Glutathione and ascorbate are negatively correlated with oxidative DNA damage in human lymphocytes

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Intracellular antioxidants, glutathione and ascorbate, and two molecular markers of oxidative DNA damage, 5-hydroxy-2’-deoxycytidine (5-OH-dCyd) and 8-oxo-7,8-dihydro-2’-deoxyguanosine (8-oxo-dGuo), were measured in lymphocytes from 105 healthy volunteers. The analysis of 5-OH-dCyd and 8-oxo-dGuo was carried out by HPLC with electrochemical detection such that both compounds were detected on the same chromatography run. There was no significant difference in oxidative DNA damage when the extraction of DNA from cells using phenol was carried out under anaerobic conditions or in the presence of metal ion chelators. This indicates that auto-oxidation of DNA during sample preparation was minimal. Using the above methods, the average level of oxidative DNA damage in lymphocytes was 2.9 ± 1.4 for 5-OH-dCyd and 4.5 ± 1.8 for 8-oxo-dGuo lesions per 10⁶ dGuo (n = 105). It is unlikely that artificial oxidation contributed to the observed damage because the level of 5-OH-dCyd was comparable with that of 8-oxo-dGuo in lymphocyte DNA, whereas 8-oxo-dGuo outnumbers 5-OH-dCyd by a ratio of >5:1 when DNA is exposed to various oxidants, including ionizing radiation or Fenton reagents. Rather, the nearly equal levels of 5-OH-dCyd and 8-oxo-dGuo in cellular DNA implies that 8-oxo-dGuo may be more efficiently removed by DNA repair. Finally, and most importantly, the correlation of our endpoints revealed that the naturally occurring level of intracellular antioxidants was negatively correlated to the level of oxidative DNA damage with the strongest correlation observed for glutathione and 8-oxo-dGuo (r = −0.36; P < 0.001). These results strongly suggest that intracellular glutathione and ascorbate protect human lymphocytes against oxidative DNA damage.

Introduction

Free radicals and oxidants are generated by diverse biological processes that include oxidative phosphorylation, phagocytosis, fatty acid metabolism and the synthesis and degradation of biomolecules (1). The status of oxidants and antioxidants in part regulates vital processes including cell proliferation and apoptosis (2–4). However, the reaction of free radicals and oxidants with lipid, protein and DNA produces potentially harmful damage. In particular, oxidative DNA damage probably largely contributes to aging and cancer (5).

The formation of endogenous oxidative DNA damage in cells involves a Fenton reaction that requires two basic components: H₂O₂ and metal ions (e.g. Fe²⁺ or Cu⁺). The resulting damaging species of this reaction appear to be hydroxyl radicals, although metal ion peroxide complexes cannot be ruled out (6). On the basis of model studies with ionizing radiation, the reaction of hydroxyl radicals with nucleic acids leads to a multitude of base and sugar modifications (7,8). In a biological setting, DNA damaging Fenton reactions must occur in close proximity to DNA because hydroxyl radicals react rapidly with nearly all biological compounds and thus do not diffuse very far from their site of generation. Therefore, the formation of oxidative DNA damage in cells is expected to depend on the rate of production of free radicals and oxidants, the probability that these species diffuse to DNA and the movement of reactive metal ions from cellular sources to DNA. Additionally, free radicals and oxidants induce the release of metal ions, which in turn generate more reactive species. For instance, during oxidative stress, reactive iron is liberated from heme-containing compounds, iron storage proteins and proteins containing iron-sulfur clusters, and this can contribute to oxidative damage (9,10).

