Ascorbic acid: Chemistry, biology and the treatment of cancer★

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Abstract

Since the discovery of vitamin C, the number of its known biological functions is continually expanding. Both the names ascorbic acid and vitamin C reflect its antiscorbutic properties due to its role in the synthesis of collagen in connective tissues. Ascorbate acts as an electron-donor keeping iron in the ferrous state thereby maintaining the full activity of collagen hydroxylases; parallel reactions with a variety of dioxygenases affect the expression of a wide array of genes, for example *via* the HIF system, as well as *via* the epigenetic landscape of cells and tissues. In fact, all known physiological and biochemical functions of ascorbate are due to its action as an electron donor. The ability to donate one or two electrons makes $AscH^-$ an excellent reducing agent and antioxidant. Ascorbate readily undergoes pH-dependent autoxidation producing hydrogen peroxide (H_2O_2). In the presence of catalytic metals this oxidation is accelerated. In this review, we show that the chemical and biochemical nature of ascorbate contribute to its antioxidant as well as its prooxidant properties. Recent pharmacokinetic data indicate that intravenous (i.v.) administration of ascorbate bypasses the tight control of the gut producing highly elevated plasma levels; ascorbate at very high levels can act as prodrug to deliver a significant flux of H_2O_2 to tumors. This new knowledge has rekindled interest and spurred new research into the clinical potential of pharmacological ascorbate. Knowledge and understanding of the mechanisms of action of pharmacological ascorbate bring a rationale to its use to treat disease especially the use of i.v. delivery of pharmacological ascorbate as an adjuvant in the treatment of cancer.

Keywords

Ascorbate; Oxidative stress; Hydrogen peroxide; Cancer; Pharmacology; Nutrition

1. Introduction

In the 80 years since the discovery of vitamin C (ascorbic acid, $AscH_2$; ascorbate, $AscH^-$) [1,2], the number of its known biological functions is continually expanding. Because of the ease of oxidation of ascorbate, gaining the first understanding of its role as the antiscorbutic vitamin was a major challenge. As a water-soluble reducing agent and donor antioxidant, $AscH^-$ can undergo two consecutive, one-electron oxidations resulting in the formation of ascorbate radical ($Asc^{\bullet-}$) and dehydroascorbic acid (DHA) (Fig. 1). Ascorbate can be

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regenerated from ascorbate radical and DHA, either enzymatically or non-enzymatically. Ascorbate readily undergoes pH-dependent autoxidation producing hydrogen peroxide [3]. In the presence of catalytic metals this oxidation is accelerated [4]. Ascorbate can also have pro-oxidant effects. In fact the combination of iron and ascorbate has long been used as an oxidizing system; the combination of these two reagents is referred to as the Udenfriend system and is used for the hydroxylation of alkanes, aromatics, and other oxidations [5,6]. In addition, ascorbate serves as a reducing cofactor for many enzymes, for example hydroxylases that belong to the Fe^{2+}–2-oxoglutarate-dependent families of dioxygenases.

The uptake of ascorbate from the intestinal tract is very tightly controlled [7]. However, recent pharmacokinetic data indicate that intravenous administration of ascorbate can bypass this tight control resulting in highly elevated plasma levels [8]. Because ascorbate readily oxidizes to produce H_2O_2, pharmacological ascorbate has been proposed as a prodrug for the delivery of H_2O_2 to tumors [9–12]. This new knowledge brings insights into the controversial role of ascorbate in the treatment of cancer. In this review we present the fundamental chemistry and biochemistry of ascorbate that may have a role in the mechanisms of high dose, i.v. ascorbate in the treatment of cancer.

2. Chemistry and biochemistry

Ascorbic acid ($AscH_2$, vitamin C) is a water-soluble ketolactone with two ionizable hydroxyl groups. It has two pK_a's, pK_1 is 4.2 and pK_2 is 11.6; thus, the ascorbate monoanion, $AscH^-$, is the dominant form at physiological pH (Fig. 1). Ascorbate is an excellent reducing agent and readily undergoes two consecutive, one-electron oxidations to form ascorbate radical ($Asc^{\bullet-}$) and dehydroascorbic acid (DHA). The ascorbate radical is relatively unreactive due to resonance stabilization of the unpaired electron; it readily dismutes to ascorbate and DHA ($k_{obs} =2\times105$ M^{-1} s^{-1}, pH 7.0) [4].

$$2Asc^{\bullet-}+H^+ \leftrightarrow AscH^-+DHA. \quad (1)$$

These properties make ascorbate an effective donor antioxidant [13].

Pure ascorbic acid is a white crystalline powder, extremely soluble in water making a colorless solution. Sodium ascorbate often contains significant quantities of oxidation products departing a yellow color. At pH values between 6 and 7.8, the purity and concentration of an ascorbate solution can be easily determined by its absorbance, $\varepsilon_{265} =14,500$ M^{-1} s^{-1} [14].

Ascorbate oxidizes readily. The rate of oxidation is dependent on pH and is accelerated by catalytic metals [14]. In the absence of catalytic metals, the spontaneous oxidation of ascorbate is quite slow at pH 7.0 [15]. This autoxidation, *i.e.* oxidation in the absence of catalytic metals, occurs *via* the ascorbate dianion, Asc^{2-} [16]. At pH 7.0 the dominant species for vitamin C is $AscH^-$ (99.9%) with low concentrations of $AscH_2$ (0.1%) and Asc^{2-} (0.005%). The amount of Asc^{2-} will increase by a factor of ten with a one unit increase in pH; this will increase the rate of autoxidation by a factor of 10. In an air-saturated phosphate buffer (pH 7.0), it has been estimated that the observed pseudo-first-order rate constant for the autoxidation of ascorbate is on the order of 10^{-7}–10^{-6} s^{-1} at pH 7.0 [15], consistent with a rate constant of ≈300 M^{-1} s^{-1} for the true autoxidation of Asc^{2-}, Reaction 2 [17].

$$k=300\,M^{-1}\,s^{-1}$$
$$Asc^{2-}+O_2 \rightarrow Asc^{\bullet-}+O_2^{\bullet-}. \quad (2)$$
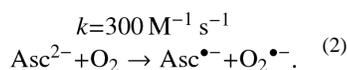
Thus, true autoxidation is indeed very slow. In most laboratory settings, oxidation of ascorbate is catalyzed by adventitious catalytic metals in the buffers as well as metals that enter the buffer for laboratory equipment [15,18]. In near-neutral buffers with contaminating metals, the oxidation and subsequent loss of ascorbate can be very rapid.

3. Biosynthesis and uptake

Plants and most animals synthesize ascorbate from glucose. In primitive fish, amphibians and reptiles, ascorbate synthesis takes place in kidney, whereas liver is the site of synthesis in mammals. Humans, other primates, guinea-pigs and a few species of fruit-eating bats cannot synthesize ascorbate because the gene encoding L-gulonolactone oxidase (GLO), the enzyme required for the last step in ascorbate synthesis, is not functional [19]. Thus, dietary intake becomes vital, *i.e.* it is a vitamin.

The typical human diet contains both ascorbate and DHA; absorption occurs in the enterocytes of the small intestine. While ascorbate is accumulated in cells by Na^+-dependent vitamin C transporters (SVCTs), DHA is absorbed *via* a Na^+-independent facilitative glucose transporters (GLUTs) followed by intracellular reduction [20–24]. Under physiological conditions, DHA concentrations in plasma are very low, 2 μM or less, while plasma glucose levels are significantly higher (2–5 mM). Thus, high intracellular ascorbate concentrations are mostly due to the uptake of ascorbate by SVCT1 (SLC23A1) and SVCT2 (SLC23A2) [25,26]. Human SVCT1 ($K_m \approx$ 65–240 μM) resides largely in brush-border surfaces of intestinal and renal tubular cells to mediate absorption and re-absorption [21]. Intestinal absorption and renal re-absorption determines the bio-availability of ascorbate. Because of the limited affinity of SVCT1 for ascorbate, plasma ascorbate concentrations are tightly controlled [8]. SVCT2 ($K_m \approx$ 8–62 μM) is found in a range of neural, neuroendocrine, exocrine, and endothelial tissues as well as in osteoblasts [25,27]. Both isoforms of SVCT are sensitive to the changes in ascorbate levels [28,29]; it has been observed that high intracellular ascorbate lowered SVCTs in human platelets and in an intestinal epithelial cell line Caco-2TC7, while low levels up-regulated SVCTs. In addition, both SVCT1 and SVCT2 are remarkably specific for L-ascorbate; ascorbate-2-O-phosphate, DHA, glucose, and 2-deoxyglucose are not transported by SVCTs. The SVCTs have a greater affinity for L-ascorbate than D-isoascorbate; they depend on sodium to create the electrochemical gradient across the plasma membrane, thereby providing the energy required for uptake of ascorbate [27]. Recently an ascorbate analog 6-bromo-6-deoxy-L-ascorbic acid has been identified as completely specific for SVCTs but not GLUT1 or -3, the authors demonstrated that 6-bromo-6-deoxy-L-ascorbic acid was taken up through the SVCTs of human skin fibroblasts but not by neutrophils, which accumulate DHA through GLUTs [30]. It has been hypothesized that the SVCT pathway maintains intracellular levels of ascorbate under normal physiologic conditions, whereas the GLUT-mediated DHA uptake is restricted to specialized oxidizing conditions, such as those found in the surrounding milieu of the activated neutrophils [30]. Given that cancer development and progression is considered to have an inflammatory component [31], it is highly possible that ascorbate, is oxidized in the extracellular environment, and then taken up by tumor as well as stromal cells [32].

