Correlation of DNA Adducts in Blood Mononuclear Cells with Tobacco Carcinogen-induced Damage in Human Lung

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ABSTRACT

The formation of carcinogen-DNA adducts within the respiratory epithelium is thought to be a critical factor in the induction of lung cancer from tobacco smoke. A reliable surrogate measure of carcinogen damage to the lung would be of great value in molecular epidemiological studies of cancer risk. The validity of measurements of DNA adducts formed from hydrophobic aromatic hydrocarbons in peripheral blood mononuclear cells (MNCs) was investigated by comparing the levels of aromatic DNA adducts detected in lung tissue from 31 lung cancer patients with those detected in MNCs from the same individuals using the 32P-postlabeling assay. The associations of smoking history and intake of dietary antioxidants with adduct levels also were assessed. Time-specific, as well as common DNA adducts were detected in lung and blood; total MNC adduct levels were highly correlated with total lung adducts. After smoking cessation, adduct levels appeared to decay in both tissues at similar rates. Multivariate analyses (Poisson regression modeling) indicated that dietary antioxidant intake (carotenoids, vitamin A, and retinol) modified the levels of aromatic DNA adducts in both the lungs and blood. Of all models tested, the optimal one for predicting lung adduct levels included the measure of blood MNC adduct levels only. Therefore, blood MNCs are a valid surrogate tissue for estimating the burden of DNA adducts in respiratory tissue in molecular epidemiological studies.

INTRODUCTION

Epidemiological studies have established the causal link between tobacco smoking and the lung cancer epidemic of the 20th century (1–3). Recent studies indicate the evolving nature of the epidemic, and new approaches that incorporate molecular markers have been proposed to address these new challenges for lung cancer prevention and control. Decreases in tobacco consumption in middle-aged males have led to decreases in lung cancer mortality (4–6); former smokers, however, may be at excess risk for up to 30 years after smoking cessation (7). Increases in smoking among women have been associated with striking increases in lung cancer incidence and mortality (8). Dramatic shifts in the histological type and location of lung tumors have occurred during the last two decades (9). Life-style factors, including changes in dietary fat or antioxidant intake, also could affect future trends in lung cancer mortality (10, 11).

Biomarkers of the biologically effective dose of tobacco carcinogens within the respiratory epithelium figure prominently in molecular studies of lung carcinogenesis. These markers estimate the formation of stable complexes of tobacco carcinogens with DNA, which are thought to be a crucial step in cancer induction (12, 13). The most widely studied class of smoking-induced DNA adducts is derived from polynuclear aromatic hydrocarbons. Hydrophobic aromatic DNA adducts detected by the ultrasensitive 32P-postlabeling assay have been identified in tissues obtained at surgery or autopsy that are known targets of tobacco smoke carcinogenesis: lung, bronchus, larynx, oral cavity, and bladder (14–24). Smoking exposure indices such as the cumulative pack/year history of tobacco use among current smokers have been found to be correlated linearly with aromatic DNA adduct levels in human lung (14, 16, 25) and bronchus (15). After smoking cessation, aromatic DNA adduct levels decrease in lung (26), although the kinetics of the disappearance of these lesions have not been well characterized.

A crucial issue for molecular epidemiologists is the validity of surrogate tissue sources of DNA adducts that are obtained from the general population. Previous attempts to validate peripheral blood white cells as a surrogate tissue source for lung adduct determinations used DNA isolated from total white blood cells. Approximately 70% of total white blood cells are short-lived granulocytes, whereas the remaining 30% are longer-lived MNCs.3 Total white blood cell aromatic DNA adduct levels were found to be associated neither with active cigarette smoking (14, 16, 26) nor with adduct levels measured in lung tissue from the same individuals (27). In other studies, however, aromatic DNA adduct levels from MNCs were found to be increased in current smokers (28) and were reproducibly measured by the 32P-postlabeling assay over time (29). Consequently, we propose that long-lived MNCs should be a valid surrogate tissue for assessing chronic exposure to tobacco carcinogens.

