THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 19, Issue of May 10, pp. 16895–16899, 2002
Printed in U.S.A.

Vitamin C Prevents DNA Mutation Induced by Oxidative Stress*

Received for publication, February 4, 2002, and in revised form, March 5, 2002
Published, JBC Papers in Press, March 7, 2002, DOI 10.1074/jbc.M201151200

Eugene A. Lutsenko, Juan M. Cárcamo, and David W. Golde‡

From the Memorial Sloan-Kettering Cancer Center, New York, New York 10021

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 chloramphenicol 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 chloramphenicol 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 H_2O_2. Depletion of glutathione led to cytotoxicity and an increase in H_2O_2-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-guanine measured 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 H_2O_2, O_2^-, and $^{\cdot}OH$ 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)[1] (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 H_2O_2. 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, 1% L-glutamine, and 1% sodium pyruvate. 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% CO_2 at 37 °C. Cells (293) were transfected by Ca^{2+}-phosphate method at 1.5×10^6 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 $CaCl_2$, and 0.8 mM $MgCl_2$). 1.5×10^6 cells were added to incubation buffer containing 100 μM ascorbic acid and 0.2 μCi of L-[^{14}C]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 Ca^{2+}/Mg^{2+}-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 radioactivity (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 ^{3}H-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 Ca^{2+}/Mg^{2+}-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 spec-

* This work was supported by grants from the National Institutes of Health and New York State. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Memorial Sloan-Kettering Cancer Center, 1275 York Ave., New York, NY 10021. Tel.: 212-639-8483; E-mail: d-golde@ski.mskcc.org.

[1] The abbreviations used are: 8-oxo-dG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; DHA, dehydroascorbic acid; AA, ascorbic acid; PBS, phosphate-buffered saline; HPLC, high pressure liquid chromatography; ECD, electrochemical detection; Cm, chloramphenicol; Carb, carbenicillin; Cm^s, Cm-sensitive allele; CAT, Cm acetyltransferase; OMG, 3-oxy-methyl-glucose.

trometry. The amount of radioactivity accumulated inside the cells is in direct proportion to the intracellular volume.

Construction of pCAT19-Cm^s—Tyrosine 33 codon (TAT) within the CAT gene of plasmid pCAT19 was changed to a TAG stop codon by site-directed mutagenesis using the unique restriction site elimination method (18). The change in the sequence was confirmed by sequencing. The following primers were used in mutagenesis: mutagenic primer (amber mutation is *underlined*), GCT CAA TGT ACC TAG AAC CAG ACC GTT CAG C; selection primer (eliminates *Aat*II site, *underlined*), GGT TTC TTA GAC Gta AGG TGG CAC TTT TCG.

Depletion of Glutathione—293T cells were depleted of glutathione as previously described (19). Briefly, 293T cells (2×10^6/100-mm plate) transfected with 6 μg of pCAT19-Cm^S were incubated with 200 μM DL-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 as described below.

Enzymatic Digestion of DNA—Total genomic DNA from 50×10^6 HL-60 cells was isolated using the blood and cell culture kit from Qiagen. The isolated DNA was digested by nuclease P_1 (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 P_1 (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.

Determination of 8-oxo-dG by HPLC-ECD—Digested DNA was injected onto a modified C18 column (catalog no. 70-4106, ESA, Chelmsford, MA). Equal amounts of digested DNA isolated from untreated or treated cells were injected (based on absorbance at 260 nm). The column was equilibrated with the following buffer on the mobile phase (100 mM lithium acetate, 10% methanol, pH 5) at 30 °C. The flow rate was 0.5 ml/min. Under these conditions the retention time of 8-oxo-dG was 4 min. Peak areas for 8-oxo-dG were determined using a dominant potential for 8-oxo-dG of 270 mV. Based on calibration curve (obtained with standard solution of 8-oxo-dG) the amounts of 8-oxo-dG in total genomic DNA were determined.

