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ORIGINAL ARTICLE

The antioxidant ascorbic acid mobilizes nuclear copper leading to a prooxidant breakage of cellular DNA: implications for chemotherapeutic action against cancer

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Abstract
Purpose Ascorbic acid is an essential micronutrient and is considered to have an antioxidant function in living systems. For the past several decades, ascorbic acid has been the subject of considerable interest as an anticancer agent. Several studies have shown that ascorbic acid is cytotoxic to a variety of cancer cells, whereas normal cells are relatively resistant to such cytotoxic action. In this study, we propose a putative molecular mechanism that accounts for the preferential cytotoxicity of ascorbic acid against cancer cells.
Methods Standard and lysed version of alkaline single-cell gel electrophoresis (Comet assay); ferrous oxidation–xylenol orange (FOX) assay.
Results We show that ascorbic acid acts as a prooxidant and leads to oxidative DNA breakage in lymphocytes and lymphocyte nuclei. Scavengers of reactive oxygen species were able to inhibit ascorbic acid-induced DNA breakage, suggesting the involvement of reactive oxygen species in this reaction. We further show that such DNA breakage is inhibited by both iron and copper chelators in cells, whereas in nuclei, similar inhibition was achieved only by copper chelators, indicating an important role of chromatin-bound copper in the prooxidant cellular DNA breakage by ascorbic acid.
Conclusion We propose that the copper-dependent cellular redox status is an important element in the cytotoxic action of ascorbic acid against cancer cells. It is well established that cellular copper levels are considerably elevated in various malignancies. Therefore, cancer cells may be more subject to electron transfer between copper and ascorbate to generate reactive oxygen species. In light of these observations and those in literature, in this paper we explain that the preferential cytotoxicity of ascorbic acid against cancer cells is the result of elevated copper levels in such cells. Further, this study identifies nuclear copper as a novel molecular target for cytotoxic action of ascorbic acid, which has implications for its chemotherapeutic properties against cancer.

Keywords Ascorbic acid · Endogenous copper · Prooxidant DNA breakage · Comet assay · Redox cycling

Introduction

In recent years and earlier, a number of studies have appeared demonstrating that ascorbic acid, an essential micronutrient in living systems, possesses chemopreventive and therapeutic properties against cancer [1–6]. However, the anticancer effect of ascorbic acid and its mechanism of action have remained controversial. Earlier studies by Cameron and Pauling reported clinical benefits and improved survival using both oral and intravenously administered ascorbic acid in the treatment of terminal cancer [6, 7]. Later, in two double blind, placebo-controlled trials, investigators at the Mayo clinic found that a high-dose oral administration of vitamin C had no

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effect on cancer survival [8, 9]. These trials were considered definitive possibly because the difference in the in vivo levels of ascorbic acid achieved between the oral and intravenous administration was not adequately appreciated. Plasma levels of ascorbic acid are tightly controlled and are around 50 μM [10]. However, Padayatty et al. have shown that intravenous administration of ascorbic acid bypasses such tight control and results in concentrations as much as 70 folds higher than those achieved by maximum oral consumption [11].

Recently, Levine and coworkers have demonstrated that pharmacologic ascorbic acid concentrations achievable through intravenous administration were cytotoxic to many types of cancer cells in vitro and significantly impeded tumor progression in vivo without toxicity to normal tissues [12]. Although an interesting mechanism of action was suggested that involved ascorbic acid-induced formation of H_2O_2 in the extracellular fluid leading to the observed cytotoxic effects, the authors were not clear about the molecular basis for the relative resistance of normal cells. Moreover, it is well known that H_2O_2 is freely permeable to both normal and cancer cells [13–15].

