Mechanisms by which Vegetable Consumption Reduces Genetic Damage in Humans

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Abstract
A previous intervention study had shown that consumption of carotenoid-containing vegetable juices reduces oxidative DNA damage in lymphocytes of 23 male subjects. It was the aim of this study to elucidate the potential mechanisms involved. Specifically, we studied the modulation of protein expression and determined susceptibility factors. Cryopreserved lymphocytes from the study were analyzed for genetic polymorphisms of glutathione S-transferase (GSTM1, GSTP1, and GSTT1) using multiplex PCR, GSTP1-protein with an ELISA, total protein by a colorimetric enzyme reaction, and DNA-repair enzymes with the Comet Assay. Analyses of the genotoxicity data revealed a more steady state of protection for GSTM1*+ than for GSTM1*0 (15 and 8 of 23, respectively) genotypes. Increased expression of cytosolic protein was observed in 23 subjects, increased expression of GSTP1 in 6 of 23 subjects, and capacity of repair of oxidized DNA bases in 9 of 21 subjects. GSTP1 induction was independent of the GSTP1 genotype (GSTP1a or GSTP1b/c alleles). Kinetics of induction of cytosolic protein and of GSTP1 were compared in one GSTM1*+ and one GSTM1*0 subject and showed an efficacy of tomato and carrots, but not of spinach. Reduced genetic DNA damage in lymphocytes may be due to the enhancement of cytosolic GSTP1, and DNA-repair proteins by tomato and carrot juices. Enhancement of cytosolic proteins may be indicative of increased gene expression by vegetable juices, some of which may be associated with protective activities.

Introduction
Research is needed to assess the impact of antioxidant plant ingredients in healthy humans and especially to improve understanding of the cancer-preventive functions of whole fruits and vegetables (1). Thus, we have reported first results from a human intervention study with 23 healthy male volunteers (nonsmokers), ages 27–40 (33.8 ± 4.08), to assess the anti-genotoxic, antioxidative potencies of three vegetable products (2). Analysis of carotenoids in plasma showed that the levels of lycopene, α- and β-carotene, and lutein were a direct reflection of the juices being consumed (3). Also, peripheral lymphocytes isolated from these subjects during the intervention study showed a significant reduction of strand breaks and of oxidized pyrimidine bases, especially after intervention with carrot juice (2). Thus, biomarkers of DNA damage have offered a focused and economical approach to answer the question of whether these vegetable juices have a protective potential in humans.

One major effect of the juices could be a direct scavenging attack of individual carotenoids on the reactive intermediates causing the observed DNA damage. Indeed our new studies on the detection of elevated levels of oxidized lycopenes are supportive for this mechanism (2). However, in addition to these plasma effects, carotenoids may also alter cellular parameters in the lymphocytes. We have determined additional data to clarify some of the potential mechanisms.

One major indirect mechanism of the juices could be an enhancing of the endogenous defense systems that protect against DNA-damaging factors (4). It has been shown that the chemopreventive system is inducible via a common ARE (5). As a surrogate for such more general types of protein induction, we have, therefore, assessed the modulation of total lymphocytic protein.

Secondly, the super gene family of GSTs (consisting of the cytosolic classes α, μ, π, θ, and a microsomal form) are specifically one of the most important cellular detoxification systems. They act either by conjugating electrophiles with glutathione, or by a glutathione-dependent peroxidase activity (6). They also may participate in the repair of damaged DNA bases. For studies in nutritional toxicology, it is important to recognize that some isomers of GST are inducible by oxidants and antioxidants. This has been shown in humans for the π-form after broccoli consumption in model systems with rats or in vitro systems using human cells (7–9). GST π is reportedly also the most abundant GST-isoenzyme in peripheral lymphocytes followed by μ and α (10, 11). Therefore, we have determined the expression of the GSTP1-protein subunit in the cryopreserved lymphocytes to assess whether it could be induced by carotenoid-rich vegetable juices.

