Markers for Cytogenetic Damage in Smokers: Associations with Plasma Antioxidants and Glutathione S-Transferase $\mu$

Geert van Poppel, Hans Verhagen, Pieter van 't Veer, and Peter J. van Bladeren
TNO Toxicology and Nutrition Institute, Post Office Box 360, 3700 Al Zeist, The Netherlands

Abstract

Biomarkers for increased cytogenetic damage in smokers include sister chromatid exchanges (SCE) in peripheral lymphocytes and micronuclei in sputum cells. These markers may reflect increased cancer risk. Increased cancer risk has also been associated with lower blood levels of the antioxidants $\beta$-carotene and vitamin C and with genetic deficiency of the detoxification enzyme glutathione $S$-transferase $\mu$ (GST-$\mu$). We therefore evaluated the associations of plasma antioxidants, GST-$\mu$ phenotype, and indices for tobacco exposure with SCEs and micronuclei in a group of 156 male cigarette smokers and 38 nonsmokers.

As expected, smokers as compared with nonsmokers had higher SCE levels (5.08 versus 4.71 SCE/lymphocyte) and lower levels of plasma $\beta$-carotene (0.31 versus 0.48 $\mu$mol/liter) and blood vitamin C (36.6 versus 33.8 $\mu$mol/liter). In smokers, SCEs were weakly correlated with plasma cotinine ($r = 0.186$) but not with plasma antioxidants (all $r < 0.04$). Micronuclei in smokers were not correlated with either cotinine or antioxidants (all $r < 0.14$). As reported previously, SCEs were higher (5.24 versus 4.97 SCE/lymphocyte) in GST-$\mu$-deficient smokers than in nondeficient smokers. Micronuclei, however, were similar in both GST-$\mu$ phenotypes (4.3 versus 4.9 micronuclei/3000 cells). No correlation was observed between micronuclei and SCEs ($r = -0.025$).

Large random variations in both SCEs and micronuclei make it difficult to interpret the absence of relations unambiguously. The results indicate that SCEs and micronuclei have only limited sensitivity to variations in cigarette smoke exposure. The association between GST-$\mu$ and cancer risk may be mediated through increases in certain forms of smoking-induced DNA damage in GST-$\mu$ deficiency.

Introduction

Biomarkers are increasingly used in cancer epidemiology to estimate exposure to carcinogens or putative anticarcinogens, preclinical biological effects, as well as genetic factors that may determine individual susceptibility (1). Markers for DNA damage are of special interest since DNA damage is a crucial step in carcinogenesis (2). Markers for DNA damage such as SCE (3) and micronuclei (4) are increased in smokers, who have a known increased risk of cancer (5).

Despite a large risk, a majority of smokers do not develop cancer. Smokers may be partially protected by dietary antioxidants such as $\beta$-carotene and vitamin C (6, 7). Also, genetic differences in detoxification of tobacco constituents may determine individual cancer risk (8-10). The possible protection against cancer of antioxidants has been hypothesized to involve decreased DNA damage (11). Likewise, a more efficient detoxification of tobacco smoke could offer protection by limiting DNA damage (8-10). It can therefore be hypothesized that increased antioxidant status and more successful detoxification will correspond with less DNA damage in smokers. We have previously used the SCE measure to demonstrate an association between deficiency in the detoxification enzyme GST-$\mu$ and increased cytogenetic damage in smokers (12). We now have new data on blood levels of antioxidants and micronuclei in expectorated sputum from the same cross-sectional study. This allowed us to evaluate whether the two biomarkers for cytogenetic damage are inversely related with markers for antioxidant status and positively associated with markers for cigarette smoke exposure. Also, the association of GST-$\mu$ phenotype with micronuclei was studied, and we evaluated whether SCE and micronuclei are associated.

