels through GLUT-mediated vitamin C accumulation (24). This may also protect against ROS generated from mitochondrial respiration, which is of particular interest in human nutrition, because oxidative respiration in mitochondria is the major source of biological ROS in the cell. As oxidative

TABLE 1 Major mechanisms for vitamin C transport¹

Type	Transporter	Distribution	Regulation
A	GLUT1	Osteoblast (13), muscle cells (14), and retinal cells (15)	Glucose: competitive inhibition (11,12,19)
	GLUT3		
	GLUT4		
B	SVCT1	Intestinal, renal, and liver epithelial cells (25)	Ascorbate: Substrate feed back inhibition of SVCT expression (5,32)
	SVCT2	Brain (14), retinal (25), and placental cells (35)	

¹ Vitamin C is transported by GLUT in an energy-independent, three-step mechanism (Type A) or by secondary active SVCT in an ATP-dependent manner (Type B).

damage is a key contributor to age-related degenerative diseases, these findings support the therapeutic potential of intracellular vitamin C and implicate DHA, in conjunction with the GLUT transport system, as potential targets in the treatment of these diseases.

Active transport by SVCT transporters

In addition to the facilitated mechanism, vitamin C is also transported by active SVCT, which transport ascorbate directly into the cell. Based on K_m values, SVCT have higher affinity for ascorbate than do GLUT for DHA and thus are considered high-affinity vitamin C transporters (25). The SVCT system transports ascorbate at the expense of the sodium electrochemical gradient across the cell membrane and, as such, are classified as secondary active transporters (26) (Fig. 1).

There are 2 isoforms of SVCT transporter: hSVCT1 (*slc23a2*) and hSVCT2 (*slc23a1*). However, the Human Genome Organization gene names for these 2 transporters have recently been reassigned: SVCT1 and SVCT2 are encoded by *SLC23A1* and *SLC23A2*, respectively (27). A comparison of the 2 isoforms reveals that SVCT2 has a higher affinity (28) but lower transport capacity (29) for ascorbate than SVCT1. The distribution and functions of the 2 SVCT isoforms are distinct (Table 1). SVCT1 is predominantly expressed in epithelial cells, including those of the intestine, kidney and liver, and can transport amounts of ascorbate exceeding the internal requirement of these cells (25). Hence, it is often referred to as the “bulk” transporter of ascorbate. In contrast, SVCT2 is localized to metabolically-active and specialized cells, such as those of the brain, eye, and placenta (25,27), and has been implicated in the maintenance of intracellular vitamin C levels vital for neuronal function and the protection against oxidative stress (30).

Both isoforms of SVCT are subject to substrate feedback inhibition by ascorbate. The expression of SVCT1 is attenuated by high concentrations of ascorbic acid in vitro (5). As SVCT1 is the high-capacity, bulk transporter of vitamin C, its down-regulation by ascorbate effectively limits the maximum achievable concentration of plasma vitamin C by oral ingestion (26) and is a major obstacle in high-dose vitamin C strategies (6). Similar to its isoform, SVCT2 is sensitive to the changes in intracellular ascorbate levels (31), which may play a regulatory role in maintaining ascorbate homeostasis inside the cell (26). Indeed, the SVCT2 transporter is regulated by intracellular ascorbate at the translational level (32). This feedback mechanism presents a similar challenge to that of using SVCT1 to accumulate intracellular vitamin C, as pharmacologically increased intracellular ascorbate will attenuate the rate of transport (32) and, in effect, restore intracellular ascorbate to its normal physiological levels (31).

In addition to substrate inhibition, age-related decline in SVCT1 expression in rat liver cells has been observed (33). If this is subsequently found to occur in humans, it may help explain the observation that elderly individuals require higher levels of

dietary vitamin C to reach serum ascorbate concentrations comparable to those of younger individuals (34). As the effect of this decline can be compensated by increased vitamin C intake (33), clinical or nutritional treatment leading to moderately increased serum vitamin C levels might be beneficial for elderly individuals (26). Unlike SVCT1, age-related decline is not observed in SVCT2 levels in the liver, perhaps as a result of low abundance of this transporter in the liver (33). Future studies examining tissues rich in SVCT2, such as brain and retina, may reveal potential roles for aging on this transporter, as well as consequent changes in vitamin C accumulation and physiological abnormalities that might contribute to age-related diseases.

SVCT2 is, surprisingly, essential for perinatal survival of mice (35). It is required for vitamin C transport across the placenta as well as prenatal distribution of ascorbate into various tissues of the unborn mouse (35). Newborn mice carrying null mutation of *SLC23A2* die of respiratory failure and brain hemorrhage shortly after birth, suggesting a vital but unknown role for vitamin C in lung and brain tissues during early development (35). The phenotypic difference between *SLC23A2*^{+/-} and wild-type mice reflects the delicate correlation between SVCT2 activity and the intracellular ascorbate levels (35), which may be important for maintaining optimal intracellular vitamin C required for certain tissues. For example, overexpression of human SVCT2 transporter in mice leads to abnormal elevation of vitamin C levels in the retina, which results in damage to the eye (36). A number of single-nucleotide polymorphisms at the *SLC23A2* locus have been identified among human populations (37) and certain allelic variants associate with preterm birth in humans (38), raising the possibility that vitamin C may be implicated in some premature births in humans.