In an earlier publication, we have shown that at normally achievable concentrations, ascorbic acid leads to oxidative breakage of cellular DNA [16]. Further, it has been proposed that most clinically used anticancer drugs can activate late events of apoptosis (DNA degradation and morphological changes), and the essential signaling pathways differ between pharmacological cell death and physiologic induction of cell death [17]. As a further confirmation of our earlier results [16], using a lysed version of Comet assay (alkaline single-cell gel electrophoresis), in this paper we show that ascorbic acid is able to mobilize nuclear copper from lymphocyte nuclei and exhibits a prooxidant effect leading to cellular DNA breakage and consequent cell death. It is known that copper ions occur naturally in chromatin and can be mobilized by metal-chelating agents [18]. The concentration of copper in various tissues ranges from 10 to >100 μM with 20% found in the nucleus [19]. Further, serum [20], tissue [21] and cellular [22] concentrations of copper are known to be considerably elevated in various malignancies. Therefore, cancer cells may be more subject to electron transfer between copper ions and ascorbate than normal cells to generate reactive oxygen species such as hydroxyl radicals that act as proximal DNA-cleaving agents. These observations and the results presented in this paper explain the findings of Levine and coworkers [12], toward the sensitivity of cancer cells and relative resistance of normal cells against the cytotoxic action of ascorbic acid and thus have implications for the anticancer mechanism of action of ascorbic acid.

Materials and methods

Materials

Ascorbic acid, neocuproine, bathocuproine disulfonate, superoxide dismutase (SOD), agarose, low melting point agarose (LMPA), RPMI 1640, Triton X-100, trypan blue, Histopaque1077 and phosphate-buffered saline (PBS) Ca^{++}- and Mg^{++}-free were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade. Ascorbic acid was dissolved in 3 mM NaOH before use as a stock of 1 mM ascorbic acid solution (pH 8). The volumes of stock solution added did not lead to any appreciable change in the pH of reaction mixtures (pH 7.5). Upon addition to reaction mixtures, in the presence of buffers mentioned and at concentrations used (as indicated in figures), ascorbic acid remained in solution.

Isolation of lymphocytes

Heparinized blood samples (2 ml) from a single healthy non-smoking donor were obtained by venepuncture and diluted suitably in Ca^{++}- and Mg^{++}-free PBS. Lymphocytes were isolated from blood using Histopaque 1077 (Sigma), and the cells were finally suspended in RPMI 1640. A single donor donated blood for all experiments (first author).

Viability assessment of lymphocytes

The lymphocytes were checked for their viability before the start and after the end of the reaction using trypan blue exclusion test [23]. The viability of the cells was found to be greater than 93%.

Treatment of lymphocyte nuclei with ascorbic acid and evaluation of DNA breakage by using lysed version of comet assay

Treatment of nuclei by ascorbic acid was carried out by lysing the lymphocytes embedded in agarose on slides and subsequently treating the nuclei in such slides by ascorbic acid and other agents. Such lysed version of the comet assay has been used to study the direct interaction of various agents with cell nuclei as it eliminates the effect of cell membrane as a barrier and the intracellular environment [24]. Lysed version of comet assay was performed as described by Kasamatsu et al. [25] with some modifications. Lymphocytes isolated from 2-ml blood were diluted to the count of 2×10^5 cells/2 ml. Approximately, 10,000 of these cells were mixed with 75 μl of prewarmed LMPA in PBS and immediately applied to a frosted microscopic slide layered with 75 μl of 1% standard agarose in PBS.

The slides were allowed to gel at 4°C for 10 min. Lysis of cells was then performed by submerging the slides in a tank containing lysis solution in the absence of light for 1 h at 4°C. The use of a tank instead of a coplin jar allowed simultaneous processing of a number of slides. The lysis solution (pH 10) consisted of 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris and 1% Triton X-100 added just prior to use. After lysis, slides were transferred to another tank containing 0.4 M phosphate buffer (pH 7.5) for 10 min. Each slide was then transferred to a rectangular dish (8 cm × 3 cm × 5 mm) that contained a reaction mixture of ascorbic acid and other additions as mentioned in various legends to figures and tables. The slides with the reaction mixture were incubated at 37°C. The slides were then washed twice by placing in 0.4 M phosphate buffer pH 7.5 for 5 min at room temperature. DNA unwinding and expression of alkali-labile sites were done by leaving the slides in the high-pH electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH > 13 prepared in PBS) at 4°C for 30 min. Subsequently, the electrophoresis, neutralization and staining of the slides were carried out as described earlier [16]. Comet images were observed at 100× magnification with a fluorescence microscope (Olympus CX41) and COHU 4910 (equipped with a 510- to 560-nm excitation and 590-nm barrier filters) integrated CC camera. Fifty images were randomly selected from each sample, and their lengths (diameter of the nucleus plus migrated DNA) were measured on the screen as automatically generated by Komet 5.5 image analysis system of Kinetic Imaging, Liverpool, UK.

