Antioxidant Supplementation Decreases Oxidative DNA Damage in Human Lymphocytes

Susan J. Duthie, Aiguo Ma, Marion A. Ross, and Andrew R. Collins

Rowett Research Institute, Greenburn Road, Bucksburn, Aberdeen AB2 9SB, Scotland, United Kingdom

ABSTRACT

The association between high intake of fruit and vegetables and low incidence of certain cancers is well established. Dietary antioxidants present in these foods are thought to decrease free radical attack on DNA and hence to protect against mutations that cause cancer, but this causal mechanism remains conjectural. We have adopted a molecular epidemiological approach to this question, based on a modified alkaline single-cell gel electrophoresis assay ("comet assay") which specifically detects oxidation of pyrimidines in the DNA of human lymphocytes. In a survey of men 50–59 years of age living in the northeast of Scotland, smokers showed significantly more base damage than nonsmokers. Correlations between oxidative base damage and plasma concentrations of various antioxidants were generally negative but not statistically significant.

Supplementation of the diet for 20 weeks with vitamin C (100 mg/day), vitamin E (280 mg/day), and β-carotene (25 mg/day) resulted in a highly significant (P < 0.002) decrease in endogenous oxidative base damage in the lymphocyte DNA of both smokers and nonsmokers. In addition, lymphocytes of antioxidant-supplemented subjects showed an increased resistance to oxidative damage when challenged in vitro with H2O2.

These findings strongly support the hypothesis that fruit and vegetables exert a cancer-protective effect via a decrease in oxidative damage to DNA.

INTRODUCTION

Endogenous damage to DNA, caused by oxygen-free radicals liberated during normal respiration, may be significant in the aetiology of cancer (1). DNA damage can account for the genetic changes that occur at the different stages in the progression from anaplastic growth to metastasis (2), and therefore dietary factors that reduce the impact of free radical attack are likely to protect against cancer. Epidemiological studies indicate that a diet rich in fruit and vegetables is indeed associated with lower incidence of various forms of cancer (3–6). This protection is commonly attributed to the dietary content of antioxidants such as vitamin C, vitamin E, and various carotenoids (7–10). Few intervention trials have been carried out to test this hypothesis, since large numbers of volunteers are needed, and several years must elapse before sufficient cases of disease have arisen. The recent Lixinan trial demonstrated a significant effect of a combination of vitamin E, β-carotene, and selenium on mortality, particularly from esophageal cancer (11). At the same time, however, conflicting results have been reported, notably in the recent Finnish study (12), in which smokers were given supplements of vitamin E, β-carotene, or both for 40 weeks to determine DNA damage in lymphocytes using SCGE.

We have carried out a double-blind supplementation study on small, well-defined groups of smokers and nonsmokers, half of each group receiving a daily antioxidant supplement, and the other half a placebo, for up to 40 weeks. At intervals, we measured antioxidant concentrations in plasma and biomarkers of genetic change. The "comet assay," or SCGE, was used to estimate DNA strand breaks and oxidized bases. We report here the substantial protective effect provided by antioxidant supplementation, as indicated by (a) a decrease in endogenous oxidative damage to bases in DNA and (b) an increase in the resistance of lymphocytes to oxidative damage inflicted in vitro.

MATERIALS AND METHODS

Materials. Histopaque 1077 and RPMI 1640 medium without phenol red and bovine serum albumin fraction V were obtained from Sigma (Poole, England). Ultrapure low melting point agarose and standard melting point agarose and bovine serum albumin fraction V were obtained from Sigma (Poole, England). Ultrapure low melting point agarose and standard melting point agarose (both electrophoresis grade) were supplied by Gibco Life Technologies (Paisley, Scotland). Fully frosted glass microscope slides were obtained from Richardson Supply Co. (London, England), and 4′,6-diamidino-2-phenylindole dihydrochloride was obtained from Boehringer Mannheim (Lewes, England). FCS was obtained from Globepharm Ltd. (Essex, England). Dr. R. Cunningham (Department of Biological Sciences, State University of New York, Albany, NY) generously supplied the Escherichia coli strain producing endonuclease III. Capsules containing vitamin C, vitamin E, and β-carotene in hydrogenated coconut oil and placebo capsules containing just oil were a gift from BASF (Ludwigshafen, Germany).

