Serum carotenoids and oxidative DNA damage in human lymphocytes

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Carotenoids are thought to act as antioxidants in vivo, decreasing oxidative damage to biomolecules and thus protecting against coronary heart disease and cancer. However, human intervention studies with β-carotene have given equivocal results in terms of cancer incidence. In an alternative molecular epidemiological approach, we have employed the ‘comet assay’ (single cell alkaline gel electrophoresis) to measure strand breaks, oxidized pyrimidines and altered purines in the DNA of lymphocytes from volunteers supplemented with αβ-carotene, lutein, lycopene or placebo. In addition, we measured concentrations of the main serum carotenoids, and vitamins E and C, by HPLC. We report a significant negative correlation between basal concentrations of total serum carotenoids and oxidized pyrimidines. A similar correlation was seen between individual carotenoids (notably lutein and β-carotene) and oxidized pyrimidines. However, carotenoid supplementation did not have a significant effect on endogenous oxidative damage. This suggests that there are some factors in the basal diet, probably found in fruit and vegetables, that decrease oxidative damage to DNA. In this case, basal serum carotenoids may simply be markers of consumption of fruit and vegetables, they themselves having little or no protective value.

Introduction

The evidence that carotenoids, obtained from fruit and vegetables, can protect against coronary heart disease (CHD) and cancer comes mainly from epidemiological studies of the observational kind, i.e. cross-country comparisons, case-control studies and prospective studies. Peto et al. (1), having analysed the accumulated data relating to human cancer, advocated the setting up of intervention trials with β-carotene, and several such studies have been undertaken in the period since. In Linxian, China, a region with very high incidence of oesophageal and stomach cancers, the study group of marginally malnourished people showed a significant decrease in cancer incidence over a 5.25 year period of supplementation with a mixture of β-carotene, vitamin E and selenium (2). In contrast, in a large trial carried out in Finland, a significant increase of 18% in lung cancer incidence was seen among those participants (all smokers) who received β-carotene over a period of 5–8 years, compared with those not receiving the carotenoid (3). Soon after the publication of this report, another trial [the Beta-carotene and Retinol Efficacy Trial (CARET), investigating a high-risk population of smokers and/or asbestos workers] was prematurely halted when a trend towards increased incidence of cancer with β-carotene supplementation became evident (4). A fourth trial, the Harvard Physicians Health Study, reported no significant effect of β-carotene supplementation on cancer incidence, in a low-risk population group (5). In retrospect, dietary intervention with antioxidant supplements in high-risk groups may have been premature, since there is a need for more fundamental information about the mechanism of action of these compounds.

With the aim of producing such information, we have adopted an alternative approach based on the measurement of oxidative DNA damage in lymphocytes as a marker of oxidative stress, which is widely believed to play a role in diseases such as cancer and CHD (6,7). The comet assay (single cell gel electrophoresis), a convenient and sensitive method for measuring DNA strand breaks in human cells such as lymphocytes, has been modified for the detection of oxidized pyrimidines and purines, which are more specific indicators of oxidative damage than strand breaks alone (8). We found a significant decrease in base oxidation in lymphocyte DNA in smokers and non-smokers receiving β-carotene, vitamin C and vitamin E for 20 weeks, compared with those receiving a placebo (9).

In the present study, we specifically investigate the effects of supplementation with individual carotenoids on levels of oxidative DNA damage in lymphocytes, and the relationship between DNA damage and basal concentrations of serum antioxidants (resulting from normal dietary intake, without supplements).