Subjects and Methods

Subjects. We studied healthy male volunteers, employed at the AMEV Insurance Company, the taxation office, and the power company at Utrecht. The study was approved by an external medical ethical committee and all participants gave their informed consent. Smokers were studied if they reported consumption of more than 15 cigarettes/day for more than 2 years. Nonsmokers were included in this study if they reported never to have smoked and, in addition, did not work or live with smokers. None of the participants used vitamin preparations containing retinol or carotenoids or medications known to influence SCE levels. Moreover, they reported not to be exposed to xenobiotic chemicals through their occupation or hobbies. Initially, 163 smokers and 38 nonsmokers volunteered and were eligible for the study. The smokers were studied as part of an intervention trial (13, 14). The present analysis is limited to the 156 smokers and 38 nonsmokers for whom SCE data are available. The GST-$\mu$ assay was ambiguous for one smoker and missing for another smoker. Sputum samples were only collected by smokers since nonsmokers do not spontaneously produce sputum.

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1 To whom requests for reprints should be addressed.

2 The abbreviations used are: SCE, sister chromatid exchange; GST-$\mu$, glutathione $S$-transferase $\mu$.
the smokers group, 29 participants failed to produce sputum samples, whereas insufficient sputum cells could be evaluated in another 7 smokers.

**Blood Parameters.** Nonfasting blood samples were collected between 8:00 a.m. and 12:00 a.m. Directly after venipuncture blood samples were stored in the dark at 0 to 4°C. After 20 to 23 h overnight dark storage at 4°C, a separate evacuated tube containing heparin as anticoagulant was opened to determine the sum of ascorbic acid + dehydroascorbic acid (vitamin C) in whole blood by high performance liquid chromatography with fluorometric detection (15) (variation coefficient = 7.3%). All-trans-retinol, α-tocopherol, β-carotene, and total carotenoids were assayed in EDTA plasma (stored at −80°C) by high performance liquid chromatography with colorimetric detection (16). For these assays, coefficients of variation for samples from a plasma pool were 4.3, 4.1, 7.0, and 5.7%, respectively. Plasma cotinine levels were determined by gas chromatography (17) (variation coefficient = 3%). Presence or absence of GST-μ was established in heparinized whole blood using an enzyme-linked immunosorbent assay (MUNKIT, Medlabs, Dublin, Ireland).

**Sister Chromatid Exchanges.** Blood cultures for determination of SCEs in lymphocytes (18) were set up within 2 to 6 h after venipuncture, after the blood had reached room temperature for 30 min. For the total study group, blood cultures were set up on 18 separate days; nonsmokers were studied on 10 of these 18 days. Heparinized whole blood (0.5 ml) was added to 4.4 ml prewarmed RPMI 1640 (Flow) containing 20% fetal calf serum (inactivated for 30 min at 56°C), 2.5% phytohaemagglutinin (HA-15; Welcome), 100 international units/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, and 10 μg/ml 5-bromo-2-deoxyuridine. The blood was cultured in the dark at 37°C in T-25 culture flasks (Costar) in 5% CO2 for 68 ± 1 h. Colcemid was added to a final concentration of 0.2 μg/ml for the last 2 h of incubation. The cells were collected by centrifugation, treated with hypotonic KCl (0.075 M) incubation. The cells were collected by centrifugation, treated with hypotonic KCI (0.075 M) for 8 min to spread the chromosomes and to hemolyse the RBC, and fixed 3 times with methanol-acetic acid (3:1). After overnight storage in the dark at 4°C, cells were transferred to microscopic slides and air-dried. Preparations aged for 3 days and were stained by the Fluorescence plus Giemsa Technique (18) to obtain harlequin chromosomes. For each subject SCEs in 50 second-division metaphases were scored as color changes in the longitudinal direction of the chromatid, excluding the centromere. Only metaphases with 46 chromosomes were scored. Individual data are the mean counts of 50 metaphases. SCEs were scored by a single observer in the nonsmoker group, whereas an additional observer assisted in scoring SCEs in the smoker group.

