Vitamin C Prevents DNA Mutation Induced by Oxidative Stress*

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The precise role of vitamin C in the prevention of DNA mutations is controversial. Although ascorbic acid has strong antioxidant properties, it also has pro-oxidant effects in the presence of free transition metals. Vitamin C was recently reported to induce the decomposition of lipid hydroperoxides independent of metal interactions, suggesting that it may cause DNA damage. To directly address the role of vitamin C in maintaining genomic integrity we developed a genetic system for quantifying guanine base mutations induced in human cells under oxidative stress. The assay utilized a plasmid construct encoding the cDNA for chloramphenical acetyl transferase modified to contain an amber stop codon, which was restored to wild type by G to T transversion induced by oxidative stress. The mutation frequency was determined from the number of plasmids containing the wild type chloramphenical acetyl transferase gene rescued from oxidatively stressed cells. Cells were loaded with vitamin C by exposing them to dehydroascorbic acid, thereby avoiding transition metal-related pro-oxidant effects of ascorbic acid. We found that vitamin C loading resulted in substantially decreased mutations induced by H₂O₂. Depletion of glutathione led to cytotoxicity and an increase in H₂O₂-induced mutation frequency; however, mutation frequency was prominently decreased in depleted cells preloaded with vitamin C. The mutation results correlated with a decrease in total 8-oxo-dG found in genomic DNA of cells loaded with vitamin C and oxidatively stressed. These findings directly support the concept that high intracellular concentrations of vitamin C can prevent oxidation-induced mutations in human cells.

DNA damage caused by reactive oxygen species such as H₂O₂, O₂-, and •OH radicals has been implicated in mutagenesis, oncogenesis, and aging (1). Oxidative lesions in DNA include base modifications, sugar damage, strand breaks, and abasic sites. In vitro studies suggest that the hydroxyl radical is highly reactive toward DNA (2). One of the most common oxidized adducts in human cells and tissues is 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG) (3). This adduct results from exposure to oxidizing agents as well as from γ irradiation of DNA (4), and quantitation of 8-oxo-dG has been used as a marker of DNA damage (5). 8-oxo-dG “mis-pairs” with adenine during replication (6), resulting in G to T transversions in 50% of the replicated DNA (7).

The role of vitamin C in protecting against oxidatively induced DNA mutations is controversial. Although numerous studies demonstrate the antioxidant effects of vitamin C (8, 9), in vitro studies are often confounded by the pro-oxidant effects of ascorbic acid in the presence of free transition metals (10). We circumvented this problem using dehydroascorbic acid to load cells with vitamin C. Vitamin C is transported into most cells in the oxidized form, dehydroascorbic acid (DHA), via facilitative glucose transporters (11, 12) and as ascorbic acid in specialized cells by sodium-dependent ascorbic acid transporters (13). When transported as DHA it is rapidly reduced inside the cells and accumulated as ascorbic acid (14).

We developed a new genetic system to quantify oxidative DNA damage and resulting mutagenesis in human cells to determine directly the role of vitamin C in maintaining genomic integrity. We found that vitamin C markedly decreased mutations induced by H₂O₂. The mutation results correlated with a decrease in total 8-oxo-dG found in genomic DNA of cells that were loaded with vitamin C and oxidatively stressed.

MATERIALS AND METHODS

Cell Culture and Transfections—Human kidney 293T cells were cultured in Dulbecco's high glucose medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% l-glutamine. Human myeloid HL-60 cells were cultured in Iscove's modified Dulbecco's medium containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1% l-glutamine. Cells were maintained in an incubator with 5% CO₂ at 37 °C. Cells (293) were transfected by Ca²⁺-phosphate method at 1.5 × 10⁶ cells per 100-mm plate (15). Plates were incubated overnight, and the cells were collected.

