Age-related Negative Associations between Parameters of Cytogenetic Damage and \textit{ex Vivo} (±)-\textit{anti}-Benzo(a)pyrene Diolepoxide-induced Unscheduled DNA Synthesis in Smoking Humans\textsuperscript{1}

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Abstract

Chemical or physical modification of DNA may cause an increase in genomic mutations or other genetic alterations, which may ultimately result in the onset of cancer. To avoid these deleterious effects of DNA damage, humans possess DNA repair mechanisms. Decreased DNA repair, induced \textit{ex vivo} by UV light or ionizing radiation in human peripheral blood lymphocytes (PBLs), has been associated with aging. The aim of this study was to investigate whether repair of DNA damage, after \textit{ex vivo} exposure of PBLs obtained from smokers (\(n = 20\)) to (±)-\textit{anti}-benzo(a)pyrene diolepoxide [(±)-\textit{anti}-BPDE], which is a mixture of reactive metabolites from the environmental carcinogen benzo(a)pyrene, is also associated with age. Furthermore, age-related associations between \textit{ex vivo} (±)-\textit{anti}-BPDE-induced DNA repair and the frequency of endogenous cytogenetic damage (sister chromatid exchange: SCE, micronuclei: MN) were evaluated. A statistically significant negative association was observed between \textit{ex vivo} (±)-\textit{anti}-BPDE-induced unscheduled DNA synthesis and age of the donors. Also, parameters of endogenous lymphocytic cytogenetic damage were negatively associated with \textit{ex vivo} (±)-\textit{anti}-BPDE-induced unscheduled DNA synthesis and positively associated with age in this population. It is concluded that, with increasing age, a decrease in lymphocytic excision repair capacity may be responsible for increased lymphocytic DNA damage in smokers.

Introduction

Humans are continuously exposed to various agents that may chemically or physically modify cellular DNA. It is believed that such modifications result in a time-dependent accumula-

\textsuperscript{1}The abbreviations used are: PBL, peripheral blood lymphocyte; (±)-\textit{anti}-BPDE, (±)-\textit{anti}-benzo(a)pyrene diolepoxide; UDS, unscheduled DNA synthesis; BER, base excision repair; NER, nucleotide excision repair; SCE, sister chromatid exchange; MN, micronuclei; PHA, phytohemagglutinin.

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Here, we report age-related associations between DNA repair and DNA damage in this carcinogen-exposed population. As parameter of DNA repair, (±)-anti-BPDE-induced UDS was determined ex vivo in PBLs. (±)-anti-BPDE, a mixture of metabolites from the carcinogen benz(a)pyrene, which is present in cigarette smoke, induces a variety of DNA lesions that are substrates for BER or NER mechanisms (13-15). These repair mechanisms are expected to also act on cigarette smoke-induced DNA damage in vivo. To compare this estimate of DNA repair capacity with the extent of in vivo (smoking-induced) DNA damage, SCE frequencies and MN frequencies were determined in untreated PBLs, after stimulation to proliferation ex vivo.

Materials and Methods
All chemicals were purchased from Merck (Darmstadt, Germany), unless otherwise specified. Cell culture media and supplements were obtained from Life Technologies, Inc., (Paisley, Scotland). Twenty healthy male human smokers who smoked at least 10 cigarettes per day were included in this study. The age of the subjects ranged from 21 to 55 years. Mean (± SD) values for number of cigarettes smoked per day, pack-years (pack of cigarettes smoked per day × number of years of smoking) were: 19.6 ± 5.8 cigarettes/day; 14.8 ± 9.8 pack-years; and 12.3 ± 9.3 glasses/week and 5.3 ± 2.6 cups/day, respectively. All volunteers agreed to participate to this study by giving their written informed consent. The study protocol was approved by the Medical Ethical Commission of the University of Limburg (Maastricht, The Netherlands).

