Immunoperoxidase Detection of Polycyclic Aromatic Hydrocarbon-DNA Adducts in Oral Mucosa Cells of Smokers and Nonsmokers

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Abstract

An immunoperoxidase method using a polyclonal antiserum which recognizes benzo(a)pyrene and structurally related polycyclic aromatic hydrocarbon diol epoxide-DNA adducts has been developed for the detection and quantitation of DNA damage in single cells. The method was used initially on 10T½ cells treated with [3H]anti-benzo(a)pyrene diol epoxide then applied to the detection of adducts in oral mucosa cells of smokers and nonsmokers. Levels of DNA damage were elevated in each of 16 smokers (mean relative staining, 503 ± 252; P < 0.0001). There was an approximately 3-fold range in relative staining in both smokers (252 ± 125 to 663 ± 189) and nonsmokers (157 ± 72 to 431 ± 269) suggesting the importance of individual differences in capacity to metabolize the carcinogens and/or repair damaged DNA. This noninvasive method, requiring small numbers of cells, will be useful for routine monitoring of DNA damage in intervention studies as well as for biofeedback in smoking cessation programs.

Introduction

A number of methods have been developed for quantitation of DNA damage resulting from environmental or occupational exposures to PAH3 including immunassays, gas chromatography-mass spectroscopy, fluorescence, and 32P postlabeling (reviewed in Refs. 1–5). Most methods require the isolation of bulk DNA from tissue or blood samples and thus do not allow the detection of adducts in specific cell types. In addition, some methods require relatively large amounts of DNA for analysis, limiting their application. We have used an ELISA to monitor PAH-DNA in foundry workers and coke oven workers (6, 7), smokers (8), and individuals with environmental exposure (7, 9). Anti- sera used in ELISA are sometimes applicable to immunohistochemical detection of DNA adducts in single cells.

Major advantages include the detection of adducts in specific cells and the requirement for small numbers of cells making the method applicable to biopsy samples. Immunohistochemical methods have been used extensively to monitor DNA damage in animals, and limited studies have been carried out on humans exposed to PAH, aflatoxin, and 8-methoxypsoralen (reviewed in Refs. 1, 10). We have used the same polyclonal antiserum (Subject 29), raised against BPDE-I-DNA (11), used in the ELISA for immunofluorescence detection PAH-DNA in skin biopsies of coal tar-treated psoriasis patients (12). Application of a similar method to tissues and bronchial cells from smokers has also been reported (13, 14).

Here we report on the development of an immunohistochemical method for direct quantitation of DNA damage in oral mucosa cells, a target tissue for smoking-induced cancers and a tissue which can be readily and repeatedly collected by noninvasive methods. Although we initially attempted to use the immunofluorescence method to detect damage in oral cells, background autofluorescence interfered with the assay. Thus, an immunoperoxidase method using biotinylated secondary antiserum and streptavidin-conjugated peroxidase was used in conjunction with direct quantitation of staining for detection of damage in oral cells. A similar method was used to monitor cisplatin modified DNA in oral cells of chemotherapy patients (15).

Materials and Methods

Treatment of 10T½ Cells. To develop the quantitative immunoperoxidase method, 10T½ cells cultured in 8-chambered slides (Nunc, Naperville, IL) were treated with 0, 5, 10, 20, and 40 μM [3H] 7-r,8-t-dihydroxy-9,10-oxy-7,8,9,10-tetrahydrobenzo[a]pyrene (444 mCi/mmol; Chemsyn Science Laboratories, Lenexa, KS) in DMEM (GIBCO-BRL Life Technologies, Gaithersburg, MD) for 1 h at 37°C. BPDE-I was dissolved in DMSO before addition to the media with a final DMSO concentration of 0.05%.