Treatment of whole lymphocytes with ascorbic acid and evaluation using the standard comet assay

Treatment of whole lymphocytes with ascorbic acid and the subsequent Comet assay was performed essentially as described earlier [16]. However, since, the DNA breakage had to be compared with that in lymphocyte nuclei, the treatment of cells with ascorbic acid was done on slides rather than in Eppendorf tubes. Further, the lysis of cells was carried out after the treatment. The other conditions remained the same as described earlier.

Detection of H_2O_2 generation by ascorbic acid in the incubation medium of nuclei

The ferrous oxidation–xylenol orange (FOX) assay [26] was adapted to detect and quantify the generation of H_2O_2 in the incubation medium (PBS 0.04 M, pH 7.5) by ascorbic acid and compared with a known generator of H_2O_2 tannic acid [27]. The simplified reaction sequence involves the oxidation of ferrous (Fe^{2+}) to ferric (Fe^{3+}) ions by H_2O_2 with the subsequent binding of the Fe^{3+} ion to the ferric-sensitive dye xylenol orange, yielding an orange to purple complex, which is measured at 560 nm. The reaction mixture contained ascorbic acid and PBS (incubation medium used in the treatment of nuclei). After incubation for 2 h at 37°C, an aliquot of 200 µl was analyzed for H_2O_2 formation.

Statistics

The statistical analysis was performed as described by Tice et al. [28] and is expressed as ±SEM of three independent experiments. A student's *t* test was used to examine statistically significant differences. Analysis of variance was performed using ANOVA. *P* values <0.05 were considered statistically significant.

Results

DNA breakage by ascorbic acid in whole lymphocytes and lymphocyte nuclei as measured by comet assay

We have earlier shown that ascorbic acid causes oxidative DNA breakage in intact human peripheral lymphocytes [16]. Since in the lysed version of the Comet assay, membrane and cytoplasmic barriers are eliminated, it would be reasonable to assume that the ascorbic acid is able to directly interact with the cell nuclei. Thus, considerably greater DNA breakage should be observed in the lysed version when compared with the standard version where intact lymphocytes are used. Increasing concentrations of ascorbic acid (25, 50, 100 µM) were tested for DNA breakage in intact lymphocytes (standard version of Comet assay) and compared with that observed with lymphocyte nuclei (lysed version). Photographs of comets obtained with 50 µM ascorbic acid in the standard and lysed version of Comet assay are shown in Fig. 1. In Fig. 2, the data are plotted as tail lengths of comets with increasing concentration of ascorbic acid. It is seen that the rate of tail formation is considerably greater in the case of lysed version, suggesting that ascorbic acid is able to directly interact with the nuclei when the lysed version of comet assay is used. Similar results have also been reported by Kasamatsu et al. [25] using H_2O_2 and bleomycin.