Heparinized venous blood for assaying cytogenetic damage and DNA repair was collected from each donor on 2 different days, within a period of 8 weeks. Methods for determination of SCE an MN have been described elsewhere (16). Briefly, for determination of endogenous SCE levels, 0.4 ml of blood were added to 5.0 ml of RPMI 1640, supplemented with 10% FCS, 100 μg/ml streptomycin, 100 units/ml penicillin, 5 mM L-glutamine, and 50 units/ml heparin (RPMI 1640 complete medium). PHA (0.2 ml) was added (final concentration, 52 μg/ml), and cells were cultured for 24 h at 37°C. Bromodeoxyuridine (Serva, Europe) was added (final concentration, 58 μM), and cultures were incubated for another 48 h. One h prior to harvesting, 100 μl of colcemid were added. Then cells were hypotonized in 75 mM KCl for 10–15 min and fixed with methanol:acetic acid (3:1, v/v). Metaphase slides were prepared and stained by means of the Hoechst-plus-Giemsa technique (17). Before microscopic evaluation, the slides were encoded. Slides were evaluated by a well-trained observer, and SCE scores were checked by a second independent observer. From each subject, for each time point, at least 20 metaphases, containing 40 chromosomes as a minimum, were analyzed for SCEs.

Endogenous MN frequencies were determined using the assay of Fenech and Morley (18). Venous heparinized blood (0.4 ml) was cultured in 5 ml of RPMI 1640 complete medium and 0.2 ml of PHA (final concentration, 52 μg/ml) to stimulate T-cell proliferation. To block cytokinesis, 44 h after culturing, cytochalasin B (Sigma, Axel, The Netherlands) was added to a final concentration of 6 μg/ml. After an additional culture period of 28 h, cultures were harvested as described above, and slides were prepared and stained with 3% Giemsa for 20 min. Prior to microscopic analysis, slides were encoded. Per individual, 1000 binucleated cells were analyzed for the presence of MN by a well-trained observer and checked by a second independent observer.

To estimate in vivo DNA repair capacity, (±)-anti-BPDE-induced UDS was determined in whole-blood cultures. (±)-anti-BPDE [(±)-7β,8α-dihydroxy-9α,10α-epoxy-7,8,9,10-tetrahydrobenz(a)pyrene; Midwest Research Institute, Kansas City, MO] stock solutions were prepared in anhydrous DMSO and stored at ~20°C in the dark prior to single use. (±)-anti-BPDE (or DMSO for solvent control-treated cultures) was added to a large volume of RPMI 1640, supplemented with 10% FCS, 100 μg/ml streptomycin, 100 units/ml penicillin, 5 mM L-glutamine, and 50 units/ml heparin, of which 2.5 ml were subsequently added to 0.2 ml of blood, to a final concentration of 1.0 μM (final concentration of DMSO, 0.4%). Within 10 min after addition of the carcinogen, [methyl-3H]thymidine (Amer- sham; specific activity, ~80 Ci/mmol) was added to a final concentration of 10 μCi/ml. Twenty-four h after initiation of the cultures, cells were hypotonized in 75 mM KCl for 10–15 min and fixed with methanol:acetic acid (3:1, v/v). Part of the cell suspension was pipetted on slides. Autoradiography and determination of the number of net grains/nucleus were performed as described elsewhere (19), except that quantification was done with the aid of an Artrek Counter model 880 (New Brunswick Scientific, Edison, NJ; adjusted to count mode) in combination with a Sony CCD/RGB Video Camera (Sony, Tokyo, Japan), interfaced to a Zeiss Axioskop microscope. The net number of grains/nucleus from a culture was obtained by correcting the number of nuclear grains for background grains (by subtracting the mean number of grains in two areas equal in size to the nucleus, situated to the right and left of it). The average number of net grains/nucleus observed in (±)-anti-BPDE-treated cultures corrected for the average number of net grains/nucleus observed in DMSO-treated cultures is referred to as (±)-anti-BPDE-induced UDS. Possible age-related correlations between ex vivo (±)-anti-BPDE-induced UDS and parameters of cytogenetic damage monitoring in vivo smoking-induced DNA damage were statistically analyzed by means of simple and multiple regression.