In summary, the 2 major vitamin C transporters, GLUT and SVCT, regulate the tissue-specific vitamin C levels and must be considered in treatments aiming to achieve high intracellular ascorbate levels. Indeed, a major difficulty in achieving high effective concentrations of vitamin C by oral administration is attributable to inhibition of these transporters. Alternative administration methods, such as i.v. injection which bypasses the renal system, can temporarily raise serum vitamin C to pharmacological levels (6). Alternatively, treatments altering the activity of a specific vitamin C transporter may potentiate localized accumulation of vitamin C and may be utilized when specific tissue is targeted for therapy. However, such strategies require better understanding of the physiological activities and tissue distributions of various vitamin C transporters in vivo. Transgenic animals harboring knockout mutations of SVCT2 (35) or overexpressing this transporter (36) are excellent in vivo models for studying the function of this transporter. Mice defective in SVCT1 have not yet been constructed and, given the fact that wild-type mice do not rely on vitamin C absorption for survival, may not be suitable for modeling the nutritional requirement for this vitamin in humans. A double knockout mouse that carries SVCT1 null mutation and is defective in

vitamin C biosynthesis would be an invaluable tool and may yield insight into the function of SVCT1 in humans. An alternative strategy using a chemical knock-out substrate that is exclusively recognized and transported by 1 specific system has also been devised (39). The advantage of this substrate analogue, 6-bromo-6-deoxy-L-ascorbic acid, is that it is specific for the SVCT system and, as such, allows the contributions of the GLUT and SVCT pathways in vitamin C transport to be assessed independently (39).

Vitamin C bioavailability

Bioavailability, or the effective concentration, of ascorbic acid is dependent on both intestinal absorption and renal excretion. Vitamin C, consumed either with diet or dietary supplements, is absorbed by the epithelial cells of the small intestine by the SVCT1 transporter and, subsequently, diffuses into the surrounding capillaries and then the circulatory system (27,40–42). In the kidney, circulating ascorbic acid is filtered from the glomerulus capillary bed into the Bowman's capsule through a general filtration mechanism. Ascorbic acid, while passing through the proximal convoluted tubule, is reabsorbed into the capillary bed surrounding this portion of the renal tubule through renal epithelial cells by the SVCT1 transporter (27). The difference between the amount of ascorbic acid filtered and the amount reabsorbed constitutes renal excretion (43).

Together, intestinal absorption and renal excretion determine the serum level of vitamin C and hence its bioavailability. At low concentrations, most vitamin C is absorbed in the small intestine and reabsorbed from the renal tubule (44). However, at high concentrations, SVCT1 becomes saturated, which, combined with ascorbate-mediated SVCT1 downregulation (5), limits the amount of ascorbic acid absorbed from the intestine and reabsorbed from the kidney (26). This imposes a physiological restriction on the maximal effective serum vitamin C concentration (or its bioavailability) that is attainable by oral consumption (6). This value has been determined to be $\sim 200 \mu\text{mol/L}$ (6), although "normal" physiological serum concentrations of ascorbate in healthy humans range from 60 to $100 \mu\text{mol/L}$ (45). However, vitamin C levels in circulating blood cells, such as platelets, are much higher than those in the plasma (45), as these cells express the SVCT2 transporter (32), which mediates intracellular ascorbate accumulation.

CVD

CVD is multifactorial with many identifiable risk factors, including diet, tobacco smoking, diabetes, and hypertension (46). Diet, as a modifiable determinant, is important in the prevention of CVD. While some studies reported that consumption of vitamin C-rich foods, such as fruits and vegetables, is correlated with a reduced risk of CVD (47–49), others have reported contradictory results (50). Apart from well-recognized confounding phenomena, the inconsistency is due at least in part to our limited understanding of the mechanisms of action of this vitamin on different pathophysiological variables contributing to cardiovascular complications and, as such, more focused mechanistic studies on the interaction of ascorbic acid with contributors of specific vascular pathology are required. In this section, both epidemiological and experimental evidence pertaining to the roles for vitamin C on the prevention and treatment of CVD is reviewed, with a focus on the mechanisms of action that may contribute to the potential benefits of vitamin C.

Epidemiological evidence

High dietary intake of vegetables and fruits reduces the risk of heart disease (47–49). This association is partially attributable to antioxidants, such as vitamin C and vitamin E, present in these foods, which protect biological molecules from oxidative damage. This is supported by compelling evidence that oxidative damage due to ROS is a major cause of CVD (51). Many epidemiological studies, including observational studies and randomized controlled trials, have examined the relationship between antioxidants and incidence of CVD. However, the results and conclusions of these studies are not consistent. Whereas some observational studies report a negative correlation between dietary intake of vitamin C, in itself or in combination with other antioxidant vitamins, and the risk of cardiovascular complications (51–54), this association is not seen in randomized controlled trials (55,56). The findings of these epidemiologic studies have been systematically reviewed and potential causes of their discrepancy discussed (57,58).