Effect of active oxygen scavengers on ascorbic acid-induced DNA breakage in lymphocyte nuclei

We have previously shown [16] that ascorbic acid-induced DNA breakage in intact lymphocytes is inhibited to significant degrees by various scavengers of reactive oxygen species. Table 1 gives the results of an experiment where three scavengers (superoxide dismutase, catalase and

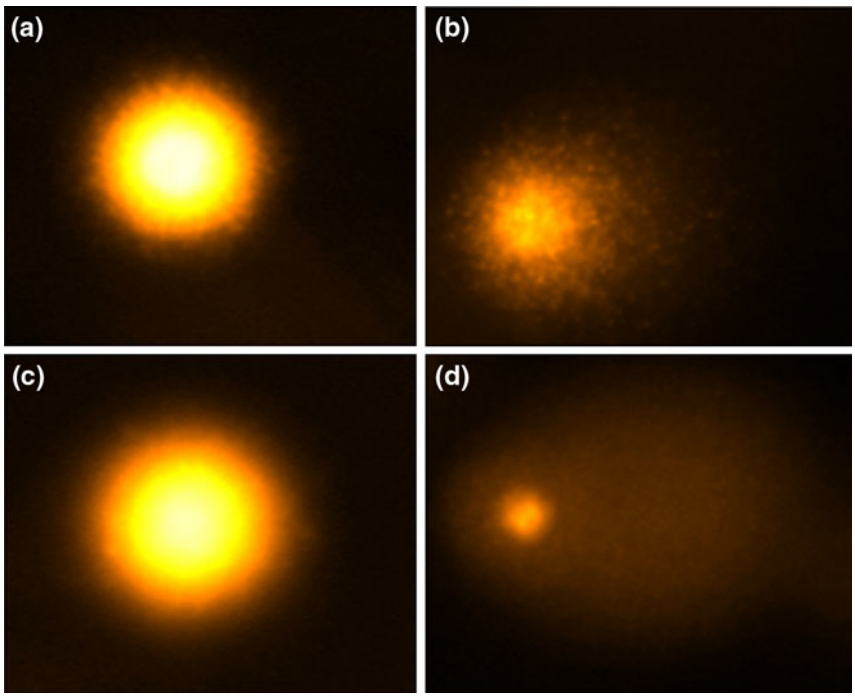

Fig. 1 Single-cell gel electrophoresis of human peripheral lymphocytes and lymphocyte nuclei showing comets (100×) after treatment with ascorbic acid (50 μM); **a** lymphocyte control, **b** treated lymphocyte, **c** lymphocyte nucleus control and **d** treated lymphocyte nucleus. The incubation of lymphocytes and lymphocyte nuclei with ascorbic acid was for 1 h at 37°C

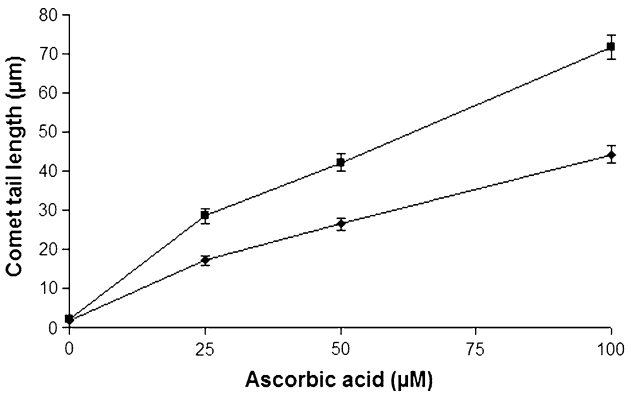

Fig. 2 A comparison of DNA breakage in intact lymphocytes (*filled diamond*) and lymphocyte nuclei (*filled square*) using increasing concentrations of ascorbic acid. The data obtained in the experiment given in Fig. 1 are plotted. Values reported are ±SEM of three independent experiments. $P < 0.01$ by comparison with control (in the absence of ascorbic acid)

Table 1 Effect of scavengers of reactive oxygen species on ascorbic acid-induced DNA breakage in lymphocyte nuclei

Dose	Tail length (μm)	Inhibition (%)
Untreated	02.09 ± 0.12	–
Ascorbic acid (50 μM)	41.76 ± 2.12#	–
+Catalase (100 μg/ml)	24.09 ± 1.16*	42.3
+SOD (100 μg/ml)	21.92 ± 1.43*	47.5
+Thiourea (1 mM)	20.12 ± 1.11*	51.8