### Results and Discussion
In Table 1, means (± SD) and ranges of (±)-anti-BPDE-induced UDS, SCE frequencies, and MN frequencies in PBLs obtained from male smokers.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDS (net grains/nucleus)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(±)-anti-BPDE-treated cultures</td>
<td>24.9 ± 6.5</td>
<td>14.0-34.7</td>
</tr>
<tr>
<td>DMSO-treated cultures</td>
<td>1.81 ± 0.6</td>
<td>0.7-3.0</td>
</tr>
<tr>
<td>(±)-anti-BPDE-induced UDS</td>
<td>23.1 ± 6.2</td>
<td>12.2-32.5</td>
</tr>
<tr>
<td>SCE/cell (nontreated cultures)</td>
<td>6.1 ± 1.0</td>
<td>4.7-8.1</td>
</tr>
<tr>
<td>MN/1000 binucleated cell (nontreated cultures)</td>
<td>5.3 ± 2.0</td>
<td>2.5-9.5</td>
</tr>
</tbody>
</table>

To compare this estimate of DNA repair capacity with the extent of in vivo (smoking-induced) DNA damage, SCE frequencies and MN frequencies were determined in untreated PBLs, after stimulation to proliferation ex vivo.
anti-BPDE-induced-UDS = \((-0.30 \times \text{age}) + 33.31\). On the basis of the regression equation, average decrease in the extent of \((\pm)-\text{anti-BPDE}\)-induced DNA repair was 1.1% per year between 20 and 60 years of age. Other studies also report negative associations between repair, induced \textit{ex vivo} in PBLs with UV, and age (5–7). Pero and Ostlund (5) and Lambert et al. (6) observed decreases in \textit{ex vivo} UV-induced UDS of approximately 0.75 and 0.43% per year, respectively, between 20 and 60 years of age. Wei et al. (4) reported a reduction in removal of \textit{ex vivo} UV-induced DNA damage of approximately 0.6% per year, within a similar range of age. However, both positive associations and no associations between DNA repair, determined as UV- or \(N\)-acetoxy-\(N\)-acetylaminofluorene-induced UDS, and age have been reported as well (5, 20–22). This may be attributed to differences in either the duration of exposure of PBLs to carcinogens or the treatment of exposed cells with radiolabeled nucleotide precursors and thus, in fact, to differences in observed repair kinetics.

\((\pm)-\text{anti-BPDE}\) treatment of mammalian cells \textit{ex vivo}, including human PBLs, has been shown to result predominantly in formation of \(N^2\)-deoxyguanosine adducts \([\pm]-\text{anti-BPDE}-\(N^2\)-dG; Refs. 14 and 23–25]. Repair of these \((\pm)-\text{anti-BPDE}-\(N^2\)-dG adducts may proceed through transcription-coupled NER mechanisms (14, 15, 26]. Recently, a mechanism for transcription-coupled human NER was proposed (27). It is assumed that at least 17 proteins are involved in damage recognition, local DNA unwinding, dual incision around the lesion, release of the damaged oligonucleotide and refilling of the excision gap. In addition, \((\pm)-\text{anti-BPDE}\) treatment of isolated DNA has been shown to cause formation of base adducts like \(N^2\)-guanine and its unstable imidazole ring open product (28), which may be substrates for BER proteins \textit{in vivo}. With increasing age, DNA repair may decline as a consequence of intracellular decrease in activity or levels of one or more of these proteins involved in NER or BER.

In the same individuals, no significant association was observed between age and the rate of \((\pm)-\text{anti-BPDE}-\(N^2\)-dG adduct removal, as determined by \(^{32}\)P-postlabeling of DNA obtained at different time points from isolated PBLs after a 15-min exposure to \(0.5 \mu M \pm\text{anti-BPDE}\)\(^4\) (relative mean removal over 24 h compared to initial level, 7.6 ± 37%). Given the fact that UDS reflects the activity of all enzymes involved in excision repair, these data suggest that enzymes that are involved in later steps of repair, following the initial recognition and removal of \((\pm)-\text{anti-BPDE}\)-induced DNA damage, may be affected by aging.

Associations between age and initial \((\pm)-\text{anti-BPDE}-\(N^2\)-dG adduct formation have also not been observed in the same individuals\(^4\) \[mean \((\pm)-\text{anti-BPDE}-\(N^2\)-dG adduct level, within 15 min after \textit{ex vivo} exposure of PBLs to \(0.5 \mu M \pm\text{anti-BPDE}, 21.2 \pm 18.5\) per \(10^8\) nucleotides\]. Thus, it is likely that the observed age-dependent decrease in UDS is not related to age-dependent decreased initial \((\pm)-\text{anti-BPDE}\)-DNA adduct formation. Besides, because it is conceivable that aging results in decreased efficiency of detoxification mechanisms, an increase rather than a decrease in initial \((\pm)-\text{anti-BPDE}\)-DNA binding with aging would have been expected.