DHA is taken up with lower affinity (K_m =0.8 mM) than ascorbate (K_m =0.2 mM), but the maximal rates of uptake by adult human jejunum are similar for DHA and ascorbate (V_{max} 28 *vs.* 35 pmol s^{-1} mg-protein^{-1}) when glucose is absent [33]. Although the structure of DHA has similarities to glucose, the influence of glucose on DHA uptake varies between cell types. In adipocytes, neutrophils, osteoblasts and smooth muscle cells, the uptake of DHA is largely inhibited by physiological concentrations of glucose, which occurs to a lesser extent in astrocytes, enterocytes, and renal tubular cells [27]. Mammalian facilitative

glucose transporters GLUT1 and GLUT3 mediate DHA transport with a similar efficiency to that of glucose. GLUT4 transports DHA with a higher affinity than 2-deoxyglucose (K_m =0.98 *vs.* 5.2 mM) but a much lower V_{max} [34]. Given that: (1) cellular uptake of ascorbate is regulated by both glucose and insulin [35,36]; and (2) ascorbate infusion has been shown to enhance glucose uptake and whole body glucose disposal in healthy subjects and diabetics [37], it is clear that more research is needed on the effects of ascorbate infusion and tissue uptake.

Human erythrocytes express a high number of GLUT1, but have no SVCT proteins [38]. The ascorbate level in erythrocytes is similar to the plasma from which they were taken [39,40]. It is speculated that erythrocytes recycle and release ascorbate to help maintain plasma levels of ascorbate. With the exception of erythrocytes, intracellular ascorbate concentrations are higher than extracellular fluids. Studies have shown that ascorbate accumulates to millimolar concentrations in neutrophils, lymphocytes, monocytes, and platelets (Table 1). In circulating lymphocytes, 4 mM can be achieved in healthy young women with oral ascorbate supplementation [41]. Intracellular ascorbate not only contributes to the intracellular reducing environment, it can also quench extracellular oxidants by transferring electrons across the plasma membrane [42]. In addition, there appears to be regulated ascorbate efflux from astrocytes to neurons, thereby bolstering antioxidant defense [43]. Furthermore, the release of ascorbate from cells may also reduce non-transferrin-bound iron, thereby stimulating the uptake of iron by cells [44].

In human and animal tissues, the highest concentrations of ascorbate are in the adrenal and pituitary glands (Table 1) [45–48]. Ascorbate is a physiological reductant in the dopamine β-hydroxylase-catalyzed reaction that converts dopamine to norepinephrine in the chromaffin granules of adrenal medulla. Likewise, peptidyl glycine α-amidating monooxygenase in the pituitary glands also requires high concentrations of ascorbate to catalyze the amidation reaction of carboxyl-terminal glycine residue of a number of peptides. In addition, millimolar levels of ascorbate also found in eye tissues [46,49]; this may protect these tissues from potential damage caused by solar radiation. Since tissues from lungs are constantly exposed to relatively high concentrations of oxygen and ROS, antioxidant enzymes and low-molecular-weight antioxidants, such as ascorbate, serve to protect cells and tissues from oxidative damage. Ascorbate levels in respiratory tract lining fluids are similar or lower than those in plasma [50]. However, there appears to be increased ascorbate content in the epithelial lining fluid and alveolar macrophage of smokers who have a relatively high dietary intake of vitamin C [51]. This increased content of ascorbate suggests that there is an adaptive defense mechanism against the ROS derived from cigarette smoke.

Human cell culture experiments usually lack ascorbate, as these cells are incapable of synthesizing this vitamin; in addition, there is no source of vitamin C in typical cell culture media formulations. However, cultured cells take up ascorbate, ascorbate-2-phosphate, as well as DHA when supplemented in the media. DHA is rapidly transported into cells by glucose transporters over a period of minutes while ascorbate and its phosphate require SVCTs and can take several hours [52]. Ascorbate 2-phosphate is most likely hydrolyzed by membrane esterases with the resulting ascorbate being transported into cells [53]. Compared to the same concentration of ascorbate, cells accumulate intracellular ascorbate *via* ascorbate 2-phosphate at a somewhat slower rate to reach the same intracellular concentration [54]. However, ascorbate 2-phosphate does not oxidize in the media and thus does not produce H_2O_2 as does ascorbate. Thus, the use of ascorbate 2-phosphate is preferred when studying the biology of vitamin C in cell culture. Millimolar concentrations of ascorbate can be achieved intracellularly by carrier-mediated mechanisms (Table 2). There is no detectable

level of DHA in cells as intracellular DHA is readily reduced by an array of biological reductants and enzyme systems [55,56].

At physiological conditions DHA rapidly hydrolyzes to 2,3-L-diketogulonate (2,3-DKG), which is quite unstable [57]. 2,3-DKG subsequently decarboxylates yielding L-xylonate and L-lyxonate; these compounds can enter the pentose phosphate pathways [58]. Alternatively, L-erythrulose and oxalate can be produced from 2,3-DKG degradation. It has been proposed that highly reactive L-erythrulose rapidly glycates and crosslinks proteins. In fact, ascorbate-dependent modification of proteins was proposed to occur in human lens during diabetic and cataract formation. Oxalate monoalkylamide, one of the advanced glycation end products (AGEs) is a major product induced by incubation of human lens protein with DKG and other degradation product of ascorbate [59]. Oxalate is one of the major end products of ascorbate breakdown in humans, and it has the potential to crystallize as calcium oxalate in the urinary space in susceptible people [60,61]. However, in a study involving 16 cancer patients who had normal renal function, intravenous administration of ascorbate ranging from 0.2 to 1.5 g kg^{-1} body weight, resulted in approximately 30 to 80 mg of oxalic acid being excreted during the 6 h after the infusion [62]. While in primary hyperoxaluria, oxalic acid excretion ranged between 100 mg d^{-1} and 400 mg d^{-1}. Oxalate nephrocalcinosis and calcium oxalate stones develop over months to years [63]. Overall, these studies suggest that patients with normal renal functions and a time-limited course of ascorbate infusions would not have an immediate risk of oxalate stone formation.

4. Biological functions

4.1. Ascorbate as enzyme cofactors of hydroxylases

In addition to the monooxygenases mentioned above, ascorbate is involved in many physiological and biochemical processes involving enzymatic reactions that are catalyzed by members of the Fe^{2+}–2-oxoglutarate-dependent family of dioxygenases. These dioxygenases use 2-oxoglutarate as a co-substrate and as a source for two of the four electrons needed for the reduction of O_2; they transfer one oxygen atom from O_2 to the succinate product and one atom to the protein substrate. Ascorbate is required for maintaining iron in the ferrous state, thereby maintaining full activity of this class of enzymes. Reaction 3 [64,65].

$$Fe^{3+}-\text{dioxygenase (inactive)}+AscH^- \rightarrow Fe^{2+}$$
$$-\text{dioxygenase (active)}+Asc^{\bullet -}. \qquad (3)$$
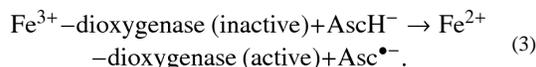
Evidence that the Fe^{2+}–2-oxoglutarate-dependent dioxygenases can modify proteins was gathered from the studies of hydroxylases in collagen metabolism. Located within the lumen of the endoplasmic reticulum, collagen prolyl-4-hydroxylase and prolyl-3-hydroxylase are responsible for the insertion of the hydroxyl groups onto prolyl residues, which is essential for the proper assembly of collagen [66]. There are at least three isozymes of collagen prolyl hydroxylase in humans each with distinct α subunits; but they all have protein disulfide isomerases (PDIs) to serve as β subunits, which catalyze the formation of intra- and inter-chain disulfide bonds during collagen synthesis [67]. Collagens are major constituents of the extracellular matrix (ECM), constituting ≈30% of total protein mass. Type I collagen is the major structural protein in the interstitial ECM, while type IV is prevalent in basement membrane [68]. Collagens in the extracellular matrix are important components of the physical barrier against invasion and metastasis of cancer cells [88,89]. Ascorbate has been shown to stimulate the production of types I and III collagen in human skin fibroblasts [70]. Cameron and Pauling proposed that mega doses of ascorbate would inhibit cancer growth by preventing cancer cell invasion [71]. More recently, it has been shown that a type IV

collagen domain, produced as a recombinant protein, inhibits the proliferation of capillary endothelial cells and blood vessel formation, resulting in the suppression of tumor growth [72]. However, it is not known if very high levels of ascorbate will influence cancer growth by mechanisms related to collagen synthesis.