Apart from reliance on subject self-report, susceptibility to measurement error, and the short intervention duration commonly associated with these studies, the inconsistency is also caused by confounding effects (57). In addition, epidemiological studies often do not consider the specific physiological conditions of the subjects and because vitamin C may have opposing effects (antioxidant vs. pro-oxidant) under different physiological conditions (59,60), cancellation of positive and negative outcomes within a pooled sample population may result in the lack of treatment effect. This further underscores the importance of understanding the mechanisms of action of this vitamin and its interaction with other physiological variables in the biological system. Indeed, research into the therapeutic effects of vitamin C on CVD has refocused on the elucidation of potential mechanisms of action that may contribute to its therapeutic potentials in CVD.

Oxidative stress, vitamin C, and CVD

Oxidative stress induced by both ROS and reactive nitrogen species (RNS) plays a major role in the initiation and progression of CVD (51). In ROS and RNS, superoxide is the most biologically relevant radical in vasculature, as it is naturally produced by most vascular cells (61) and can mediate the generation of other ROS and RNS, leading to augmentation of oxidative damage (51).

The effects of oxidative stress on the cardiovascular system are multifold and include: 1) ROS-induced apoptosis of endothelial cells (62,63); 2) induction of inflammation by oxidative modification of the expression of proinflammatory genes (64) and cell adhesion (65); 3) reduction of intracellular bioavailability of vasodilator nitric oxide (NO) (66); and 4) oxidative modification of LDL (67). All of these contribute to clinical manifestations of CVD.

Biological antioxidants can sequester free radicals and thus prevent oxidative damage to the cardiovascular system (68,69). In the following section, the mechanisms by which vitamin C can influence cardiovascular health are reviewed with emphasis on interaction with key molecules/pathways of the vascular system, including LDL, vitamin E, and the NO synthetic pathway.

Oxidative modification of LDL

Oxidative modification of LDL by ROS, such as superoxide and hydroxyl radicals, generated by subendothelial cells transforms native LDL into highly bioreactive oxidized LDL (oxLDL), which initiates a sequence of atherogenic events in the subendothelial space. These include: 1) increased intake of oxLDL by

macrophages and the consequent formation of cholesterol lipid-laden foam cells (70,71); 2) upregulation of intercellular adhesion molecule (ICAM) and vascular cell adhesion molecule by activated macrophages, leading to influx of monocytes into subendothelial space and further accumulation of macrophages (72,73); 3) subsequent elicitation of multiple inflammatory responses (74,75); and 4) induction of apoptosis of endothelial cells (76,77). These events lead to the initiation and progression of atherosclerosis and other symptoms of heart disease. Because oxidative modification of LDL occurs in the early stages of vascular dysfunction and is instrumental in the progression of atherosclerosis, strategies to reduce LDL modification may help prevent the onset of atherosclerosis (78–80).

Because the origin of oxLDL formation can be traced to the elevation of ROS and RNS, a reduction in the concentration of these species and restoration of vascular redox balance by water-soluble antioxidants such as ascorbic acid may be effective in attenuating oxLDL-mediated endothelial dysfunction. For instance, physiological concentrations of ascorbic acid (50–100 $\mu\text{mol/L}$) in vitro attenuate oxidative modification of LDL induced by transition metals (81,82), homocysteine (83), and myeloperoxidase-derived HOCl (84,85), as well as those naturally produced by human vascular endothelial cells (86). Two key mechanisms are responsible for these actions: 1) ascorbate quenches aqueous ROS and RNS, decreasing their bioavailability in the plasma (87); and 2) ascorbate reduces the affinity of LDL-bound apolipoprotein B protein for transition metal ions and this, in effect, enhances the resistance of LDL to metal ion-dependent oxidation (87). In addition to preventing oxLDL formation, vitamin C also counteracts the damaging effects of existing oxLDL on different vascular components. For example, vitamin C protects arterial smooth muscle (88) and mature human macrophages (89) from oxLDL-induced apoptosis. It also attenuates the atherogenic inflammatory response by inhibiting oxLDL-related ICAM-1 overexpression and monocyte adhesion (90–93) and spares intracellular glutathione from oxLDL-stimulated modulation (94). This further increases the antioxidant capacity of the cell (94). Moreover, synergistic anti-atherogenic effect can be achieved when vitamin C is given with other antioxidants. For example, ascorbic acid can interact with estradiol in vitro, enhancing its ability to inhibit oxidation of LDL (95,96). In combination with vitamin E, vitamin C prevents oxLDL-induced overexpression of vascular endothelial growth factor (VEGF) and its receptor responsible for atherosclerotic plaque formation (97,98) and decreases plasma vascular cell adhesion molecule-1 and ICAM-1 responsible for monocyte adhesion and inflammation (99). The synergism between vitamin C and vitamin E can at least in part be ascribed to the ability of ascorbic acid to regenerate vitamin E from α -tocopherol radical (100), therefore restoring and augmenting the intrinsic antioxidant property of vitamin E.