The majority of biological studies dealing with oxidative DNA damage have focused on 8-oxo-7,8-dihydro-2’-deoxyguanosine (8-oxo-dGuo), a major oxidation product of dGuo in DNA (11). This modification probably contributes to spontaneous mutagenesis because enzymes with similar substrate specificity appear to function in the removal of 8-oxo-dGuo in bacteria, yeast and mammalian cells, and disruption of this repair system leads to a specific mutator phenotype (12,13). In comparison, the biological consequences of 5-hydroxy-2’-deoxycytidine (5-OH-dCyd), which arises from the oxidation of dCyd in DNA, are not clearly understood. The yield of 5-OH-dCyd is at least 5-fold lower than that of 8-oxo-dGuo when DNA is exposed to various oxidants (14). DNA containing 5-OH-dCyd is a substrate for endonuclease III and VIII and probably for homologous enzymes in yeast and mammalian cells (15,16). Previous studies suggested that 5-OH-dCyd is potentially mutagenic because it mismatches during DNA synthesis with synthetic oligonucleotides using polymerase I Klenow fragment (17) and causes C→T transitions (2.5%) when incorporated into bacteria (18). In contrast, a recent study (19) suggested that the frequency of mutations for 5-OH-dCyd is relatively low in bacteria (0.05%). It should also be noted that mammalian cells can tolerate a level of nuclear 5-OH-dCyd that is 20-fold above baseline without any adverse effects with respect to cell growth, which suggests that this lesion is not very genotoxic (20). Thus, the potential mutagenesis of 5-OH-dCyd lesions remains to be established. Nevertheless, 5-OH-dCyd may be considered as a marker of oxidative DNA damage related to mutagenesis because its formation and DNA repair are similar in many ways to the formation

Abbreviations: EC, electrochemical detection; FBS, fetal bovine serum; GC–MS, gas chromatography–mass spectrometry; ODS, octadecylsilyl; 5-OH-dCyd, 5-hydroxy-2’-deoxycytidine; 8-oxo-dGuo, 8-oxo-7,8-dihydro-2’-deoxyguanosine; PBS, phosphate-buffered saline.

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and DNA repair of 5-OH-dUrd and dUrd 5.6-glycols. The latter lesions are highly mutagenic since, in contrast to 5-OH-dCyd, they have lost the exocyclic amino group that is involved in base pairing with G in duplex DNA.

Epidemiological studies consistently point to an inverse association for the consumption of fruits and vegetables and cancer incidence, an effect that can be attributed to a variety of anticarcinogenic ingredients including dietary fiber, folic acid, vitamin A and various antioxidants (vitamin C, vitamin E, carotenoids, selenium) (21). The majority but not all of epidemiological studies support a beneficial effect of antioxidants micronutrients (22,23). In the present study, we report on the analysis of intracellular antioxidants, including glutathione and ascorbate, and oxidative DNA damage, including 8-oxo-dGua and 5-OH-dCyd, as measured from freshly separated lymphocytes obtained from 105 healthy subjects. The results reveal an inverse correlation between intracellular antioxidants and oxidative DNA damage in human lymphocytes.

Materials and methods

Water was extensively purified first by double distillation through a Fi-stream II glass still and then by ion exchange and filtration through an Easypure RF water system equipped with ultrapure and high purity Low Toc cartridges giving a final resistance of no less than 18.3 MΩ cm (Barnstead Thermolyne, Dubuque, IA). The buffers were made from chemicals with the highest available purity. Phosphate-buffered saline (PBS) was treated with 1 g/l of chlex 100 (Bio-Rad, Richmond, CA) and filtered before use.