**Micronuclei in Sputum.** Sputum was collected and processed as described in detail by Saccomanno et al. (19). Each participant received a careful individual instruction on how to produce a specimen from “deep in the lungs.” Sputum was collected at home on three consecutive mornings, directly after rising and after carefully rinsing the mouth. The three or, minimally, two samples collected in preservative (50 ml 50% ethanol with 2% polyethylene glycol) (Carbowax 1540; Merck) were mixed, homogenized, centrifuged, and smeared onto slides. Sputum was collected on the days following the venipuncture. The slides were stained with Feulgen and fast green, which is specific for DNA and strongly highlights micronuclei (20). For each subject, 3000 cells were examined and evaluated on the basis of the following criteria: shape and size typical of epithelial cells; a well-defined nucleus; and a clearly defined cytoplasm. The criteria in defining a micronucleus were chromatin structure and color intensity similar to those of the main nucleus; on focusing, the micronucleus must be on the same level as the nucleus and must be roundish and clearly included in the cytoplasm. The dimensions should be less than one-fifth that of the main nucleus, and the micronucleus should not be connected to it. Slides were screened at ×400 magnification, and micronucleated cells were examined at ×1000 magnification. Slides were read coded/blinded by a single observer. Repeated blinded scoring of 9 samples yielded a good correlation (Pearson r = 0.86), with 2 of the 9 samples showing a difference of more than 1 micronucleus upon rescoring.

**Data Analysis.** Smoking and nonsmoking groups, as well as GST-μ-deficient and nondeficient groups were compared using the Student t test and the χ2 test. Associations between variables were evaluated using simple and multiple linear regression. For micronuclei counts, square roots of observations were taken to stabilize variances before data analysis. In addition to the linear regression techniques, the untransformed micronuclei data were also analyzed using Poisson regression. Data analysis were performed using the BMDP package (21). For the Poisson regression, the GENSTAT program was used (22).

**Results**

Table 1 shows the data for the smoking and nonsmoking group. Plasma cotinine levels reflect the large contrast in cigarette consumption between both groups. Age, body mass index, and plasma retinol are similar in both groups, although slightly higher in the smoking group. In the smoking group, there are more alcohol users (78 versus 63%) and the mean alcohol consumption is substantially higher. All plasma antioxidants are higher in nonsmokers than in smokers, except for plasma α-tocopherol which is slightly though significantly higher in smokers. The SCE measure is about 10% increased in smokers. Adjustment for alcohol consumption did not alter this difference (ΔSCE = 0.37 before...
adjustment, 0.33 after adjustment). The distribution of SCEs is given in Fig. 1, whereas the distribution of micronuclei counts is given in Fig. 2.

The correlations for SCEs, micronuclei, and plasma cotinine for the smokers group are given in Table 2. Plasma cotinine is clearly positively associated with the reported cigarette consumption and weakly though significantly with age, smoking years, and, unexpectedly, plasma β-carotene. SCE levels show a weak significant association with both plasma cotinine and reported cigarette consumption but no association whatsoever with plasma antioxidants or alcohol consumption. No association was observed between SCEs and micronuclei in the smokers group (r = -0.025; Fig. 3). For the nonsmokers group, there were no significant associations of SCEs with any of the parameters in Table 2 (all r < 0.14). Micronuclei counts were not associated with any of the indices for tobacco consumption or antioxidant status. Poisson regression models for the micronuclei counts yielded results similar to those in Table 2: none of the associations with the listed parameters was significant.

Of all subjects, 45% were deficient in GST-μ. We observed no association between GST-μ and any of the blood parameters or characteristics given in Table 2. The association of GST-μ deficiency with SCEs and micronuclei is given in Table 3. In nonsmokers, there is no relation between GST-μ deficiency and SCE levels. In GST-μ deficient smokers, SCE levels are higher than in nondeficient smokers. This μ-related difference is more pronounced in “heavy” smokers (plasma cotinine above the median), whereas it is absent in “light” smokers. In the heavy smokers, the μ-related difference was similar in multiple regression analysis controlling for age, body mass index, duration and quantity of smoking, and cotinine levels (5.47 versus 4.99; P = 0.02). In the light smokers, the multivariate adjustment also yielded similar results (4.94 versus 4.98; P = 0.87). Similar analyses for micronuclei in smokers did not reveal any associations with GST-μ phenotype, either in light or heavy smokers (Table 3).