In Vitro DNA Mutation Assay—One microgram of plasmid pCAT19-Cm^S 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_2O_2 and Cu^{2+} (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 on LB plates supplemented with carbenicillin (Carb) at 50 μg/ml. Plates were incubated at 37 °C for 16 h. Mutation frequencies were determined as ratios between the number of colonies on Cm plates over the total number of colonies on Carb plates.

In Vivo DNA Mutation Assay—293T cells (2×10^6/100-mm plate) transfected with 6 μg of pCAT19-Cm^S were incubated with 500 μM DHA for 1 h and treated with 0.1, 0.5, and 5 mM H_2O_2 with Cu^{2+} (100 μM) or with 10 mM H_2O_2 without Cu^{2+} 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 (0.3×10^6/ml) were incubated for 2 days prior to the experiment. Cells were washed in PBS, incubated with different amounts of DHA for 1 h, and treated with H_2O_2/Cu^{2+} for 22 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^S) 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 by site-directed mutagenesis at codon 33 from TAT (tyrosine) to TAG (stop) as outlined under "Materials and Methods." The resulting plasmid (pCAT19-Cm^S) expresses a truncated chloramphenicol acetyl transferase; therefore, bacterial cells with this allele are sensitive to chloramphenicol. 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

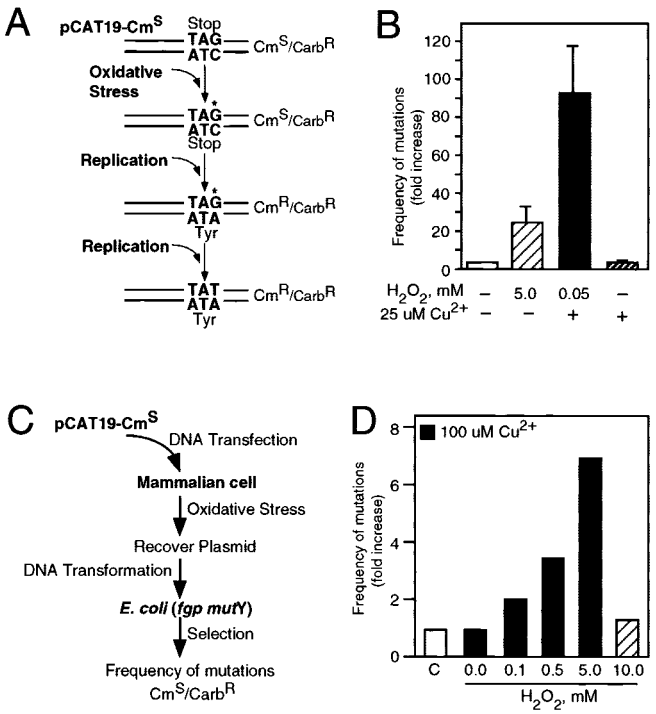

FIG. 1. **Mutations induced by H_2O_2 in naked DNA and 293T human kidney cells.** *A*, conversion of 2′-deoxy-guanine to 8-oxo-dG by oxidation. A plasmid containing the CAT-sensitive allele, pCAT19-Cm^S, was constructed by introducing the amber TAG mutation at codon 33 of the CAT gene. Oxidation of dG into 8-oxo-dG (*) during DNA replication introduces 50% of G to T transversions generating functional CAT gene. *B*, frequency of mutations induced by hydrogen peroxide in naked DNA. The plasmid pCAT19-Cm^S was incubated with 5 mM H_2O_2 or with 50 μM H_2O_2 and 25 μM Cu^{2+}. The plasmid was transformed into the *E. coli* strain PR195, and the frequency of mutations was calculated as the ratio of colonies Cm^R/Carb^R. Results are given as fold increase over mutation frequency of untreated DNA. The absolute mutation frequency in untreated DNA was about 4×10^{-5}. Values represent the mean ± standard deviation from triplicate measurements. The data are statistically significant at $p < 0.05$ using the Student's *t* test. *C*, schematic representation of the oxidative DNA damage assay in human cells. *D*, 293T cells transfected with pCAT19-Cm^S were either left untreated (*c*), incubated with H_2O_2, or incubated with H_2O_2 and Cu^{2+} for 1 h. The plasmid was extracted and electroporated into PR195. The frequency of mutations was determined by the ratio of colonies Cm^R/Carb^R. Results are given as fold increase over mutation frequency of DNA isolated from untreated cells.