The criteria for acceptance of subjects into our study were that they did not smoke, nor take medication or antioxidant supplements, and generally, the subjects were required to be in good health as ascertained during an interview. The average age of the group was 43 years old with a range of ages from 20 to 86 years. The group consisted of 68 women and 37 men. Blood donations were collected by venipuncture in the morning from fasting individuals using five 20 ml vacutainer tubes that contained citrate buffer as anticoagulant (total 100 ml). The lymphocytes were immediately isolated from the blood by techniques used in general-practice today by immunologists (24). First, blood was divided into three fractions (~30 ml each) into 50 ml Falcon tubes, and the tubes were centrifuged at 300 g for 15 min to remove plasma. The plasma was deproteinized by the addition of CaCl₂ (33 mM, final concentration), coated onto 250 ml Falcon tissue culture flasks, and incubated for at least 30 min before used for the removal of monocytes (below). To the blood sample, 10–15 ml of PBS (completing the volume to 30 ml) and 15 ml of a solution of 2% dextran were added, and the sample was mixed thoroughly. The erythrocytes were allowed to sediment out for 30 min at 37°C. The resulting cell suspension was layered onto Ficol-Hypaque (Pharmacia Biotech Uppsala, Sweden), with a density of 1.077, and centrifuged at 900 g for 30 min at 4°C. The mononuclear layer was collected and the cells were washed twice with RPMI (Roswell Park Memorial Institute) media (Gibco BRL, Grand Island, NY) at 300 g for 15 min. The cells (lymphocytes and monocytes) were then incubated at 37°C in RPMI media with 10% fetal bovine serum (FBS) (ICN, Costa Mesa, CA) for 50 min using plasma coated flasks (above) and the lymphocytes were removed from the monocytes that remained attached to the surface of the flask. The resulting lymphocytes were <90% viable as determined by trypan blue exclusion. In addition, it should be noted that there was no indication of hemolysis throughout purification and there was no visible trace of red blood cells in the final preparation of lymphocytes.

Ten million lymphocytes were washed with PBS and nuclei buffer solution (0.25 M sucrose, 10 mM EDTA and 1 mM CaCl₂) and then lysed with 0.2% Triton X-100 in the latter buffer (10 min at 4°C). The nuclei were spun down at 2000 g and stored at ~80°C. To extract DNA, the nuclei were first treated with 0.8 mg/ml proteinase K (Boehringer #745 723, Indianapolis, IN) for 1 h at 37°C in lysis buffer composed of urea (8 M), NaCl (0.4 M), Tris-Cl (0.2 M; pH 7.9), Na-laurolylsarcosine (1%) and CaCl₂–EDTA (20 mM) (Applied Biosystems #743 876). The DNA sample was purified by liquid–liquid extraction with a mixture of ultrapure phenol, chloroform and water (80:10:10) (Applied Biosystems #743 125, Foster City, CA). After the addition of 0.3 M (pH 4.5) acetate buffer (Aldrich #38,012-1, Milwaukee, WI) and an equal volume of cold HPLC grade isopropanol (~15°C), the mixture was held at ~15°C for 1 h and then centrifuged at 10 000 g for 30 min at 4°C. DNA was recovered and subsequently dried and stored at ~80°C until analysis.

Intracellular antioxidants glutathione and ascorbate were measured according to the method of Rose and Bode (25). Ten million cells were washed three times with ice-cold chlex treated PBS, suspended in 200 mM phosphate buffer pH 2 containing 0.1 mM EDTA, and subjected to three freeze–thaw cycles (4 to ~80°C). Glutathione and ascorbtime were separated by HPLC using an octadecylsilil (ODS) Inertsil 5 µm 150×4.6 mm internal diameter column (Supelco, Bellefonte, PA) with 200 mM phosphate buffer (pH 3.0). The HPLC system consisted of a M6000 pump (Waters, Milford, MA) and a L-EC96A amperometric electrochemical detector (Shimadzu, Kyoto, Japan) set at 1.1 V versus AgCl reference electrode. The concentration of protein in cell lysates was determined by the Bradford assay (Bio-Rad), which was automated using an AS 3000 autosampler (Hitachi, Tokyo, Japan) and a U 3000 spectrophotometer (Hitachi). The standards were stored at ~80°C and the same ones were used for the entire study.