Materials and methods

Supplementation trial design

Forty volunteers in Madrid, Spain (healthy non-smokers, 25–45 years of age, equal numbers of men and women) received vitamin E (100 mg/day) for 4 weeks. (The vitamin E supplementation was to allow a comparison of carotenoid effects with vitamin E effects on susceptibility of low density lipoprotein to oxidation, and is irrelevant to the present study.) The volunteers were randomly assigned to four groups and, over the next 12 weeks, received 15 mg/day of (i) ‘palm oil carotenes’ (a mixture of α- and β-carotene, 30 and 66.5% respectively), (ii) lutein (80% trans-lutein, 20% 13-15-cis-lutein), (iii) lycopene (containing ~10% of β-carotene), or (iv) a placebo. Capsules were prepared by R.P.Scherer International (St Petersburg, FL), with lutein or carotenes from Quest International (Cork, Ireland), or lycopene from Makhtesh Chemical Works (Beer-Sheva, Israel), or corn-oil placebo. Blood samples were taken at the start of the trial (week 0) and at week 16. Lymphocytes were isolated by centrifugation on a Ficoll-based density gradient (10), suspended in 90% calf serum/10% dimethylsulphoxide, frozen slowly and stored at −70°C until being assayed for DNA damage using the comet assay (32 lymphocyte samples being recovered in a suitable state). Serum samples were frozen and stored at −70°C for carotenoid and vitamin E analysis; plasma became evident (4). A fourth trial, the Harvard Physicians Health Study, reported no significant effect of β-carotene supplementation on cancer incidence, in a low-risk population group (5). In retrospect, dietary intervention with antioxidant supplements in high-risk groups may have been premature, since there is a need for more fundamental information about the mechanism of action of these compounds.

Abbreviations: CARET, Beta-carotene and Retinol Efficacy Trial; CHD, coronary heart disease; DAPI, 4,6-diamidino-2-phenylindole; FPG, formamidopyrimidine glycosylase.

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Fig. 1. Base damage in lymphocyte DNA measured with the comet assay; distribution, within each group of individual scores for (A) endonuclease III-sensitive sites and (B) FPG-sensitive sites. Data shown here are mean values (after subtraction of scores for strand breaks, without enzyme) for each volunteer (six in the carotenes group, nine in the lutein group, eight in the lycopene group and nine in the placebo group).

<table>
<thead>
<tr>
<th>Correlation coefficient</th>
<th>Week 0</th>
<th></th>
<th>Week 16</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Endo III</td>
<td>FPG</td>
<td>Endo III</td>
<td>FPG</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>0.04</td>
<td>-0.15</td>
<td>-0.14</td>
<td>-0.06</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.14</td>
<td>-0.03</td>
<td>0.10</td>
<td>-0.07</td>
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<tr>
<td>Total carotenoids</td>
<td>-0.44**</td>
<td>-0.04</td>
<td>-0.41**</td>
<td>-0.21</td>
</tr>
<tr>
<td>β-Cryptoxanthin</td>
<td>-0.13</td>
<td>-0.03</td>
<td>-0.22</td>
<td>-0.02</td>
</tr>
<tr>
<td>Lutein</td>
<td>-0.44**</td>
<td>-0.20</td>
<td>-0.15</td>
<td>-0.16</td>
</tr>
<tr>
<td>Lutein-supplemented group</td>
<td>-0.39</td>
<td>-0.74**</td>
<td>-0.39</td>
<td>-0.74**</td>
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<tr>
<td>Excluding lutein group</td>
<td>-0.46*</td>
<td>-0.20</td>
<td>-0.46*</td>
<td>-0.20</td>
</tr>
<tr>
<td>Lycopene</td>
<td>-0.34</td>
<td>0.07</td>
<td>-0.06</td>
<td>-0.21</td>
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<td>Lycopene-supplemented group</td>
<td>-0.42</td>
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<td>-0.42</td>
<td>-0.62</td>
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<tr>
<td>Excluding lycopene group</td>
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<td>-0.02</td>
<td>-0.04</td>
<td>-0.02</td>
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<td>β-Carotene</td>
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<td>0.02</td>
<td>-0.33*</td>
<td>-0.09</td>
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<td>-0.51</td>
<td>0.04</td>
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<tr>
<td>Excluding carotene group</td>
<td>-0.37*</td>
<td>-0.02</td>
<td>-0.37*</td>
<td>-0.02</td>
</tr>
</tbody>
</table>

For week 16, in the case of supplemented carotenoids, correlations are given for all samples, for the group receiving the corresponding supplement and for the other three groups combined.

*P < 0.05; **P < 0.02.

