Advances in Brief

In Vitro Induction of Benzo(a)pyrene Diol Epoxide-DNA Adducts in Peripheral Lymphocytes as a Susceptibility Marker for Human Lung Cancer

Donghui Li, Mianying Wang, Lie Cheng, Margaret R. Spitz, Walter N. Hittelman, and Qingyi Wei

Departments of Clinical Investigations [D. L., M. W., W. N. H.] and Epidemiology [L. C., M. R. S., Q. W.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Abstract

Given the same exposure, DNA adduct profiles can be considered as a phenotypic marker for carcinogen metabolism and DNA repair, which may reflect individual susceptibility to chemical carcinogenesis. Based on this notion, we have established a straightforward assay that measures induced DNA adducts in peripheral lymphocytes exposed in vitro to a model carcinogen, benzo(a)pyrene diol epoxide (BPDE) by 32P-postlabeling. To test the hypothesis that the levels of induced DNA adducts are a predictor for cancer risk, we conducted a pilot study of 21 lung cancer patients and 41 healthy frequency-matched controls. We found that the peripheral lymphocytes of cancer patients tended to accumulate higher levels of BPDE-DNA adducts than controls did (mean ± SE of relative adduct labeling × 107 value: 59.6 ± 12.0 versus 39.4 ± 6.1 for cases and controls, respectively; P = 0.09). Using the tertile relative adduct labeling value of controls (10 adducts/107 nucleotides) as the cutoff point, 18 of 21 cases and 23 of 41 controls distributed above this level (odds ratio, 4.7; 95% confidence interval, 1.2—18.5). In logistic regression analysis, the level of induced adduct was an independent risk factor (odds ratio, 6.4; 95% confidence interval, 1.3—29.4) after adjustment for the potential confounding factors, i.e., age, sex, ethnicity, and smoking. Stratified analyses showed that greater differences in DNA adduct levels induced by BPDE between cases and controls were observed in individuals younger than 65 years and in nonsmokers. Despite the small sample size, the significant association between the level of BPDE-induced DNA adducts and risk for lung cancer suggests that this assay is a promising method for further investigations.

Introduction

Lung cancer is the leading cause of cancer-related death for both men and women in the United States (1). Although cigarette smoking as the major risk factor is associated with 80% of lung cancer cases, only 10—15% of heavy smokers ultimately develop lung cancer (2). Inter-individual variation in cancer susceptibility to similar carcinogen exposure may depend on genetic polymorphisms in carcinogen metabolism and DNA repair. Biomarkers reflecting such susceptibility may, therefore, be useful for identifying high-risk individuals for cancer prevention.

Lung cancer is a genetic disease that is associated with the accumulation of genetic damage induced by carcinogen exposure. Most chemical carcinogens present in cigarette smoke exist in a procarcinogen form and require metabolic activation to form electrophilic species that covalently bind to DNA, resulting in DNA adduct formation. If DNA adducts are not repaired efficiently before DNA replication, they may cause mutations, DNA strand breaks, and other genetic alterations. Hence, carcinogen-DNA adduct formation is thought to play a central role in the early stages of chemical carcinogenesis and to contribute to tumor development through accumulated genetic damage (3). The working hypothesis that DNA adduct profiles as a sum of carcinogen activation/detoxification reactions and DNA repair activities under the same exposure conditions are risk factors for developing smoking-related cancers is attractive. Indeed, two studies of aromatic DNA adducts in the peripheral WBCs of lung cancer patients and healthy controls have demonstrated that given similar current smoking patterns, cases tend to have higher levels of adducts than controls do (4, 5). However, due to the difficulties in accurately measuring exposure in population-based studies, it is often not clear whether the levels of DNA adducts mainly reflect the ambient exposure level or individual variation in response to the exposure. To evaluate the importance of intrinsic determinants, such as carcinogen metabolism (detoxification) and DNA repair capacities, on DNA adduct formation, we established the current assay that compares DNA adduct levels in the readily accessible peripheral blood lymphocytes in vitro exposed to a model carcinogen, BPDE2 (the activated form of BP) in different individuals. In this assay, the same dose of BPDE is given to each test sample; therefore, the levels of BPDE-induced DNA adducts reflect individual variation in response to the carcinogen challenge. Using this assay in a pilot case-control study, we demonstrated that lung cancer patients tended to have a higher level of BPDE-induced DNA adducts than cancer-free controls did.