Selection of Subjects and Blood Sampling. Our volunteers were normal healthy males between the age of 50 and 59 years. Individuals on drug therapy or health supplements or with a history of heart disease were excluded. About 50 smokers and 50 nonsmokers were recruited. Nonsmokers were defined as those who reported never having smoked; ex-smokers were not used in this study. They were assigned at random to the supplement or placebo group. The supplements of vitamin C (100 mg), vitamin E (α-tocopherol, 280 mg), and β-carotene (25 mg) or placebo were taken daily, and blood samples were collected by venipuncture (after overnight fasting) at 0, 5, 10, 20, and, in some cases, 40 weeks to determine DNA damage in lymphocytes using SCGE: plasma antioxidants including vitamins C and E and β-carotene were also measured.

The study was approved by the Joint Ethical Committee of the Grampian Health Board and the University of Aberdeen.

DNA Damage Measured by SCGE. Thirty μl whole blood were mixed with 1 ml RPMI 1640 medium supplemented with 10% (v/v) FCS and kept on 18 U.S.C. Section 1734 solely to indicate this fact.

1 Supported by the Ministry of Agriculture, Fisheries, and Food and the Scottish Office Agriculture and Fisheries Department.

2 To whom requests for reprints should be addressed.

Received 9/25/95; accepted 1/12/95.

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.

1 The abbreviation used is: SCGE, single-cell gel electrophoresis.

Downloaded from cancerres.aacrjournals.org on January 13, 2018. © 1996 American Association for Cancer Research.
ice for 30 min. Lymphocytes were isolated after centrifuging over 100 μl Histopaque 1077 at 200 × g for 3 min at 4°C, washed once in PBS (pH 7.4), and centrifuged again. The cells recovered were confirmed to be 95% mono-nuclear cells (data not shown).

DNA damage was induced in vitro by exposing lymphocytes to H₂O₂ in PBS for 5 min on ice. Treatment on ice minimizes the possibility of cellular DNA repair.

DNA breaks and oxidized pyrimidine bases were measured using the alkaline comet assay as described previously (15–17). In brief, lymphocytes (from 30 μl blood, with or without H₂O₂ treatment) were suspended in 80 μl 1% low melting point agarose in PBS at 37°C and pipetted immediately onto a frosted glass microscope slide precoated with 80 μl 1% high melting point agarose, similarly prepared in PBS. The agarose was allowed to set by incubating at 4°C for 10 min, and the slides were then immersed in lysis solution (1% Triton X-100, 2.5 mM NaCl, 0.1 mM Na₂EDTA, 10 mM Tris, pH 10.0) at 4°C for 1 h to remove cellular membranes, proteins, and so forth. Slides were then placed in a single row in a 260-mm wide horizontal electrophoresis tank containing 0.3 M NaOH and 1 mM Na₂EDTA at 4°C for 40 min before electrophoresis in the same solution at 25 V for 30 min at an ambient temperature of 4°C (temperature of running solution not exceeding 15°C). The slides were washed three times for 5 min each with neutralizing buffer (0.4 M Tris-HCl, pH 7.5) at 4°C before staining with 20 μl 4′,6-diamidine-2-phenylindole dihydrochloride (5 μg/ml).

The comet assay as described detects strand breaks in DNA. The presence of breaks allows supercoiled loops of DNA to relax and move out to form a tail (hence, the name comet assay), and the fraction of DNA in the tail reflects the frequency of breaks (18). A modification of the assay permits detection of oxidized bases in addition to breaks (17). In this case, following lysis, the slides were washed three times for 5 min each in endonuclease III buffer (40 mM HEPES-KOH, 0.1 mM KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8.0), drained, and the agarose covered with 50 μl of either endonuclease III in buffer (hence, the name comet assay), and the fraction of DNA in the tail reflects the extent of damage for subjects in the placebo groups were examined for any correlation with the individual plasma antioxidant levels, similarly averaged from the four determinations made during the trial (Table 2). A positive correlation is seen between strand breaks and α-tocopherol in nonsmokers but not smokers. Almost all of the correlations between oxidized bases and antioxidant concentrations are weakly negative; the only significant associations are seen with retinol (nonsmokers) and lycopene (smokers). The multiple correlation coefficients of strand breaks or oxidized bases with antioxidant concentrations were not significant.