* $P < 0.05$ when compared with control (#). Data represent ±SEM of three independent experiments

thiourea) were tested for their effect on ascorbic acid-induced DNA breakage in lymphocyte nuclei using the lysed version of Comet assay. All three caused significant inhibition of DNA breakage as evidenced by decreased tail lengths. It may be mentioned that due to the site-specific nature of the reaction of hydroxyl radicals with DNA, it is difficult for any trapping molecules to intercept them completely [29]. We conclude that superoxide anion and H_2O_2 are essential components in the pathway that leads to the formation of hydroxyl radical and other species which would be the proximal DNA-cleaving agents. Since the results are similar to those seen with whole lymphocytes, it is suggested that the same mechanism involving reactive oxygen species is responsible for DNA breakage irrespective of whether intact lymphocytes or lymphocyte nuclei are treated with ascorbic acid.

Effect of metal-specific chelators on the ascorbic acid-induced DNA breakage in lymphocyte and lymphocyte nuclei

Iron and copper are the most redox active of the metals present in living cells. In the experiments shown in Fig. 3a, b, we have used various metal-specific chelators, which selectively bind to copper, iron and zinc, in order to study their effect on ascorbic acid-induced DNA degradation in whole lymphocytes and in lymphocyte nuclei, respectively. In whole cells (Fig. 3a), a clear inhibition of DNA degradation was observed with neocuproine (a cell membrane–permeable Cu (I)-specific chelator) and to a lesser extent

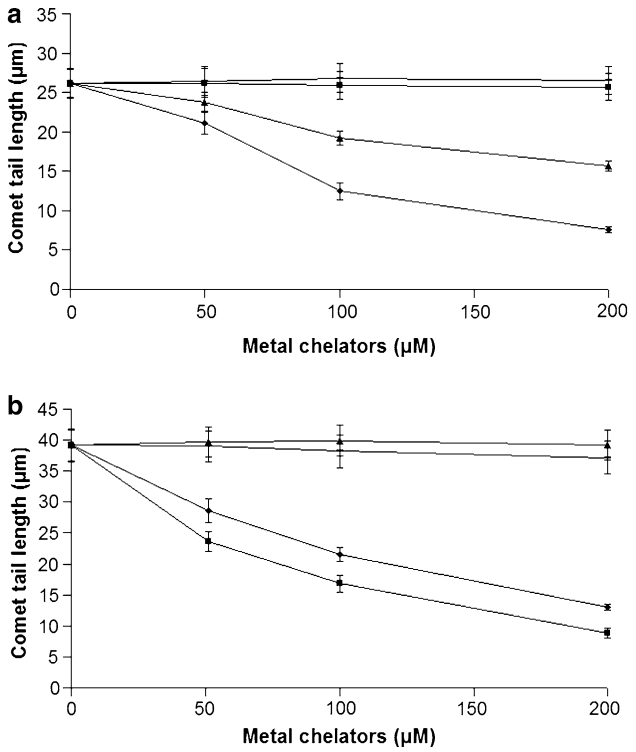

Fig. 3 Ascorbic acid (50 μM)-induced DNA degradation in **a** lymphocytes **b** lymphocyte nuclei in the presence of copper chelators neocuproine (*filled diamond*); bathocuproine (*filled square*), iron chelator desferrioxamine mesylate (*filled triangle*) and zinc chelator histidine (*black rectangle*). The concentration of chelators used was as indicated, and incubation was performed for 1 h at 37°C. Values reported are ±SEM of three independent experiments. $P < 0.01$ by comparison with control (in the absence of chelators)