Thirdly, the host susceptibility to genotoxic stress and to antigenotoxic protection is expected to be substantially influenced by the genetic polymorphisms of enzymes involved in

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1 The abbreviations used are: ARE, antioxidant responsive element; GST, glutathione S-transferase; HPLC, high-performance liquid chromatography; GSTM1, GSTP1, and GSTT1, GST genes; GSTP1, GST π protein; GST π, GST π-enzyme (GSTP1-homodimer).
vegetables abate DNA ruin and induce proteins in humans

Table 1 Susceptibility factors of subjects of the intervention study: genetic polymorphisms and modulation of protein expression (see details of responsiveness in "Materials and Methods")

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<th>GSTP1 (genotype)</th>
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* samples 2 and 6 were not available. DNA repair was analyzed with samples 4 and 8 instead.

Finally, the extent of DNA damage is not only dependent on exogenous exposure to genotoxins and antigenotoxins, or the efficiency of endogenous defense systems like GST, but also the cell's inherent DNA repair capacities. Therefore, we have isolated proteins from the cryopreserved lymphocytes to investigate whether the capacity to repair oxidative damage was modulated during the intervention.

Fig. 1. Agarose gels with DNA of subjects with intact GSTTI and GSTM1, and with various genotype patterns. Lane 1, DNA molecular weight marker (100-bp ladder; AGS); Lanes 3, 4, 6, 7, and 11 show DNA with intact GSTTI and GSTM1 genes; Lanes 5, 9, and 10 show GSTM1*0 genotype in the presence of GSTTI; Lane 2, GSTM1*+ and GSTTI*0; Lane 8, GSTM1*0 and GSTTI*0; Lane 12, negative control. β-Globin (268-bp fragment) was coamplified in all samples. Fragment length of GSTTI: 480 bp. Fragment length of GSTM1: 215 bp.

Materials and Methods

Intervention Study. As has been described previously, the intervention study was carried out with 23 healthy male volunteers (nonsmokers), ages 27–40 (mean ± SD, 33.8 ± 4.08), to assess the antigenotoxic, antioxidative potencies of three vegetable products. Two-week intervention periods were chosen to ensure sufficient time for the absorption and distribution of the micronutrients. Commercially available juices from tomatoes (a gift from Walter Schoenenberger of Pflanzensaftwerk GmbH and Co. and Völpel GmbH and Co. KG) and

Compound metabolism, including GSTs (12, 13). Examples are null genotypes for GSTM1, GSTTI, and single bp substitutions in GSTP1, each probably leading to different phenotypical GST activities. Therefore, we have determined GSTM1, GSTTI, and GSTP1 genetic polymorphisms in our subjects of the intervention study. On the basis of this genetic background, we have reassessed their susceptibility for genetic damage, and for responsiveness to antigenotoxic effects of vegetable juices.

Altogether, these factors (protective actions of carotenoids, efficacy of endogenous defenses, genetic susceptibility, and extent of DNA repair) are expected to explain mechanisms by which vegetables can lead to protection of genetic damage in humans.
carrots (Volpel GmbH and Co. KG) were chosen on account of their respective contents of lycopene and β-carotene. Spinach powder (to be ingested as a suspension in milk or water) was taken as a source of lutein because an appropriate “juice” equivalent with high lutein content was not available. The subjects were asked to maintain their normal dietary habits with the exception of refraining from consuming carotinoid-containing foods. After a 2-week period of this restricted diet (depletion period), the vegetable products were given with the subjects’ individual and normal lunch consecutively for a 2-week period each. The daily uptake was 330 ml of tomato juice with 40 mg of lycopene (weeks 3 and 4); 330 ml of carrot juice with 21.6 mg of β-carotene, and 15.7 mg of α-carotene (weeks 5 and 6); and 10 g of dried spinach powder with 11.5 mg of lutein, dissolved in water or milk (weeks 7 and 8). Blood was collected weekly from subjects before breakfast. Analysis of carotenoids in plasma showed that the levels of lycopene, α- and β-carotene, and lutein were a direct reflection of the juices being consumed (3).