A.R.Collins et al.

study was obtained from the Ethical Committee of the Clínica Puerta de Hierro and the Spanish Ministry of Health.

Antioxidant analysis

The carotenoids lutein, zeaxanthin, lycopene, β-cryptoxanthin, α- and β-carotene, and vitamin E were measured in serum by HPLC (11). Vitamin C was measured in acidified plasma by HPLC (12).

Comet assay for DNA damage

DNA damage was measured using the comet assay (8,13). Briefly, lymphocytes isolated by centrifugation on a Ficoll-based density gradient were embedded in agarose on a microscope slide and lysed in a solution containing Triton X-100 and 2.5 M NaCl. The resulting nucleoids were electrophoresed under alkaline conditions: the presence of breaks in the DNA allows it to extend towards the anode, forming a comet-like image when stained with 4,6-diamidino-2-phenylindole (DAPI) and viewed by fluorescence microscopy.

To measure DNA base damage in addition to strand breaks, the nucleoids were incubated after lysis with a lesion-specific repair endonuclease, either endonuclease III, recognizing oxidized pyrimidines, or formamidopyrimidine glycosylase (FPG), acting on altered purines including 8-oxo-guanine. The additional breaks formed at the sites of base oxidation increase the relative amount of DNA in the tail of the comet. One hundred comets in each gel were analysed visually; the score of between 0 and 400 arbitrary units reflects, approximately linearly, the frequency of DNA breaks, and covers the range up to $12 \times 10^3$ breaks/cell (14). Visual scores correspond accurately to the relative intensity of tail fluorescence as determined by computer image analysis (8).

Statistical analysis

The effects of supplementation and baseline (week 0) carotenoid concentrations on the indices of DNA damage were analysed as a linear model in Genstat 5.
Antioxidant effects of carotenoids

Results

Samples of lymphocytes prepared from blood taken at week 16 of the carotenoid intervention study were assessed for DNA damage by the comet assay; we were looking for effects of carotenoid supplements compared with placebo. Oxidized pyrimidines and altered purines (mainly 8-oxo-guanine) were estimated from the additional DNA breaks detected when nucleoids are digested with endonuclease III and FPG, respectively. Figure 1 shows the distribution of both kinds of damage among the individuals from the four supplementation groups: there are no statistically significant effects (although high levels of damage are not seen in the samples from carotene- or lutein-supplemented volunteers). The lack of significant protective effects of carotenoid supplements against oxidative DNA damage is supported by direct measurements (by HPLC) of concentrations of 8-oxo-deoxyguanosine in lymphocyte DNA samples from this trial (15).

The influence of supplementation on serum carotenoid concentrations will be reported in full elsewhere. To summarize, we found highly significant increases in α- and β-carotene and lutein, and smaller (still significant) increases in lycopene. Greater increases in lutein levels were seen in those individuals with initially higher baseline levels of lutein, which is an effect not found for other carotenoids.

When individual values for serum carotenoid concentrations are compared with the corresponding measures of lymphocyte DNA damage (oxidized bases), negative correlations are found (Table I). As an example, Figure 2A shows the association between total carotenoids (i.e. the sum of all carotenoids assayed individually by HPLC) and the frequency of oxidized pyrimidines in the 32 volunteers. The right-hand panel indicates a statistically significant negative correlation between endonuclease III-sensitive sites and carotenoids, both measured at week 16. We also had carotenoid data (but not DNA damage measurements) at week 0 and, to our surprise, found that the correlation between week 0 carotenoids and week 16 oxidized pyrimidines is just as strong as that for week 16 carotenoids. When looking at the carotenoids used for supplementation, it is informative (for the week 16 carotenoid concentrations) to look separately at the group of subjects receiving that supplement and at the other groups combined; this is illustrated for lutein in Figure 2B (oxidized pyrimidines) and C (altered purines). Correlations are stronger with lutein and β-carotene than with lycopene.