4.2. Ascorbate as a cofactor in the HIF system

As novel members of the dioxygenase family, cytoplasmic prolyl hydroxylases have been shown to regulate hypoxia-inducible transcription factor (HIF) [73]. These enzymes have no PDI subunits but have higher values of K_m for oxygen than collagen prolyl hydroxylases. HIF protein is composed of an oxygen-sensitive HIF-α subunit and an oxygen-insensitive HIF-β subunit. In hypoxia, HIF-α is induced, enters the nucleus, and forms a heterodimer with HIF-β, which is constitutively expressed. In normoxia, HIF-α is efficiently hydroxylated by prolyl hydroxylases. Hydroxylated HIF-α then binds to the von Hippel–Lindau (pVHL) protein, which is part of an ubiquitin ligase complex, leading to ubiquitination and proteasomal degradation of the protein [74]. All three of the HIF prolyl hydroxylases (PHD1, PHD2, and PHD3) recognize the C-terminal hydroxylation site with a higher affinity than the N-terminal hydroxylation site [75]. In addition, an asparaginyl hydroxylase inhibits HIF through the hydroxylation of a C-terminal asparagine residue, blocking coactivator recruitment. Given that there are more than 800 direct HIF target genes yielding more than 60 gene products that are regulated by HIF [76–78], it is likely that the availability of ascorbate will have a profound effect on many cell functions such as angiogenesis, cell survival, glucose uptake, glycolysis and iron homeostasis.

As members of the Fe^{2+}–2-oxoglutarate-dependent dioxygenase families, HIF prolyl- and asparaginyl hydroxylases require ascorbate as a reducing agent to maintain iron in the ferrous state. Values of K_m for ascorbate with the HIF prolyl hydroxylases range from 140 to 300 μM [65], well below the millimolar concentrations of ascorbate in most of cells in healthy individuals [55]. Supplementation of ascorbate in a lymphoma cell model in SCID mice has been shown to diminish HIF levels and the rate of tumor growth [79]. Lu et al. demonstrated that the antitumorigenic effect of ascorbate is dependent on the activity of HIF hydroxylases [80]. By analyzing endometrial tumor tissues for ascorbate and three of its targets GLUT-1, BNIP3 and VEGF, Kuiper et al. [81] found that HIF-1α and its targets were all up-regulated in human tumor tissue. Tumors with higher ascorbate levels had low HIF-1 activation, while tumors with high HIF-1 activation and aggressive characteristics had lower ascorbate levels. These important findings indicate that having high tissue levels of ascorbate may protect against HIF-1 activation and aggressive tumor behavior.

As a downstream factor produced upon activation of HIF, VEGF plays an important role in tumor angiogenesis. In addition to direct cytotoxicity effects, pharmacological ascorbate has been shown to suppress capillary-like tube formation on cells grown on Matrigel [82]. However, it is still unknown whether the suppression of angiogenesis by pharmacological ascorbate is due to the production of H_2O_2 [83] or through the inhibition of HIF [84].

4.3. Ascorbate as a cofactor in histone demethylation

The newly discovered histone demethylases containing Jumonji catalytic domains, which can demethylate trimethylated lysines, also belong to the Fe^{2+}–2-oxoglutarate dioxygenase family [85]. As with other members of this family, the Jumonji proteins have the ability to bind Fe^{2+}, use 2-oxoglutarate as substrate, and require ascorbate for full catalytic activity. Furthermore, ascorbate supplementation also leads to increased histone acetylation as well as increased expression of HIF histone demethylases JMJD 1 and 2 in human embryonic stem cells [86]. Thus, we can speculate that epigenetic status of tissue may also depend on ascorbate to keep Fe^{2+}–2-oxoglutarate dioxygenases functional [87]. Indeed, Esteban et al.

[88] also postulated that ascorbate supplementation may enhance the function of epigenetic modifiers during reprogramming of induced pluripotent stem cells (iPSCs), as many of these modifiers are ascorbate-dependent dioxygenases. Considering that cancer cells have shared features of normal stem cells [89] and DNA hypermethylation is associated with cancer development, ascorbate and other nutritional supplements could prevent cancer by modulating the components of the systems that set the epigenetic code [90].

The necessity of ascorbate as a reducing agent to maintain full functioning of an array of enzymes shows the importance of vitamin C in maintaining the biochemical machinery of cells and tissues. These functions are often overlooked as many researchers focus only on the antioxidant abilities of ascorbate.

4.4. Ascorbate and cancer prevention

Epidemiologic evidence suggests that vitamin C-rich foods play a protective role against development of cancer [91–94], plasma concentrations of ascorbate have been shown to be inversely associated with cancer risk [95,96]; however, large scale randomized intervention trials comparing antioxidants (vitamins A, C, E and β-carotene) supplements given alone or in combination have not shown protective effects [97–99]. One of the drawbacks of randomized control trials (RCT) for nutrients is that most nutrients such as vitamins have threshold behavior [100]. For example, scurvy only develops when serum ascorbate levels are on the order of 8 μM or less [8]. The recommended dietary allowance (RDA) for vitamin C is 75–125 mg daily; consistent intake of 60 mg d^{-1} will in general prevent scurvy for 30–45 days once vitamin C intake ceased [101,102]. The fact that ascorbate is required to maintain full function of an array of enzymes indicates optimizing intake would optimize metabolism as well as prevent cancer and other degenerative disease [103,104].

5. Recycling of ascorbate

5.1. Reduction of ascorbate free radical

There are several enzymatic systems involved in the recycling of ascorbate radical (Asc$^{•-}$) and DHA. The reduction of ascorbate radical by NADH-cytochrome b_5 reductase has been shown in rat liver [105], where most of the reductase activity is localized in the outer mitochondria membrane. As a membrane-bound protein, NADH-cytochrome b_5 reductase is also associated with the endoplasmic reticulum and the plasma membrane [106]. Cytochrome b_5 donates an electron to ascorbate radical, which in turn, draws electrons from NADH. On the other hand, the transmembrane ascorbate radical reductase in erythrocytes can use electrons from NADH or intracellular ascorbate to regenerate extracellular ascorbate from ascorbate radical [107]. The plasma membrane ascorbate radical reductase reduces ascorbate radical generated in blood during oxidative stress as well as the ascorbate radical generated by reduction of α-tocoperoxyl radical during lipid oxidation. Reduction of ascorbate radical by thioredoxin reductase (TrxR) purified from rat liver suggests that TrxR can also function as a cytosolic ascorbate radical reductase, which may complement cellular ascorbate recycling. Thus, there are several biochemical routes for the one-electron reduction of ascorbate radical to regenerate ascorbate.

5.2. Reduction of dehydroascorbate

DHA is unstable at physiological pH with a half-life is 5–15 min at 37 °C [108–110]. It is estimated that the concentration of DHA is less than 2 μM in healthy humans; many reported measurements may have overestimated DHA levels due to the ease of oxidation of AscH$^-$ during sample processing. Extracellular DHA can be imported into cells using glucose transporters present in cells, where DHA can be reduced to ascorbate by glutathione (GSH) directly [111] or more efficiently by glutaredoxin in the cytoplasm [112]. Purified rat

liver thioredoxin reductase also catalyzes NADPH-dependent reduction of DHA [113]. Thus, electrons from NADPH can find their way to DHA *via* thiols.

Purified bovine liver PDI has been found to react directly with DHA and GSH to catalyze the reduction of DHA to ascorbate [112]. The finding that PDI has DHA reductase activity is interesting since human collagen prolyl 4-hydroxylase has PDI as a β subunit [69]. It is possible that the β subunit also serves as DHA reductase to generate ascorbate from DHA since collagen hydroxylases require ascorbate to maintain iron in its ferrous state.

3α-Hydroxysteroid dehydrogenase belongs to the family of oxido-reductases that catalyze NADPH-dependent oxidoreduction of a variety of substrates. Del Bello et al. demonstrated that the NADPH-dependent DHA reductase from rat liver cytosol is identical to 3α-hydroxysteroid dehydrogenase [114]. The enzyme has a low affinity for DHA ($K_m = 4.6$ mM) but a high affinity for NADPH ($K_m < 5$ μM). Under pathological conditions, decreases in GSH and increases in DHA could activate 3α-hydroxysteroid dehydrogenase.

Thus, DHA has many routes for its two-electron reduction to regenerate ascorbate. Because of the relative instability of DHA, rapid reduction to ascorbate also prevents loss of vitamin C. Ascorbate is involved in a variety of biochemical functions that are fundamental to the functioning of a wide variety of enzyme systems; thus efficient recycling maintains the pool of ascorbate in cells and tissues.

The regeneration of ascorbate from ascorbate radical and DHA will allow many cycles of ascorbate oxidation, thus one molecule of ascorbate can produce many molecules of H_2O_2. This mechanism is an important one when considering pharmacological ascorbate in cancer treatment that will be discussed later.