Vitamins C and E

Vitamin E, in the form of α -tocopherol, is a key lipophilic antioxidant in human circulation and the vasculature and plays a role in many key processes contributing to the onset and progression of atherosclerosis (101). As a lipophilic antioxidant, vitamin E can interact with the lipid components in the vascular systems, notably LDL, and protects them from atherogenic oxidative modification (102). Conversely, the lipid-bound α -tocopherols can be oxidized by aqueous-phase radicals and transformed into reactive tocopherol radicals, which, in turn, react with the unsaturated lipids of the lipoprotein, initiating lipid oxidation by a tocopherol-mediated peroxidation reaction

(103,104). Oxidized vitamin E can be reduced back to its antioxidant form by other aqueous-phase reductants (104).

Ascorbic acid reacts rapidly with the tocopherol radical, reducing it back to its native form (105). As such, it may be conducive for vitamin E regeneration (100). In addition, ascorbate may sequester aqueous radicals in the plasma before they can oxidize vitamin E in the lipid phase and affords preemptive protection for lipid-bound tocopherols. In cigarette smokers, the rate of the blood vitamin E oxidation caused by increased oxidative stress is substantially attenuated by vitamin C supplementation (106,107), indicative of a vitamin E-recycling role for vitamin C and a potential cooperative relationship between vitamins C and E. Indeed, this cooperativity against oxidation of lipoproteins has been shown both in vitro (108) and in vivo (108–111). Furthermore, vitamins C and E can interact synergistically in protection against the development of CVD (112). For example, when applied in combination, they synergistically attenuate copper-mediated LDL oxidation in vitro (113), downregulate the expression of endothelial VEGF and its receptor VEGF-2 (97,98), and decrease the activation of NADPH oxidase while increasing that of superoxide dismutase, leading to reduced levels of oxidative stress (114). These cooperative interactions between these 2 vitamins have important clinical ramifications, because they provide a mechanistic basis for combined therapy (co-antioxidant therapy) in treatment of CDV. Indeed, the therapeutic value of multivitamin treatment has been supported by a growing body of clinical evidence (99,115–118).

Endothelial NO and vitamin C

NO is produced from L-arginine by NO synthase (NOS) in the presence of NOS cofactor (6R)-5,6,7,8-tetrahydro-L-biopterin (BH4). Endothelial NO produced by endothelial NOS (eNOS) modulates cardiovascular homeostasis and protects the vascular system by several mechanisms. As an endothelial signal molecule, NO stimulates vascular smooth muscle relaxation, allowing vasodilatation and unhindered blood flow (119). It also participates in more complex regulatory pathways of vascular inflammation (120). For example, it prevents adhesion of leukocytes to the interior wall of blood vessels (121), activation of proinflammatory adhesion molecules and cytokines (122,123), and suppression of endothelial cell apoptosis (123,124). These mechanisms contribute to a broad spectrum of physiological effects that inhibit atherosclerosis (120,125).

However, the NO biosynthetic pathway is sensitive to oxidative modification by ROS and its alteration has detrimental consequences for the vascular system. In the presence of superoxide produced by NAD(P)H oxidase and uncoupled eNOS, NO is oxidized to peroxynitrite, which, in turn, oxidizes eNOS cofactor BH4, leading to the inactivating uncoupling of eNOS from BH4. The uncoupled eNOS exhibits altered enzymatic activity, reducing molecular oxygen to superoxide, which, in effect, leads to significant reduction of endothelial NO and accumulation of ROS and RNS in the vasculature (126). The former is manifest by impaired endothelium-dependent vessel relaxation commonly observed in hypertensive animal models (127,128) and patients with endothelial dysfunction (129). The latter causes further NO oxidation and eNOS modification, leading to augmented oxidative stress to the vascular system. Indeed, aberrant NO metabolism is closely correlated with, and may be instrumental to, the development of CVD.

Vitamin C protects normal NO synthesis by modulating the redox states of its components. It stabilizes endothelial BH4 level by regenerating BH4 from its oxidized form in a series of

reduction reactions (65,130–132). As BH₄ is an essential cofactor for eNOS and its oxidative inactivation is the major contributing factor to NO pathway aberration and consequent clinical manifestations (133–135), the stabilization and reactivation of the endothelial BH₄ by ascorbic acid, and the consequent restoration of the normal biological activities of eNOS (136,137) and endothelial NO accumulation, may represent a key mechanism by which vitamin C impacts overall endothelial health.