Cm^S allele. Subsequent replication introduced adenine across the 8-oxo-dG. The second round of replication restored the wild type TAT codon in 50% of the replicated DNA (7) (Fig. 1*A*), and consequently those cells became resistant to Cm. Thus, the degree of oxidative DNA damage was expressed as a function of Cm-resistant colonies that appeared over Carb-resistant colonies. Mutations outside the TAG stop codon in the mutated CAT gene were not detected by this assay. The probability of two mutations occurring in the same plasmid (as two independent events) is approximately 10^{-9} and is beyond the detection sensitivity of the assay.

To test the plasmid for the induction of mutations by oxidative stress, pCAT19-Cm^S was incubated with either 5 mM H_2O_2 or 25 μM Cu^{2+} as well as with 50 μM H_2O_2 in the presence of 25 μM Cu^{2+} for 1 h at 37 °C. Treated DNA was electroporated into *Escherichia coli* strain PR195 (*fpg mut*Y) along with untreated DNA as a control. The PR195 strain was deficient in the removal of both 8-oxo-dG (*fpg*) (21) and adenines across 8-oxo-dG (*mut*Y) (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

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 4×10^{-5}. DNA treated with 5 mM H_2O_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. 1*B*). Copper ions alone, however, did not induce mutagenesis (Fig. 1*B*). 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^S has no mammalian origin of replication and therefore cannot be replicated inside 293T cells. Cells were transfected with pCAT19-Cm^S and were treated with H_2O_2 24 h later (Fig. 1*C*). 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_2O_2 in the presence of 100 μM Cu^{2+} (Fig. 1*D*). The addition of Cu^{2+} ions presumably led to increased oxidation and G to T transversion mutations in the plasmid due to $^{\cdot}OH$ generation via the Fenton reaction. Cells treated with 5 mM H_2O_2 and 100 μM 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. 1*D*). Hydrogen peroxide without added copper did not cause a significant increase in mutations under these conditions even at concentrations of 10 mM (Fig. 1*D*). We therefore used 5 mM H_2O_2 and 100 μM 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 facilitative glucose transporters (11). Once inside the cell DHA is rapidly reduced to ascorbic acid (14). We found that 293T cells incubated with 500 μM DHA for 60 min accumulated 7 nmol of ascorbic acid/10^6 cells (Fig. 2*A*). We estimated the internal volume of 293T cells as 1.0 μl/10^6 cells from tritiated methylglucose equilibrium studies (see "Materials and Methods"). Based on this internal volume 293T cells incubated with 500 μM DHA for 60 min accumulated 7 mM ascorbic acid. However, cells incubated with 500 μM AA for 60 min accumulated only 0.7 mM ascorbic acid (Fig. 2*A*).

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. 2*B*). Cells incubated with 500 μM DHA for 60 min prior to H_2O_2/Cu^{2+} treatment showed a markedly reduced mutation frequency similar to the control level (Fig. 2*B*). This result indicated that vitamin C inhibits mutagenesis induced by oxidative stress *in vitro*. Under these conditions, cells with an intracellular concentration of 7 mM vitamin C were resistant to mutagenesis when treated with 5 mM H_2O_2 and 100 μM 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_2O_2. Depletion of glutathione substantially increased the toxicity of H_2O_2. Glutathione-depleted cells did not survive treatment with 5 mM H_2O_2 and 100 μM Cu^{2+} for 1 h (data not shown). Cells depleted of glutathione showed a 2- to