Materials and Methods

Materials. Benzo[a]pyrene-7,8-dihydriodiol-9,10-epoxide (anti-BPDE, 99%) was purchased from NCI Chemical Carcinogenesis Repository (Midwest Research Institute, Kansas City, MO) and was dissolved in tetrahydrofuran (Sigma Chemical Co., St. Louis, MO). The 5 mg/ml (16.7 mM) stock solution was further diluted to 1 mM for the working solution. All solutions were prepared in a dark room and kept at −20°C to avoid photooxidation.

Study Population. The study subjects included in this report were participating in an ongoing case-control study of lung cancer in minority populations (African Americans and Mexican Americans). The demographic characteristics of the study subjects are summarized in Table 1. The average ages of the study subjects (± SD) were 68.7 ± 8.5 and 66.7 ± 12.3 for cases and controls, respectively. Mexican Americans older than 65 years of age were overrepresented in this study, and there were more male than female smokers (data not shown). The 21 cases had newly diagnosed, histologically confirmed lung cancer and had not had prior radiotherapy or chemotherapy. Tumors of the 21 cases included 11 squamous carcinomas; 4 adenocarcinomas; 2 small cell, 1 large cell, and 1 non-small cell carcinoma; and 2 unspecified lung cancers. The 41 controls were healthy individuals without prior history of cancer and were frequency-matched to the cases by age, sex, and ethnicity. A short questionnaire that elicited information about demographic variables and smoking history was completed by each study subject.

Cell Culture and BPDE Treatment. The cell culture and BPDE treatment conditions were optimized in a previous study where chromosome aberrations...
were measured by the mutagen sensitivity assay (6). Briefly, 1 ml of whole
blood (usually containing 10–15 million cells) was inoculated into each of two
T-25 flasks (containing 9 ml of standard RPMI 1640, supplemented with 15%
chromosomal damage along with DNA adduct measurement (6). The cells
cytotoxicity related to prolonged exposure (i.e., 24 h) and allow evaluation of
37°C for 72 h. It is known that unstimulated lymphocytes have little DNA
blood (usually containing 10–15 million cells) was inoculated into each of two
were measured by the mutagen sensitivity assay (6). Briefly, 1 ml of whole
blood (usually containing 10–15 million cells) was inoculated into each of two
T-25 flasks (containing 9 ml of standard RPMI 1640, supplemented with 15%
fetal bovine serum and 112.5 μg/ml phytohemagglutinin), and incubated at
37°C for 72 h. It is known that unstimulated lymphocytes have little DNA
repair activity (7); therefore, the 72 h of cell stimulation was necessary to
demonstrate differences in DNA repair capacity (8). One flask of cells was
treated with 4 μM BPDE for 5 h, and a control flask received tetrahydrofuran
alone. This dose and duration of BPDE treatment was chosen to prevent
cytotoxicity related to prolonged exposure (i.e., 24 h) and allow evaluation of
chromosomal damage along with DNA adduct measurement (6). The cells
were then harvested by using published procedures (9) and stored at -80°C
before DNA isolation.