Uptake of Ascorbic Acid (AA) and DHA—Cells were washed with PBS and incubated for 30 min in incubation buffer (15 mM HEPES pH 7.4, 135 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, and 0.8 mM MgCl₂). 1.5 × 10⁶ cells were added to incubation buffer containing 100 μM ascorbic acid and 0.2 μCi of L-[14C]ascorbic acid (PerkinElmer Life Sciences) for AA uptake or to a mixture containing ascorbic acid and ascorbate oxidase for DHA uptake. Following incubation samples were washed twice with cold Ca²⁺/Mg²⁺-free PBS. After lysis in 10 mM Tris-HCl (pH 8.0) containing 0.2% SDS, the incorporated radioactivity was determined by liquid scintillation spectrometry.

Cell Volume Determination—Intracellular volume was estimated as described previously (11, 16) with 30% correction for trapped extracellular material (17). Briefly, five million cells were incubated for 60 min at room temperature in 200 μl of incubation buffer containing 1 mM 3-oxy-methyl-glucose (OMG) and 5 μCi of H₂-OMG. During incubation equilibrium (zero-trans) was established between intra- and extracellular concentrations of OMG. After incubation 2 μl of 2 mM cytochalasin B was added to the cells to prevent efflux of trapped OMG during washing, and the mixture was incubated at room temperature for 5 min. Cells were then washed three times with cold Ca²⁺/Mg²⁺-free PBS containing 20 μM cytochalasin B to remove unincorporated radioactivity. After lysis in 10 mM Tris-HCl (pH 8.0) containing 0.2% SDS the incorporated radioactivity was determined by liquid scintillation spectrometry.

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RESULTS

The chloramphenicol-sensitive allele (Cm<sup>s</sup>) of the chloramphenicol acetyl transferase (CAT) gene was used as a marker for the determination of 8-oxo-dG-related mutagenesis in DNA. The wild type sequence of the CAT gene in plasmid pCAT19<sup>−</sup> was changed to the chloramphenicol-resistant allele (Cm<sup>R</sup>) by site-directed mutagenesis using the unique restriction site elimination method (19). The change in the sequence was confirmed by sequencing.

Enzymatic Digestion of DNA—Total genomic DNA from 50 × 10<sup>6</sup> HL-60 cells was isolated using the blood and cell culture kit from Qiagen. The isolated DNA was digested by nuclease P1 (Sigma) and calf alkaline phosphatase (Roche Molecular Biochemicals). Briefly, 5–15 μg of DNA (200 μl) in 25 mM sodium acetate, 1 mM zinc chloride (pH 4.8) was boiled for 5 min and quickly chilled on ice. Nuclease P1 (0.1 units/μg of DNA) was added to DNA and incubated at 37 °C for 1 h. 20 μl of 1.5 M Tris-HCl (pH 8) was added, and the solution was briefly vortexed. Calf alkaline phosphatase (0.075 units/μg of DNA) was added, and the solution was incubated for another 30 min at 37 °C. Digested DNA was filtered through a 0.2-μm Nalgene 4-mm nylon syringe filter. Samples were further analyzed by HPLC-ECD.

Depletion of Glutathione—293T cells were depleted of glutathione as previously described (19). Briefly, 293T cells (2 × 10<sup>6</sup>/100-mm plate) transfected with 6 μg of pCAT19-Cm<sup>R</sup> were incubated with 200 μM N-buthionine-(S,R)-sulfoximine (Sigma) for 16 h, followed by a 1-h incubation with 1 mM diethyl maleate (Sigma). Cells were washed with PBS and used in the in vivo DNA damage assay described below.

In Vitro DNA Mutation Assay—One microgram of plasmid pCAT19-Cm<sup>s</sup> was incubated at 37 °C for 1 h in 15 mM potassium phosphate buffer (pH 7.2) in the absence or presence of 0.05 and 5 mM H<sub>2</sub>O<sub>2</sub> and 25 μM Cu<sup>2+</sup> (25 μM). DNA was ethanol precipitated, resuspended in 5 μl of water, and electroporated into PR195. Bacteria were plated on LB plates supplemented with chloramphenicol (Cm) at 20 μg/ml as well as carbamethicillin (Carb) at 50 μg/ml to select for the transformation of untreated DNA. The absolute mutation frequency in untreated DNA was about 4 × 10<sup>−9</sup>. Values represent the mean ± standard deviation from triplicate measurements. The data are statistically significant at p < 0.05 using the Student's t test.