**DNA Damage and Oxidized DNA Bases.** DNA damage was measured with the single-cell gel electrophoresis assay, also known as the “Comet” test (14, 15). We used our protocol of the Comet assay and, additionally, the modification with endonuclease III, to detect levels of oxidized pyrimidine bases (16, 17). A lymphocyte suspension (20 μl; 2 × 10⁶ cells) was distributed with 75 μl of low melting point agarose on microscope slides and after 10 min was covered with another layer of agarose. Three slides from each donor were placed in on ice-cold racks, treated with 50 μl of physiological NaCl for 5 min, washed with lysis solution (100 mM Na₂EDTA, 1% Triton X-100, 2.5 mM NaCl), and then placed into a lysis bath for 2 h. Three slides were placed into the lysis solution for 1 h, washed three times with endonuclease buffer (40 mM HEPES-KOH, 0.1 M KCl, 0.5 mM Na₂EDTA, 0.2 mg/ml BSA fraction V, pH 8.0), and then incubated with endonuclease III in a buffer sealed with a coverslip for 45 min at 37°C. Subsequently, all slides of one donor were placed into an electrophoresis chamber containing alkaline solution (1 mM Na₂EDTA, 300 mM NaOH) for DNA unwinding. After 20 min, the current was switched on and electrophoresis carried out at 25 V, 300 mA for 40 min. The slides were removed from the alkaline buffer, placed on a tray, and washed three times, 5 min with neutralization buffer (0.4 mM Tris, pH 7.5). Subsequently, the slides were stained with 100 μl of ethidium bromide solution (2 μg/ml). Thus, for each donor and week, triplicate values for DNA strand breaks (NaCl-control slides) and oxidative DNA damage (slides treated with endonuclease III) were available for evaluation. All steps beginning with the isolated lymphocytes were conducted under red light.

Evaluation of the images on the slides was performed by...
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**Fig. 3.** HPLC profiles of GST subunits in subjects 2 (a) and 3 (b) detected at 214 nm. Each curve represents the results of GST subunits isolated from lymphocytes and purified by affinity chromatography. Identification of the PI subunit (major peak) was achieved by cochromatography with an internal standard. The second peak could not be identified because no compatible internal standard was available.

microscopical analysis. Using the imaging software of Perceptive Instruments (Halstead, United Kingdom), 50 images were evaluated/slide and the percentage of fluorescence in the tail (“tail-intensity”) was scored. The value for NaCl-treated slides without endonuclease reflects the amount of DNA breaks, alkali labile sites, apurinic and apyrimidinic sites, and single-stranded DNA resulting from repair or replication. The percentage of fluorescence in the tail for slides with endonuclease III minus the values from corresponding NaCl-control slides is the measure for oxidized DNA pyrimidine bases. The absolute values had been shown previously for all subjects (2). In this study, we present values for differently responsive subgroups (GSTM1++ and GSTM1+0 genotypes).

**Isolation of DNA for Genotyping.** Cryopreserved lymphocytes (5 x 10^6 cells) were resuspended in 1.5 ml of buffer (8.0 g/l NaCl, 0.2 g/l KCl, 1.75 g/l Na_2HPO_4, 0.2 g/l KH_2PO_4/l, 10 mM EDTA, pH 8.0) and supplemented with 1.5 ml of protease K solution (2% w/v) SDS, 100 mM EDTA, pH 7.5, 2 mg/ml Protease K) and incubated either overnight at 37°C or for 3 h at 50°C. DNA was obtained by standard phenol/chloroform extraction and ethanol precipitation. The extracted pellet was air-dried and resuspended in 20–50 μl of sterile water.