**Plasma Antioxidant Levels: Effect of Supplementation.** Table 1 gives the mean plasma concentrations of vitamin C, α-tocopherol, and β-carotene. At the start of the trial, smokers had lower levels of vitamin C and β-carotene than nonsmokers (P<0.003 and P<0.03, respectively).

**DNA strand breaks and oxidized pyrimidines were measured in lymphocytes using the comet assay. Mean values of endogenous DNA damage for subjects in the placebo groups were examined for any correlation with the individual plasma antioxidant levels, similarly averaged from the four determinations made during the trial (Table 2).**

**RESULTS**

**Endogenous DNA Damage in Relation to Antioxidant Status.** Table 1 gives the mean plasma concentrations of vitamin C, α-tocopherol, and β-carotene. At the start of the trial, smokers had lower levels of vitamin C and β-carotene than nonsmokers (P<0.003 and P<0.03, respectively).

DNA strand breaks and oxidized pyrimidines were measured in lymphocytes using the comet assay. Mean values of endogenous DNA damage for subjects in the placebo groups were examined for any correlation with the individual plasma antioxidant levels, similarly averaged from the four determinations made during the trial (Table 2). A positive correlation is seen between strand breaks and α-tocopherol in nonsmokers but not smokers. Almost all of the correlations between oxidized bases and antioxidant concentrations are weakly negative; the only significant associations are seen with retinol (nonsmokers) and lycopene (smokers). The multiple correlation coefficients of strand breaks or oxidized bases with antioxidant concentrations were not significant.

**Plasma Antioxidant Levels: Effect of Supplementation.** Table 1 gives the mean plasma concentrations of antioxidants after supplementation for 20 weeks. Both smokers and nonsmokers demonstrated substantial increases in plasma concentrations of all three supplemented antioxidants by the end of the supplementation program.

**Modulation of Endogenous DNA Damage by Supplementation.** DNA strand breaks were assessed at 0, 5, 10, and 20 weeks and oxidized pyrimidines at 5, 10, and 20 weeks after the start of the trial. Fig. 1 shows the values for strand breaks, for smokers and nonsmokers.

**Table 1 Plasma antioxidant levels: effects of supplementation for 20 weeks**

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Smokers</th>
<th>Nonsmokers</th>
<th>Smokers</th>
<th>Nonsmokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Tocopherol/cholesterol (μM/mg)</td>
<td>4.20 ± 0.15 (n=16)</td>
<td>4.79 ± 0.18 (n=20)</td>
<td>4.91 ± 0.15 (n=25)</td>
<td>6.59 ± 0.24 (n=23)</td>
</tr>
<tr>
<td>β-Carotene (μg/ml)</td>
<td>0.21 ± 0.02 (n=16)</td>
<td>0.31 ± 0.03 (n=21)</td>
<td>0.33 ± 0.02 (n=24)</td>
<td>1.93 ± 0.17 (n=23)</td>
</tr>
<tr>
<td>Ascorbate (μM)</td>
<td>26.4 ± 5.4 (n=18)</td>
<td>38.2 ± 3.2 (n=25)</td>
<td>37.2 ± 3.5 (n=30)</td>
<td>52.1 ± 2.7 (n=28)</td>
</tr>
</tbody>
</table>

**Table 2 DNA damage correlated with plasma antioxidant concentrations in smokers and nonsmokers.**

<table>
<thead>
<tr>
<th>Antioxidant</th>
<th>Smokers</th>
<th>Nonsmokers</th>
<th>Smokers</th>
<th>Nonsmokers</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Tocopherol</td>
<td>+0.02</td>
<td>-0.15</td>
<td>-0.03</td>
<td>-0.08</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>+0.02</td>
<td>-0.15</td>
<td>-0.03</td>
<td>-0.08</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>-0.30</td>
<td>-0.17</td>
<td>-0.20</td>
<td>-0.05</td>
</tr>
<tr>
<td>γ-Tocopherol</td>
<td>+0.01</td>
<td>-0.03</td>
<td>+0.10</td>
<td>-0.02</td>
</tr>
<tr>
<td>Retinol</td>
<td>+0.20</td>
<td>+0.02</td>
<td>-0.21</td>
<td>-0.43</td>
</tr>
<tr>
<td>α-Carotene</td>
<td>-0.00</td>
<td>-0.05</td>
<td>-0.18</td>
<td>+0.01</td>
</tr>
<tr>
<td>β-Cryptoxanthin</td>
<td>+0.22</td>
<td>+0.29</td>
<td>+0.08</td>
<td>-0.34</td>
</tr>
<tr>
<td>Lycopene</td>
<td>+0.29</td>
<td>-0.03</td>
<td>-0.50</td>
<td>+0.06</td>
</tr>
<tr>
<td>Phytofluene</td>
<td>+0.19</td>
<td>+0.10</td>
<td>-0.37</td>
<td>-0.19</td>
</tr>
<tr>
<td>Lutein/zeaxanthin</td>
<td>+0.33</td>
<td>+0.10</td>
<td>+0.10</td>
<td>-0.10</td>
</tr>
</tbody>
</table>