Vitamin C attenuates the activity of NAD(P)H oxidase and the production of superoxide *in vitro* (138) and *in vivo* (114,139). As NAD(P)H oxidase is the major source of endothelial superoxide (140) responsible for the initial uncoupling of eNOS, its inactivation by vitamin C and E suggests that these 2 vitamins may prevent the onset of eNOS uncoupling-induced endothelial dysfunction by inhibiting the early events of this process. As a free radical scavenger, ascorbic acid, at high concentrations, reacts directly with superoxide, decreasing its availability and inhibiting superoxide-mediated NO inactivation (141). Ascorbic acid may also preserve the normal enzymatic activity of eNOS by preventing the S-nitrosylation of the regulatory cysteine residues on eNOS (142).

In addition, ascorbate directly mediates the production of NO by reducing nitrite compounds, thereby maintaining high levels of local tissue NO concentrations independent of the NO synthesis (142). It potentiates the responsiveness to NO stimulation by increasing the sensitivity of guanylyl cyclase to NO signaling (143). However, the biological relevance of these *in vitro* phenomena is still unknown.

The protective role for vitamin C on NO and its biosynthetic pathway is supported by clinical evidence that administration of vitamin C improves endothelium-dependent vasodilatation in patients with endothelial dysfunction (144,145). Because endothelial dysfunction generally marks the onset of atherosclerosis and CDV, vitamin C supplementation may be beneficial during early stages of CVD.

Vitamin C and collagen

Animal studies with *Gulo*^{-/-} mice, which are unable to produce vitamin C, show that ascorbic acid deficiency gives rise to structural abnormalities in the wall of the aorta, which is caused by defects in collagen and elastin synthesis (146). Bitransgenic *Gulo*^{-/-} mice that carry an *Apoe* null mutation (*Gulo*^{-/-}*Apoe*^{-/-}) have lower collagen content in atherosclerotic plaques when fed a low-vitamin C diet (147). Lower collagen content leads to instability of plaques, facilitating rupture and making them high risk for secondary plaque formation (147). However, whether these collagen deficiency-associated abnormalities are applicable in humans is not yet clear.

Cancer

The idea of using vitamin C to treat and prevent cancer was first proposed in 1949 and later supported by Cameron et al. who, in a controversial study, showed that administration of high-dose ascorbic acid improved the survival of patients with terminal cancer (148–150). Their results led to the proposal of using megadoses of vitamin C to combat degenerative diseases, including cancer and CVD.

One of the most important modifiable determinants of cancer risk is diet. Several research panels and committees have independently concluded that high fruit and vegetable intake decreases the risk of many types of cancer (151,152). Because

vitamin C is present in large quantities in these foods, it is plausible that the reduction in cancer risk associated with the consumption of fruits and vegetables may be, at least in part attributable to dietary vitamin C. This is supported by 2 large prospective studies that showed that plasma vitamin C concentration is inversely related to cancer mortality in human subjects (153,154). However, contradictory results have also been reported (155,156). The inconsistency of the vitamin C-cancer correlation and lack of validated mechanistic basis for its therapeutic action has critically undermined the feasibility of using vitamin C in clinical treatment or prevention of cancer (157).

One of the most critical findings that has cast doubt over the effectiveness of vitamin C in treating cancer is the Moertel study (158), a randomized, placebo-controlled clinical study in which a high dose of vitamin C was given orally to advanced cancer patients with no effect detected. It contradicted the findings of early studies conducted by Cameron et al. (148–150) in which clear improvements in the health status of terminal cancer patients were shown after high-dose *i.v.* vitamin C treatment. The discrepancy between these studies may be explained by the differences in the plasma vitamin C concentrations achieved by different administration methods. The former administered vitamin C exclusively orally, whereas the latter used both oral and *i.v.* administrations. Maximum plasma vitamin C concentrations achievable by oral administration are limited by the kidney, which eliminates excess ascorbic acid through renal excretion. In contrast, because *i.v.* injection bypasses the renal absorptive system, it results in elevated plasma concentrations to high levels (6). This pharmacokinetic property of ascorbic acid was demonstrated recently in healthy subjects. *I.v.* administration resulted in substantially higher (~70-fold) plasma vitamin C levels than those attainable by oral dose (6). In light of these results, it is likely that higher plasma concentrations were achieved in the Cameron study (148–150), which used both *i.v.* and oral administrations, but not in the Moertel study (158), in which only oral administration was used. The difference in effective vitamin C concentrations may have, in turn, contributed to the observed discrepancy in therapeutic outcomes reported. Indeed, a recent case study examining the clinical history of 3 cancer patients and the treatment they received supports the notion that high-dose vitamin C administration through *i.v.* injection has potential anti-tumor effects for certain types of cancer (157).

Newly available pharmacokinetic data, improved understanding of the regulation of vitamin C transport, and the growing evidence on the therapeutic efficacy of vitamin C have stimulated interest to reassess the feasibility of using vitamin C in the prevention and treatment of cancer. Though different in their methodologies, most recent studies on vitamin C and cancer have been conducted around 2 central themes: 1) the effects of high-dose ascorbic acid on the development and progression of tumors; and 2) the mechanisms of action that may contribute to the anti-cancer effect of this vitamin.