**Determination of GSTM1 and GSTT1 Genotypes.** A multiplex PCR method was used to detect the presence or absence of the GSTM1 and GSTT1 genes [modifed according to Pemble et al. (1994) and Bell et al. (1993); Refs. 18 and 19]). Primers were synthesized at Genset (Paris, France) and used without further purification. The primer sequences used were:

- GSTM1-F: 5′-GAACTCCTGAAAGCTAAGC-3′
- GSTM1-R: 5′-TTAGGCTCTAAATATAACGT-3′
- GSTT1-F: 5′-TTTCTTATCGGCCTCATCATC-3′
- GSTT1-R: 5′-TCCACGGATCATGGCCAGC-3′
- β-globin-F: 5′-CACTTTCATCCACGTTACC-3′
- β-globin-R: 5′-GAGAGCCAAGAGCACGTAC-3′

A fragment of the β-globin gene was coamplified as an internal positive control in the PCR reaction.

For GSTM1/GSTT1 determination, 10–100 ng of genomic DNA (1 μl) were amplified in PCR-buffer (AGS, Heidelberg, Germany) including 1.5 mM MgCl_2 in a total volume of 30 μl containing 250 μM of each deoxyribonucleotide triphosphate, 2 units of Taq DNA polymerase (AGS), 30 pmol of GSTM1-F and GSTT1-R, 50 pmol GSTM1-F and GSTM1-R, and 20 pmol of β-globin-F and β-globin-R primers, respectively. PCR mixtures were overlaid with three drops of mineral oil and placed into a Perkin-Elmer Thermal Cycler 480. PCR conditions were 7 min, 94°C (initial denaturation), followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and a final elongation step at 72°C for 5 min. This reaction mixture (5 μl) was subsequently separated on a 2% agarose gel in TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.3) and stained with ethidium bromide after electrophoresis. The presence of the GSTM1 and GSTT1 genes indicated the respective null-polymorphisms.

**Determination of GSTP1 Genotypes.** For GSTP1 we used the PCR/RFLP protocol from Harries et al. (20), to identify the A1w26 I restriction site in exon 5 and, thus, to discriminate between GSTP1*a and GSTP1*b/c. PCR reaction was conducted in a total volume of 40 μl using 250 μM deoxyribonucleotide triphosphate, 40 pmol of each GSTP1 primer (GSTP1-F: 5′-ACCACGGGCTCTATGGA-3′ and GSTP1-R: 5′-GAGGCGACAAGAGCGCC-3′; Genset), 10–100 ng of genomic DNA (1 μl) and 2 units of Taq DNA polymerase in PCR buffer, including 1.5 mM MgCl_2 (AGS). Reaction mixtures were overlaid with three drops of mineral oil and initially denatured for 7 min at 94°C, followed by 30 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s. The terminal polymerization step was 5 min at 72°C.

The reaction product was then submitted to electrophoresis in 2% agarose, and the gel was stained in ethidium bromide to visualize the amplified PCR product with an expected length of 176 bp.

To determine the A1w26 I-RFLP, 20 μl of the PCR-product were incubated with 5 units of A1w26 I (AGS) in a total volume of 25 μl, for 4 h at 37°C.

The digest (10 μl) was then loaded on a 3.5% agarose gel and stained with ethidium bromide after electrophoresis. The DNA sequence corresponding to GSTP1*a will not be cut by...
A1w26 I, but the A-G transition at nucleotide + 313 will cleave to yield two fragments of 91 bp and 85 bp (corresponding to GSTP1* b or c).

**Determination of Total Protein and of GST Proteins in Lymphocytes.** Cryopreserved lymphocytes were taken up in 2.5 ml of buffer comprising of 25 mM Tris, 250 mM sucrose, 1 mM EDTA, pH 7.8, 1 μg/ml leupeptin and pepstatin, and 1 mM phenylmethylsulfonyl fluoride (10.4 × 10^6 ± 4.4 cells/ml). The cells were disrupted by vigorous treatment with an ultra-turrax at 4°C. Total cytosolic protein content was determined with a homogenate of 0.1–0.2 × 10^6 lymphocytes/100 μl on microtiter plates. The reaction mixture (100 μl) was incubated in the wells of microtiter plates and color development immediately measured at 585 nm (21). Induction of total cytosolic protein was defined as an increase of over 5 μg (equal to overall SD) in sample 7 (after 2 weeks of carrot juice) as compared with sample 3 (after 2 weeks depletion).