Oxidative DNA damage was measured by HPLC with electrochemical and UV detection using a similar procedure as described (14). Samples were processed in sets of 12. Between 40 and 60 µg samples of DNA were dissolved in 60 µl of acetic buffer (10 mM, pH 4.5; Aldrich #38,012-1) and digested down to deoxyribonucleosides, first by treatment with Nuclease P1 (10 U, Pharmacia Biotech #27-0852-01) for 20 min at 30°C, and then by treatment with alkaline phosphatase (10 U, Boehringer #405 612) in ammonium acetate buffer (100 mM pH 7.0, Aldrich #37,233-1) for 40 min at 37°C. The amount of Nuclease P1 was in excess of that required to completely release 8-oxo-dGuo and 5-OH-dCyd from freshly extracted DNA. After digestion, the buffer was adjusted to pH 5.5 by the addition of 5 µl 0.2 M phosphoric acid. The samples were transferred into glass 150 µl inserts and immediately frozen at ~80°C. They were analyzed on the same day of digestion in two groups of six samples. Before analysis, the samples were maintained at 4°C inside an autosampler. Both oxidative lesions, 5-OH-dCyd and 8-oxo-dGuo were quantitated by HPLC on the same chromatography run using a step gradient such that 5-OH-dCyd was eluted at the first 20 ml with 0.1% methanol in phosphate buffer (25 mM, pH 5.5) whereas 8-oxo-dGuo was subsequently eluted in 57 ml with 6.0% methanol in the same buffer. The separation was achieved using an ODS-AQ 5 µm 250×4.6 mm internal diameter column (YMC, Wilmington, NC). The HPLC system consisted of the following modules: 6005 controller, 616 pump, 717 plus autoinjector, 510 UV detector (Waters), and Coulotron II detector equipped with a 5011 analytical cell (ESA Associates, Chelmsford, MA), controlled and operated by millennium 2010 chromatography manager (Waters). In order to optimize the detection of both 8-oxo-dGuo and 5-OH-dCyd, the electrochemical cell was set at 0.05 (cell #1) and 0.35 V (cell #2) versus Pt reference electrode. Oxidative DNA damage was determined from the amount of 5-OH-dCyd or 8-oxo-dGuo divided by the amount of dCyd, measured by electrochemical and UV detection, respectively. Every fourth analysis, synthetic standards were injected to correct for any changes in the response of the electrochemical detector.

For statistical analysis, data were fit to a line using least square regression analysis, with the aid of Microsoft Excel software. The statistical significance was determined using a two-tailed test of the null hypothesis that the true correlation is zero. A probability of <0.05 was considered significant. Grouped data are expressed as the mean ± SD.

Results

The analysis of 8-oxo-dGuo in DNA by HPLC with electrochemical detection (EC) has been widely used as a measure of oxidative DNA damage. Furthermore, this method has been extended to 5-OH-dCyd and 5-hydroxy-2′-deoxyuridine, which are derived from the oxidation of dCyd in DNA exposed to ionizing radiation or various chemical oxidants (14). In the present work, we have optimized the HPLC conditions in order to combine the analysis of 5-OH-dCyd and 8-oxo-dGuo on the same chromatographic run. To achieve adequate separation of both compounds in biological samples, it was necessary to first elute 5-OH-dCyd in phosphate buffer with 0.1% methanol and then change the mobile phase to buffer with 6% methanol to elute 8-oxo-dGuo (Figure 1). The percentage of methanol in the mobile phase was optimized to allow for the electrochemical signal to stabilize after it deflected off-scale because of the change in solvent. Based on comparison of the chromatographic and electrochemical features with standard compounds, we conclude that the peaks corresponding to 5-OH-dCyd and 8-oxo-dGuo do not contain electrochemical
Lymphocytes (n = 105) c
Lymphocytes (n = 5) d
Granulocytes (n = 3) d
Monocytes (n = 4) d
Transformed Jurkat T (n = 5)

Table I. Average levels of intracellular antioxidants and oxidative DNA damage in human cells

<table>
<thead>
<tr>
<th>Antioxidants</th>
<th>Glutathione</th>
<th>Ascorbate</th>
<th>Oxidative DNA damage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>8-oxo-dGuo</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>26.9 ± 7.7</td>
<td>20.9 ± 7.5</td>
<td>4.5 ± 1.8</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>35.3 ± 1.3</td>
<td>28.7 ± 9.1</td>
<td>3.9 ± 1.3</td>
</tr>
<tr>
<td>Granulocytes</td>
<td>27.6 ± 11.5</td>
<td>7.0 ± 3.3</td>
<td>5.5 ± 1.1</td>
</tr>
<tr>
<td>Monocytes</td>
<td>59.6 ± 9.1</td>
<td>31.8 ± 1.5</td>
<td>6.9 ± 2.4</td>
</tr>
<tr>
<td>Jurkat T</td>
<td>25.1 ± 1.8</td>
<td>Not detected</td>
<td>3.9 ± 1.3</td>
</tr>
</tbody>
</table>

a In units of nmol/mg protein.
b In units of lesion/10^6 dGuo residues.
c Taken over a 12 month period.
d Taken from the same group of individuals during the summer.