6. Ascorbate as an antioxidant

The typical concentration of ascorbate in plasma from healthy humans is about 40–80 μM (Table 1). At these levels ascorbate functions as an endogenous antioxidant; for example, it serves as a co-antioxidant with vitamin E to protect LDL from detectable oxidative damage induced by aqueous peroxyl radicals [115]. Thermodynamically ascorbate readily donates an electron to potentially damaging oxidizing radicals such as hydroxyl radical (HO$^\bullet$), alkoxyl radical (RO$^\bullet$), peroxyl radical (LOO$^\bullet$), thiol radical (GS$^\bullet$), and tocopheroxyl radicals (TO$^\bullet$) [13]; this one-electron oxidation of AscH$^-$ results in the production of the ascorbate radical (Asc$^{\bullet-}$). Ascorbate radical is relatively unreactive, and can be reduced back to ascorbate by NADH- and NADPH-dependent reductases [116]; Asc$^{\bullet-}$ can also undergo a pH-dependent disproportionation reaction, resulting in the formation ascorbate and DHA, Reaction 1 [117,118].
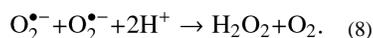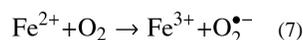
Physiological concentrations of ascorbate have been shown to inhibit LDL oxidation induced by endothelial cells, macrophages, copper ions, and AAPH (2,2′-azo-*bis* (2-amidinoproprane) dihydrochloride) [119,120]. An important feature of the action of ascorbate is its synergistic interaction with vitamin E [121]. Vitamin E is lipid-soluble; it is a primary antioxidant in LDL and lipid membrane oxidation [122]. Its one-electron oxidation product, the α-tocoperoxyl radical, can be reduced by ascorbate. The two-electron oxidation product, tocopherol quinone, undergoes ring breakage and thus vitamin E is lost [123]. In this context, ascorbate is important for maintaining vitamin E and inhibiting lipid oxidation. It has been shown that doubling plasma ascorbate concentrations by supplementation results in decreased rates of disappearance of vitamin E in smokers [124]. This is the first *in vivo* demonstration that ascorbate maintains vitamin E, most likely through "recycling".

$$LOO^{\bullet}+TOH \rightarrow LOOH+TO^{\bullet} \quad (4)$$

$$AscH^{-}+TO^{\bullet} \rightarrow Asc^{\bullet-}+TOH. \quad (5)$$
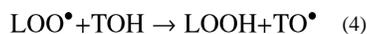
Ascorbate is clearly a co-antioxidant protecting lipid oxidation even in iron-loaded human plasma [125]. In human plasma lipid peroxides (cholesteryl ester hydroperoxides) were detectable only when serum ascorbate was depleted; iron-induced lipid peroxidation was inhibited by ascorbate in a concentration dependent manner [126]. Guinea pigs with iron overload had greater oxidative lipid damage that is suppressed with oral supplementation of ascorbate [127]. Thus, in the presence of catalytic iron, robust nutritional levels of ascorbate protect from oxidative damage.

7. Pro-oxidant effects of ascorbate

In the presence of catalytic metal ions, ascorbate can also exert pro-oxidant effects [14,128,129]. Ascorbate is an excellent one-electron reducing agent that can reduce ferric (Fe^{3+}) to ferrous (Fe^{2+}) iron, while being oxidized to ascorbate radical (Reaction 6). Depending on co-ordination environment, Fe^{2+} can readily react with O_2, reducing it to superoxide radical (Reaction 7), which in turn dismutes to H_2O_2 and O_2 (Reaction 8).

$$AscH^{-}+Fe^{3+} \rightarrow Asc^{\bullet-}+Fe^{2+} \quad (6)$$

$$Fe^{2+}+O_2 \rightarrow Fe^{3+}+O_2^{\bullet-} \quad (7)$$

$$O_2^{\bullet-}+O_2^{\bullet-}+2H^{+} \rightarrow H_2O_2+O_2. \quad (8)$$
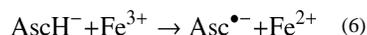
In a classic Fenton reaction, Fe^{2+} reacts with H_2O_2 to generate Fe^{3+} and the very oxidizing hydroxyl radical, Reaction 9. The presence of ascorbate can allow the recycling of Fe^{3+} back to Fe^{2+}, which in turn will catalyze the formation of highly reactive oxidants from H_2O_2.

$$Fe^{2+}+H_2O_2 \rightarrow Fe^{3+}+HO^{\bullet} (Fenton\ reaction). \quad (9)$$

Depending on concentrations, the effects of ascorbate on models of lipid peroxidation can be pro- or antioxidant [14,130]. There is considerable variability in the literature; this variability appears to be a result of the different concentrations and form of transition metal ions in the experiments and the media [131].

The prooxidant effects of ascorbate may be important *in vivo* depending on the availability of catalytic metal ions. In healthy individuals, iron is largely sequestered by iron binding proteins such as transferrin and ferritin [132,133]. Transferrin is a glycoprotein that is synthesized in the liver. It is the major circulating iron binding protein with a high affinity, but low capacity for iron; this iron is essentially redox inactive [134]. Transferrin avidly binds to the transferrin receptor on the cell surface. The transferrin–transferrin receptor complex is internalized to an endosome, which releases iron in acidic conditions. The ferric iron released is reduced and transported to the cytoplasm where it is either utilized for synthesis of iron-containing proteins or bound in ferritin, an iron storage protein. Ferritin is capable of sequestering up to 4500 atoms of iron, but is normally only 20% saturated. Iron stored in ferritin can be released by appropriate reductants in the presence of a chelator or by degradation of ferritin in the lysosome.

Iron can be released from ferritin by biological reductants such as thiols, ascorbate and reduced flavins [135]. The released iron enables cells to synthesize cytochromes and iron-containing enzymes. However, uncontrolled release of iron from ferritin has the potential to form HO$^\bullet$, which can damage critical cellular components [136–138].

In pathological situations, such as thalassaemia or hemotochromatosis, non-transferrin-bound iron is present. Thus, supplemental ascorbate without administration of an iron chelator can lead to deleterious effects [14]. Tissue damage resulting from ischemia/reperfusion is another example of increased availability of catalytic metal occurring *in vivo* [139]. Intravenous ascorbate prior to vascular surgery increased concentrations of ascorbate radical and lipid hydroperoxides suggesting that catalytic iron released into the circulation during the ischemic phase of the surgery with ascorbate may promote iron-induced lipid peroxidation [140,141].

Elevated levels of catalytic metal ions have also been demonstrated in chronic inflammatory diseases [142]. There is an increased deposition of iron proteins in the synovial membranes in rheumatoid arthritis. Ascorbate radical has been detected in synovial fluid from patients with synovitis disease indicating that catalytic iron is in part responsible for the decreased levels of ascorbate and increased levels of DHA [141]. In addition, ascorbate concentrations were decreased while levels of catalytic iron increased in patients with sepsis, compared to healthy subjects [143].

Although there is no direct evidence that catalytic iron is increased in tumors, many patients with malignant disease have elevated serum or tissue ferritin concentrations [144–146]. Raised levels of circulating ferritin are found in childhood Hodgkins lymphoma and are associated with poor survival [147]; serum ferritin levels have been shown to be related to the stage of the disease and tumor volume in cervical cancer [148]. Furthermore, studies by Feng et al. [149] have shown that the peritoneal and subcutaneous microvessels of normal mice were largely impermeable to circulating ferritin, but in mice bearing solid or ascites tumors, circulating ferritin was found in the basal lamina and extravascular space, suggesting that tumor vessels are hyperpermeable to circulating macromolecules such as ferritin. In fact, ferritin staining was detected in stroma and histiocytes surrounding neoplastic cells of breast carcinoma tissue of patients [150]. Extracellular metal-containing proteins have been proposed to be essential for the pro-oxidant effects of ascorbate [10,129], iron-saturated ferritin could be a potential candidate as source of catalytic iron. A recent study by Deubzer et al. [151] demonstrated that ferritin released by neuroblastoma cells enhanced pharmacologic ascorbate induced-cytotoxicity, indicating that ferritin with high iron-saturation could be a source of catalytic iron [152]. Consistent with this, ascorbate has also been shown to be capable of releasing iron from cellular ferritin [158]. Ferritin is only one candidate as a source of catalytic iron; extracellular iron chelates are present in tissue and seem to be increased under pathological conditions [159]. It is clear that only low levels of catalytic metals are needed to substantially increase the rate of oxidation of ascorbate [15,18]. These many observations provide insights on the mechanism by which pharmacologic concentrations of ascorbate have potential in treating certain types of cancer.