DNA Isolation and Adduct Analysis. The cell pellets were incubated in
digestion buffer [10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 25 mM EDTA, 0.5%
SDS, and 0.1 mg/ml proteinase] at 50°C for 12 h. The cell lysates were then extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1
v/v/v). Each aqueous phase was transferred to a fresh tube and adjusted to 150
mM sodium acetate and 10 mM MgCl₂, and DNA was precipitated with 3 volumes of ethanol at -20°C for 12 h. The DNA pellet was dissolved in 0.01 X
SSC, digested with RNAse A and RNAse T1 at 37°C for 2 h, and extracted
with an equal volume of phenol:chloroform:isoamyl alcohol. The DNA was
then precipitated with ethanol and dissolved in 0.01 X SSC, and its concen-
tration was measured by spectrophotometry. The nuclease P1-enhanced ver-
sion of the 32P-postlabeling assay (10) was used in the DNA adduct analysis,
and 2.5 μg DNA of each sample were used.

Statistical Analysis. DNA adduct levels were analyzed as a continuous
variable. Student’s t test was used to compare the differences in the means of
adduct levels between groups. Correlation analyses were performed to com-
pare the level of induced adducts with selected host factors. To test for asso-
ciations between smoking, DNA adduct level, and cancer risk, crude ORs
were calculated as estimates of the relative risk by using both the median
and tertile adduct levels in controls as cutoff values. Ninety-five % CI was
computed as described previously (9). Logistic regression was used to estimate
risks adjusted for other covariates. All statistical analyses were performed by
using Statistical Analysis System Software (Version 6; SAS Institute, Inc.,
Cary, NC).

Results

Detection of BPDE-DNA Adducts. By using the nuclease P1-
enhanced version of 32P-postlabeling, four major BPDE-DNA adducts
were detected in peripheral lymphocytes exposed in vitro to BPDE for
5 h (Fig. 1). Spot 1, which accounted for 73% of the total adducts, is
the stable BPDE-N²-deoxyguanosine adduct, as confirmed by com-
parison with an adduct standard by high-performance liquid chroma-
tography (data not shown). The levels of total BPDE-DNA adducts
ranged from 0.6 to 161.9 adducts per 10⁷ nucleotides in the test
samples (data not shown). Because 63% of the study subjects were
smokers, we expected to detect cigarette smoking-related DNA ad-
ducts in their lymphocytes. To determine whether the background
adduct levels would affect the levels of BPDE-induced DNA adducts,
we also analyzed DNA adducts from the vehicle-treated lymphocytes
of 13 cases and 20 controls under the same experimental conditions.
The average RAL × 10⁷ value (± SD) for mock-treated specimens
was 0.2 ± 0.1 and 0.1 ± 0.1 for cases and controls, respectively.
These levels were negligible compared with those observed following
BPDE exposure, and no correlation was found between the levels of
background adducts and BPDE-induced adducts (P > 0.05). The low
level of DNA adducts found in the unexposed cells was expected
because the adducts initially present in the DNA may have been
diluted by DNA synthesized during the 72 h of culture before treat-
ment. In addition, a large portion of the initial DNA adducts contain-
ing aromatic amine compounds may have been lost as a result of the
nuclease P1 digestion and the strong chromatography conditions used
for BPDE adduct analysis.

DNA Adducts and Cancer Risk. The total BPDE-DNA adduct levels were
different between cases (range, 0.6–161.9/10⁷; median, 50.7/10⁷) and controls (range, 1.0–118.1/10⁷; median, 17.2/10⁷) (Fig. 2). The RAL × 10⁷ values of total adducts were 59.6 ± 12.0
(mean ± SE) and 39.4 ± 6.1 for cases and controls, respectively
(P = 0.09 by t test). Stratified analysis showed that the differences in
adduct levels between cases and controls were larger in nonsmokers

Table 1 Adduct levels for select patient categories (RAL × 10⁷)

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control</th>
<th>Case</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &gt;65</td>
<td>24</td>
<td>14</td>
</tr>
<tr>
<td>Age ≤65</td>
<td>17</td>
<td>7</td>
</tr>
<tr>
<td>Sex Male</td>
<td>25</td>
<td>18</td>
</tr>
<tr>
<td>Sex Female</td>
<td>16</td>
<td>3</td>
</tr>
<tr>
<td>Ethnicity African</td>
<td>12</td>
<td>10</td>
</tr>
<tr>
<td>Ethnicity Mexican</td>
<td>29</td>
<td>11</td>
</tr>
<tr>
<td>Smoking Ever</td>
<td>21</td>
<td>18</td>
</tr>
<tr>
<td>Smoking Never</td>
<td>20</td>
<td>3</td>
</tr>
</tbody>
</table>