MATERIALS AND METHODS

Surgically resected noninvolved lung tissue was obtained from patients (21 male and 10 female) undergoing surgery for histopathologically confirmed lung cancer. The protocol used was approved by the Committees on the Use of Human Subjects in Research at the Massachusetts General Hospital and the Harvard School of Public Health (Boston, MA). Tissue specimens were frozen immediately on dry ice and maintained frozen at −70°C until DNA adduct studies. Blood samples (30 ml heparinized whole blood) were obtained from each subject and applied to Ficoll-Hypaque density gradients to separate MNCs from erythrocytes and granulocytes. In addition, a questionnaire was administered to each patient, eliciting information on his or her smoking history, occupation, and other demographic factors. Detailed dietary histories were collected using the Harvard-Willett food frequency questionnaire (30).

Frozen tissues or MNCs were homogenized in 0.1 M Tris, 0.1 M NaCl, 5 mM EDTA (pH 8.0), and 10% SDS on ice and then extracted twice with equal volumes of chloroform:isoamyl alcohol (24:1). The aqueous supernatant was incubated with RNase A and RNase T1 (250 µg/ml, Sigma Chemical Co.) at 37°C for 60 min followed by digestion with proteinase K (10 µg/ml, Merck) at 37°C for 60 min. The digest was extracted twice with chloroform:isoamyl alcohol, then sodium acetate (4.0 M final solution) was added to the aqueous supernatant. DNA was precipitated with ethanol at −4°C and dissolved in water. The quantity of DNA was determined by a fluorometric method. The 32P-postlabeling method has been described (31). Four µg purified DNA were

Received 5/19/95; accepted 8/31/95.

1 Supported by Grants PO1-ES06409 and P42-ES04705 from the National Institute for Environmental Health and Safety and Grant CA06409 from the National Cancer Institute.

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3 The abbreviations used are: MNC, mononuclear cell; DRZ, diagonal radioactive zone.
digested enzymatically to 3'-deoxynucleotide monophosphates with micrococcal nuclease (Worthington Biochemicals) and spleen phosphodiesterase. Samples were then treated with P<sub>1</sub> nuclease. The modified nucleotides were converted into 32P-labeled 3',5'-deoxynucleotide bisphosphates by incubation with 150 µCi of [32P]ATP (6000 Ci/mmol; DuPont-NEN) and 2.5 µL T<sub>4</sub> polynucleotide kinase. The total volume of 32P-labeled 3',5'-deoxynucleotide bisphosphates was applied to each 10 × 10 cm polyethyleneimine cellulose plate. Plates were developed overnight in 1 M NaH<sub>2</sub>PO<sub>4</sub> (pH 6), and the plates were washed twice (7 min) with H<sub>2</sub>O, followed by a 10-min wash in 0.15 M ammonium formate (pH 3.5). The plates were air dried and developed in 3.0 M lithium formate and 7.0 M urea (pH 3.5) from the bottom to the top of the plate. The plates were then washed twice (7 min) in H<sub>2</sub>O, air dried, and developed at a right angle to the previous direction of development in 0.72 M NaH<sub>2</sub>PO<sub>4</sub>, 0.45 M Tris-HCl, and 7.6 M urea (pH 8.2). The plates were then washed twice with H<sub>2</sub>O and subsequently developed in the same direction with 1.7 M NaH<sub>2</sub>PO<sub>4</sub> (pH 6.0). The adducts were located by autoradiography using Kodak XAR-5 film (Eastman Kodak) and a Dupont Chronex-Lightning Plus intensifying screen (DuPont-NEN). The film was exposed at -70°C for 3-4 days. The areas of the radioactive spots on the polyethyleneimine cellulose sheets were measured, and the spots were then scraped into liquid scintillation vials containing 5 ml scintillation mixture (Safety Solve; Research Products, Inc.); radioactivity was determined by liquid scintillation counting. Regions adjacent to the radioactive spots of equal area also were scraped, placed into scintillation vials, and counted for background determination. Adduct levels were corrected for background counts after adjusting for the area of the TLC sample. The level of modification was calculated as described. For example, assuming that 4 µg of DNA = 1.21 × 10<sup>12</sup> pmol of 3'-deoxynucleotide monophosphates and that the specific activity of the [32P]ATP is 9.36 × 10<sup>6</sup> cpm/pmol, adduct levels were calculated as follows: relative adduct level (RAL) = cpm in adducts/11.32 × 10<sup>18</sup> cpm.