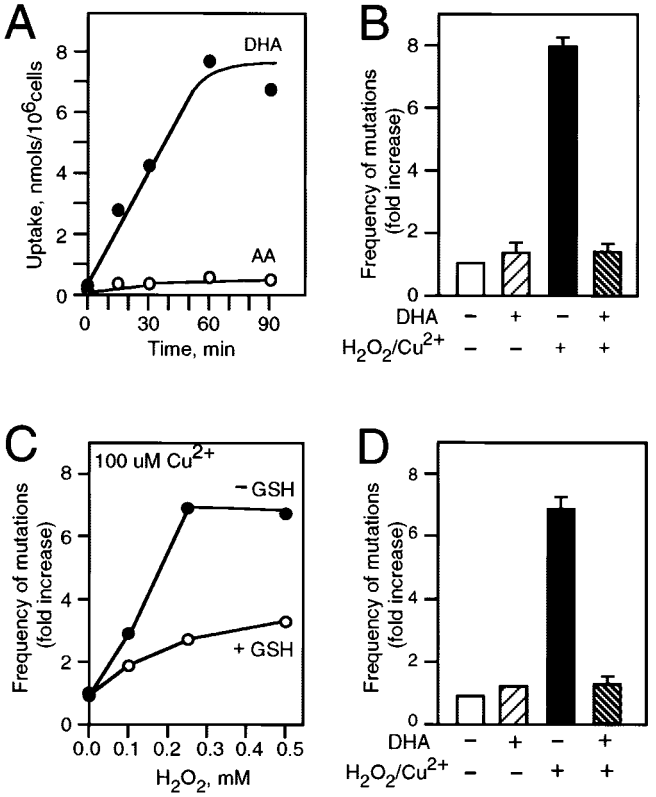

FIG. 2. **Vitamin C protects DNA from mutations induced by oxidation in normal and glutathione-depleted cells.** *A*, uptake of AA and DHA by human 293T kidney cells. Cells were incubated with either radiolabeled AA or radiolabeled DHA for the periods indicated. Cells were washed, and the incorporated radioactivity was determined. Results are shown as accumulation in nmol of vitamin C/10^6 cells. *B*, frequency of mutations in human 293T kidney cells. Cells transfected with pCAT19-Cm^S were incubated with 500 μM DHA for 60 min followed by treatment with 5 mM H_2O_2 and 100 μM Cu^{2+} for 1 h. The mutation frequencies were resolved in *E. coli* as outlined in the legend to Fig. 1*C*. Results are shown as fold increase over mutation frequency of DNA isolated from untreated cells. Values represent the mean ± standard deviation from triplicate measurements. Results are statistically significant at $p < 0.05$ using the Student's *t* test. *C*, 293T cells transfected with pCAT19-Cm^S were depleted of glutathione and incubated with 100 μM Cu^{2+} and H_2O_2 for 1 h. The mutation frequencies were resolved in *E. coli* as outlined in the legend to Fig. 1*C*. Results are shown as fold increase over mutation frequency of DNA isolated from H_2O_2 untreated cells (*closed circles* represent cells depleted of glutathione, and *open circles* represent cells with glutathione). *D*, frequency of mutations in glutathione-depleted human 293T kidney cells preloaded with DHA. The transfected cells were pre-incubated with 500 μM DHA for 60 min followed by treatment with 250 μM H_2O_2 and 100 μM Cu^{2+} for 1 h. The mutation frequencies were resolved in *E. coli* as outlined in the legend to Fig. 1*C*. Results are shown as fold increase over mutation frequency of DNA isolated from untreated cells. Values represent the mean ± standard deviation from triplicate measurements. Results are statistically significant at $p < 0.05$ using the Student's *t* test.