We have used an ELISA to monitor PAH-DNA in foundry workers and coke oven workers (6, 7), smokers (8), and individuals with environmental exposure (7, 9). Antiserum raised against BPDE-I-DNA (11), used in the ELISA for immunofluorescence detection PAH-DNA in skin biopsies of coal tar-treated psoriasis patients (12). Application of a similar method to tissues and bronchial cells from smokers has also been reported (13, 14).

Human Subjects. Volunteers were recruited, after approval by the Institutional Review Board by advertisements, around the Columbia Presbyterian Medical Center. After informed consent was obtained, oral mucosa cells were collected by gently scraping the inside of the cheek with a
DNA Adducts in Oral Cells of Smokers

wooden tongue depressor. Samples were obtained from 16 smokers of at least 1 pack/day (range, 20–30 cigarettes/day) and 16 race-, sex-, and age-matched (within 5 years) nonsmokers and were coded for analysis. A questionnaire was administered collecting information on smoking history as well as dietary consumption of charcoal broiled or smoked foods containing high levels of PAH over the previous 2 weeks. A subset of 6 smokers provided repeat samples 3 months after original sample collection. Cells were smeared on slides precoated with 0.2% poly-D-lysine, air dried, fixed in −20°C acetone for 20 min, and stored at −80°C until staining.

Immunoperoxidase Staining. Slides were washed with PBS, treated with RNase (100 μg/ml; Sigma Chemical Co., St Louis, MO) at 37°C for 1 h, washed with PBS, treated with proteinase K (10 μg/ml; Sigma) at room temperature for 10 min and washed. To denature the DNA, slides were incubated with 4n HCl for 10 min at room temperature and then with 50 mM Tris base for 5 min at room temperature. After washing with PBS, slides were incubated with 0.3% H2O2 in methyl alcohol at room temperature for 30 min to quench endogenous peroxidase activity. Nonspecific binding was blocked with 1.5% normal horse serum and then slides were incubated with the anti BPDE-I-DNA polyclonal antiserum #29 (11) (1:800 dilution in 1.5% horse serum) overnight at 4°C. This antiserum was obtained from animals immunized with BPDE-I-DNA, but cross-reacts with DNA modified by several other PAH-diol epoxides (16). Thus, the antiserum recognizes a class of adducts rather than just those of BPDE-I. Elite rabbit or mouse ABC and DAB kits (Vector Laboratories, Burlingame CA) were used for visualization of bound antiseras as directed by the manufacturer.

Slides were dehydrated and cleaned in serial ethyl alcohol in −20°C acetone for 20 min, and stoned at −80°C until staining. Immunoperoxidase method was then applied to treated 10T1/2 cells in culture with an antiserum recognizing PAH-DNA adducts in human cells. To standardize the assay for possible day to day variations, an 8-chambered slide of treated 10T1/2 cells was stained with each batch of human samples. The coefficient of variation for the different doses ranged from 9–25% (n = 4). One-way analysis of variance indicated there was no significant difference in repeat staining of the 10T1/2 cells.

Representative staining in a smoker (subject 12; Table 2) and nonsmoker (subject 32) are illustrated in Fig. 2, A and B, respectively. Quantitative staining intensity data for each subject are given in Table 2. Preabsorption of primary antiserum with BPDE-I-DNA before use decreased staining in smoker 12 from 520 ± 146 to 109 ± 49 (Fig. 2C) and from 237 ± 58 to 125 ± 41 for nonsmoker 32 (Fig. 2D). Staining with a nonspecific antiserum recognizing DNA damage produced by 8-methoxypsoralen gave a value of 118 ± 95 in smoker 12 (Fig. 2E) and 68 ± 42 in nonsmoker 32 (Fig. 2F). Pretreatment of slides from smoker 12 with DNase also decreased relative staining (139 ± 70) as did omission of primary antiserum (94 ± 26; not shown). Background staining with these control conditions demonstrate the specific-immunoperoxidase staining and BPDE-I-DNA adduct levels, the slope was less than 1 (Fig. 1).