Blood (85 ml) was collected from the two representative isolated from 46–50 weeks after intervention. Lymphocytes were taken up in 100 ml of buffer comprising of 90–100/ml blood were used with HPLC-analysis to determine the profile of GST subunits in these specific cells. GST subunits were isolated and purified by affinity chromatography. An aliquot of 100 μl of the eluate was subjected to HPLC and detected by measuring absorption at 214 nm. A special CH3CN (TFA)/H2O gradient program on RP18 was used. Internal standards (human GST subunits A1, A2, M1a, M2, and P1) were obtained from Biotrin (Sinheim-Reihen, Germany). Data were analyzed with MILLENIUM 153 chromatography Manager Software, Version 2.10 (Waters, Eschborn, Germany).

For determinations of GSTP1-protein (= n subunit) in the lymphocytes of the intervention study, each homogenate, equivalent to approximately 4 × 10^6 cells in a 100-μl volume, was added to the wells of an ELISA kit from Biotrin. Absorbance determinations were carried out according to the protocol, and absorption of the ensuing color was measured at 450 nm (reference at 650 nm). Calibration was carried out by defined concentrations of the kit-standard, and optimal measurement was between 3–100 μg GSTP1/1 (or 0.3–10 ng/well). Each homogenate was assessed in triplicate. Two homogenates were prepared from each individual, one of sample time 3 and one of sample time 7. For individuals 3 (GSTM1*0) and 2 (GSTM1*+), proteins were also isolated from samples 1, 3, 5, 7, and 9. Proteins of each donor were analyzed on the same plate. A550 nm/A280 nm was calculated for each individual sample and means from replications were determined by excluding outliers according to Nalimov (22). Statistical analysis was performed with two-tailed paired or unpaired t tests (Graphpad Prism Software). Inducibility of the GSTP1-protein was defined as an increase of >7 ng/10^6 lymphocytes in sample 7 as compared with sample 3, which is equivalent to the mean sample deviation found in all subjects for sample 3 or sample 7.

**Determination of DNA-Repair Capacity by Proteins of Lymphocytes.** To isolate proteins from lymphocytes of the intervention study, the cells from 10 ml of blood were supplemented with extract buffer [45 mM Hepes, 0.4 M KCl, 1 mM EDTA, 1 mM DTT, and 10% (v/v) glycerol adjusted to pH 7.8 with KOH] to give 10 × 10^6 cells/100 μl, and cryopreserved at −80°C until use in the DNA repair assay (procedure from A. R. Collins, Rowett Research Institute, Aberdeen, Scotland). This cell protein extract (50 μl) was supplemented with 12 μl of extract buffer including 1% Triton X. The lystate was then centrifuged for 5 min (400 × g at 4°C), and the supernatant (equal to protein extract) was mixed with 10 volumes of a reaction buffer (2.5 mM ATP, 10 μg/ml creatine phosphokinase, 50 mM phosphocreatine in Hepes buffer supplemented with 10% DTA, 2% glycerol and 0.3 mg/ml BSA: pH 7.8) containing appropriate cofactors to facilitate repair of DNA. Human indicator cells (HT-29 clone 19A) suspended into agarose on microscope slides were treated with NaCl (to reveal DNA breaks) or with 150 μM H2O2 (to reveal oxidative DNA damage) and were processed until lysis in the Comet Assay (see above). The protein extracts (50 μl) or 50 μl of the reaction buffer were added to the damaged DNA on the slides. The slides were incubated for 45 min at 37°C, and the procedure of microelectrophoresis (alkaline unwinding, electrophoresis, neutralization, staining, and evaluation) was carried out as described above. The proteins of each individual were evaluated from sample times 2 and 6 and/or sample times 4 and 8 of the study (depending on availability of remaining protein; see Table 1). The respective repair capacities were a reflection of the percentage of fluorescence in the tail, and the data of the H2O2 slides treated with protein extract were compared with the slides treated only with the corresponding buffer. Subjects with induced repair capacity were defined by differences of the percentage of tail intensity >10 before intervention (sample 2) and during intervention (samples 6 or 8). This value is the maximum mean deviation of all investigated samples 2.