8. Ascorbate and cancer treatment

The use of high-dose ascorbate in treating cancer patients began in the 1970s. These early studies demonstrated beneficial effects of high-dose ascorbate [155–158]. However, two double-blinded, randomized clinical trials at the Mayo Clinic did not show any benefit [169,160]. Subsequently, use of ascorbate for cancer treatment was considered ineffective and dismissed by the research and medical communities. However, a marked difference existed in these studies. Cameron's group gave patients ascorbate intravenously as well as

orally, while patients in the Mayo Clinic trials received only oral ascorbate. Some years later, clinical data were generated that demonstrated that when ascorbate is given orally, plasma concentrations are tightly controlled [101]. At oral doses of 200 mg, the steady-state plasma concentrations are ≈80 μM. As doses exceed 200 mg, relative absorption decreases, urine excretion increases and the fraction of bioavailable ascorbate is reduced [7]. Peak plasma values do not exceed ≈220 μM even after maximum oral dose of 3 g 6 times daily [8]. In contrast, when ascorbate is administered intravenously, millimolar concentrations can be achieved. In fact, infusion of 10 g of ascorbate in cancer patients results in plasma concentrations of 1 to 5 mM [161,162]. Thus, only intravenous administration of ascorbate can yield high plasma levels, *i.e.* pharmacological levels.

8.1. The role of hydrogen peroxide generation and removal

In vitro, the toxicity of ascorbate centers on the generation of H_2O_2 by ascorbate upon its oxidation [9,10,12,163,164]. The true autoxidation of ascorbate, *i.e.* in the absence of catalytic metals, *via* reaction 2 will generate considerable amounts of H_2O_2 when ascorbate is at millimolar levels. For example, in an aqueous solution containing 20 mM ascorbate at pH 7.4, the concentration of ascorbate dianion, Asc^{2-}, will be on the order of 1 μM. This will result in a flux of H_2O_2 on the order of 10 nM s^{-1}, in a typical cell culture experiment.

Catalytic iron (and perhaps copper) in the media and serum will increase the rate of ascorbate oxidation and associated generation of H_2O_2. Clement et al. found that Dulbecco's modification of Eagle's MEM (DMEM) generates more H_2O_2 than RPMI 1640 during a 6-hour incubation with increasing concentrations of ascorbate [165]. In addition to adventitious iron, DMEM has 0.25 μM Fe $(NO_3)_3$ in its formulation, which could contribute to this greater production of H_2O_2 in DMEM than that from RPMI. Serum usually contains total iron at a range of 10 to 50 μM, which could also be another factor that causes the variable toxicity of ascorbate in different media; however, some of this iron will be redox inactive as it will be sequestered in transferrin [134]; there will undoubtedly be a highly variable amount of iron present that has nonspecific peroxidase activity [166]. This iron will actively catalyze the oxidation of ascorbate. Thus, the amount of catalytic iron present in typical cell culture media is highly variable. Even with careful attention to detail, this can lead to considerable variability in experimental results, even in the same set of experiments.

α-Keto acids such as pyruvate and α-ketoglutarate directly react with H_2O_2 when present in cell culture media [172];

$$CH_3(=O)(=O)OH + H_2O_2 \rightarrow CH_3C(=O)OH + CO_2 \quad (10)$$
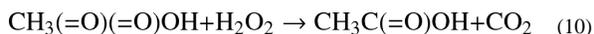
this will result in an apparent lowering of the toxicity of ascorbate [168]. Thus, the presence of α-keto acids in media must be taken into consideration in the design and interpretation of data from experiments where the oxidation of ascorbate and associated production of H_2O_2 are central to the study.

Because cells rapidly and efficiently remove extracellular H_2O_2, observed toxicities or cell killing induced by ascorbate is an inverse function of cell density [151,153,169]. There is an array of intracellular antioxidant enzymes involved in the removal of H_2O_2, such as catalase, glutathione peroxidase, and the peroxiredoxins. Although modest catalase activity has been detected in heart mitochondria [170], catalase is predominantly located in peroxisomes of mammalian cells. Concomitant administration of the catalase inhibitor amino-1,2,4-triazole has been shown to enhance ascorbate toxicity to Ehrlich ascites cells *in vitro* [171]. Using adenovirus constructs containing human catalase cDNA, Du et al. [12] demonstrated that overexpressing intracellular catalase protected cells from ascorbate-induced cytotoxicity.

These results provide evidence supporting a fundamental role for H_2O_2 in ascorbate-induced toxicity.

Cytosolic glutathione peroxidase (GPx1), another antioxidant enzyme that removes peroxide, is widely distributed in tissues. GPx1 catalyzes GSH-dependent reduction of H_2O_2 to water. Gaetani et al. [172,173] have shown that the intracellular concentration of H_2O_2 in human erythrocytes is inversely proportional to the activity of catalase and GPx-1. GPx1 is responsible for eliminating low concentrations of H_2O_2; however, at high fluxes of H_2O_2 recycling of GPx1 by GSH becomes rate-limiting [174]; at high fluxes of H_2O_2 catalase is the enzyme that is principally responsible for removal of H_2O_2 [175].

Another antioxidant system that contributes to removal of H_2O_2 within cells is the family of peroxiredoxins, in particular, peroxiredoxin 2 (Prx2). Prx2 is the third most abundant protein in erythrocytes [176]. The rate constant of human Prx2 reacting with H_2O_2 is 1.3×10^7 M^{-1} s^{-1} [177] which is similar to that of catalase (1.7×10^7 M^{-1} s^{-1} [178]). These complementary enzymes systems for the removal of intracellular H_2O_2 in RBCs will also make these cells efficient sinks for H_2O_2.

8.2. Ascorbate-induced cytotoxicity in vitro

Chen et al. [9,11] have demonstrated that some cancer cells have increased sensitivity to ascorbate-induced cytotoxicity compared to normal cells. In a complementary study, Du et al. [12] demonstrated that pancreatic cancer cells are more sensitive to pharmacological concentrations of ascorbate than their normal cell counterparts. The difference in sensitivity between normal and cancer cells towards ascorbate may be due to low levels of antioxidant enzymes and high endogenous levels of ROS in cancer cells [179–181]. The relative lower activities of catalase, glutathione peroxidase, and peroxiredoxins in cancer cells could potentially contribute to less efficient removal of H_2O_2 and increased sensitivity to ascorbate-induced cytotoxicity.

The rate of oxidation of ascorbate is typically a function of the level of catalytically active iron and copper in solution. Iron in cell culture media contributes significantly to the rate of H_2O_2 generation. Deferoxamine (Desferal® or DFO) is an iron-chelating agent that renders iron catalytically inactive with respect to ascorbate oxidation [15]. Although hydrophilic, DFO is cell-permeable; short-term exposure to DFO is not effective in accessing cytosolic labile iron pool (LIP) of cells from different origins [182]. However, short-term pre-incubation of cells to DFO appeared to protect cells from ascorbate-induced toxicity [184]. These observations indicate that DFO either is effective in accessing endosomal iron [182] or iron associated with cellular membranes [183]. Other cell culture studies have demonstrated that Fe^{2+} in the media can actually protect cells from the oxidative damage resulting from exposure to extracellular H_2O_2 [184,185]. Although hydroxyl radicals are produced *via* the Fenton reaction, most will not induce cell damage, but rather disappear by reactions with components of the media. The key is that H_2O_2 is removed. Thus, interpretation of data from experiments with iron, ascorbate, H_2O_2 or some combination is not always straightforward.

Oxidative stress has been shown to increase the levels of catalytic iron in tissues. A series of electron paramagnetic resonance (EPR) studies demonstrated that ultraviolet light increased the levels of labile iron in skin [186]; heat stress-induced oxidative events increased the level of labile iron in liver [187]; and ischemia reperfusion increased the level of catalytic iron in the heart [188]. In addition, ionizing radiation and some chemotherapeutic drugs have been shown to increase catalytic free iron levels [189–191]. Since it takes only very low concentrations of catalytic metals to bring about the rapid oxidation of ascorbate

[192,193], approaches that increase catalytic iron could potentially enhance the cytotoxicity of pharmacologic ascorbate *in vivo* [194].

Porphyrin-based SOD mimics have a redox-active metal (Mn, Fe, and Cu) center and a stable porphyrin complex. The dismutation of $O_2^{\bullet -}$ by Mn porphyrin complexes involves two steps in which the Mn center cycles between Mn(III) and Mn(II): the first step is the reduction of Mn(III) by $O_2^{\bullet -}$ to yield Mn(II) and O_2 and the second step the oxidation of Mn(II) by $O_2^{\bullet -}$ to yield H_2O_2 and return the manganese to its resting state as Mn(III) porphyrin [195]. However, in the presence of a reductant such as ascorbate, Mn porphyrins function as superoxide reductases rather than dismutases; Mn(III) can be reduced to Mn(II) by ascorbate while Mn(II) can react with O_2 forming $O_2^{\bullet -}$, which subsequently forms H_2O_2 and O_2, Reaction 8. The prooxidant effects of Mn porphyrin and ascorbate have been studied by several groups. Gardner et al. [196] have demonstrated that MnTM-4-PyP^{5+} catalyzes the oxidation of ascorbate *in vitro*. Zhong et al. [197] have shown synergistic killing of human prostate cancer cell RWPE-2 by MnTM-4-PyP^{5+} and ascorbate. Most recently, Tian et al. [198] have shown that MnTM-4-PyP^{5+} synergizes with ascorbate inhibiting the growth of prostatic, pancreatic, and hepatic cancer cells. Furthermore, the cytotoxic effects of two Mn(III) alkylpyridylporphyrins (MnTE-2-PyP^{5+} and MnTnHex-2-PyP^{5+}) and ascorbate have been demonstrated in Caco-2, HeLa, HCT116, and 4T1 cells [199,200]. The more lipophilic MnTnHex-2-PyP^{5+} appeared much more effective. Given that several Mn porphyrins have already been tested *in vivo* as SOD mimetics, and by themselves have shown low toxicities at micromolar levels [195], there is great potential for using Mn porphyrins as an adjuvant to enhance the efficacy of pharmacologic ascorbate.