3-fold increase in frequency of mutations as compared with unmodified cells (Fig. 2*C*). To study the role of vitamin C in the prevention of mutagenesis in glutathione-depleted cells, the concentration of H_2O_2 was lowered to the micromolar level. At 250 μM H_2O_2 and 100 μM Cu^{2+} there was a 7-fold increase in mutation frequency over control (Fig. 2*D*). Under these conditions a prominent antimutagenic effect of vitamin C was observed in glutathione-depleted cells. Cells incubated with 500 μM DHA for 60 min prior to oxidative stress had mutation frequencies similar to those of untreated cells (Fig. 2*D*). 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

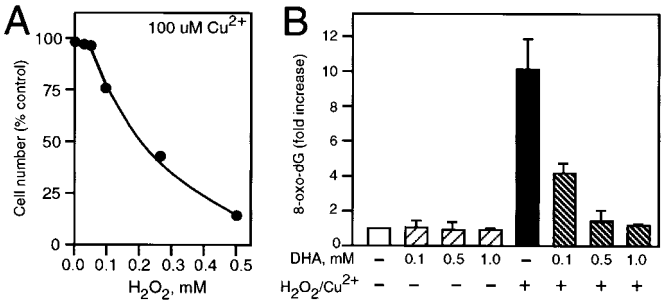

FIG. 3. **Vitamin C protects genomic DNA from oxidation.** *A*, HL-60 cells were treated with different concentrations of H_2O_2 in the presence of 100 μM Cu^{2+} for 22 h, and the cell number was determined. Cell viability is presented as a percentage of survivors over control untreated cells. *B*, HL-60 cells were incubated with different concentrations of DHA for 1 h and treated with 0.1 mM H_2O_2 in the presence of 100 μM Cu^{2+} for 22 h. The total genomic DNA was isolated and digested with nuclease P_1 and calf alkaline phosphatase. Samples were separated by HPLC, and areas of the peaks corresponding to 8-oxo-dG were determined by coulometric detection. Results are shown as fold increase over the basic amount of 8-oxo-dG in control DNA isolated from untreated HL-60. The absolute level of 8-oxo-dG was approximately 50 μmol of 8-oxo-dG/mol of G. Values represent the mean ± standard deviation from triplicate measurements. The results are statistically significant at $p < 0.05$ using the Student's *t* test.

glutathione-depleted cells were treated with 500 μM H_2O_2 100 μM Cu^{2+} (data not shown).

Because generation of 8-oxo-dG on the plasmid DNA induces G to T transversions, we sought to determine whether vitamin C directly inhibits formation of 8-oxo-dG in genomic DNA. A time course analysis demonstrated that to generate 8-oxo-dG in genomic DNA by oxidative stress, prolonged incubation for at least 20 h with H_2O_2 and Cu^{2+} was necessary (data not shown). We chose HL-60 for these studies because 293T cells could not survive prolonged incubation with H_2O_2 and Cu^{2+}. HL-60 cells were incubated with DHA for 1 h and treated with 100 μM H_2O_2/100 μM Cu^{2+} for 22 h. Cells treated under these conditions expressed ~75% viability (Fig. 3*A*).

In oxidatively stressed cells there was a 10-fold increase in 8-oxo-dG over the control. Cells incubated with either 500 μM or 1 mM DHA for 60 min prior to oxidative stress showed a complete inhibition in formation of 8-oxo-dG (Fig. 3*B*). This protective effect of vitamin C loading was dose-dependent. 100 μM DHA reduced 8-oxo-dG by ~50% (Fig. 3*B*). Cells incubated with 0.1, 0.5, or 1.0 mM DHA showed no increase in 8-oxo-dG (Fig. 3*B*). Although dead cells are not excluded by the assay, and vitamin C could protect cells from death caused by oxidative challenge, such an effect would not change the conclusions based on 8-oxo-dG measurements.