The immunoperoxidase method was then applied to the detection of damage in oral mucosa cells. To standardize the assay for possible day to day variations, an 8-chambered slide of treated 10T1/2 cells was stained with each batch of human samples. The coefficient of variation for the different doses ranged from 9–25% (n = 4). One-way analysis of variance indicated there was no significant difference in repeat staining of the 10T1/2 cells.

Table 1 Comparison of [3H]BPDE-I dose, DNA adduct level, and quantitative immunoperoxidase staining in 10T1/2 treated in culture

<table>
<thead>
<tr>
<th>[3H]BPDE-I dose (μM)</th>
<th>DNA adducts/10⁵ nucleotides</th>
<th>Relative staininga</th>
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<tr>
<td>0</td>
<td>0</td>
<td>116 ± 8</td>
</tr>
<tr>
<td>5</td>
<td>2.8b</td>
<td>134 ± 9</td>
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<tr>
<td>10</td>
<td>4.4b</td>
<td>143 ± 25</td>
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<tr>
<td>20</td>
<td>11.1b</td>
<td>176 ± 12</td>
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<tr>
<td>40</td>
<td>18.4c</td>
<td>198 ± 10</td>
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</table>

a Mean of four separate staining experiments in which 50 cells/sample were counted.
b Mean adduct level from treatment of cells with [3H]BPDE-I at two different time points.
c Adduct level from a single treatment of cells with [3H]BPDE-I.

Fig. 1. Relationship between relative intensity of immunoperoxidase staining with an antiserum recognizing PAH-DNA adducts in 10T1/2 cells treated with [3H]BPDE-I and DNA adduct levels determined by measurement of specific activity. Points, mean; bars, SD.

Results
Specific nuclear staining was observed in 10T1/2 cells treated with [3H]BPDE-I but not in control cells (data not shown). Quantitation of staining, obtained on a total of 50 randomly selected cells, indicated a dose-related increase in relative staining intensity (Table 1). DNA adduct levels, determined from the specific activity of DNA isolated from treated cells, also increased with increasing dose of BPDE-I (Table 1). While a linear relationship was observed between DNA adduct level from a single treatment of cells with [3H]BPDE-I and DNA adduct levels determined by measurement of specific activity. Points, mean; bars, SD.
within the past week. Analysis of repeat samples from 6 oven the previous 2 weeks and only 6 subjects reported this nonsmokers
(P > this group of heavy smokers (20 cigarettes/day; range, time frame of cell migration from the basal layer to exfoli-
折 the carcinogenic effects of cigarette smoking in a target A simple, noninvasive method for monitoring humans for
± 189). Mean level of relative staining was elevated 2-fold 
3-fold variation in staining in both nonsmokers (range, 157
(19). The immunohistochemical assay can also detect dam-
result in unrealistically high values. The high relative stain-
level using an extrapolated standard curve (Fig. 1) would result in unrealistically high values. The high relative stain-
ing intensity in the oral cells may be a result of the different cell types or the presence of mucus in the oral samples. In
addition, the 10T½ cells contain only BPDE-I-DNA adducts while the human samples may contain a range of PAH adducts with different affinity for the antiserum. Nevertheless, although absolute adduct levels cannot be determined,
Table 2 Immunoperoxidase staining of oral mucosa cells from smokers and nonsmokers for polycyclic aromatic hydrocarbon-DNA damage

<table>
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<tr>
<th>Race a</th>
<th>Sex b</th>
<th>ID</th>
<th>Age</th>
<th>Mean ± SD</th>
<th>Race</th>
<th>Sex</th>
<th>ID</th>
<th>Age</th>
<th>Cigarettes/day</th>
<th>Mean ± SD</th>
<th>1st sample</th>
<th>Mean ± SD</th>
<th>2nd sample</th>
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<td>377 ± 119</td>
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<td>20</td>
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<td>H M</td>
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<td>43</td>
<td>180 ± 77</td>
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* a, African-American; C, Caucasian; H, Hispanic.
* b, M, male; F, female.
* c, ID, subject number.
* d, Relative staining intensity measured in 50 cells/subject.
* e, Subjects resampled 3 months after initial sample collection.
* f, This subject decreased the number of cigarettes smoked to 5-7/day 1 month before sample collection.