Fig. 1. Typical 32P-labeled BPDE-DNA adduct profiles in cultured peripheral blood
cells treated with 4 μM BPDE or vehicle (C) for 5 h. Spot 1 is the BPDE-deoxyguanosine
adduct, S internal standard DNA adduct from skin DNA of ICR mice treated
with dibenzo[a]pyrene.
than smokers ($P = 0.07$) and in individuals younger than 65 ($P = 0.07$; Table 1).

Because cases and controls were matched for age, sex, and ethnicity, the univariate analysis focused on the selected risk factors, smoking, and adduct levels. When the median value (17.2/10^7) of total DNA adducts in controls was used as a cutoff point, 14 of 21 cases had levels of total adducts above this value (OR, 1.9). However, when the tertile of the control values (10.0/10^7) was used as the cutoff point, 18 of 21 cases and 23 of 41 controls had levels above this value (OR, 4.7; Table 2). When multivariate logistic regression analysis was performed, having a DNA adduct level above the tertile level of controls remained a significant and independent risk factor for lung cancer after adjustment for age, sex, ethnicity, and smoking status (adjusted OR, 6.4; 95% CI, 1.4–30.5; $P < 0.02$).

**Discussion**

Several human cancers, including cancers of the lung and upper aerodigestive tract, are known to be related to cigarette smoking. Although smoking cessation may be the most effective way to reduce the risk of such cancers, using biomarkers to identify individuals at high risk, especially those who cannot quit smoking, may be of potential importance in cancer prevention.

Many of the carcinogens present in cigarette smoke can be metabolically activated in vivo and covalently bind to DNA, resulting in DNA adduct formation. If DNA adducts are not repaired before DNA replication, they could cause mutation, DNA strand break, and other genetic alterations that directly contribute to carcinogenesis. Therefore, genetically determined individual variation in carcinogen metabolism and DNA repair may modulate the risk of developing smoking-related cancers. Nevertheless, many genes are involved in carcinogen metabolism and DNA repair; it would not be practical to analyze every gene involved to satisfactorily characterize the susceptibility profiles of an individual. In general, DNA adduct formation represents a sum of carcinogen activation/detoxification reactions as well as DNA repair. Therefore, the measurement of DNA adducts would be a more informative downstream indicator of the capacity of cells to respond to carcinogen challenge than would a single measurement of enzyme activity or genetic polymorphism.

Molecular epidemiological study usually involves a large number but a little amount of tissue samples and, therefore, requires a simple and sensitive laboratory method. Several previous studies (11–17) have used isolated peripheral lymphocytes or monocytes in vitro exposed to BP or BPDE to investigate individual variations in DNA adduct formation and DNA repair. The protocol used in the current study was different from these studies and has several advantages for epidemiological applications. For example, we used 1 ml of a whole blood sample instead of isolated lymphocytes, which usually require 20 ml of blood for each assay. This approach not only reduced the amount of blood needed but also simplified the experimental procedure. The use of 1 ml of whole blood was possible because of the small amount of DNA samples required for adduct analysis. The high levels of DNA adducts formed during continuous treatment of stimulated cells with the activated carcinogens, along with the high sensitivity of the 32P-postlabeling assay, allowed us to perform the adduct analysis using 1–2.5 µg of DNA. Furthermore, under this experimental condition, BPDE induced a substantial number of chromosome aberrations without severe cytotoxicity (as determined by the mitotic index). Therefore, the individual differences in response to carcinogen exposure can be examined at both DNA and chromosome levels in the same sample, which increased the efficiency of the study. Although it does not provide information on which particular genes or enzymes are associated with the detected levels of DNA adducts, the simplicity and efficiency of this type of assay make it more desirable for epidemiological studies.