RESULTS

Table 1 summarizes the characteristics of the 31 lung cancer cases; of significance is the fact that 50% of the subjects were former smokers who had not smoked for at least 1 year. The average time since smoking cessation in these subjects was nearly 15 years. Despite the length of time since active smoking, all subjects had detectable levels of lung aromatic DNA adducts (i.e., above the limit of detection, which is 1 adduct/10<sup>10</sup> nucleotides). Samples used for DNA adduct studies were uninvolved tissues adjacent to the tumor. Because of variations in the sizes of the resected tissue, the specimens varied in size (0.2-2.0 g). No correlation was observed between the size of the sample and the resultant DNA adduct level (data not shown).

Although no attempt was made to correlate adduct values with the anatomic location of the tissue samples, we did analyze adduct values in relationship to tumor histology and found no association of cell type with DNA adduct levels.

A diffuse band of radioactivity, the DRZ, and discrete adducts were observed (Fig. 1). The structures of the individual adducts were not determined. However, we developed a composite adduct map (Fig. 1A) and assigned the same adduct number to chromatographic spots, which, when reisolated, cochromatographed in several different buffer systems to the same location or region. Overall, total relative adduct values (DRZ plus discrete adducts) were 2.5-fold higher in lung compared with blood MNCs (Table 1). However, these differences between tissues cannot be considered absolute, because the labeling efficiencies and recoveries of the adducts detected are not known.

Among the nine discrete adducts that were detected, tissue-specific associations were noted. Adducts that chromatographed to regions 3, 4, and 5 were not detected in any MNC analyses; adducts in the regions 7, 8, and 9 were detected only in MNCs; and adducts observed in regions 1, 2, and 6 were found in both lung and MNC samples.

The DNA adduct found in region 2, which was found in both lung and blood MNCs, cochromatographed with 32P-labeled (+)-anti-BPDE-N<sup>2</sup>-deGuanine. This is the major stable adduct formed from benzo(a)pyrene in many cells and tissues, including human blood MNCs (31). Adducts in regions 3, 4, and 5 cochromatographed with DNA isolated from MNCs treated with benzo(a)pyrene-4,5-oxide (31). DNA adducts with chromatographic properties similar to those of adducts observed in regions 3, 4, and 5 also have been isolated from the lung of 9-hydroxy-benzo(a)pyrene-treated rats; these adducts were postulated to be derived from the 4,5-epoxide of 9-hydroxy-benzo(a)pyrene (32).

Initial analysis indicated a significant correlation between total aromatic DNA adducts in blood MNCs and total adduct levels in lung (Spearman’s correlation r = 0.74; P < 0.001; Fig. 2). We tested whether individual blood DNA adducts or combinations showed a stronger association with total lung adduct levels than the total blood adduct value. None of the individual blood adduct indices correlated more highly with total lung adducts than did total blood adduct level. Of interest, however, was a significant correlation of MNC-specific adducts with lung adducts. For example, the sum of MNC adducts in regions 7, 8, and 9 correlated well with total lung adducts (Spearman’s correlation r = 0.54; P < 0.002) and with lung specific adducts in regions 3, 4, and 5 (Spearman’s correlation r = 0.76; P < 0.001).

Because so many of the subjects were former smokers, and because aromatic DNA adducts have been shown to decrease after smoking cessation, we examined lung and blood adduct levels in relation to years since smoking cessation. Fig. 3 illustrates the cross-sectional analysis of adduct levels in former smokers. The data suggest a rapid decrease in adduct levels in both lung and blood within the first 2 years after smoking cessation, although DNA adducts were detectable even in individuals who had quit smoking 40 years before our study. Although all data comparing lung and blood adduct levels are derived from smokers, three additional lung samples were obtained from lung cancer patients who reported never smoking tobacco. Very low adduct levels were found in lung specimens from never-smokers; total adduct values of 37, 3, and 50 adducts/10<sup>10</sup> nucleotides were observed for the three subjects. The relatively low adduct values in never-smokers is consistent with the idea that the majority of adducts detected in the 31 current and former smokers are smoking-related.