Antioxidant concentrations were determined on the placebo groups of smokers and nonsmokers on four occasions during the trial. The means of the four determinations were assessed against mean values for strand breaks (four determinations) or oxidized pyrimidines (three determinations). The sample size varied between 18 and 25. Full details of antioxidant concentrations will appear elsewhere.
ers. There is no effect of supplementation on strand break levels; the only significant difference is a slight increase in strand breakage in smokers compared with nonsmokers at week 20 (supplement and placebo groups combined, P<0.02). The data for oxidized pyrimidines are presented in Fig. 2. Early in the trial, smokers had a significantly higher level of oxidized bases (P<0.002) than nonsmokers. After 20 weeks of supplementation, the differences between smokers and nonsmokers had disappeared, and smokers and nonsmokers taken together show a highly significant effect (P<0.002) of antioxidants on the level of base oxidation; the mean for the placebo group (in arbitrary units of damage) is 60.5 ± 7.0 (SE) compared with the mean for the supplemented group of 38.0 ± 5.9 (SE).

Resistance of Lymphocytes to Exogenous DNA Damage. If the antioxidant status of lymphocytes is enhanced by supplementation, they would be expected to show increased resistance to oxidative damage when challenged in vitro with an agent such as H₂O₂. We tested lymphocytes from randomly selected subsets of nonsmoking volunteers after 20 (or in some cases 40) weeks of administering antioxidants or placebo. They were treated with different doses of H₂O₂ and immediately processed by SCGE to determine the extent of DNA strand breakage induced (Fig. 3). Damage is significantly decreased in the supplemented samples given treatment with 0.1 mM H₂O₂ (P<0.001) or 0.3 mM H₂O₂ (P<0.001). The mean values of plasma antioxidants, measured at the time of the in vitro challenge, are shown for both subsets in Table 3.

DISCUSSION

The aim of this study was two-fold: first, to look for correlations between natural (i.e., unsupplemented) levels of antioxidants and various indicators of genetic damage; and, second, to see whether antioxidant supplementation could significantly affect these markers. To examine the relationship between oxidative DNA damage and preexisting antioxidant status (without supplementation), the data obtained from samples within the placebo group, taken at intervals during the study, were combined to calculate mean values for the whole period in relation to both DNA damage and antioxidants. This longitudinal approach was considered preferable to the snapshot view which would have been provided by assessing all volunteers (supplement and placebo groups) once before the start of the trial, since it may help to accommodate random or seasonal variations in both the diet and the assays.

The alkaline comet assay, as used here, detects strand breaks and oxidized pyrimidines. As an index of endogenous oxidative damage to DNA, strand breaks are unreliable. The steady-state level of breaks is expected to be very low, since they can be rejoined very quickly by cellular repair (22). Furthermore, under the conditions of the assay, apurinic/apyrimidinic sites, which are alkali labile, will be revealed as breaks. Finally, both apurinic/apyrimidinic sites and actual strand breaks occur as intermediates in the cellular processing of a variety of DNA lesions via either base excision repair or nucleotide excision...
The occurrence of oxidized pyrimidines in lymphocyte DNA was used as a specific index of oxidative damage. There was no difference in this parameter between subjects on supplement and those on placebo (either smokers or nonsmokers) at week 10, but by week 20 there was a very significant decrease in oxidized pyrimidines in smokers and nonsmokers receiving supplement compared with those on placebo. This indicates protection of DNA by antioxidants in the supplement against attack by endogenously produced reactive oxygen species. Fraga et al. (25) measured 8-hydroxydeoxyguanosine as a marker of oxidative damage in DNA of human sperm. The level of damage was significantly increased following depletion of dietary vitamin C and decreased on subsequent supplementation with vitamin C. Our study made use of a cocktail of antioxidants, and therefore it is not possible to attribute protective effects to individual compounds; it is, however, unlikely that vitamin C is responsible for the decrease in damage, since there is so small a difference in plasma concentrations between nonsmokers on supplement or placebo at this time.