**Results**

The ingestion of vegetable juices caused a significant reduction of endogenous DNA breaks in lymphocytes of 23 healthy human subjects, as we had shown previously. Also, the ingestion of carrot juice reduced the levels of oxidized DNA bases during intervention (2). Plasma analyses have, meanwhile, revealed that the levels of individual carotenoids are a close reflection of the carotenoid-levels in the juices being consumed (3). Thus, lycopene is high (approximately 2–3-fold) after tomato juice intervention, whereas elevated levels of carotenoids (<4-fold) and luteins (2-fold) are each found after intervention with carrot and spinach juices, respectively. During the carrot juice intervention, the levels of oxidized pyrimidines were particularly low (>50% reduction). However, when attempting to correlate individual serum levels (α-, β-carotene, carotene diepoxide, and cis-carotene, or sum of all carotenes) with data on genetic damage (percentage of fluorescence in the tail for slides with and without endonuclease III), no significant associations were found (data not shown).

The 23 subjects were further analyzed for genotypes, to assess which impact endogenous defense systems could have on exogenous antioxidant activities. Eight of 23 subjects were GSTM1*0 (35%), and 2 of 23 subjects were GSTT1*0 (9%); 13 of 23 subjects were homozygote GSTP1*0 (57%), heterozygote GSTP1*a/GSTP1*b/c (30%), or homozygote GSTP1*b/c (13%), respectively. These data are shown in Table 1. Fig. 1 shows an example of an agarose gel with different GSTP1/GSTM1 genotypes.

In Fig. 2, the data on genetic damage were analyzed according to genotypes of the individuals. The subjects with the functional GSTM1 gene show relative steady levels of both general DNA damage as well as of oxidized DNA bases during the depletion period (sampling times 1–3). The tomato juice intervention (sampling times 4 and 5) causes a reduction of DNA breaks that, however, did not reach significance for DNA

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damage. Oxidized DNA bases were increased in GSTM1*0 after tomato juice intervention and reduced in GSTM1*+ genotypes. The carrot juice intervention (sampling times 6 and 7) resulted in a reduction of both damage types. Interestingly, the trend observed previously for all subjects, that spinach juice ingestion is less protective, is also very apparent for each subgroup of subjects (sampling times 8 and 9).

Larger volumes of blood were collected from the GSTM1*+ and GSTM1*0 individual to determine GST protein profiles in the lymphocytes. Fig. 3 shows that GSTP1 was most abundant with 110 ng/10⁶ cells. This amount of HPLC protein corresponds to 8 ng of immunoreactive GST as determined by ELISA (below). Additionally, there was an unidentified peak (R+) not identical to our other available standards: A1, A2, M1a, and M2, respectively (realized through standard addition procedures).

The expression of total cytosolic protein and GSTP1 was studied in the lymphocytes of all subjects for sampling time 3 (after depletion, before intervention) and for sampling time 7 (after 2 weeks of intervention with carrot juice). Fig. 4 shows that there was a significant increase in cytosolic proteins for all subjects and trend for increased GSTP1. When discriminating between GSTM1*+ and GSTM1*0 genotypes, differences in induction of GSTP1 were not observed. The increased expression of GSTP1 was observed in seven subjects irrespective of genotypes (Table 1), and was more than 2-fold for these, which are classified as responders (Fig. 4). This increase of GSTP1 in responders, however, does not lead to a more effective reduction of genetic damage (4) than observed for all 23 subjects (Fig. 4).