Discussion

A simple, noninvasive method for monitoring humans for the carcinogenic effects of cigarette smoking in a target tissue for smoking-induced cancers has been developed. The 2-fold increase in damage levels is comparable to the 3-fold difference observed in DNA from mononuclear cells of smokers and nonsmokers using an ELISA with the same antiserum (8). Repeat sampling of smokers 3 months after initial sampling indicated similar damage levels. It would be of interest to analyze a smoker who quit smoking. The time frame of cell migration from the basal layer to exfoli-

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the immunohistochemical method provides useful information on relative levels of DNA damage in specific cell types. Oral mucosa cells have been used previously to monitor exposure to PAH by 32P postlabeling (25–28). An early study (25), using the carrier-free [32P]ATP method, in groups at high risk for oral cancer, (including betel nut chewers, inverted smokers, and tobacco chewers) and Canadian controls found similar adducts in exposed and unexposed individuals. Estimated levels of adducts ranged from nondetectable to > 1 / 107. The butyl alcohol extraction method has also been used to demonstrate similar adducts in both smokers and nonsmokers in the range of 1/105 to 6/105 (26). No adduct was detected in the samples of tobacco users that was not present in the controls. Another study using the butyl alcohol extraction method also found no adduct spots consistently associated with exposure to alcohol or tobacco (27). Relative adduct labeling values ranged from 7.7/1011 to 1.6/106. Adduct levels in

Fig. 2. Immunohistochemical staining of oral mucosa cells from a smoker and nonsmoker with a polyclonal antiserum recognizing PAH-DNA adducts. Staining of cells from smoker 12 (A) and nonsmoker 32 (B); cells from smoker 12 (C) and nonsmoker 32 (D) stained with antiserum 29 that was preabsorbed with BPDE-I-DNA before use; cells from smoker 12 (E) and nonsmoker 32 (F) stained with an antiserum recognizing 8-methoxypsoralen-DNA (x 400).
smokers (median 4.8/10^6) were 1.71-fold higher (P < 0.001) than levels in nonsmokers (2.9/10^6). Oral biopsies of clinically normal tissue from smoking and nonsmoking patients undergoing surgery for intraoral squamous cell carcinoma have been assayed by the butyl alcohol extraction and nuclease P1 enrichment methods (28). The butyl alcohol extraction method revealed a wider range and higher level of adducts than the nuclease P1 method. Adduct levels in smokers, exsmokers, and nonsmokers were 3.75, 2.59, and 2.18/10^6, respectively. The differential results with the butyl alcohol and nuclease P1 methods suggest that aromatic hydrocarbons-DNA adducts in peripheral mononuclear cells. Cancer in Genes (Lond.), 13: 2041–2045, 1992.

A single study has used immunohistochemical methods to detect DNA damage in oral mucosa cells (15). Cells from cancer patients receiving carboplatin and cisplatin combination chemotherapy were analyzed with an antisera recognizing cisplatin-modified DNA and peroxidase-rabbit anti-peroxidase staining. All patients demonstrated increased nuclear staining but large individual differences in intensity were observed, suggesting differences in adduct formation and/or repair.

The immunoperoxidase method developed here will be useful to further investigate interindividual differences in damage and, potentially, of risk for cancer development. Because samples can be so easily collected it will be useful for repeated analysis of subjects. It can also be used as an intermediate biomarker in intervention studies in which modulation of DNA damage is an endpoint. Finally, feedback of specific individual DNA damage information to smokers may provide additional motivation for smoking cessation.

Acknowledgments

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References

DNA Adducts in Oral Cells of Smokers


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