Impurities. Using this method, we determined the levels of 8-oxo-dGuo and 5-OH-dCyd in the DNA of lymphocytes from 105 healthy subjects (Table I). In addition, the levels of oxidative DNA damage for the different categories of white blood cells are reported in the same individuals as well as the level of damage in Jurkat T cells in culture.

The effect of either removing oxygen or reactive metal ions as mediators of auto-oxidation was examined using Jurkat T cells. To study the effect of oxygen, the entire procedure including cell lysis, DNA extraction and digestion was carried out in a glove-box under N2. DNA samples processed in the glove-box under N2 were analyzed side-by-side with control samples processed in air using HPLC–EC. Thereby, we observed no significant difference of 8-oxo-dGuo in the two samples. The level was 4.4 ± 1.1 (n = 5) 8-oxo-dGuo/10^6 dGuo and 3.9 ± 1.3 (n = 5) 8-oxo-dGuo/10^6 dGuo under deaerated and aerated conditions, respectively. These results indicate that molecular oxygen does not contribute to auto-oxidation of DNA in our samples. Next, we examined the effect of the addition of metal ion chelators to the DNA extraction buffer including desferrioxamine or 1,10-phenanthroline. Again, the level of 8-oxo-dGuo did not go down upon addition of these metal chelators. In fact, the level of 8-oxo-dGuo in cellular DNA appeared to slightly increase. The level was 5.5 ± 1.2 (n = 3) 8-oxo-dGuo/10^6 dGuo with desferrioxamine (100 µM) and 5.4 ± 0.8 (n = 5) 8-oxo-dGuo/10^6 dGuo with 1,10-phenanthroline (5 mM), compared with 3.9 ± 1.3 (n = 5) 8-oxo-dGuo/10^6 dGuo for control samples.

Similarly, removing oxygen or adding metal chelators resulted in no significant changes in the level of 5-OH-dCyd. Together, the above studies indicate that auto-oxidation is reduced to the point that it is no longer dependent on the removal of oxygen or the elimination of free metal ions. Thus, we conclude that auto-oxidation during sample preparation has been minimized. In contrast, Nakajima et al. (26) reported a reduction in the level of 8-oxo-dGuo in leukocyte DNA by 6-fold when the extraction procedure was carried out under anaerobic conditions. Also, the level of 8-oxo-dGuo in cellular DNA has been shown to be affected by the addition of antioxidants or metal chelators under certain conditions. For example, Kvam and Tyrrell (27) reported a decrease in the level of 8-oxo-dGuo in the DNA of primary human skin fibroblasts by ~3-fold when initial cell lysis was carried out in the presence of desferrioxamine, histidine and glutathione. However, auto-oxidation was clearly a problem in the latter studies in view of the high baseline levels of 8-oxo-dGuo in unprotected samples. Thus, it is not surprising that the level of 8-oxo-dGuo will decrease by taking appropriate measures to minimize this problem.