A kinetic study was performed with proteins from samples 1, 3, 5, 7, and 9 of subjects 2 (GSTM1*+) and 3 (GSTM1*0). The inducibility of cytosolic proteins increases at sample time 5 or 7 (Fig. 5) in the subjects. Similar profiles were obtained for subjects 12 (GSTM1*+) and 9 (GSTM1*0), respectively (data not shown). Marked individual differences, however, were observed for the expression of GSTP1. In subject 2, a clear-cut induction was observed throughout intervention, which after 48 weeks fell back to the values observed before the beginning of the trial. Subject 3 was less responsive to this parameter. No association can be found for protein induction and damage reduction (Fig. 5).

Repair of oxidative DNA damage is achieved by the protein extract of lymphocytes. This is apparent for some of the subjects by the reduced genetic damage of samples 4, 6 or 8 in comparison with the controls (slides treated with H2O2 and following lysis, incubated only with the protein buffer). The repair enzymes may be increased during intervention, because damage is reduced by the protein extracts obtained during intervention (Table 1). There was a trend for a difference between means ± SEM of sample 2 (34.7 ± 2% fluorescence in the tail; n = 20) and sample 6 (29.6 ± 2.6; n = 22) that, however, was not statistically significant (data not shown).

Discussion

An increased ingestion of fruits and vegetables is associated with a decreased risk for cancer in various tissues (23, 24). The protective activities of these plant foods are expected to be due to their high fiber content, complex carbohydrates, and phytochemicals (25–28). Especially carotenoids, one of the antioxidant groups of phytochemicals, have been implicated as potentially contributing to risk reduction (29–31). Recent intervention studies with supplements, however, have failed to verify these protective properties (32, 33) and more attention is being focused on understanding the role of phytochemicals.

Fig. 4. Modulation of total cytosolic protein, GSTP1, and DNA damage in all individuals and in a subset of individuals (six responders, defined as having an enhanced expression of GSTP1 after vegetable consumption; see "Materials and Methods"). *P <0.05, **P <0.005, ***P <0.001; paired one-tailed t tests.

Damage is reduced by the protein extracts obtained during intervention, repair enzymes may be increased during intervention, because damage is reduced by the protein extracts obtained during intervention (Table 1). There was a trend for a difference between means ± SEM of sample 2 (34.7 ± 2% fluorescence in the tail; n = 20) and sample 6 (29.6 ± 2.6; n = 22) that, however, was not statistically significant (data not shown).
available in plant foods, instead of supplements (34, 35). Thus, we have performed an intervention study with carotenoid-rich vegetable juices. Over 20 carotenoids were analyzed in plasma of this human intervention study (3). Interestingly, the levels of lycopene, lutein, and α- or β-carotene are close reflection of the juices being consumed. When attempting to correlate individual levels of DNA damage or oxidized DNA bases with plasma levels of individual carotenoids or with groups of carotenoids (lycopenes, carotenes, or luteins) the associations are not clear-cut. However, for sample 7 (after 4 weeks of intervention with juices), β-carotene levels are high and oxidized DNA bases are low. This may point to an association, but it may also be coincidental, if the mechanisms leading to a lower level of oxidative DNA damage were active before the availability of this particular carotene. There have been no other reports correlating levels of oxidized DNA bases in lymphocytes with carotene ingestion through vegetables. Recently, Clevid et al. (36) presented a preliminary report on the excretion of 8-hydroxyguanidine after 3 weeks consumption of carotenoid-rich vegetables. They did not find this parameter to be modulated. However, because they did not measure oxidized bases directly in lymphocytes, but only in the urine, a direct comparison with our study is not possible.