High-dose *i.v.* vitamin C administration

Because achieving high levels of ascorbic acid by *i.v.* injection are feasible *in vivo* (157), research has refocused on the implications and applicability of high-dose *i.v.* vitamin C administration in cancer therapy. Pharmacological concentrations of ascorbic acid (0.3–20 mmol/L) that are comparable to those attained by *i.v.* administration selectively target and kill tumor cells *in vitro* (159). In contrast, physiological concentrations of ascorbic acid (0.1 mmol/L) do not have any effect on either tumor or normal

cells (159). This tumor-killing phenomenon is attributable to the pro-oxidant property of vitamin C, which, at high concentrations, mediates the production of hydrogen peroxide (159). This provides a potential mechanism of action for the anti-tumor effect of vitamin C and implicates it as a pro-drug in cancer treatment (6,156,159). The manifestation of this effect in a real clinical setting has also been examined (157). A case study examined the treatment effects of i.v. vitamin C administration on cancer progression in patients with well-documented case histories (157). In all 3 cases, high-dose i.v. vitamin C therapy effectively reduced the progression of malignant tumor and improved the health status of these patients (157). Unfortunately, the information on the plasma vitamin C concentrations of these patients is not available to establish a causal relationship between the route of administration, the resultant effective concentrations, and the observed therapeutic effect. Nonetheless, this association can be reasonably assumed based on findings of a previous pharmacokinetic study, which shows that i.v. injection leads to high concentrations of serum vitamin C (6). However, it is difficult to assess the precise contribution of vitamin C in the clinical outcome, because all subjects under examination were receiving other forms of therapeutic treatments concurrent with high-dose vitamin C therapy (160). Moreover, alternative explanations for this outcome cannot be readily ruled out. As pointed out by the authors (6) and others (160), the observed remission of cancer in these cases may be attributable to spontaneous remission or as the consequence of prior treatments rather than ascorbic acid administration (160). Therefore, the therapeutic value of high-dose vitamin C administration in cancer progression or remission is not unequivocally supported by this study.

When administered in high doses by i.v. injection, vitamin C also improves the health-related quality of life in terminal cancer patients (161). After 1 wk of high-dose therapy, the global health/quality of life on both the functional (such as emotional and cognitive) and the symptom scales (such as fatigue and pain) were significantly improved in 39 terminal cancer patients (161). Though not curative, vitamin C treatment in this case successfully fulfilled an equally important goal in treating cancer—the improvement in the quality of life, which is particularly critical in patients at the terminal stages of this disease. Although showing a direct relationship between vitamin C treatment and therapeutic benefits, the results of this study were not unequivocal. For example, like the case study, the plasma ascorbic acid concentrations that resulted from the treatment were not assessed. In many cancer patients, especially those at the terminal stages, the absorption and excretion of certain drugs, including vitamin C, may be altered due to physiological abnormalities, which in turn may influence bioavailability. Thus, the plasma vitamin C concentrations in these patients may not be comparable to those measured in healthy subjects in the early pharmacokinetic study (6). For this reason, it is imperative to obtain direct information on plasma vitamin C concentrations in future clinical studies, especially when cancer patients are employed as test subjects. Because of a lack of control groups, it is unclear whether the improved status in these patients is a direct result of vitamin C treatment. Nevertheless, the encouraging findings of these clinical (161) and case studies (157) have stimulated new interests for more systematic research. Phase I trial studies are being conducted to collect preliminary data on the efficacy, safety, and pharmacokinetics of high-dose i.v. therapy and systematically examine its potential application in cancer treatment (160).

Mechanism of action

Parallel to clinical case/prospective studies examining the anti-cancer effects of high-dose vitamin C, experimental studies designed to investigate the mechanisms of action contributing to the therapeutic effect of vitamin C are concurrently being conducted, including its antioxidant or pro-oxidant function, its ability to modulate signal transduction and gene expression, and its potential role in tumor metastasis.

Antioxidant and pro-oxidant. At physiological concentrations, vitamin C is a potent free radical scavenger in the plasma, protecting cells against oxidative damage caused by ROS (162). The antioxidant property of ascorbic acid is attributed to its ability to reduce potentially damaging ROS, forming, instead, resonance-stabilized and relatively stable ascorbate free radicals (163). This mechanism is manifest in a number of cytoprotective functions under physiological conditions, including prevention of DNA mutation induced by oxidation (164–167), protection of lipids against peroxidative damage (168,169), and repair of oxidized amino acid residues to maintain protein integrity (168,170,171). The effects of vitamin C on these 3 classes of biological molecules have been reviewed (162). As DNA mutation is likely a major contributor to the age-related development of cancer (172,173), attenuation of oxidation-induced mutations by vitamin C constitutes a potential anti-cancer mechanism. Plasma vitamin C at normal to high physiological concentrations (60–100 $\mu\text{mol/L}$) decreases oxidative stress-induced DNA damage by neutralizing potentially mutagenic ROS (164–167). Consumption of vitamin C-rich foods is inversely related to the level of oxidative DNA damage *in vivo* (172,174–176).