The average level of 8-oxo-dGuo was 4.5 ± 1.8 fmol per 10^6 dGuo and that of 5-OH-dCyd was 2.9 ± 1.4 fmol per 10^6 dGuo for lymphocytes obtained from 105 healthy subjects (Table I). The observed level of 8-oxo-dGuo is comparable with that reported by others using similar methods of DNA digestion and HPLC analysis with EC. In particular, Collins et al. (28) observed a level of 4.3 ± 3.0 (n = 36) 8-oxo-dGuo/10^6 dGuo in lymphocyte DNA. Similarly, Nakajima et al. (29) reported 2.7 ± 1.2 (n = 92) 8-oxo-dGuo/10^6 dGuo whereas Asami et al. (30) reported 3.1 ± 1.6 (n = 10) 8-oxo-dGuo/10^6 dGuo when taking white blood cells as the source of DNA. The lower value of 8-oxo-dGuo reported by Asami et al. compared with our value, may be caused by the extraction procedure. The extraction of DNA using high concentrations of NaI has been shown to reduce the level of 8-oxo-dGuo in the DNA of rat liver by ~50% compared with the common phenol-based method (31). Similarly, we observed lower levels of 8-oxo-dGuo as well as 5-OH-dCyd for the analysis of DNA damage in lymphocytes when comparing our method using phenol with the NaI method using the DNA Extractor WB kit (Wako Pure Chemical Industries, Japan). However, there was an indication from preliminary experiments that NaI or its byproducts destroyed 8-oxo-dGuo and 5-OH-dCyd in DNA during extraction. In particular, NaI extraction of previously extracted calf thymus DNA that contained a pre-existing quantity of damage led to a significant decrease in the level of 8-oxo-dGuo (20%) and 5-OH-dCyd (90%).
The level of 8-oxo-dGuo was 4.5 ± 1.8 for men and 4.6 ± 1.8 for women, whereas the corresponding average level of 5-OH-dCyd was 3.1 ± 1.3 for men and 2.7 ± 1.4 for women in units of lesions per 10^6 dGuo. Interestingly, there was a strong effect of season for the 105 subjects (note that the subjects were recruited over a 12 month period beginning in May). The average level of ascorbate in lymphocytes declined by ~40%, and that of glutathione by 25% from subjects recruited in the winter (November to April) and returned to the initially high level observed in the summer (May to October). The variation with season of ascorbate can probably be attributed to a decrease in the consumption of fruits and vegetables in the winter compared with the summer when these commodities are readily available.

**Discussion**

The level of 8-oxo-dGuo in cellular DNA reported in the literature varies by greater than three orders of magnitude depending on the method used for its determination (28,33). Basically, the problem is that DNA extraction and subsequent manipulation can result in artifactual oxidation of DNA bases mediated by intrinsic oxidants such as H_2O_2 in combination with reactive transition metal ions. This leads to an overestimation of the actual damage (in contrast, damage is much lower in cellular DNA because of antioxidants and DNA repair). The original method of analysis using acid hydrolysis of DNA followed by derivatization and gas chromatography–mass spectrometry (GC–MS) leads to extensive auto-oxidation of DNA bases, apparently during the derivatization step that is performed at high temperature under slightly basic conditions (34). The level of 8-oxo-dGuo (detected as the nucleobase) in extracted lymphocyte DNA using acid hydrolysis and GC–MS is ~60-fold higher than that observed in the present study using enzymatic digestion followed by HPLC–EC analysis (300 lesions compared with 4.5 lesions per 10^6 dGuo; ref. 35).

Recently, it has been shown that the level of 8-oxo-dGuo observed by GC–MS can be reduced to about the same level as that observed by HPLC–EC after taking appropriate precautions, which include pre-purifying the lesions before derivation, removing oxygen and/or adding antioxidants (34,36). Thus, one can conclude that the majority of oxidative DNA damage observed by GC–MS is caused by auto-oxidation, unless the appropriate corrective measures are taken.

It is unlikely that HPLC–EC grossly overestimates the level of 8-oxo-dGuo. Firstly, auto-oxidation is minimal in our study because the removal of oxygen or the addition of metal chelators had no significant effect on the level of 8-oxo-dGuo in cellular DNA. Secondly, it is doubtful that 8-oxo-dGuo and 5-OH-dCyd arise from artifactual oxidation during sample preparation because the ratio with 8-oxo-dGuo:5-OH-dCyd is 1.4 in cellular DNA whereas the ratio is at least 5-fold greater when DNA undergoes oxidation in solution induced by ionizing radiation or Fenton reagents. The nearly equal levels of 8-oxo-dGuo and 5-OH-dCyd in cellular DNA implies that the former may be more efficiently removed by DNA repair. Finally, the measurement of 8-oxo-dGuo in cellular DNA by either the comet assay or the alkali-elution method, in combination with purified DNA repair enzymes, leads to levels of damage that are ~10-fold lower than by HPLC–EC (28,33). The reason for this discrepancy is not clear. Although formamidopyrimidine DNA N-glycosylase (fapy) efficiently removes 8-oxo-dGuo from oxidized DNA, it is not clear whether this...
occurs to the same extent for DNA that contains endogenous damage. A reason why oxidative damage persists in cellular DNA may be because it is partly resistant to removal by DNA repair depending on the specific context or sequence of the damage. For example, it is known that fapy efficiently excises 8-oxo-dGuo from 8-oxo-dGuo-C base pairs in duplex DNA but not from 8-oxo-dGuo-A base pairs, which may persist to some extent in cellular DNA (37). Thus, the comet assay or the alkali elution method with DNA repair enzymes may underestimate 8-oxo-dGuo in cellular DNA.