8.3. High dose ascorbate in animal studies

As mentioned above, the uptake of oral ascorbate in humans is tightly controlled by the gut and kidney filtration [7]. Rats receiving ascorbate by gavage (0.5 mg g^{-1}) increased blood and extracellular concentration to peak levels <150 μM [10]. By contrast, concentrations reached peak levels of nearly 3 mM in rats receiving intraperitoneal injections, while intravenous injection increased peak levels to >8 mM. In a similar study, mice receiving bolus intravenous injections of ascorbate (1 g kg^{-1}), resulted in plasma concentrations of 15 mM [164]. Supplementation of ascorbate in drinking water at 1 g kg^{-1} only increased plasma concentrations to <50 μM. These results clearly indicate that pharmacologic concentrations of ascorbate cannot be obtained by oral administration.

In addition to the finding that millimolar levels of ascorbate were achieved by parenteral administration, Chen and colleagues [10] demonstrated that ascorbate radical was formed in extracellular fluid but was not detectable in whole blood. Moreover, H_2O_2 was detected in extracellular fluid, but not in the blood; H_2O_2 correlated with ascorbate radical concentration. These results indicate that plasma membrane associated ascorbate radical reductase and high levels of catalase, glutathione peroxidase, and peroxiredoxin enable erythrocytes to act as a sink for extracellular ascorbate radical and H_2O_2 [176,107]. On the other hand, ascorbate could be oxidized by catalytic iron associated with damaged proteins in the extracellular space [201–203, 209]. Considering that the permeability of tumor vessels is much higher compared to normal endothelium, tumor endothelium may permit the outflow of macromolecules, such as albumin, to interstitial fluid [169,204]. The fluid that accumulated in the peritoneal cavities of ascites tumor-bearing mice had a protein concentration several-fold greater than that of normal peritoneal fluid, with approximately 85% of the protein level of plasma, including albumin, in a proportion similar to that found in plasma [205,206]. Human albumin is the most abundant protein in the plasma; and has the capacity to bind to metal ions such as Cu^{2+} [207]. The first four amino acids of the N-terminus of human albumin Asp-Ala-His-Lys forms a tight-binding site for Cu^{2+} [207]. In fact, almost thirty years ago, Linus Pauling and his colleagues [194] designed a

copper:glycylglycylhistidine complex that mimics the binding of albumin to copper; it enhanced the antitumor activity of ascorbate against Ehrlich ascites tumor cells. In addition, extracellular fluids contain little or no catalase activity, and low levels of SOD and GPx [208]. Thus, the H_2O_2 generated by ascorbate oxidation in the extracellular space could accumulate to a concentration greater than the intracellular level resulting in net rate of diffusion across the cell membrane into cells, resulting in toxicity to cancer cells [10].

Pharmacological ascorbate inhibits tumor growth in mice. In mice bearing tumor xenografts of pancreatic cancer, treatment with 4 g kg^{-1} ascorbate (i.p., twice daily) significantly decreased the rate of tumor growth [11,12,164] (Table 4). Upon cessation of treatment with pharmacological ascorbate, the rate of tumor growth increased. This rate was similar to the rate observed in the control group, *i.e.* no ascorbate (Du et al., unpublished results). In this tumor model pharmacologic ascorbate as a single agent was cytostatic, not cytotoxic. Obviously, the use of intravenous high dose ascorbate as a single agent for cancer was not curative. To test the combination effect of standard chemotherapy with ascorbate, Espey et al. [209] have shown that synergistic cytotoxic effects can be achieved with gemcitabine and ascorbate in pancreatic cancer both *in vitro* and in a nude-mouse model. These data provide support for investigating the use of pharmacologic ascorbate as an adjuvant for conventional cancer chemotherapies.

8.4. Clinical studies

Although the two clinical trials using oral ascorbate at the Mayo Clinic failed to show any benefit, numerous case reports have provided encouraging results with intravenous ascorbate therapy [162,210,211]. However, most of the cases were reported without sufficient detail or follow-up for evaluation. Padayatty et al. [212] examined three well-documented cases in accordance with National Cancer Institute (NCI) Best Case Series guidelines. These cases suggest that high-dose, intravenous ascorbate therapy prolonged the survival of the patients with advanced cancer.

Intravenous ascorbate therapy is likely to be safe in most patients except under certain conditions [213]. Because the removal of H_2O_2 generated in blood requires glutathione, which in turn requires NADPH generated by G6PD, the rate-limiting enzyme of the pentose phosphate pathway [214], high-dose ascorbate can induce intravascular hemolysis in patients with glucose-6-phosphate dehydrogenase (G6PD) deficiency [215–217]. One end-product of ascorbate oxidation is oxalic acid, which has the potential to crystallize as calcium oxalate in the urinary space in patients with pre-existing renal insufficiency [218]. In addition, rare cases of massive tumor hemorrhage have been reported in patients with advanced cancer following high-dose intravenous ascorbate [155,219]. Thus, as always, caution is needed, but there are clear indications for a potential role for ascorbate in cancer treatment.

Three phase I clinical trials of intravenous ascorbate in patients with advanced cancer measured plasma concentrations of ascorbate and evaluated clinical consequences [162,220,221]. In the study by Riordan and colleagues, patients were given continuous infusion of 0.15 to 0.7 g kg^{-1} day^{-1} for up to eight weeks; in the Hoffer study, ascorbate was administrated three times per week at fixed doses of 0.4, 0.6, 0.9 and 1.5 g kg^{-1}; and in the most recent study by Monti et al. [221], patients received 50, 75 and 100 g per infusion (three infusions per week) for 8 weeks with concomitant i.v. gemcitabine and oral erlotinib. In the Riordan study, ascorbate concentrations in serum during therapy ranged from 0.28 to 3.8 mM. In the Hoffer study, peak plasma ascorbate concentrations ranged from 2.4 to 26 mM. When 1.5 g kg^{-1} was administered, plasma ascorbate concentrations exceeded 10 mM for ≈4.5 h [220], levels that have been shown to induce cell killing in a variety of cancer cells [9,11,12,164]. Finally in the Monti study, patients that received 100 g of ascorbate

achieved peak plasma concentrations between 25 and 32 mM. All three trials demonstrated that high-dose intravenous ascorbate was well tolerated in cancer patients with normal renal function; the combination of ascorbate infusion with standard of care chemotherapies did not increase toxicity.

8.5. Ascorbate and conventional cancer therapy

The cytotoxicity of high-dose intravenous ascorbate is dependent on the ascorbate-induced production of H_2O_2. Hydrogen peroxide can deplete intracellular GSH, causing oxidative stress. In high doses ascorbate acts as a pro-oxidant to kill cancer cells. Initial concerns that ascorbate might reduce the effectiveness of standard chemotherapy and/or radiation therapy have not been demonstrated. Numerous reports suggest that pharmacological ascorbate may actually increase the efficacy of several chemotherapeutic drugs and radiation *in vitro* [164,222–224] (Table 3). Espey et al. [209] demonstrated that pharmacologic concentrations of ascorbate with gemcitabine resulted in a synergistic cytotoxic response in pancreatic tumor cell lines; gemcitabine–ascorbate combinations administrated to mice bearing pancreatic tumor xenografts consistently enhanced inhibition of growth compared to gemcitabine alone. Case reports [161,212] indicate that intravenous ascorbate can be safely used with chemotherapy and radiotherapy and could potentially enhance the effects of conventional cancer therapy. Furthermore, intravenous ascorbate has been shown to improve the quality of life in breast cancer patients during chemo-/radiotherapy and aftercare [225]. Clinical trials using combinations of chemotherapy and intravenous ascorbate are underway at several institutions (www.clinicaltrials.gov).

9. Perspectives

The inhibition effects of pharmacologic ascorbate on tumor growth have been confirmed in many laboratories (Table 4). In murine models elevated ascorbate levels in plasma were verified following i.p. administration [11,164]. It is still unknown whether cells and tissues levels are also elevated [226,227]. Human platelets supplemented with 500 μM Asc for 30 min showed a decrease in SVCT2 levels, while in Asc depleted platelets SVCT2 expression was higher than native ones [29]. Thus *in vivo* studies for possible changes in SVCT expression levels following ascorbate infusion are needed. Another issue is a possible transient withdrawal effect upon cessation of treatment with pharmacologic ascorbate [228]. Male guinea pigs that received ascorbate 1 g kg^{-1} body weight per day by i.p. for 4 weeks had elevated plasma and urinary levels; however, in the weeks after the treatment withdrawn, mean plasma and urinary ascorbate were significantly lower than normal [229]. It is unknown if a similar rebound effect would occur in human subjects following high doses i.v. ascorbate. F_2-isoprostane is a biomarker for lipid peroxidation *in vivo*, and its quantification in plasma and urine has emerged as the most reliable method to assess systemic oxidative stress in humans and animals [230–232]. For healthy young women taking oral doses of ascorbate 30–2500 mg daily, plasma and urine F_2-isoprostanes were unchanged [41]. It is unclear whether plasma F_2-isoprostanes will change following pharmacologic ascorbate infusion in cancer patients. Furthermore, since pharmacological ascorbate is a prodrug for H_2O_2 generation, there should be many applications where delivery of H_2O_2 *via* pharmacological ascorbate may have clinical benefit, such as infectious diseases caused by viruses, bacteria, and other pathogens [10,233].