In Vivo DNA Mutation Assay—293T cells (2 × 10<sup>6</sup>/100-mm plate) transfected with 6 μg of pCAT19-Cm<sup>s</sup> were incubated with 500 μM H<sub>2</sub>O<sub>2</sub> or with 50 μM Cu<sup>2+</sup> for 16 h and treated with 0.1, 0.5, and 5 mM H<sub>2</sub>O<sub>2</sub> or with Cu<sup>2+</sup> (100 μM) or with 10 μM H<sub>2</sub>O<sub>2</sub> without Cu<sup>2+</sup> for 1 h. The plasmid was isolated using the SDS-NaOH lysis method (20) and electroporated into PR195. Mutation frequencies were determined as outlined above.

Fifty million HL-60 cells (0.3 × 10<sup>6</sup>/ml) were incubated for 2 days prior to the experiment. Cells were washed in PBS, incubated with different amounts of DNA for 1 h, and treated with H<sub>2</sub>O<sub>2</sub>/O<sub>2</sub> or with Cu<sup>2+</sup> (100 μM) or with 10 μM H<sub>2</sub>O<sub>2</sub> without Cu<sup>2+</sup> for 1 h. Total genomic DNA was isolated using the blood and cell culture kit from Qiagen. DNA was enzymatically digested for HPLC-ECD assays as described above.

RESULTS

The chloramphenicol-sensitive allele (Cm<sup>s</sup>) of the chloramphenicol acetyl transferase (CAT) gene was used as a marker for the determination of 8-oxo-dG-related mutagenesis in DNA. The wild type sequence of the CAT gene in plasmid pCAT19 was changed to a chloramphenicol-sensitive allele (Cm<sup>R</sup>) by site-directed mutagenesis using the unique restriction site elimination method (19). The change in the sequence was confirmed by sequencing.

The plasmid pCAT19 also carries the carbenicillin resistance gene (Carb) used for positive selection for the plasmid.

Under oxidative stress 8-oxo-dG can be created throughout the DNA molecule including the mutated codon 33 within the DNA as a control. The PR195 strain was deficient in the removal of both 8-oxo-dG (fpg) (21) and adenosines across 8-oxo-dG (mutY) (22). The latter mutation ensured that all 8-oxo-dG created in human cells were counted. Bacteria were plated on LB plates with chloramphenicol at 20 μg/ml as well as on LB plates with carbenicillin at 50 μg/ml. Mutation frequencies

![Image](https://via.placeholder.com/150)
were determined as ratios of the number of colonies on Cm plates to the number of colonies on Carb plates. The absolute mutation frequency in untreated DNA was approximately $4 \times 10^{-5}$. DNA treated with 5 mM H$_2$O$_2$ showed a 27-fold increase in mutation frequencies, and the addition of Cu$^{2+}$ ions induced further increases in mutation frequencies even at low concentrations of hydrogen peroxide (Fig. 1B). Copper ions alone, however, did not induce mutagenesis (Fig. 1B). These experiments demonstrated that the system was suitable for quantifying the frequency of mutations induced by oxidative stress.