Whether the separation of lymphocytes from blood has an effect on the level of oxidative DNA damage is difficult to determine. During separation, lymphocytes are exposed to various potentially damaging agents or conditions, including changes in volume and pressure caused by centrifugation, changes in temperature (37–22°C) and changes in oxygen tension, i.e. the concentration of oxygen may be expected to increase 5-fold in going from in vivo to in vitro. Nevertheless, it should be noted that the separation of lymphocytes from blood is not sufficient by itself to induce either proliferation or apoptosis in cell culture (in contrast, granulocytes or monocytes are much more sensitive toward activation). The work of Collins et al. (28) suggests that the transition of going from oxygen tension in vivo to that in vitro has only a minor effect on the level of oxidative DNA damage in lymphocytes. They compared the level of oxidative DNA damage for lymphocytes isolated from blood taken before and 2 h after consumption of 1 g of vitamin C on the hypothesis that an increased level of antioxidants would protect lymphocytes against oxidative stress in vitro. However, the data showed no significant difference in the initial level of either single strand breaks or oxidized pyrimidines estimated by the comet assay, although there were noticeable changes in the initial level of H2O2-induced damage and the subsequent removal of this damage. Another important point is whether the incubation of lymphocytes in culture has an effect on oxidative DNA damage. The final step in the separation of lymphocytes from blood involves the removal of monocytes, which requires the incubation of cells in culture for 50 min. However, it is doubtful that such a short period of incubation has any effect on oxidative DNA damage since there is no observable effect on damage even for lymphocytes in culture for as long as 24 h on the basis of single strand breaks or oxidized pyrimidines measured by the comet assay, as well as fapy-sensitive sites measured by the alkali elution method (28,33). In contrast, primary lymphocytes in cell culture for 18–24 h are more vulnerable to H2O2-induced DNA damage (38). The effects at long incubation times though can probably be attributed to a dramatic drop in the activity of several antioxidant enzymes, glutathione reductase, glutathione peroxidase and catalase. Interestingly, the addition of antioxidants, particularly flavonoids, to primary lymphocytes in cell culture significantly inhibits the formation of oxidative DNA damage as measured by the comet assay (38,39). This indicates that antioxidants are able to protect lymphocytes against H2O2-induced oxidative DNA damage.

The levels of intracellular glutathione and ascorbate as well as oxidative DNA damage were different in lymphocytes, granulocytes and monocytes (Table I). In particular, the level of 8-oxo-dGuo in granulocytes and monocytes was significantly higher than that in lymphocytes. This may be caused in part by the activation and increased oxidant production of granulocytes and monocytes during their separation from blood. However, the ratio of 8-oxo-dGuo:5-OH-dCyd was different in all categories of white blood cells, which may possibly reflect differences in DNA repair between these cell types. Intriguingly, there was no apparent correlation between the levels of glutathione and ascorbate with oxidative DNA damage in these cells. For example, the level of glutathione was 70% higher in monocytes than in lymphocytes whereas the former showed slightly higher levels of 8-oxo-dGuo. Also, it was surprising that Jurkat T cells in culture have about the same level of nuclear 8-oxo-dGuo as primary lymphocytes whereas the former have no detectable ascorbate. These results underline the importance of separating white blood cells into their different major groups in order to evaluate antioxidants and oxidative DNA damage.

