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Immunohistochemical Quantitation of Polycyclic Aromatic Hydrocarbon-DNA Adducts in Human Lymphocytes

Grazyna Motykiewicz, Ewa Mahusecka, Ewa Grzybowska, Mieczyslaw Choraży, Yu-Jing Zhang, Frederica P. Perera, and Regina M. Santella

Department of Tumor Biology, Institute of Oncology, 44-100 Gliwice, Poland (G. M., E. M., E. G., M. C.); and Cancer Center/Division of Environmental Sciences, Columbia School of Public Health, New York, New York 10032 (Y.-J. Z.; F. P. P.; R. M. S.)

Abstract

The formation of polycyclic aromatic hydrocarbon-DNA adducts was studied in peripheral blood lymphocytes obtained from men with occupational and environmental exposure. Subjects included dock workers, residents from the vicinity of the dockery, and rural region inhabitants (16 individuals in each exposure group). Adducts were determined by immunohistochemical analysis using a polyclonal antiserum recognizing benzo(a)pyrene and related polycyclic aromatic hydrocarbon diol epoxide-DNA adducts, a biotinylated secondary antiserum, and streptavidin-conjugated FITC. Propidium iodide was used to quantitate nuclear DNA. Dual fluorescence intensities were simultaneously measured with a Zeiss Axiosvert microscope and a Bio-Rad MRC-600 argon laser scanning confocal attachment. Adducts were significantly elevated (P < 0.001) in both occupational and environmental groups, as compared to the rural control group by Mann-Whitney U test. The distribution of the data indicated the existence of cells with relatively higher adduct levels. The percentages of these so-called “higher adduct-level cells” were 13.6, 11.5, and 3.7 in dockery workers, environmentally exposed individuals, and rural controls, respectively. The immunohistochemical method allows visualization and relative quantitation of polycyclic aromatic hydrocarbon-DNA adducts in individual lymphocytes. It requires a much smaller amount of blood than the previously used 32P-postlabeling and ELISA methods, which used isolated bulk DNA. It can also be used for adduct quantitation in biopsy material. The results of this pilot study indicate that this technique is a promising addition to biomonitoring studies.

Introduction

PAH are common environmental pollutants, and their uptake has been causally associated with the induction of cancer. As a result of coal burning and/or processing, PAH are among the most prevalent mutagenic air pollutants in Silesia, Poland (1). The highest levels are found in the occupational environment, i.e., coke-oven plants (0.12–89.58 μg of B[a]P/m³ of air). Chronic exposure to lower levels of PAH (1.0–55.3 ng of B[a]P/m³) occurs in the general environment of Silesia and mainly originates from coal-based heavy industry, power plants, and individual domestic heating, as well as automobile traffic.

Among the known molecular effects of PAH is the ability to bind covalently to DNA, giving rise to PAH-DNA adducts. A number of methods have been developed for quantitation of adducts, resulting from occupational or environmental exposure to PAH (reviewed in Refs. 2–5). They include 32P-postlabeling, gas chromatography/mass spectrometry, fluorescence, and immunoassays (ELISA) using specific antisera. Most methods are not applicable to small amounts of blood or tissue, a major limitation in biomonitoring studies.

Antisera directed against PAH-DNA adducts can also be used for the immunocytochemical localization of adducts in individual cells. This approach has been successfully applied to mouse cells (6) and tissues (7, 8) as well as human WBC treated in vitro with B[a]P (8). Pilot immunofluorescence studies on bronchial cells from a smoker have also been reported (9). Application of immunoperoxidase techniques have been applied to tissues and oral mucosa cells of smokers and nonsmokers (10, 11).

Here we report on an immunofluorescence technique for visualization and quantitation of PAH-DNA adducts in individual human lymphocytes. The formation of BPDE and related PAH-DNA adducts was studied in lymphocytes obtained from coke-factory workers in Silesia, Poland, residents from the vicinity of the dockery, and inhabitants of a rural region in Poland with PAH levels estimated to be 10-fold lower than in Silesia. This study builds upon our earlier results on aromatic adducts and PAH-DNA adducts measured on isolated blood DNA by 32P-postlabeling and ELISA (12). Both methods showed DNA adducts to be significantly elevated in the Silesian groups (dockery workers and subjects living in the surrounding area) compared to rural controls, with only a slight difference between dockery workers and local controls. In addition to DNA adducts, chromosomal aberrations and sister chromatid exchanges were significantly elevated in occupationally and environmentally exposed residents of Silesia compared to controls (13, 14).

Materials and Methods

Chemicals. BPDE (444 mCi/mmol) was supplied by Chemsyn Science Laboratories (Lenexa, KS). RNase, protease K, PI, Histopaque-1077, and 1,4-phenylenediamine were purchased from Sigma Chemical Co. (St. Louis, MO). Biotinylated goat anti-rabbit IgG and fluorescein-conjugated streptavidin were from Boehringer Mannheim (Indianapolis, IN). FCS was obtained from HyClone Laboratories, Inc. (Logan, UT), and DMEM medium was purchased from GIBCO-BRL Life Technologies (Gaithersburg, MD). Tissue culture chambers were supplied by NUNC (Naperville, IL).