To test whether dietary intake of antioxidants affects lung DNA adduct levels and to compare these effects with adduct levels in blood, we performed multivariate analyses that controlled for intensity of recent smoking and the time since smoking cessation in former smokers. These analyses were carried out after testing for potential effects of the patients’ age, sex, or tumor histology, none of which were associated with adduct levels. The most important smoking
variables identified (cigarettes/day during the last year and years since smoking cessation) were entered into Poisson regression models (33) with a series of antioxidant variables: total vitamin A, retinol, carotenoids (those with provitamin A activity), vitamin C, and vitamin E. Stepwise elimination of variables yielded carotenoid intake as the most significant independent variable in the lung. Vitamin A and retinol, which were highly correlated with carotenoids, also were significant in the lung and when the same model was applied to the blood MNC data. Vitamins C and E were the least significant in the lung, although, in the blood model, vitamin E was highly significant (data not shown). Table 2 illustrates the similarity in both direction and magnitude of the regression coefficients for years since smoking cessation, and carotenoid intake in the lung and blood models.

**DISCUSSION**

Molecular epidemiology has been proposed to be an important adjunct to conventional studies that will provide the basis for understanding the role of environmental tobacco smoke in lung cancer (34), the efficacy of chemoprevention and dietary interventions (35), and the contribution of genetic susceptibility (36, 37) in smoking-related cancers. Our results indicate that DNA adducts measured by $^{32}$P-postlabeling in blood MNCs may be a useful marker to address these issues. Our finding of a positive correlation between blood and lung adduct levels suggests that MNCs are a valid surrogate for the lung. Recent studies indicate that a large fraction of blood lymphocytes, which represents approximately 70–90% of the MNC fraction, migrates to the lung and slowly redistributes to peripheral blood and other tissues (38, 39). Such intimate contact with the pulmonary capillary network would be expected to result in significant exposure of lymphocytes to inhaled tobacco carcinogens. The dose to these cells would not be expected to be as great as that to the directly exposed respiratory epithelium. In addition, blood MNCs may express a distinct complement of metabolic activities toward tobacco carcinogens (40, 41). Therefore, blood MNCs exposed *in vivo* could be expected to produce a cell-specific pattern of reactive electrophiles and DNA adducts. These observations may help to explain the major findings of our studies: (a) Aromatic DNA adduct levels in blood MNCs, although lower in concentration, were highly correlated with levels in lung; and (b) tissue-specific patterns of adducts were observed; however, MNC-specific adducts still correlated with the lung measurements. The fact that MNCs are longer-lived cells than are blood granulocytes may explain why we observed positive correlations between blood and lung adduct levels, whereas earlier studies, in which total white cells were used, reported no significant associations (17). Short-lived granulocytes, which are the predominant nucleated cell type within whole blood, may not persist long enough to accumulate detectable levels of smoking-related DNA adducts. The higher relative adduct levels in lung compared with white blood cells is
consistent with previous studies using the $^{32}$P-postlabeling assay (42). Differences in adduct levels between tissues also could be attributed to differences in the efficiency of phosphorylation of tissue-specific DNA adducts. To address these issues, additional characterization of the chemical structures of tobacco-related DNA-adducts is required, and new analytical strategies must be developed (21).

Perhaps the most important determinant of individual differences in DNA adduct levels uncovered in this study was the time since smoking cessation in former smokers. The negative correlation between adduct levels and smoking cessation is consistent with previous studies (26) and supports the notion that DNA adducts are related to lung cancer risk, because epidemiological studies have demonstrated decreases in risk after smoking cessation. Cross-sectional studies, such as this one, are limited in their ability to define the rate of loss of adducts from the lung. Longitudinal studies of lung DNA adducts, however, are not feasible, given the requirement for repeated invasive procedures, such as lung biopsy. The apparent similarity in the kinetics of decay of aromatic DNA adducts in lung and MNCs that we observed indicates that longitudinal studies using blood DNA adduct levels, which are less invasive, may be useful for estimating the kinetics of adduct loss in lung after smoking cessation. In long-term former smokers, low levels of DNA adducts were detectable even decades after smoking cessation. Although these adducts could represent highly persistent forms of smoking-related DNA damage, they also may arise from sources other than cigarette smoking. Consumption of charbroiled food has been shown to modify polycyclic aromatic hydrocarbon-DNA adduct levels in peripheral white blood cells (43); we did not assess the recent consumption of dietary sources of polycyclic aromatic hydrocarbons in this study.