At the individual level, total WBC count was not significantly correlated with \((\pm)-\text{anti-BPDE}\)-induced UDS, excluding the possibility that intrindividual differences in effective cellular dose of \((\pm)-\text{anti-BPDE}\) was of influence on the observed correlation.

From this population, no information is available regarding T-cell subset CD4:CD8 ratios, which may be associated with aging (reviewed in Ref. 29). Therefore, we cannot exclude that the observed correlation may, in case T-cell subsets differ in their sensitivity toward \((\pm)-\text{anti-BPDE}\), in fact, reflect altered CD4:CD8 ratios.

In this population of smokers, interindividual variation was observed in the extent of \((\pm)-\text{anti-BPDE}\)-induced UDS, which could not be explained by smoking and alcohol and coffee consumption. Thus, with regard to possible effects of smoking on DNA repair, the number of cigarettes smoked per day did not correlate significantly with \((\pm)-\text{anti-BPDE}\)-induced UDS. Pack-years correlated positively with \((\pm)-\text{anti-BPDE}\)-induced UDS [simple regression, \(r = 0.46\), \(P = 0.042\]; regres-
Fig. 2. Correlation between SCE frequencies and ex vivo (±)-anti-BPDE-induced UDS in PBLs obtained from male human smokers. Numbers, ages of individual donors.

Both SCE [simple regression, \( r = -0.45, P = 0.048 \); regression equation, \( \text{SCE} = (-7.00 \times 10^{-2} \times (\pm)-\text{anti-BPDE-induced UDS}) + 7.73 \)] and MN [simple regression, \( r = -0.44, P = 0.052 \); regression equation, \( \text{MN} = (-0.14 \times (\pm)-\text{anti-BPDE-induced UDS}) + 8.48 \)] negatively correlated with the extent of (±)-anti-BPDE-induced UDS (Figs. 2 and 3). Smoking habits (cigarettes/day) did not influence these associations.

In conclusion, age-related decrease in the capacity of an individual’s DNA repair, determined ex vivo in (±)-anti-BPDE-treated resting PBLs, may be indicative of the capacity of those repair mechanisms in resting PBLs in vivo, which remove those lesions that are responsible for induction of cytogenetic damage in PHA-stimulated PBLs ex vivo.

In the same population, SCE frequencies were significantly positively correlated with age [simple regression, \( r = 0.45, P = 0.044 \); regression equation, \( \text{SCE} = 0.041x + 4.70 \)]. MN frequencies were also positively (nonsignificantly) associated with age [simple regression, \( r = 0.39, P = 0.09 \); regression equation, \( \text{MN} = 0.072x + 2.81 \)]. Smoking habits (cigarettes/day) did not influence these associations.

Previous studies also report positive associations between SCE frequencies or MN frequencies, respectively, and age. Smoking habits (cigarettes/day) did not influence these associations. Thus, the capacity of an individual’s DNA repair, as determined ex vivo in (±)-anti-BPDE-treated resting PBLs, may be indicative of the capacity of those repair mechanisms in resting PBLs in vivo, which remove those lesions that are responsible for induction of cytogenetic damage in PHA-stimulated PBLs ex vivo.

In conclusion, age-related decrease in the capacity of an individual’s DNA repair, determined ex vivo in (±)-anti-
BPDE-treated unstimulated PBLs, may resemble age-related decrease in the capacity of repair mechanisms, acting on smoking-induced DNA damage in vivo. Consequently, smoking-induced DNA damage may accumulate with increasing age and may be revealed as an age-associated increase in SCE and MN, after untreated PBLs are stimulated to proliferation ex vivo. PBLs do not themselves represent target organs for carcinogenic factors in vivo. However, in a recent study, increased PBL chromosome aberrations were found to be associated with increased overall risk for cancer (12). Therefore, age-dependent decreased DNA repair and increased cytogenetic damage in PBLs may reflect similar processes that are involved in age-related development of cancer in target organs in human populations exposed to environmental carcinogens like tobacco smoke.

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