10. Summary

More than eighty years since its discovery, our understanding of the functions of ascorbic acid has evolved from the prevention of scurvy to its potential use as a therapeutic drug for cancer treatment. Ascorbate maintains Fe^{2+} of collagen hydroxylases in an active state; therefore it plays a pivotal role in collagen synthesis; parallel reactions with a variety of

dioxygenases affect the expression of a wide array of genes, for example *via* the HIF system, and possibly the epigenetic landscape of cells and tissues. The ability to donate one or two electrons makes ascorbate an excellent reducing agent and antioxidant. However, in the presence of catalytic metals, ascorbate also has pro-oxidant effects, where the redox-active metal is reduced by ascorbate and then in turn reacts with oxygen, producing superoxide that subsequently dismutes to produce H_2O_2.

Apart from its biochemical functions that are met by healthy nutritional levels, recent pharmacokinetic data indicate that intravenous administration of ascorbate bypasses the tight control of the gut and renal excretion; thus, intravenous administration of ascorbate will produce highly elevated plasma levels; this ascorbate will autoxidize resulting in a high flux of extracellular H_2O_2. This H_2O_2 will readily diffuse into cells challenging the intracellular peroxide-removal system, initiating oxidative cascades. These high fluxes of H_2O_2 appear to have little effect on normal cells but can be detrimental to certain tumor cells. Knowledge and understanding of these mechanisms brings a rationale to the use of high-dose ascorbate to treat disease and thereby is reviving interest in the use of i.v. ascorbate in cancer treatment. The full potential of pharmacological ascorbate in cancer treatment will only be realized with greater understanding of its basic mechanism of action in conjunction with the appropriate design of clinical trials.

Abbreviations

Asc	a general abbreviation to include all forms, *i.e.* $AscH_2$ +$AscH^-$+Asc^{2-}+DHA
AscH$_2$	ascorbic acid, vitamin C
AscH$^-$	ascorbate monoanion
Asc^{2-}	ascorbate dianion
Asc$^{\bullet-}$	semidehydroascorbate, *i.e.* ascorbate radical
DHA	dehydroascorbic acid
HIF	hypoxia inducible factor
PDI	protein disulfide isomerase
SVCT	sodium-dependent vitamin C transporters

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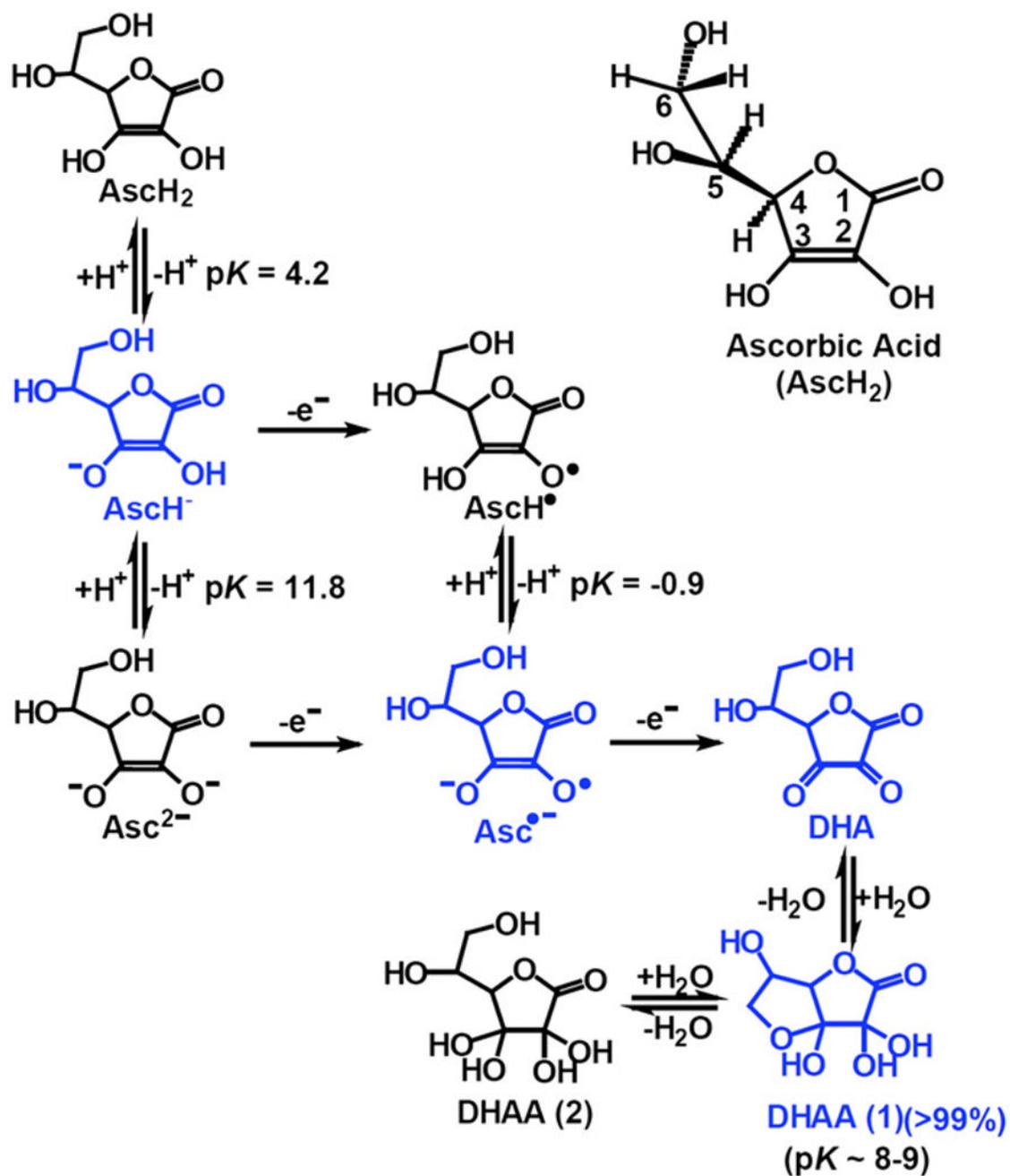
Fig. 1.
Structures of the chemical species associated with vitamin C. The structures in blue are those forms that dominate the biochemistry of vitamin C.

Table 1

Ascorbate content of human tissues.

Tissue	Ascorbate (mmol/kg wet tissue)	Ascorbate (mM)	Ref.
Adrenal glands	1.7–2.3		[45]
Pituitary gland	2.3–2.8		[45]
Liver	0.8–1		[45]
Spleen	0.8–1		[45]
Pancreas	0.8–1		[45]
Kidneys	0.28–0.85		[45]
Skeletal muscle	0.17–0.23		[45]
Brain	0.74–0.85		[45,226,227,234]
Placenta	0.23–0.72		[48]
Plasma (healthy)		0.04–0.08	[41,45,51]
Red blood cell		0.04–0.06	[39]
Neutrophil		1.2	[41]
Lymphocyte		4.0	[41]
Monocyte		3.2	[41]
Platelet		3.7	[41,56]
Cerebral spinal fluid		0.15–0.25[a]	[235–237]
Neuron		10	[238]
Glial cells		1	[238]
Lens		2.5–3.4	[46]
Corneal epithelium		12.5	[49]
Aqueous humor		0.4–1.1	[46]
Alveolar macrophage		0.32	[50]
Bronchoalveolar lavage		0.04–0.06	[51]
Saliva		0.04–0.05	[45]

[a]Ascorbate levels in cerebral spinal fluid are approximately three to four times that of plasma.

Table 2

Ascorbate in cultured cells.