In addition to intensity and duration of smoking, a number of studies indicate that intake of dietary antioxidants modifies lung cancer risk (10, 11). One postulated mechanism for this association is that antioxidants decrease the induction of DNA damage by tobacco carcinogens (44). Molecular epidemiological studies aimed at understanding the role of diet in lung carcinogenesis must use surrogate markers of lung damage that respond to differences in antioxidant intake and cigarette consumption and that accurately reflect the modification of DNA damage within the lung that is associated with these differences. This study indicates that blood MNC DNA adduct levels meet these criteria. Our multivariate analyses (Table 2) indicated a similarity in the extent of modification of adduct levels by diet and smoking cessation in the target and surrogate tissue. In addition, it is not unexpected that the regression coefficient for the recent smoking term (i.e., cigarettes/day) is positive in sign and of greater magnitude in the lung than in blood. In all subjects, lung adduct levels were higher than blood adducts, which likely reflects the higher dose of tobacco carcinogens to the respiratory epithelium. Although the validity of MNC DNA adducts as markers of the effects of dietary antioxidants on lung DNA damage requires additional study, these results are consistent with the epidemiological data that indicate that intake of carotenoids, vitamin A, and foods containing these nutrients are strongly associated with decreased lung cancer risk (10, 11). Other researchers, who used an ELISA method to measure DNA adducts, found an inverse correlation of adduct levels in white blood cells with serum antioxidant levels but not with self-reported dietary intake (45).

Differences in the analytic sensitivity and specificity of adduct detection by immunological methods and the $^{32}$P-postlabeling assay that influence assessment of dietary antioxidants warrant additional study. In any case, this study indicates that blood MNC adduct levels may prove to be useful as markers for chemoprevention and diet intervention studies of individuals at high risk for lung cancer. The correlation of blood MNC adduct levels with lung levels was found to not be improved by assessment of dietary intake of antioxidants, even in combination with smoking variables that reflected time since smoking cessation and recent tobacco consumption. These results indicate that total MNC adduct levels are a useful marker of tobacco-induced DNA damage in the lung and should be further evaluated as a potential tool for the assessment of individual lung cancer risk in molecular epidemiological studies.

Table 2 Poisson regression analysis of lung or blood MNC DNA adducts

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Lung DNA adducts</th>
<th>Blood MNC DNA adducts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Value</td>
<td>SE</td>
</tr>
<tr>
<td>Years since cessation</td>
<td>$-45 \times 10^{-2}$</td>
<td>$30 \times 10^{-4}$</td>
</tr>
<tr>
<td>Cigarettes/day&lt;sup&gt;b&lt;/sup&gt;</td>
<td>$69 \times 10^{-4}$</td>
<td>$10 \times 10^{-4}$</td>
</tr>
<tr>
<td>Carotenoids</td>
<td>$-22 \times 10^{-5}$</td>
<td>$21 \times 10^{-7}$</td>
</tr>
</tbody>
</table>

<sup>a</sup> Absolute values of t are shown.

<sup>b</sup> Average number of cigarettes smoked/day during the past year.

Fig. 3. Cross-sectional plot of total aromatic DNA adducts levels (ordinate) in lung (A) and blood MNCs (B) versus the number of years since smoking cessation (abscissa).
ACKNOWLEDGMENTS

We thank Fred Grillo, Linda Lineback, Marcia Chertok, Marlys Rogers, Lucy-Ann Prinipe-Hasan, Nick Weidemann, and Dr. H. Kazemi. We greatly appreciate critical review of the manuscript by V. Ernster, J.B. Little, N. Petrakis, D. Trichopoulos, W. Willett, and G. Wogan. We thank Maureen Morris for manuscript preparation.

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