The viability of the HL-60 cells treated with 1 mM DHA for 60 min was greater then 95%, as measured by trypan blue exclusion. We determined previously that in HL-60 cells uptake of DHA proceeds in a near linear fashion for about 30 min with no saturation by 60 min (14). The lack of toxicity to DHA may relate to its rapid uptake and conversion intracellularly to ascorbic acid. Quantification of 8-oxo-dG in genomic DNA revealed that cells treated with H_2O_2 contained 0.53 mmol of 8-oxo-dG/mol of G. Background levels of 8-oxo-dG in HL-60 cells were 0.053 mmol of 8-oxo-dG/mol of G (or 1 8-oxo-dG/19,000 G), which is higher than previously reported (1). The degree of protection against oxidative stress in HL-60 cells correlated to the quantity of vitamin C present inside the cells. We determined the intracellular volume of HL-60 as 0.6 μl per million of cells (see "Materials and Methods"). Based on DHA uptake studies (24) we found that HL-60 cells that were incubated with 100 μM, 500 μM, and 1 mM DHA for 60 min accumulated 3, 8, and 12 mM ascorbic acid, respectively.

DISCUSSION

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 showed 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 (Cu^{2+}, Fe^{3+}) (10). Many *in vitro* experiments with cells have been performed with ascorbic acid with confounding results caused by the generation of H_2O_2 and subsequently $^{\cdot}OH$ 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 uptake 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 H_2O_2-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 general in nature, inhibiting the creation of 8-oxo-dG in plasmid DNA as well as genomic DNA. Thus, direct measurement of 8-oxo-dG in genomic DNA confirmed the results of the genetic assay.

Under physiological conditions vitamin C circulates in the blood in its reduced form, ascorbic acid, at ~30–50 μM. There is

a wide range of intracellular concentrations of vitamin C in human cells and tissues. Levine *et al.* (39) found that lymphocytes accumulate 3.5 mM ascorbic acid at oral intakes of 1,000 mg of vitamin C daily (41). Under similar conditions mononuclear leukocytes were reported to accumulate 3.5–6 mM ascorbic acid (39, 41, 42). Our data show that high intracellular 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.

Acknowledgments—The PR195 strain was provided by Dr. Pablo Radicella (Centre National de la Recherche Scientifique, Fontenay-aux-Roses, France). The plasmid pCAT19 was provided by W. Claiborne Fuqua (Indiana University). We thank Dr. Jerard Hurwitz for a critical review of the manuscript.