Cell culture	Treatment	Intracellular ascorbate (mM)	Ref
A431 (human epidermoid carcinoma)	1 mM ascorbate[a], 18 h	1	[239]
Adj.PC-5 mouse plasmacytoma	200 µM Asc[b], 12 h	7	[240]
Alveolar macrophage (rat)	0.1 mM Asc, 30 min	3.2	[241]
Alveolar type II epithelial cells (rat)	0.1 mM Asc, 30 min	3.2	[241]
Astrocytes (rat)	DHA[c], 100 µM, 10 min	1.5	[248]
B lymphocytes	250 µM Asc, 4 h	0.7–1.5	[242]
EA.hy926 endothelial cells	0.3 mM Asc, 60 min	3–4	[244]
HepG2 hepatic epithelial cells	32 µM ^{14}C-Asc, 3 min	1.1	[245]
HL-60	0.4–3 mM ascorbic phosphate	0.5	[246]
HUVEC	1 mM ascorbate, 18 h	3.5	
INS-1 (rat pancreatic β-cell)	0.4 mM Asc, 16–18 h	2.4	[247]
K562 (human erythroleukemia)	500 µM DHA, 30 min	3	[248]
L6 skeletal myocytes (rat)	100 µM DHA, 10 min	12.5	[242]
MIA PaCa-2 pancreatic cancer	50 µM Asc, 4 h	6	*d*
	1 mM Asc, 4 h	8	
	15 mM Asc, 4 h	18	
Murine microvascular endothelial cells	0.5 mM Asc, 12 h	5	[249]
Osteoblasts (mouse)	1 mM Asc, 24 h	8	
Primary hepatocytes	32 µM ^{14}C-Asc, 3 min	0.41	[245]
SH-SY5Y neuroblastoma	2 mM Asc, 16–18 h	6	[250]
Skin fibroblasts	1 mM ascorbate, 18 h	0.4	[239]
U937 (human histocytic leukemia)	50 µM Asc or Asc-2-phosphate, 24 h	4	[54]
U937	1 mM Asc or Asc-2-phosphate, 24 h	7	[54]

[a] Ascorbate.

[b] L-Ascorbic acid.

[c] Dehydroascorbate.

[d] Thomas J van't Erve, personal communication.

Table 3

Pro-oxidant effects of ascorbate in cultured cells.

Cell line	Treatment	Effects	Ref
Primary human diploid fibroblasts GM5399	20–500 μM Asc	Inhibit growth, DNA damage	[251]
Normal human skin fibroblast CCD-25 SK	Sodium ascorbate, 0.56–2.8 mM	No effect	[210]
Normal human colon fibroblast CCD-18 Co	Sodium ascorbate, 0.1–0.4 mM	No effect	[210]
Human embryonic fibroblast	Sodium ascorbate, 0.2 mM	Inhibit growth	[252]
HRE human normal renal epithelial cells	Asc 1.2 mM	Promote growth	[253]
Human normal myeloid cells	L-Ascorbic acid, 0.3 mM	No effect	[254]
Primary chicken embryo fibroblast	Sodium ascorbate, 0.2 mM	Inhibit growth	[252]
AN3 CA endometrial adenocarcinoma	Sodium ascorbate, 0.56–2.8 mM	Inhibit growth	[210]
Ehrlich ascites carcinoma	Ascorbate+3-amino-triazole	Synergistic killing	[171]
5637 human bladder cancer	Asc $EC_{50} < 5$ mM	Inhibit growth	[11]
T24 human bladder cancer	Asc+vitK$_3$	Synergistic, Autoschizis, Necrosis, apoptosis	[255]
MB231 human breast cancer	5 mM Asc	Decrease clonogenic survival	[9]
MCF-7 human breast cancer	5 mM Asc	Decrease clonogenic survival	[9]
MCF-7 human breast cancer	Asc+porphyrin	Apoptosis, cell cycle disruption	[256]
Hs587t human breast cancer	5 mM Asc	Decrease clonogenic survival	[9]
HeLa human cervical cancer	Sodium ascorbate, 0.25 mM; Asc $EC_{50} > 5$ mM	No effect	[11,257]
HeLa human cervical cancer	Asc+MGd (Motexafin gadolinium)	Apoptosis, Lysosomal rupture	[257]
Normal human colon fibroblast CCD-18-Co	Sodium ascorbate, 0.1–0.8 mM	No effect	[210]
Human colon carcinoma cells	Sodium ascorbate, 0.1–3.2 mM	Inhibit growth	[210]
HEp-2 human epidermoid larynx carcinoma	Asc 500 μM+25 μM vitB$_{12b}$	Apoptosis, DNA damage	[258]
A549 human lung cancer	Asc $EC_{50} < 5$ mM	Inhibit growth	[11]
HepG2 human hepatocellular carcinoma	Asc, Asc+MnTMPyP	Decrease clonogenic survival, mitochondria damage.	[198]
DU 145 human prostate cancer cells	Sodium ascorbate, 1–10 mM; Asc +MnTMPyP	Inhibit growth	[198]
LNCaP human prostate cancer cells	Sodium ascorbate, 1–10 mM; Asc +MnTMPyP	Inhibit growth	[198]
PC-3 human prostate cancer	Sodium ascorbate, 0.56–2.3 mM; Asc +MnTMPyP	Inhibit growth	[198]
MIA PaCa-2 human pancreatic carcinoma	Sodium ascorbate, 0.56–2.8 mM;	Inhibit growth	[210]
MIA PaCa-2 human pancreatic carcinoma	Asc 1–5 mM	Cytotoxic, autophagy	[12]
PANC-1	Asc, Asc+MnTMPyP	Decrease clonogenic survival	[198]
Pan02 mouse pancreatic cancer	Asc 2.5–10 mM	cytotoxic	[11]
Human acute leukemic cells	L-Ascorbic acid, 0.3 mM	Inhibit growth	[254]
U937 human histocytic leukemia cells	L-Ascorbic acid, 50–300 μM	Inhibit growth	[163]
Human chronic lymphocytic leukemia	Asc 0.3–5 mM; Asc+ATO (Arsenic trioxide)	Cytotoxic	[259]
Kelly human neuroblastoma	0.6–5 mM	Cytotoxic	[151]
SK-N-SH human neuroblastoma	0.6–5 mM	Cytotoxic	[151]
Mouse neuroblastoma	Sodium ascorbate+5-FU Sodium ascorbate+X-irradiation	Inhibit growth	[222]

Cell line	Treatment	Effects	Ref
9 L rat glioblastoma	Asc 2.5–10 mM	Cytotoxic	[11]
LN18 human glioblastoma cell lines, human primary glioblastoma cells, GL261 mouse astrocytoma cell line	Asc+γ-irradiation	Double-stranded DNA breaks, G2/M arrest block	[224]
B16F10 murine melanoma	Asc 0.05–0.2 mM	Cell cycle arrest	[260]
Chinese hamster ovary (CHO) cells	Ascorbate+misonidazole	Inhibit growth	[261]

Table 4

Pro-oxidant effects of ascorbate in animal models.

Species	Cell type	Treatment	Effects	Ref
Balb/c nude mice	HT29 colon cancer	Asc 15 mg, 100, 1000 mg/kg, i.p., daily. 4 weeks.	7/7 mice survived at 1000 mg/kg. No carcinogenic invasion. Tumor volumes decrease. ARSs and EiFs genes down regulated.	[262]
Balb/c nude mice	K562 leukemia	Vit K_3 10 mg/kg, i.p.; Asc 1 g/kg, i.p.	Asc+vit K_3 decrease tumor growth.	[263]
Ncr nude mice	Ovcar5 ovarian cancer	Asc 4 g/kg, i.p., twice daily. 30 days.	No adverse effects. Decrease tumor growth.	[11]
Ncr nude mice	Pan02 pancreatic cancer	Asc 4 g/kg, i.p., daily. 14 days.	No adverse effects. Decrease tumor growth.	[11]
Ncr nude mice	9 L rat glioblastoma	Asc 4 g/kg, i.p., twice daily. 12 days.	No adverse effects. Decrease tumor growth. Prevent metastases.	[11]
Ncr nude mice	Pan02 pancreatic cancer	Asc 4 g/kg, i.p., daily; Gemcitabine 30, 60 mg/kg, i.p., every 4 days. 21 days.	No side effects except osmotic stress. Asc+Gem significantly decrease tumor growth.	[209]
Ncr nude mice	PANC-1 pancreatic cancer	Asc 4 g/kg, i.p., daily; Gemcitabine 30, 60 mg/kg, every 4 days. 33 days.	No side effects except osmotic stress. Asc+Gem significantly decrease tumor growth.	[209]
Nude mice	MIA PaCa-2 pancreatic cancer	Asc 4 g/kg, i.p., twice daily. 14 days.	No adverse effects. Decrease tumor growth.	[12]
NMRI mice	TLT Murine hepatoma	Asc 1 g/kg, i.p., daily. 30 days.	Decrease tumor growth.	[164]
Nude mice	H322 non-small cell lung cancer	Asc 250 mg/kg, i.p.; 101 F6 nanoparticle i.v.	Asc synergistic with 101 F6 decrease tumor growth.	[264]
SCID mice	EHMS-10 mesothelioma cells	Asc 0.88, 8.8 g/mouse, i.v. single dose.	Decrease tumor growth.	[265]
Balb/c mice	Murine sarcoma S180 cells	Asc 5.5, 30 mg/mouse, i.p., every two days.	Decrease tumor growth; inhibit bFGF, VEGF and MMP2 genes.	[266]
Balb/c mice	Murine sarcoma S180 cells	Asc 1.5 mg/g, i.p., every three days.	Inhibit tumor establishment; RKIP and annexin A5 levels increase.	[267]