We used the 293T human kidney cell line to study mutagenesis induced by hydrogen peroxide because of the high transfection frequency of these cells (80–90%). This made them suitable for the study of DNA damage under non-replicative conditions (15). The plasmid pcAT19-Cm$^8$ has no mammalian origin of replication and therefore cannot be replicated inside 293T cells. Cells were transfected with pcAT19-Cm$^8$ and were treated with H$_2$O$_2$ 24 h later (Fig. 1C). Plasmid DNA was extracted from the cells, purified and electroporated into PR195, and analyzed for mutation frequencies (ratio of Cm$^R$ to Carb$^R$ colonies). We found a roughly linear increase in mutation frequency with increasing concentrations of H$_2$O$_2$ in the presence of 100 $\mu$m Cu$^{2+}$ (Fig. 1D). The addition of Cu$^{2+}$ ions presumably led to increased oxidation and G to T transversion mutations in the plasmid due to 'OH generation via the Fenton reaction. Cells treated with 5 mM H$_2$O$_2$ and 100 $\mu$m Cu$^{2+}$ showed a 6-fold increase in mutation frequency compared with control. However, cells incubated with Cu$^{2+}$ ions alone evidenced no increase in mutation frequencies (Fig. 1D). Hydrogen peroxide without added copper did not cause a significant increase in mutations under these conditions even at concentrations of 10 mM (Fig. 1D). We therefore used 5 mM H$_2$O$_2$ and 100 $\mu$m Cu$^{2+}$ to study the role of vitamin C in the prevention of oxidative DNA damage in 293T cells.

Previously we showed that vitamin C is transported into the cells preferentially in the oxidized form as DHA through facilitated glucose transporters (11). Once inside the cell DHA is rapidly reduced to ascorbic acid (14). We found that 293T cells incubated with 500 $\mu$m DHA for 60 min accumulated 7 nmol of ascorbic acid/10$^6$ cells (Fig. 2A). We estimated the internal volume of 293T cells as 1.0 $\mu$l/10$^6$ cells from tritiated methylglucose equilibrium studies (see “Materials and Methods”). Based on this internal volume 293T cells incubated with 500 $\mu$m DHA for 60 min accumulated 7 $\mu$m ascorbic acid. However, cells incubated with 500 $\mu$m AA for 60 min accumulated only 0.7 $\mu$m ascorbic acid (Fig. 2A).

We investigated the effect of vitamin C loading by exposure to DHA in preventing mutations induced by oxidative stress in the 293T cells. Hydrogen peroxide with copper increased mutation frequency by 8-fold compared with the control (Fig. 2B). Cells incubated with 500 $\mu$m DHA for 60 min prior to H$_2$O$_2$/Cu$^{2+}$ treatment showed a markedly reduced mutation frequency similar to the control level (Fig. 2B). This result indicated that vitamin C inhibits mutagenesis induced by oxidative stress in vitro. Under these conditions, cells with an intracellular concentration of 7 $\mu$m vitamin C were resistant to mutagenesis when treated with 5 mM H$_2$O$_2$ and 100 $\mu$m Cu$^{2+}$.

Glutathione and vitamin C are the most abundant natural antioxidants in human cells, and their functions are partially overlapping (23). We investigated whether vitamin C could protect cells depleted of glutathione under oxidative stress. 293T cells were depleted of glutathione as described (19, 23) and treated with H$_2$O$_2$. Depletion of glutathione substantially increased the toxicity of H$_2$O$_2$. Glutathione-depleted cells did not survive treatment with 5 mM H$_2$O$_2$ and 100 $\mu$m Cu$^{2+}$ for 1 h (data not shown). Cells depleted of glutathione showed a 2- to 3-fold increase in frequency of mutations as compared with unmodified cells (Fig. 2C). To study the role of vitamin C in the prevention of mutagenesis in glutathione-depleted cells, the concentration of H$_2$O$_2$ was lowered to the micromolar level. At 250 $\mu$m H$_2$O$_2$ and 100 $\mu$m Cu$^{2+}$ there was a 7-fold increase in mutation frequency over control (Fig. 2D). Under these conditions a prominent antimutagenic effect of vitamin C was observed in glutathione-depleted cells. Cells incubated with 500 $\mu$m DHA for 60 min prior to oxidative stress had mutation frequencies similar to those of untreated cells (Fig. 2D). As evidenced in the results shown in Fig. 2, antimutagenic effects of vitamin C could be observed in both unmodified and glutathione-depleted cells. Similar results were obtained when the
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There is considerable lay and scientific controversy regarding the role of vitamin C in preventing oxidative DNA damage. Numerous in vitro and in vivo studies have reported on the antioxidant (25–27) and antimutagenic (28, 29) effects of ascorbic acid. Conversely, other studies have shown that under certain conditions, vitamin C functions as a pro-oxidant and can increase DNA damage (30–34). It is well known that ascorbic acid acts as a pro-oxidant in the presence of free transition metals (Cu²⁺, Fe³⁺) (10). Many in vitro experiments with cells have been performed with ascorbic acid with confounding results caused by the generation of H₂O₂ and subsequently *OH via the Fenton reaction (35). Although free transition metals (iron, for example) are essential, iron-dependent reactions are controlled in vivo through sequestration of iron in non-catalytic protein-bound forms (35). Thus, it is generally believed that the antioxidant properties of ascorbate outweigh any pro-oxidant properties in vivo.