Although the interaction of antioxidants and oxidative DNA damage is complex, it is reasonable to assume that glutathione and ascorbate play a role in neutralizing free radicals and oxidants that cause DNA damage, because they are present inside cells at relatively high concentrations and they react efficiently with reactive oxygen species. The ability of these antioxidants to protect against oxidative DNA damage has been shown in various model systems (11). For example, the administration of ascorbate has been shown to reduce oxidative damage in kidney DNA induced by potassium bromate (40). Ascorbate also reduces damage in liver DNA after treatment with redox-cycling estradiol derivatives (41). Contrastingly, Cadens et al. observed no change in the level of 8-oxo-dGuo in the DNA of rat liver despite a 60-fold diet-induced variation in the level of ascorbate in the same organ (42). The notion that glutathione protects against oxidative DNA damage in rodents is shown by the effect of buthionine sulfoximine, which reduces intracellular glutathione and significantly increases 8-oxo-dGuo in various organs (43). Also, an exceptional study by Garcia de la Asuncion et al. (44) showed that the ratio of oxidized to reduced glutathione in mitochondria was strongly correlated with the level of 8-oxo-dGuo in mitochondrial DNA and that the administration of vitamin C and vitamin E reversed the levels of oxidative damage for both glutathione and DNA in aged rats to the levels observed in young rats.

In humans, there have also been a number of interesting studies directly linking antioxidants to oxidative DNA damage. For example, dietary supplementation with a cocktail of vitamin C (0.1 g/day), vitamin E (0.28 g/day) and beta-carotene (0.025 g/day) significantly reduced oxidative DNA damage in lymphocytes in both non-smokers and smokers as measured by the modified comet assay for oxidized pyrimidines (45). The subjects with a supplement compared with those without were also more resistant to ex vivo H2O2-induced oxidative DNA damage. Similarly, an increase in the consumption of vegetable products that are high in carotenoids, such as tomato and carrot juice, was shown to reduce the baseline level of oxidized pyrimidines by as much as 3-fold (46). There have also been negative or uncertain results. For example, the level of 8-oxo-dGuo decreased whereas the level of 8-oxo-7,8-dihydrod adenine increased in lymphocyte DNA upon supplementation of donors with vitamin C. Paradoxically, these results suggest that vitamin C exerts anti- and pro-oxidant effects in vivo (35). In addition, a supplement of vitamin C together with iron was reported to increase the level of certain oxidative base lesions while decreasing the level of other lesions (47). However, the conclusions of the latter two studies should be reconsidered in view of the fact that GC–MS analysis is prone to extensive auto-oxidation (e.g. the levels of 8-oxo-
dGuo in the latter studies are 60-fold higher than those in the present study). Finally, the effect of dietary antioxidants has been evaluated by urinary 8-oxo-dGuo as a marker of oxidative DNA damage. The results of an epidemiological study indicated that vitamin C based on dietary evaluation was negatively correlated with the urinary excretion of 8-oxo-dGuo (48). In contrast, other studies have not reported a significant difference in urinary 8-oxo-dGuo upon dietary supplementation with 250 mg of vitamin C in smokers despite the fact that smokers have lower plasma vitamin C and higher urinary 8-oxo-dGuo (49,50).

In the present study, we have examined the endogenous levels of antioxidants and oxidative DNA damage in human lymphocytes from healthy volunteers. Also, we have focused on the measurement of intracellular rather than plasma levels of antioxidants because the former is more relevant to oxidative DNA damage. The results demonstrate that both glutathione and ascorbate are negatively correlated to endogenous oxidative DNA damage in human lymphocytes, which provides compelling evidence that these antioxidants protect against oxidative DNA damage. Moreover, the dependence of oxidative DNA damage on intracellular glutathione and ascorbate, and the large variation of antioxidants, suggest that this damage can be modulated in the human population. Assuming that oxidative DNA damage contributes to cancer, it should be possible to prevent cancer by maintaining sufficiently high levels of intracellular glutathione and ascorbate.

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