Discussion

DNA damage, as measured by endonuclease III-sensitive sites, shows a significant negative correlation with total serum carotenoids and with several individual carotenoids, which is consistent, at first sight, with a role for carotenoids in protecting DNA against endogenous oxidative damage. Correlations between FPG-sensitive sites and carotenoids are less strong, reaching significance only in the case of lutein, in the lutein-supplemented group. Whereas endonuclease III is specific for oxidized pyrimidines (16), FPG recognizes ring-opened purines arising, for example, as breakdown products of alkylated bases, as well as 8-oxo-guanine (17). The concentration of these altered bases in DNA is not known, but there is no reason to expect them to be present in direct proportion to oxidation products. The association of FPG sites with agents protecting against oxidative damage is therefore likely to be less pronounced than that of endonuclease III sites. However, because

Fig. 2. Negative associations of DNA base damage with serum carotenoids. (A) Mean values of endonuclease III-sensitive sites for each volunteer, measured at week 16, are plotted against total serum carotenoids measured in the same person at week 0 and week 16. (B and C) Week 16 endonuclease III- and FPG-sensitive sites, respectively, plotted against lutein concentrations; for the week 16 lutein concentrations, the lutein-supplemented group is shown separately. Correlation coefficients for these and other combinations are given in Table I.
the two enzymes share in part a specificity for oxidative damage, some association between the two is expected, and endonuclease III- and FPG-sensitive sites do in fact correlate with each other \( r = 0.33, P = 0.05 \).

DNA damage was measured only at week 16. The correlation between oxidized pyrimidines in lymphocytes at this time and serum carotenoid concentrations measured at week 0 tends to be at least as strong as with carotenoids measured at week 16. Twelve weeks is certainly a sufficient time for elevated levels of carotenoids to be established in the serum (18). Supplementation with a single carotenoid seems not to enhance significantly the protective milieu provided by the normal diet. Many food-derived phytochemicals are likely to contribute to the protection of DNA against damage, and the serum carotenoids measured here may simply be a marker for these dietary components. Pool-Zobel et al. (19), also using the comet assay, reported a significant decrease in oxidized pyrimidines during a 2 week supplementation with carrot juice following a 2 week supplementation with tomato juice. This is consistent with the idea that the complex mixture of carotenoids and other phytochemicals in real foods is more effective at protection than individual carotenoids.

The lack of association between oxidative DNA damage and concentrations of vitamins C and E may simply reflect the fact that, compared with carotenoids, each of these micronutrients shows a limited range of concentrations in the blood: three-quarters of the individual week 0 vitamin C concentrations were between 0.8 and 1.4 mM, and three-quarters of week 0 vitamin E (\( \alpha \)-tocopherol) concentrations were between 1.0 and 1.6 mg/dl. DNA damage measured with the comet assay is a valuable marker of oxidative stress. We have shown an inverse correlation between the frequency of oxidized bases in lymphocyte DNA and concentrations of carotenoids in the blood. The overall balance of natural carotenoids and/or other phytochemicals present in association with carotenoids in the diet seems to be effective at suppressing oxidative DNA damage. Our findings strongly support the hypothesis, based on results from conventional epidemiology, that fruit and vegetables, by virtue of their antioxidant content, protect us against DNA damage and, by implication, against cancer, but we do not find evidence of carotenoid supplementation having any effect, protective or otherwise, on the level of DNA damage.

Acknowledgements

We thank Mike Franklin and Graham Horgan (BioSS) for expert statistical advice, and Siobhán Griffin, E.Gil-Martínez, I.Blanco and Sharon Wood for skilled technical assistance. We are grateful to R.P.Scherer International Corp. for preparation of the capsules. The present study is part of a supplementation trial with subjects in five European countries, to determine the potential health benefits of increased intake of natural carotenoid isolates. Henk van den Berg, Bernice Corridan, Isabelle Hininger and David Thurnham were involved in the overall planning of the study. This work was supported by the European Union (AIR2-CT93-0888), the Scottish Office Agriculture, Environment and Fisheries Department, the Biotechnology and Biological Sciences Research Council (UK), and the Comisión Interministerial de Ciencia y Tecnología (CICYT, Spain, SAF96-1552.CE).

References


Received on May 1, 1998; revised on September 2, 1998; accepted on September 2, 1998.