For the SCE count in smokers, we observed an effect of culturing day variation (multiple partial r = 0.53; P < 0.001) and variation between observers (partial r = 0.51; P < 0.001) in a model that included 18 runs and 2 observers (multiple r = 0.71 for the total model). Adjustment for culturing day and observer variation did not materially alter the SCE data and correlations for SCEs given in Tables 1–3. Only the difference in SCEs between smokers and nonsmokers was more pronounced after this adjustment (4.85 versus 5.66 SCE/lymphocyte; P < 0.001). For the determination of micronuclei, we did not observe a significant effect of the 18 different runs on the micronuclei counts (multiple r = 0.42; P = 0.20 in linear regression; P = 0.20 in multiple Poisson regression).

**Discussion**

The decreased blood levels of carotenoids and vitamin C that we observe in smokers are in line with previous studies (23, 24). These differences may reflect a decreased dietary intake of vitamin C and carotenoids in smokers or a metabolic consequence of cigarette smoking (23, 24). A metabolic effect is biologically plausible, since cigarette smoke is a major source of free radicals and oxidant stress (25). Our study did not measure dietary intake, but a metabolic effect of smoking is not clearly supported by the low correlations that we observe between plasma levels of cotinine, vitamin C, and carotenoids. For plasma cotinine and plasma β-carotene, we even observe an unexpected weak positive association. The antioxidant vitamin E is slightly, although significantly, higher in smokers than in nonsmokers, but this may be explained by higher plasma lipid levels, which are

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*Fig. 1. Distribution of sister chromatid exchanges in smokers (n = 156) and nonsmokers (n = 38). Numbers on abscissa, upper boundaries of categories for SCEs.*
common in smokers (26). We had no lipid measurements to standardize the vitamin E levels (27).

An increase in SCE levels in smokers is also a well-known phenomenon, with levels being 10–88% higher than in nonsmokers (5). It should be noted that SCE levels observed in several studies cannot readily be compared, since laboratory protocols are not standardized (18). The sensitivity of the SCE determination to laboratory variations is illustrated by the significant variation over the 18 culturing days, which we observe despite adherence to a strict protocol. Nonlaboratory-related differences between the culturing days may also have attributed to this variation. In the analyses within the group of smokers (Fig. 3; Tables 2 and 3), the scorer and run variations did not bias our results, since the smokers were randomly divided over the scoring runs, and the observers were equally represented in every scoring run. The analyses adjusted for run and observer variation therefore yielded results similar to the unadjusted analyses. The potential for bias, however, is illustrated by the contrast between nonsmokers and smokers. This contrast increased after adjustment for run and scorer variation, reflecting the unequal distribution of nonsmokers over the runs and scorers. This is also exemplified by Fig. 1, showing that the overall variation in SCE counts is far less in the nonsmoker group than in the smoker group.

The correlations within the group of smokers between reported cigarette consumption or plasma cotinine are rather small. Reported cigarette consumption may not be a good measure of genotoxic exposure due to differences in brands and smoking and inhaling practices (28). Also, the relation between nicotine content and content of genotoxic agents in cigarettes is uncertain. However, since we also observe an only 10–20% increase in smokers as compared with nonsmokers, the conclusion that the SCE measure in lympho-