Paradoxically, ascorbic acid may also function as a pro-oxidant, promoting oxidative damage to DNA (177). This occurs in the presence of free transition metals, such as copper and iron, which are reduced by ascorbate and, in turn, react with hydrogen peroxide, leading to the formation of highly reactive and damaging hydroxyl radicals (177). However, the relevance of this under normal physiological conditions *in vivo* has been questioned, as most transition metals exist in inactive, protein-bound form *in vivo* (178). However, when used at pharmacological concentrations (0.3–20 mmol/L), ascorbic acid displays transition metal-independent pro-oxidant activity, which is more profound in cancer cells and causes cell death (159). This tumor cell-killing response is dependent upon ascorbate incubation time and extracellular ascorbate concentration (159). The findings of this study contradict a view that *in vitro* cancer killing by vitamin C is a mere artifact due to the presence of free transition metals in the culture medium (179,180). Transition metal chelation had no effect on preventing cell death, indicative of a metal-independent mechanism in effect (159). Extracellular ascorbate is the source of this anti-cancer effect, contrary to the conventionally held view that intracellular vitamin C is a major contributor. Although the mechanism of action for this cancer-killing effect has been identified, the reasons for the selectivity have not yet been confirmed. Nonetheless, the selective toxicity may be attributed to several intrinsic properties of cancer cells, including reduced concentrations of antioxidant enzymes, such as catalase (181,182) and superoxide dismutase (183,184), increased intracellular transitional metal availability (185), and better accumulation of DHA through GLUT transporter overexpression (186,187), all contributing to the augmented intracellular hydrogen peroxide concentrations. Therefore, a nutritional regimen resulting in increased generation of hydrogen peroxide *in vivo* may be

exploited as a means for inducing tumor-specific cytotoxicity (185).

The effective concentration of vitamin C required to mediate cancer killing can be easily achieved by i.v. injection (6,159) and maintained by repeated dosing in vivo.

Whether vitamin C functions as an antioxidant or pro-oxidant is determined by at least 3 factors: 1) the redox potential of the cellular environment; 2) the presence/absence of transition metals; and 3) the local concentrations of ascorbate (185). The last factor is particularly relevant in treatments that depend on the antioxidant/pro-oxidant property of vitamin C, because it can be readily manipulated and controlled in vivo to achieve desired effects.

Signal transduction, gene expression, and vitamin C. The intracellular redox changes caused by oxidants and antioxidants can modulate the expression of genes involved in signal transduction pathways leading to cell cycle progression, cell differentiation, and apoptosis (188). For example, cells treated with ascorbic acid at low pharmacologic concentration (1 mmol/L) increase expression of apoptotic genes that are induced by UV irradiation and DNA damage, indicating that vitamin C can modulate gene expression (189). Ascorbate enhances the expression of both MLH1, a MutL homolog required for DNA mismatch repair machinery, and p73, a p53 homolog, increasing the cellular susceptibility to apoptosis, especially in the presence of DNA-damaging agents (190). As the induction of MLH1 is a critical determinant in a cell's decision between pathways leading to either accumulation of mutation and subsequent tumorigenic progression or apoptosis (190), these data support an anticancer role for intracellular vitamin C. The therapeutic potential of vitamin C in cancer is further supported by its ability to activate the apoptotic program in DNA-damaged cells independent of the p53 tumor suppressor through an alternative pathway mediated by p73, which, in contrast, is functional in most tumor types (191). Ascorbate also stabilizes p53 and augments the apoptotic response of HeLa cells to chemotherapeutic agents (192). At pharmacological concentrations (1 mmol/L), it decreases the Bcl-2:Bax ratio in the cytosol and mediates the mitochondrial release of cytochrome C, leading to the activation of the caspase cascade and apoptotic processes (193). This provides a mechanistic basis for combined therapy of vitamin C and chemotherapeutic drugs, as vitamin C potentiates the effectiveness of such drugs and, consequently, reduces the undesirable collateral damage to healthy cells (190). However, the concurrent use of antioxidants such as ascorbic acid as chemotherapeutic agents is still controversial (194).

Vitamin C, at millimolar intracellular concentrations, inhibits the activation of nuclear factor kappa B, a rapid response transcription factor, by preventing the TNF α -mediated degradation of its inhibitor in different human cell lines as well as primary cells through independent mechanisms (195–197). As NF κ B induces transcription of genes involved in both inhibition of apoptosis and promotion of cell proliferation, its overexpression directly contributes to malignancy (198). Repression of constitutive activation of NF κ B by vitamin C can induce cell cycle arrest and apoptosis in these cells and attenuate tumor progression in different types of cancer. Moreover, in vitro overexpression of the epidermal growth factor receptor family member Her-2/neu constitutively induces NF κ B activation, which likely contributes to the transformed phenotype in mammary tumor cells (199). The recent advances in transgenic animal models facilitate the examination of these phenomena in vivo. For example, the availability of Her-2/neu mice over-

expressing this receptor (200) and *Gulo* knockout mice unable to produce vitamin C (146) makes it possible to create a strain of bi-transgenic knockout mice for examining the in vivo effects of vitamin C on breast cancer.