We chose to use BPDE instead of BP because the inducibility of aryl hydrocarbon hydroxylase (which is responsible for BP activation) in stimulated lymphocytes has not been consistently associated with risk for smoking-related cancers (14). Use of BPDE enabled us to focus on factors other than BP activation, such as carcinogen detoxification and DNA repair capacities. Due to the continuous BPDE treatment in our study, the detected adducts at 5 h may reflect a balance of DNA adduct formation and removal. However, it has been shown in rodent cells that maximum DNA binding of BPDE had already been accomplished after 15 min of exposure (18). Thus, it is likely that variation in DNA repair capacity is the major underlying mechanism responsible for the differences in induced adduct levels. Indeed, our previous study showed that reduced DNA repair capacity was more common in lung cancer patients than in controls (19). On the other hand, a large difference in the initial amounts of adduct formation in lymphocytes in vitro exposed to BPDE has been reported (15), and the level of adducts after DNA repair was proportional to the initial adduct level in repair-proficient cells (20). Therefore, the induced adduct levels in this study may also be related to the initial amount of adduct formation, which is affected by the efficiency of detoxification system, such as glutathione S-transferase and other phase II enzymes. The correlation between DNA repair capacity, phase II enzymes, and DNA adduct levels warrants further investigation.

Because BP is a representative carcinogen detected in cigarette smoke and the ambient environment, sensitivity to BPDE challenge may have a significant implication in human cancer risk assessment. Our finding that induced DNA adduct level is an independent risk factor for lung cancer in this pilot case-control study is supported by the potential importance in cancer prevention.

**Table 2 Risk analysis for select variables**

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>No. of cases</th>
<th>No. of controls</th>
<th>OR (95% CI)</th>
<th>Adjusted OR* (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Never</td>
<td>3</td>
<td>20</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ever</td>
<td>18</td>
<td>21</td>
<td>5.7 (1.5–22.4)</td>
<td>3.4 (0.7–17.6)</td>
</tr>
<tr>
<td>Adduct</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;10/10^7</td>
<td>3</td>
<td>18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt;10/10^7</td>
<td>18</td>
<td>23</td>
<td>4.7 (1.2–18.5)</td>
<td>6.4 (1.4–30.5)</td>
</tr>
</tbody>
</table>

*Adjusted for age, sex, and ethnicity.
by a previous report that patients with a presumed predisposition to lung cancer had enhanced formation of DNA adducts in their monocytes, which were treated in vitro with BP (11). These findings suggest that the induced DNA adduct assay may have the potential to identify individuals at high risk of developing smoking-related cancers. The observation that smoking status was not related to the levels of the in vitro-induced DNA adducts has also been reported in previous studies (13, 14), which suggests that the difference in the levels of induced DNA adducts was determined by intrinsic host factors. The greater differences in DNA adduct levels between cases and controls among individuals younger than 65 and among nonsmokers support the notion that in predisposed patients, carcinogen exposure might be lower, e.g., in nonsmokers or young individuals with lung cancer, but their sensitivity to the carcinogen might be higher due to endogenous factors. Our previous studies have shown that younger cancer patients are more likely than older ones to have reduced DNA repair capacities as compared with their cancer-free controls (8).

Measuring a battery of related biomarkers in the same study population would not only increase the chance of identifying individuals at high risk for developing cancer but also provide some insight into how chemical carcinogen-related human cancers arise. The usefulness of this induced adduct assay and the findings from the pilot case-control comparison need to be verified with larger samples and more ethnic groups.

References

In Vitro Induction of Benzo(a)pyrene Diol Epoxide-DNA Adducts in Peripheral Lymphocytes as a Susceptibility Marker for Human Lung Cancer

Donghui Li, Mianying Wang, Lie Cheng, et al.