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**Table 2** Univariate correlations (Pearson coefficients) of several characteristics and blood parameters with SCE, micronuclei, and plasma cotinine in male cigarette smokers (n = 156).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Plasma cotinine</th>
<th>SCE</th>
<th>Micronuclei</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.193*</td>
<td>0.152</td>
<td>0.054</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>-0.163*</td>
<td>0.083</td>
<td>0.006</td>
</tr>
<tr>
<td>Alcohol (g/day)</td>
<td>-0.085</td>
<td>0.052</td>
<td>-0.057</td>
</tr>
<tr>
<td>Cigarettes/day</td>
<td>0.368*</td>
<td>0.248</td>
<td>-0.006</td>
</tr>
<tr>
<td>Duration of smoking (yrs)</td>
<td>0.239*</td>
<td>0.109</td>
<td>0.006</td>
</tr>
<tr>
<td>Plasma cotinine</td>
<td>1.000</td>
<td>0.186</td>
<td>-0.013</td>
</tr>
<tr>
<td>Blood vitamin C</td>
<td>0.042</td>
<td>0.039</td>
<td>-0.115</td>
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<tr>
<td>Plasma retinol</td>
<td>0.058</td>
<td>0.063</td>
<td>0.069</td>
</tr>
<tr>
<td>Plasma α-tocopherol</td>
<td>0.059</td>
<td>-0.022</td>
<td>0.007</td>
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<tr>
<td>Plasma β-carotene</td>
<td>0.177*</td>
<td>-0.038</td>
<td>0.109</td>
</tr>
<tr>
<td>Plasma total carotenoids</td>
<td>-0.090</td>
<td>-0.021</td>
<td>0.136</td>
</tr>
</tbody>
</table>

* P < 0.05.

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**Table 3** SCEs and micronuclei in GST-µ-positive and GST-µ-negative nonsmokers, smokers, "light" smokers (plasma cotinine < 315 ng/ml), and "heavy" smokers (plasma cotinine ≥ 315 ng/ml).

<table>
<thead>
<tr>
<th>Smoking status</th>
<th>Biomarker</th>
<th>GST-µ-negative</th>
<th>GST-µ-positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsmokers</td>
<td>SCEs</td>
<td>4.69 ± 0.35</td>
<td>4.74 ± 0.35</td>
</tr>
<tr>
<td>(n = 38)</td>
<td>Micronuclei</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smokers</td>
<td>SCEs</td>
<td>5.24 ± 0.95</td>
<td>4.97 ± 0.98</td>
</tr>
<tr>
<td>(n = 154)</td>
<td>Micronuclei</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light smokers</td>
<td>SCEs</td>
<td>4.95 ± 0.99</td>
<td>4.97 ± 1.01</td>
</tr>
<tr>
<td>(n = 76)</td>
<td>Micronuclei</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heavy smokers</td>
<td>SCEs</td>
<td>5.50 ± 0.84</td>
<td>4.97 ± 0.97</td>
</tr>
<tr>
<td>(n = 78)</td>
<td>Micronuclei</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* A part of these data have been previously published (12).

b P value for comparison, µ* versus µ+ = 0.09.

c µ* different from µ+; P = 0.01.

d Not sampled.
cytome is not very sensitive to variations in tobacco smoke exposure seems warranted. In this respect, it is noteworthy that lymphocytes also do not seem to be very sensitive to other types of genetic damage. DNA adducts or HPRT mutant frequency are also only moderately elevated in heavy smokers (29, 30).

Micronuclei in sputum (31) or bronchial brushings (32) have been reported to be 3-fold higher in smokers than nonsmokers. Micronuclei in exfoliated epithelial cells reflect the extent of chromosome breakage due to mutagenic exposure, when the cells were dividing a few days or weeks earlier, in the basal layer of the epithelium of the tracheobronchial tree (33). The mean micronuclei counts in our study in healthy volunteers are lower than in studies using hospitalized patients (31, 32). Benner et al. (34) also observed lower micronuclei counts in healthy smokers undergoing bronchoscopy than in patients. The previous studies (31, 32) also did not describe their scoring criteria, and counts for “high certainty micronuclei” may be much lower than counts including “medium certainty micronuclei” (35). Within our group of smokers, the micronuclei parameter is not associated with smoking intensity, as was reported previously using counts in only 500 sputum cells in patients (31). The lack of sensitivity for micronuclei in sputum in our study may be attributed to a large random sampling site variation, since expectorated cells may originate from all locations in the tracheobronchial tree. Studies using buccal micronuclei (33, 35) and bronchial brushings (34) have indeed demonstrated major sampling site variations.