Subjects and Blood Samples. Subjects included 16 healthy males from each exposure group as well as 16 rural controls. Donors were selected on the basis of their occupation; environmental and control groups did not include dockery workers or any other workers connected with the processing of coal. Additional information on cigarette smoking, diet, X-ray exposure, current medication, and family history of cancer was also collected. Samples were taken in September 1992 and 1993, which is considered the summer season. Therefore, the exposure was not influenced by the additional impact of PAH from domestic heating in the winter.

Treatment of Cells with BPDE. The immunostaining method was established, and the measurements were calibrated on cultured C3H 10T½ cells treated with BPDE. Cells were plated in eight-chambered slides and maintained in DMEM medium supplemented with 10% FCS. After 24 h, cells were...
exposed to 0, 0.1, 1, 5, 10, and 20 µg/ml of BPDE (2 h in the dark), washed 2X with PBS, and fixed in 70% methanol at room temperature. BPDE was dissolved in DMSO with a final concentration of 0.05%. Two chambers were treated with DMSO alone and two with 10 µg/ml BPDE for immunostaining controls (see below).

**Immunohistochemical Detection of PAH-DNA Adducts.** Mononuclear cells were isolated from blood by centrifuging over Histopaque. Cell smears were prepared on microscope slides and, after air drying, fixed in methanol for 3 min (—20°C), followed by dipping in acetone (—20°C). Cells were then treated with RNase (100 µg/ml) for 1 h at 37°C and with proteinase K (10 µg/ml) for 10 min at room temperature. DNA was denatured with 4 N HCl (10 min at room temperature), and the pH was adjusted with 50 mM Tris-base for 5 min at room temperature. Nonspecific antisera binding was blocked by incubating slides with 10% normal goat serum for 45 min at 37°C. A newly developed primary rabbit polyclonal anti-BPDE-I-DNA antiserum (#1) was used at a 1:50 dilution, and slides were incubated at 37°C for 45 min. This antiserum was generated as described previously (15) and has cross-reactivity with structurally related PAH diol epoxide-DNA adducts similar to polyclonal antiserum #29, which was used in previous immunofluorescence studies (16). Primary antiserum was followed by biotinylated anti-rabbit IgG (1:200 dilution) and streptavidin-FITC (5 µg/ml at 37°C for 45 min). Nuclear DNA was counterstained with PI (1 µg/ml at room temperature for 45 s). To reduce fading of fluorescence, slides were mounted in PBS-glycerol (1:2) containing 0.1% of 1,4-phenylenediamine (pH 8.5).

For the 8-chambered slides, duplicate DMSO, and 10 µg BPDE/ml wells were incubated with or without the primary antiserum. Additional controls included preabsorption of the antiserum with BPDE-1-DNA (1 µg/ml; 20 min at room temperature) or treatment of slides with DNase (100 µg/ml for 1 h at 37°C) before staining.

**Measurement of Adducts.** Measurement of FITC and PI fluorescence intensities in 50 randomly selected cells per subject was performed with a Zeiss Axiovert microscope and a Bio-Rad MRC-600 argon laser scanning confocal attachment using A1 and A2 filter cubes. After defining the nuclear zone by PI pattern, fluorescence was quantified from a fixed area within the nucleus (boxes of 48 and 73 µm² for lymphocytes and C3H 10T½ cells, respectively), as well as from an adjacent area by the Bio-Rad image processing software. Background fluorescence was subtracted from the measured FITC and PI nuclear fluorescence, and the FITC:PI ratio calculated for each nucleus. To standardize the assay for possible day-to-day variations, each batch of seven human samples was assayed together with one eight-chambered slide of C3H 10T½ cells.

**Statistical Analysis.** Both descriptive statistics and frequency histograms indicated that the data were not normally distributed. Therefore, the statistical significance of the difference between each exposure group was tested by

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![Immunofluorescence staining with anti-BPDE-I-DNA antiserum #1 of cultured C3H 10T½ cells treated with BPDE 10 µg/ml (a and b); c and d, DMSO control. Primary antibody (1:50 dilution) was followed by biotinylated anti-rabbit IgG (1:200 dilution) and streptavidin-FITC (5 µg/ml). Nuclear DNA was counterstained with PI (1 µg/ml). Left (a and c) and right (b and d) sides of the photographs present images of the same cells stained with FITC and PI, respectively.](image-url)
The percentage of the cells with ratios ≥2.2 increased with exposure between 3.7, 11.5, and 13.6 for control, environmental, and occupational exposure groups (Table 2).