Podmore et al. (36) suggested that vitamin C exhibits simultaneous pro-oxidant and antioxidant properties based on in vivo studies. He administered vitamin C as a dietary supplement to healthy volunteers and found that the level of 8-oxo-dG in peripheral blood lymphocytes decreased with supplementation relative to placebo; however, there was a significant increase in 8-oxo-dA levels. These contradictory results are difficult to rationalize. On the other hand, Fraga et al. (25) showed that decreasing the dietary intake of ascorbate elevated the level of 8-oxo-dG in human sperm DNA as measured by HPLC. In another study, Brennan et al. (37) found that oral supplementation with vitamin C in human volunteers decreased H₂O₂-induced DNA damage in isolated human lymphocytes but had no effect on endogenous levels of DNA damage. A recent study by Lee et al. (38) pointed to potential pro-oxidant properties of ascorbic acid based on the decomposition of lipid hydroperoxides induced by vitamin C in vitro independent of free transition metals. Under controlled and extracellular conditions they found that vitamin C can generate 4,5-epoxy-2(E)-decenal (4,5-EDE) from lipid hydroperoxides. Because 4,5-EDE can lead to the generation of etheno-2'-deoxyadenosine, they concluded that vitamin C could cause mutations. These studies, however, did not address the question of whether this reaction occurs inside cells, and limited studies in human volunteers did not support the notion of vitamin C-induced lipid peroxidation (39).

Measurement of 8-oxo-dG is one of the most common methods of assessing DNA damage, but there is no consensus on the actual levels in human DNA. Halliwell (40) states that a common artifact in measuring 8-oxo-dG levels in DNA is artificial oxidation. We directly addressed the role of vitamin C in mutagenesis by developing a quantitative plasmid-based genetic system that allowed for the quantification of oxidatively induced mutations in human cells in vitro. The effect of extracellular ascorbic acid, generating reactive oxygen species in conjunction with free transition metals, was circumvented by loading cells with vitamin C by incubation with DHA (11, 14). Our results directly support the hypothesis that vitamin C protects against oxidative DNA damage in human cells under oxidative stress. This was true for unmodified cells as well as those depleted of glutathione, and the protection was dose-dependent. The prevention of oxidative damage by vitamin C was certain conditions vitamin C functions as a pro-oxidant and can increase DNA damage (30–34). It is well known that ascorbic acid acts as a pro-oxidant in the presence of free transition metals (Cu²⁺, Fe³⁺) (10). Many in vitro experiments with cells have been performed with ascorbic acid with confounding results caused by the generation of H₂O₂ and subsequently *OH via the Fenton reaction (35). Although free transition metals (iron, for example) are essential, iron-dependent reactions are controlled in vivo through sequestration of iron in non-catalytic protein-bound forms (35). Thus, it is generally believed that the antioxidant properties of ascorbate outweigh any pro-oxidant properties in vivo.

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Under physiological conditions vitamin C circulates in the blood in its reduced form, ascorbic acid, at ∼30–50 μM. There is
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Concentrations of ascorbic acid reduce mutations caused by oxidative stress in human cells in vitro and point to a role for vitamin C in preventing DNA mutagenesis in humans.

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