REFERENCES

1. Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 7915–7922
2. Sonntag, C. V. (1987) *The Chemical Basis of Radiation Biology*, Taylor & Francis, London
3. Wagner, J. R., Hu, C. C., and Ames, B. N. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 3380–3384
4. Dizdaroglu, M. (1985) *Biochemistry* **24,** 4476–4481
5. Kasai, H. (1997) *Mutat. Res.* **387,** 147–163
6. Shibutani, S., Takeshita, M., and Grollman, A. P. (1991) *Nature* **349,** 431–434
7. Wood, M. L., Dizdaroglu, M., Gajewski, E., and Essigmann, J. M. (1990) *Biochemistry* **29,** 7024–7032
8. Schneider, M., Diemer, K., Engelhart, K., Zankl, H., Trotter, W. E., and Biesalski, H. K. (2001) *Free Radic. Res.* **34,** 209–219
9. Lee, B. M., Lee, S. K., and Kim, H. S. (1998) *Cancer Lett.* **132,** 219–227
10. Cai, L., Koropatnick, J., and Cherian, M. G. (2001) *Chem. Biol. Interact.* **137,** 75–88
11. Vera, J. C., Rivas, C. I., Fischbarg, J., and Golde, D. W. (1993) *Nature* **364,** 79–82
12. Rumsey, S. C., Daruwala, R., Al-Hasani, H., Zarnowski, M. J., Simpson, I. A., and Levine, M. (2000) *J. Biol. Chem.* **275,** 28246–28253
13. Tsukaguchi, H., Tokui, T., Mackenzie, B., Berger, U. V., Chen, X. Z., Wang, Y., Brubaker, R. F., and Hediger, M. A. (1999) *Nature* **399,** 70–75
14. Vera, J. C., Rivas, C. I., Velasquez, F. V., Zhang, R. H., Concha, I. I., and Golde, D. W. (1995) *J. Biol. Chem.* **270,** 23706–23712
15. Pear, W. S., Nolan, G. P., Scott, M. L., and Baltimore, D. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90,** 8392–8396
16. Vera, J. C., Rivas, C. I., Zhang, R. H., Farber, C. M., and Golde, D. W. (1994) *Blood* **84,** 1628–1634
17. May, J. M., Mendiratta, S., Qu, Z. C., and Loggins, E. (1999) *Free Radic. Biol. Med.* **26,** 1513–1523
18. Zhu, L. (1996) *Methods Mol. Biol.* **57,** 13–29
19. Guaiquil, V. H., Farber, C. M., Golde, D. W., and Vera, J. C. (1997) *J. Biol. Chem.* **272,** 9915–9921
20. Sambrook, J., Maniatis, T., and Fritsch, E. F. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Vol. 1 pp. 1.25–1.29, Cold Spring Harbor, New York
21. Tchou, J., Kasai, H., Shibutani, S., Chung, M. H., Laval, J., Grollman, A. P., and Nishimura, S. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 4690–4694
22. Tsai-Wu, J. J., Liu, H. F., and Lu, A. L. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 8779–8783
23. Guaiquil, V. H., Vera, J. C., and Golde, D. W. (2001) *J. Biol. Chem.* **276,** 40955–40961
24. Rivas, C. I., Vera, J. C., Guaiquil, V. H., Velasquez, F. V., Borquez-Ojeda, O. A., Carcamo, J. G., Concha, I. I., and Golde, D. W. (1997) *J. Biol. Chem.* **272,** 5814–5820
25. Fraga, C. G., Motchnik, P. A., Shigenaga, M. K., Helbock, H. J., Jacob, R. A., and Ames, B. N. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 11003–11006
26. Frei, B., Stocker, R., and Ames, B. N. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 9748–9752
27. Frei, B., England, L., and Ames, B. N. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 6377–6381
28. Krinsky, N. I. (1993) *Ann. N. Y. Acad. Sci.* **686,** 229–242
29. Sweetman, S. F., Strain, J. J., and McKelvey-Martin, V. J. (1997) *Nutr. Cancer* **27,** 122–130
30. Galloway, S. M., and Painter, R. B. (1979) *Mutat. Res.* **60,** 321–327
31. Rosin, M. P., San, R. H., and Stich, H. F. (1980) *Cancer Lett.* **8,** 299–305
32. Speit, G., Wolf, M., and Vogel, W. (1980) *Mutat. Res.* **78,** 273–278
33. Stich, H. F., Karim, J., Koropatnick, J., and Lo, L. (1976) *Nature* **260,** 722–724
34. Stich, H. F., Wei, L., and Whiting, R. F. (1979) *Cancer Res.* **39,** 4145–4151
35. Halliwell, B., and Gutteridge, J. M. C. (1999) *Free Radicals in Biology and Medicine*, 3rd Ed. pp. 48–54, Oxford University Press, Oxford
36. Podmore, I. D., Griffiths, H. R., Herbert, K. E., Mistry, N., Mistry, P., and Lunec, J. (1998) *Nature* **392,** 559
37. Brennan, L. A., Morris, G. M., Wasson, G. R., Hannigan, B. M., and Barnett, Y. A. (2000) *Br. J. Nutr.* **84,** 195–202
38. Lee, S. H., Oe, T., and Blair, I. A. (2001) *Science* **292,** 2083–2086
39. Levine, M., Wang, Y., Padayatty, S. J., and Morrow, J. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 9842–9846
40. Halliwell, B. (2000) *Am. J. Clin. Nutr.* **72,** 1082–1087
41. Levine, M., Conry-Cantilena, C., Wang, Y., Welch, R. W., Washko, P. W., Dhariwal, K. R., Park, J. B., Lazarev, A., Graumlich, J. F., King, J., and Cantilena, L. R. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 3704–3709
42. Bergsten, P., Amitai, G., Kehrl, J., Dhariwal, K. R., Klein, H. G., and Levine, M. (1990) *J. Biol. Chem.* **265,** 2584–2587