Discussion

Adduct-specific nuclear FITC staining was dose dependent in C3H 10T½ cells treated in vitro with 0.1–10 μg/ml BPDE-I (Fig. 2). There was an approximately 3-fold difference in the mean FITC:PI ratio between control cells and those treated with 10 μg/ml BPDE-I. The mean FITC:PI ratio was significantly elevated in lymphocytes from both the occupationally and environmentally exposed groups compared to rural controls (Table 1). The difference between the two Silesian-exposed groups was not statistically significant. This is in agreement with our previous results in which only minor differences in aromatic and PAH-DNA adducts were detected by postlabeling or ELISA on DNA isolated from total WBC of cokery workers and environmental controls (12). In this prior study, mean PAH-DNA adducts by ELISA were 15.2, 13.0, and 2.3/108 in occupational, environmental, and control groups, respectively. A similar trend was observed in mean FITC:PI ratio of lymphocytes. Although in a lower range of exposure than cokery workers, Silesian residents are under continuous, long-term exposure to PAH. The formation of similar amounts of adducts in those with occupational or environmental exposure may be due to their accumulation or to the impairment of DNA repair. Our preliminary results with the bleomycin sensitivity test support the suggestion that reduced DNA repair capacity is important.

The anti-BPDE-I-DNA antiserum is suitable for in situ immunofluorescence detection of PAH-DNA adducts in human lymphocytes. The method can be applied to lymphocytes from as little as 1–2 ml of blood, which makes it convenient for biomonitoring studies. It can also be used for adduct quantitation in biopsy material. A major advantage of the present method is the simultaneous measurement of FITC and PI, which allows for the correction of quantitation of individual-cell adduct levels for the amount of nuclear DNA. A similar immunocytochemical assay was developed by Müller et al. (18) for quantitation of O6-ethylguanine in human leukemic cells. In that study, adduct-specific antiserum binding was visualized with a rhodamine isothiocyanate conjugated secondary antiserum, and data was corrected for DNA content based upon 4,6-diamidino-2-phenylindole staining.

Human WBC are easily accessible and have been used successfully for studying PAH-DNA adducts in occupationally and environmentally exposed individuals (reviewed in Refs. 2 and 4). However, the relationship between blood adduct levels and target tissue levels is still uncertain. No correlation was observed between DNA adduct levels in total WBC and normal lung tissue of lung cancer patients in a prior study by 32P-postlabeling (19). In a more recent study using ELISA to measure PAH-DNA, a modest correlation was seen between WBC DNA adduct levels and lung tumor DNA (r = 0.31; P = 0.096) but not nontumor DNA (r = 0.18; P = 0.32) (20). The heterogeneity of the white cell population may be one of the reasons for the large interindividual variability in adduct levels which has been observed. Most blood cells (40–75%) are short-lived granulocytes, which can only indicate the effects of recent exposure. The remainder are the

Fig. 2. Dose-response curve obtained for C3H 10T½ cells treated with 0, 0.1, 1, 5, 10, and 20 μg/ml of BPDE. Mean FITC:PI ratio of three separate staining experiments in which 50 cells/sample were counted; bars, SD.
Fig. 3. Immunofluorescence staining with anti-BPDE-I-DNA antiserum #1 of PAH-DNA adducts in human lymphocytes. Examples of immunofluorescence stainings of lymphocytes of cokery worker (a and b); Silesian inhabitant (c and d) and control region donor (e and f). Left (a, c, and e) and right (b, d, and f) sides of the photographs present images of the same cells stained with FITC and PI, respectively. × 60.
Table 1  Immunohistochemical quantitation of PAH-DNA adducts in human lymphocytes ratio of FITC:PI fluorescence (arbitrary units)

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cells (No. ± SD)</th>
<th>Median ± SD</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Lower quartile</th>
<th>Upper quartile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occupational</td>
<td>763 ± 1.12</td>
<td>1.04 ± 0.49</td>
<td>0.17</td>
<td>0.34</td>
<td>0.17</td>
<td>1.66</td>
</tr>
<tr>
<td>Environmental</td>
<td>744 ± 0.85</td>
<td>1.25 ± 0.62</td>
<td>0.13</td>
<td>0.39</td>
<td>0.55</td>
<td>1.76</td>
</tr>
<tr>
<td>Control</td>
<td>732 ± 0.62</td>
<td>0.81 ± 0.31</td>
<td>0.17</td>
<td>0.34</td>
<td>0.31</td>
<td>1.26</td>
</tr>
</tbody>
</table>

* * Occupational/Control, P < 0.001; Environmental/Control, P < 0.001; Occupational/Environmental, P = 0.40 by Mann-Whitney U test.

Table 2  Frequency of higher adduct-level cells in human lymphocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of cells with FITC:PI ratio ≥2.2/total cells</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Occupational</td>
<td>104/763</td>
<td>13.6</td>
</tr>
<tr>
<td>Environmental</td>
<td>86/746</td>
<td>11.5</td>
</tr>
<tr>
<td>Control</td>
<td>28/732</td>
<td>3.7</td>
</tr>
</tbody>
</table>

* * Significantly different from the controls by χ² test (P < 0.05).

Acknowledgments

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References


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