Both micronuclei and SCEs reflect DNA damage, but the exact molecular mechanisms are not known. Micronuclei are considered to reflect chromosome breakage (33), whereas SCEs are considered to be due to perturbations in the DNA that persist through DNA replication (36). In vitro studies indicate that the mechanisms may be mediated by free radicals and oxidants but can also involve other pathways (37-40). The absence of associations of the vitamins C and E or carotenoids with SCEs and micronuclei does not support an involvement of free radicals and oxidants in vivo. However, there are numerous other nonenzymatic and enzymatic antioxidants that we did not measure (41), blood levels may not reflect long-term antioxidant levels in lymphocytes or lung tissue, and the range of antioxidant levels in our study may not have been sufficiently large to demonstrate associations. Also, large random variations both in micronuclei and SCE counts may introduce bias towards the null and thus obscure a possible weak association.

The results for the GST-μ phenotype indicate that a part of the variation in SCE counts in smokers is genetically determined, since the GST-μ isozyme is inherited in an autosomal dominant fashion (42). Glutathione-S-transferases detoxify reactive electrophiles, in particular epoxides (43), and GST-μ deficiency may imply a more limited capacity for detoxification and more carcinogen-mediated DNA damage. Our results for the SCE measure support this hypothesis and suggest that increased DNA damage in GST-μ-deficient heavy smokers may be involved the association between GST-μ and lung cancer that is observed in case-control studies (8, 44-46), as we have previously discussed in more detail (12). One of the studies showed a clear inverse relation between GST-μ deficiency and lung cancer in heavy smokers but not in light smokers (18), which seems to correspond with our data. Another study (46) was more equivocal but also observed an inverse relation (although not statistically significant) only in heavy smokers. Zhong (45) observed an inverse relation for squamous carcinoma but not for adenocarcinoma of the lung. The results for the micronuclei counts do not support the hypothesis since micronuclei counts were even somewhat lower in GST-μ-deficient subjects. The different results for the GST-μ analysis suggest that micronuclei and SCE may be different biological phenomena; although

Fig. 3. Relationship between sputum micronuclei counts and lymphocyte SCE in smokers (n = 143).
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epoxides may contribute to in vivo SCE induction, this may not be the case for micronuclei.

The concept that SCEs and micronuclei reflect different biological phenomena is also supported by the lack of an association between micronuclei and SCEs, as depicted in Fig. 3. Both micronuclei and SCEs are sensitive to carcinogens in experimental in vivo and in vitro models (36, 47, 48), but there is little information on correlations between SCEs and micronuclei in these models to compare with our epidemiological observations. Here again, caution in interpretation of our data is required since unexplained random variation in both parameters may have obscured a weak association. Also, the differences in tissues and time frame that the two cytogenetic parameters reflect may have contributed to the absence of a relation.

This study has evaluated the application of a number of biomarkers in a cross-sectional study in smokers and non-smokers. SCEs and micronuclei have been successfully used in previous studies to demonstrate differences in DNA damage between smokers and non-smokers. For the SCE measure, our study confirms these previous studies. In addition, the SCE measure in our study could be used to demonstrate differences in cytogenetic damage between smokers with or without genetically determined detoxification enzyme.

Our results do, however, demonstrate that both SCE and micronuclei have only limited or no sensitivity to variations in cigarette smoke exposure within a group of smokers. This limited sensitivity may be partly attributed to large variations of a yet unknown origin that we observe between persons in both SCE and micronuclei. This presumably random variation makes it difficult to unambiguously interpret the absence of relations (e.g., between antioxidants and cytogenetic damage) that we observe in this study. More information on biological, laboratory, and design factors that determine variations is necessary to be conclusive about the absence of associations.

References


### Markers for cytogenetic damage in smokers: associations with plasma antioxidants and glutathione S-transferase mu.

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