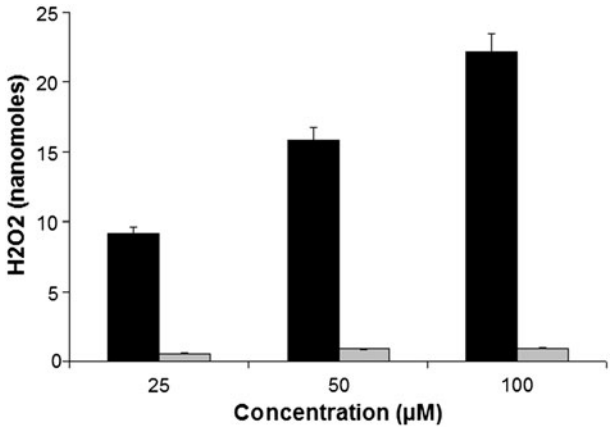

Fig. 4 A comparison of the rate of H_2O_2 formation by tannic acid (*filled square*) and ascorbic acid (*shaded square*) in the incubation medium as determined by Fox assay

with desferrioxamine mesylate (a Fe(II)-specific chelator). Further, no such inhibition was seen with bathocuproine (a water-soluble membrane-impermeable analog of neocuproine) and histidine (a zinc-specific chelator). However, in lymphocyte nuclei (Fig. 3b), both neocuproine and bathocuproine were found to inhibit the DNA breakage, while iron and zinc chelators were ineffective in causing such inhibition. It may be mentioned that the nuclear pore complex is permeable to small molecules. Bathocuproine disulfonic acid, which is impermeable to cell membrane, may freely traverse through nuclear pore complex to directly interact with the cell nuclei. It has been reported that certain agents, which are involved in the metabolism and homoeostasis of endogenous metals, can mobilize cytoplasmic content of free metals to nucleus [30]. Ascorbate is an important cofactor in many cellular metabolic processes and is intimately linked to iron homeostasis [31]. Thus, in case of whole cells, it is possible that under the homeostatic influence of ascorbate, free cytoplasmic iron migrates to the nucleus and participates in the Fenton reaction along with the chromatin-bound copper to generate hydroxyl radicals in the vicinity of DNA. Such a mechanism presumably explains the inhibition of ascorbic acid-induced DNA breakage in whole cells by desferoxamine mesylate. However, the same would not be the case with nuclei where the major metal ions present are copper and zinc. Therefore, we take these results to suggest that ascorbate mobilizes cytoplasmic iron and endogenous chromatin-bound copper, leading to oxidative DNA breakage.

H_2O_2 generation by ascorbate in the incubation medium of nuclei

Ascorbic acid is known to reduce molecular oxygen to superoxide anion, leading to the formation of H_2O_2 [32], which is known to permeate cell membranes. Such extracellular production of H_2O_2 could account for lymphocyte DNA breakage. In order to examine this possibility, we have determined the formation of H_2O_2 by ascorbate in the incubation medium of the nuclei (PBS 0.04 M/pH 7.5) and compared it with a known generator of H_2O_2 namely tannic acid [27]. In the results given in Fig. 4, we show that the rate of formation of H_2O_2 by tannic acid in the incubation medium is considerably greater whereas that by ascorbate is relatively negligible. Further, we have also compared the comet tail length formation as a function of increasing concentrations of ascorbate and tannic acid in lymphocyte nuclei. As seen in Fig. 5, whereas ascorbate shows significant tail formation in a dose-dependant manner, tannic acid is less effective in causing DNA breakage. These results indicate that cellular DNA breakage by ascorbate observed in our studies is not the result of only extraneous production of H_2O_2 as there exists no correlation between relative H_2O_2 production by tannic acid and ascorbate and their ability to cause DNA breakage.