Ascorbate and its lipophilic derivatives attenuate cell proliferation, arrest cell cycle, and induce apoptosis in human glioblastoma tumor and pancreatic cancer cells by reducing the expression of insulin-like growth factor-I receptor (201,202). Cell cycle arrest induced by vitamin C is also attributable to its ability to prevent the activation and nuclear accumulation of the mitosis-inducing phosphatase Cdc25C, hence providing a mechanism to restore cell cycle checkpoints in p53-deficient cells (203). The inhibitory effect is more potent in the lipophilic derivatives of ascorbate (201), which may have better intracellular accumulation. Therefore, it is possible that synthetic vitamin C derivatives with increased lipophilicity may have higher bioavailability in vivo and thus improved therapeutic efficacy.

Can vitamin C attenuate metastasis? The spread of cancer, or metastasis, is initiated by disrupting the physical confinement imposed by the extracellular matrix (ECM) through the primary malignant cell-induced degradation of collagen structure (204). Because vitamin C is essential for collagen maturation and stabilization, it has been suggested that ascorbic acid may reduce tumor spreading by potentiating the stability of the ECM, especially since neoplastic invasion exhibits similar pathological manifestations as vitamin C deficiency (185). Unfortunately, the effects of vitamin C deficiency on metastasis caused by reduced collagen stabilization have not yet been examined in vivo due to the lack of appropriate animal models. Interestingly, a vitamin C-independent pathway for collagen biosynthesis may exist in mice, because vitamin C restriction in *Gulo* knockout mice results in no detectable alteration in levels of angiogenesis (205), a prerequisite for en masse tumor growth that requires sufficient collagen deposition. However, whether a similar phenomenon exists in humans is not known. In addition, conflicting results have been reported. For example, in the same mouse model, vitamin C depletion significantly attenuated tumor growth by impairing angiogenesis (206), an observation that has cast some doubt on the anti-tumorigenic property of vitamin C. However, as pointed out by the authors, this finding was based on an implanted tumor that displayed unusual dependence on angiogenesis (206). Whether this mechanism is applicable for other clinical tumors in humans is uncertain. Moreover, blood vessel formation of human endothelial cells, a process that mimics blood vessel formation, is attenuated by ascorbic acid at high physiological concentrations (200 μ mol/L) but enhanced in a dose-dependent manner at normal physiological concentrations (<100 μ mol/L) (206), indicative of a dual-effect of vitamin C in blood vessel formation. However, the effects of supraphysiological (200 μ mol/L) or pharmacological levels (>1 mmol/L) of vitamin C on angiogenesis in vivo, which are more relevant in clinical vitamin C therapy, were not investigated in this study.

Though not fully understood, there are 2 opposing views on the role of the collagen-stabilizing function of vitamin C on tumor growth. First, by stabilizing collagen, ascorbic acid fortifies the ECM and stromal structures, leading to better confinement of neoplastic cells to their primary sites and preventing tumor growth and metastasis (185). Second, the same function may also facilitate the formation of new blood vessels, providing the prerequisite for malignant tumor growth (206). The interplay of these effects in vivo, especially under pharmacological levels of vitamin C, is far from clear. However,

with the availability of *Gulo* knockout mice and a better understanding of collagen biosynthesis, new research is being conducted to understand the mechanistic basis of these phenomena.

In addition to angiogenesis, cancer cells can also modify their energy metabolic pathways to adapt to the low oxygen micro-environment in the interior of a solid tumor (207,208). This is achieved by activation of hypoxia-responsive gene expression networks controlled by hypoxia-inducible factor-1 α (HIF-1 α) (209,210). The activation of HIF-1 α by cancer cells is instrumental in both tumor growth and metastasis (208,209,211,212). Ascorbate functions as a cofactor for hydroxylation of HIF-1 α (213). Proline hydroxylation targets HIF-1 α for ubiquitin-mediated degradation (214,215) and thus decreases HIF-1 α levels in the cells. Furthermore, intracellular ascorbic acid can directly attenuate basal or hypoxia-induced expression of HIF-1 α in human primary and cancer cells (216). The negative impact of ascorbate on HIF-1 α expression raises the question of whether intracellular vitamin C can inhibit the hypoxia-induced adaptation of solid tumor and thus restrict tumor growth and metastasis.

Future Perspectives

The development and availability of new animal models, the increased availability of transcriptome data, and the use of new metabolic approaches will, in the next few years, help to develop a more exhaustive portrait of the manifold roles of vitamin C in human nutrition. These reductionist approaches will reduce reliance on population studies, which are often insufficiently definitive, in confirming or refuting causal roles for vitamin C in chronic degenerative disease, enabling the resolution of the longstanding debate on the value of high levels of vitamin C in human health in normal populations. Future research focused on the potential of high-level therapy in particular cases, including treatment of cancer and in stem cell development, will yield a better understanding of potential vitamin C therapeutic benefit.

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