In general, it is believed that genetic polymorphisms will contribute substantially toward individual variations of susceptibility to environmental factors. GSTM1 is one of at least 5 genes coding for proteins of the GST μ enzyme. The polymorphisms result in homo- and heterodimeric combinations of the three known alleles GSTM1*0, GSTM1*a, and GSTM1*b. GSTM1*a and GSTM1*b differ in a single base in exon 7, leading to an amino acid exchange from the basic lysin (GSTM1*a) to the near neutral asparagine (GSTM1*b). GSTM1*0 is a complete deletion of the GSTM1 gene, and in homozygous state (null-genotype) there is no expression of the corresponding protein (37). Investigated populations (35-60%) may have a null-genotype (38, 39). Also, a null-genotype of the GST θ class is present at similar frequency because 10–65% of investigated populations carry a deletion of the GSTT1 gene (17, 39–43). In Germany, the frequencies for null-genotypes have been reported to be 51.1% and 20.8% for GSTM1*0 and GSTT1*0, respectively (44). In our study, the frequencies were 34.8 and 8.7%, respectively, and thus somewhat lower. Three variant GSTP1 cDNAs, expressed in normal and malignant cells, have been described and isolated recently (45), designated hGSTP1*a, hGSTP1*b, and hGSTP1*c. GSTP1*b and GSTP1*c contain an A-G transition at nucleotide +313, corresponding to an isoleucine to valine amino acid substitution in codon 104. GSTP1*c has an additional mutation at nucleotide +341, changing codon 113 from GCG (alanine) to GTG (valine). Both amino acid changes are in the electrophile-binding active site of the GSTP1 peptide and may be associated with alterations in the enzyme activities (46). Literature data are not yet available for the frequencies of these genotypes. German subjects had 57, 30, and 30% for GSTP1*a, ab and bb, respectively, but no associations were found with GSTP1-expression.

In our studies, we found only one association that may point to different susceptibilities on account of genetic enzyme polymorphisms, described above; namely that individuals with GSTM1*+ reacted in a more steady state manner to vegetable juice intervention and protection was more constant than in GSTM1*0-individuals. Another finding was perhaps coincidental in that during intervention, GSTP1 induction was more pronounced in the lymphocytes of the GSTM1*+ than in lymphocytes of the GSTM1*0 individual. Therefore, additional determinations will be necessary to support assumptions that different genotypes have different susceptibilities. Nevertheless, the latter finding is important because the P1 form may deactivate products of lipid peroxidation such as acrolein, oxidized DNA-bases (adenine- and guanine-prope-

Fig. 5. Kinetics of expression of total cytosolic proteins or protein GSTP1 and DNA damage in two subjects (2 and 3) during (samples 1, 3, 5, 7, and 9) and well after (sample 48) intervention study with vegetables.
GSTP1-"responders" (individuals with higher GSTP1 levels during intervention) reacted with a more distinct decrease of genetic damage. Interestingly, regardless of GST-genotype or GSTP1 inducibility, most subjects had increased levels of proteins which consequence is really not predictable. The Bradford assay is mainly specific for the arginine residues of proteins (49). Whether or not proteins of the chemopreventive system contain more arginine remains to be elucidated.

Our studies point to mechanisms by which protein induction could contribute to enhancing protection against oxidative stress. Both total lymphocytic proteins (those belonging to chemopreventive systems?) as well as DNA-repair proteins are increased. Transcriptional induction of GST, among other chemopreventive enzymes, is mediated by ARE. The necessary core sequence has been identified as TGACNNNGC, which on its own, however, is insufficient to mediate induction. Additional flanking sequences are required to yield a "functional ARE" that would be activated by a yet unknown protein, maybe a Mr 160,000 protein, as recently suggested (5). In any case, the GSTP1 induction mechanisms and especially the "cross talk" with other GST genes (and other genes of the chemopreventive system) need additional investigation.

In conclusion, our studies do show that vegetables may contribute greatly to enhancing a chemoprotective state. For the first time, we have shown that vegetables reduced genetic damage. One mechanism could probably be due to the antioxidant efficacy of the ingredients in human plasma (3). However, for a subset of individuals, additional mechanisms may occur. Thus, responsiveness may be due to induction of a variety of different proteins (e.g., cytosolic proteins, GSTP1, DNA-repair proteins). This, to our knowledge, is one of the first demonstrations in the field of ecogenetics that shows gene-environment interactions after vegetable consumption. The consequences of these interactions for human health may be a profound chemoprotection, possibly in a subset of genetically predisposed individuals, the nature of which remains to be clarified.

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References
Mechanisms by which vegetable consumption reduces genetic damage in humans.


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