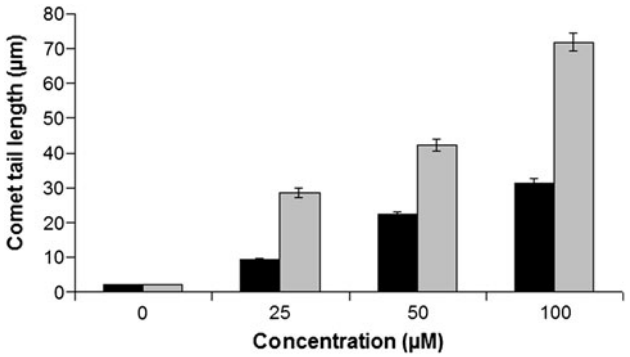

Fig. 5 A comparison of DNA breakage induced by tannic acid (*filled square*) and ascorbic acid (*shaded square*) in lymphocyte nuclei as shown by comet tail lengths. Values reported are ±SEM of three independent experiments

Discussion

The above results lead to the following major conclusion: the cellular DNA breakage in normal lymphocytes by ascorbic acid involves redox cycling of endogenous copper, possibly chromatin-bound copper either by ascorbic acid itself or through the extracellular generation of reactive oxygen species such as hydrogen peroxide and superoxide anion. Levine and coworkers have used a number of cancer cell lines to implicate a prooxidant mechanism of cytotoxic action of ascorbic acid and to show that normal cells are relatively resistant to such cell killing [12]. We believe that the preferential cytotoxic action of ascorbic acid against cancer cells is explained by the mechanism presented above where ascorbic acid is able to mobilize endogenous copper ions. In addition, we give below several lines of evidence, which suggests that this is indeed the case.

1. It is well established that in tissue, cellular and serum copper levels are considerably elevated in various malignancies [33]. Therefore, cancer cells may be more subject to electron transfer between copper ions and ascorbic acid to generate ROS. Consequently, the concentration of ascorbic acid required for a cytotoxic action against cancer cells should be lower than the concentration required against normal cells. Indeed, it has been shown that ascorbic acid is cytotoxic to leukemic cells (three to four folds higher copper levels [22]) at a much lower concentration than normal lymphocytes [34]. Moreover, there are a number of studies which have focused on determining the concentrations of four important elements; copper, zinc, iron and selenium in patients with cancer [35, 36]. These studies showed that while zinc, iron and selenium concentrations were significantly lower in patients with cancer, the copper concentrations were almost always found to be significantly elevated (up to two to three folds) compared to age-matched samples from normal tissue.

2. Fe^{3+} and Cu^{2+} are the most redox active of the metal ions in living cells. Wolfe et al. [37] have proposed that a copper-mediated Fenton reaction, generating site-specific hydroxyl radicals, is capable of inducing apoptosis in thymocytes. In a study with thiol-containing compounds, apoptosis was induced in different cell lines when either free copper or ceruloplasmin (a copper binding protein) was added; such activity was not observed, however, when either free iron or the iron-containing serum protein transferrin was added [38].

3. Among oxygen radicals, the hydroxyl radical is most electrophilic with high reactivity and therefore possesses a small diffusion radius. Thus, in order to cleave DNA, it must be produced in the vicinity of cellular DNA [39]. The location of the redox-active metal is of utmost importance because the hydroxyl radical, due to its extreme reactivity, interacts exclusively in the vicinity of the bound metal. This is also in concurrence with the observation that the ascorbic acid induces cell death, nuclear fragmentation and internucleosomal DNA cleavage in human myelogenous leukemia cell lines [40]. Such internucleosomal DNA "laddering" often used as an indicator of apoptosis may reflect DNA fragmentation by non-enzymatic processes by metal-chelating agents that promote the redox activity of endogenous copper ions and the resulting production of hydroxyl radicals [18].

4. In normal cells, there exists a balance between the free radical generation and the antioxidant defense [41]. However, it has been clearly documented that tumor cells are under persistent oxidative stress and have an altered antioxidant system [42] and thus further ROS stress in these malignant cells reaching a threshold level could result in apoptosis [33]. These observations further suggest that neoplastic cells may be more vulnerable to oxidative stress because they function with a heightened basal level of ROS due to increased rate of growth and metabolism [43]. Thus, in cancer cells, an enhanced exposure to ROS, generated through the redox activity of endogenous copper, can overwhelm the cells antioxidant capacity, leading to irreversible damage and apoptosis.

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Conflict of interest statement The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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