The other method used here to show antioxidant protection depends on challenging lymphocytes in vitro with H₂O₂. In this case, strand breaks are a valid measure of incident damage, and cellular repair is not a factor because the cells are kept cold during the brief treatment. The effect of supplementation is again striking, with a significant decrease in damage received at the higher H₂O₂ doses tested. A related effect has been reported by Green et al. (26), who treated human lymphocytes in vitro with ionizing radiation. The response, in terms of DNA strand breakage, was significantly reduced in samples of blood taken a short time after a high single dose of vitamin C compared with samples from the same subjects before ingestion of the antioxidant.

The molecular epidemiology approach is dependent on the selection of reliable and appropriate biomarkers, which ideally should have a well-established causal connection with the disease under investigation. It is generally recognized that DNA damage, resulting in base change and mutation when replication occurs, is a very early event in carcinogenesis. It seems likely that a one third fall in the level of oxidative base damage would be translated into a significantly lower risk of mutation and cancer. Such an extrapolation is justified only if peripheral nondividing lymphocytes can be regarded as a satisfactory surrogate for the actual target cells in the process of carcinogenesis, or at least as a valid indicator of the level of oxidative damage in the body overall. Application of the comet assay to other cell types such as buccal epithelial cells, urothelial cells isolated from urine, or cells isolated from biopsy material will confirm whether this is the case. Herein, we here show for the first time a highly significant modulating effect of long-term antioxidant supplementation on endogenous and exogenous oxidative DNA damage in lymphocytes, supporting the hypothesis that dietary antioxidants may protect against cancer. The ability to demonstrate such clear differences between subjects on placebo and on supplements serves to confirm the comet assay as a useful tool in screening populations for genotoxic effects.

| Table 3 Plasma antioxidant levels in nonsmokers sampled at week 20 or week 40 for the in vitro lymphocyte challenge experiment described in Fig. 3* |
|-----------------------------------------------|------------------|------------------|
|                                | α-Tocopherol/cholesterol (µM/ml) | β-Carotene (µM/ml) | Vitamin C (µM) |
| Placebo | (n=21) | (n=21) | (n=23) |
|         | 5.30 ± 0.38 | 0.36 ± 0.06 | 38.3 ± 3.6 |
| Supplement | (n=12) | (n=12) | (n=16) |
|         | 7.93 ± 0.77 | 2.19 ± 0.26 | 53.6 ± 4.0 |
| P<0.002 | P<10⁻⁶ * | P<0.01 |

*Mean values ± SE; P values refer to differences between placebo and supplemented groups for each antioxidant.

Fig. 3. Ex vivo test for antioxidant protection. Lymphocytes from subjects (nonsmokers) receiving placebo (□) or supplement (•) were challenged in vitro with H₂O₂ and DNA strand breaks were measured by SCGE. Bars, SEM; n = 24 (placebo), n = 17 (supplement).
ACKNOWLEDGMENTS

We thank Professor W. P. T. James, Dr. J. R. Arthur, and Dr. G. G. Duthie for valuable help in planning this project and in critically reviewing the manuscript; BASF for their generosity in supplying the supplements; V. L. Dobson and L. K. Crosley for skilled technical assistance; K. M. Brown for help with blood sampling; D. Hirst (Scottish Agriculture Statistics Service) for the statistical analysis of the data; and Dr. J. Beattie and colleagues of Inverurie Medical Group and all of the volunteers for their enthusiasm and commitment to the trial.

REFERENCES

Antioxidant Supplementation Decreases Oxidative DNA Damage in Human Lymphocytes

Susan J. Duthie, Aiguo Ma, Marion A. Ross, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/56/6/1291

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link http://cancerres.aacrjournals.org/content/56/6/1291.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.