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Polycyclic Aromatic Hydrocarbon-DNA Adducts in Cervix of Women Infected with Carcinogenic Human Papillomavirus Types: An Immunohistochemistry Study

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Abstract

Among women infected with carcinogenic human papillomavirus (HPV), there is a 2- to 5-fold increased risk of cervical precancer and cancer in women who smoke compared to those who do not smoke. Because tobacco smoke contains carcinogenic polycyclic aromatic hydrocarbons (PAHs), it was of interest to examine human cervical tissue for PAH-DNA adduct formation. Here, we measured PAH-DNA adduct formation in cervical biopsies collected in follow-up among women who tested positive for carcinogenic HPV at baseline. A semi-quantitative immunohistochemistry (IHC) method using antiserum elicited against DNA modified with r7, t8-dihydroxy-t-9, 10-oxy-7, 8, 9, 10-tetrahydrobenzo[a]pyrene (BPDE) was used to measure nuclear PAH-DNA adduct formation. Cultured human cervical keratinocytes exposed to 0, 0.153, or 0.331 μ M BPDE showed dose-dependent increases in r7, t8, t9-trihydroxy-c-10-(N²deoxyguanosyl)-7, 8, 9, 10-tetrahydrobenzo[a]pyrene (BPdG) adducts. For BPdG adduct analysis, paraffin-embedded keratinocytes were stained by IHC with analysis of nuclear color intensity by Automated Cellular Imaging System (ACIS) and, in parallel cultures, extracted DNA was assayed by quantitative BPDE-DNA chemiluminescence immunoassay (CIA). For paraffin-embedded samples from carcinogenic HPV-infected women, normal-appearing cervical squamous epithelium suitable for scoring was found in samples from 75 of the 114 individuals, including 29 cases of cervical precancer or cancer and 46 controls. With a lower limit of detection of 20 adducts/10⁸ nucleotides, detectable PAH-DNA adduct values ranged from 25 to 191/10⁸ nucleotides, with a median of 75/10⁸ nucleotides. PAH-DNA adduct values above 150/10⁸ nucleotides were found in 8 samples, and in 3 samples adducts were non-detectable. There was no correlation between PAH-DNA adduct formation and either smoking or case status. Therefore, PAH-DNA adduct formation as

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measured by this methodology did not appear related to the increased risk of cervical precancer and cancer among carcinogenic HPV-infected smokers.

Keywords

Automated Cellular Imaging System; BPDE-DNA antiserum; smoking; chemiluminescence immunoassay; human keratinocytes

1. Introduction

Persistent infection with carcinogenic HPV types is widely considered a necessary cause in the development of cervical cancer [1]. Numerous epidemiologic studies have reported associations between parity, the use of oral contraceptives, or smoking, and cervical cancer (reviewed in [2]). Case-control [3–5] and prospective [6] studies report that women infected with carcinogenic HPV significantly increase their risk of cervical precancer and cancer by smoking. The data regarding the risk to former smokers is mixed [3–6].

To date, approximately 4,000 compounds have been identified in tobacco smoke [7], and sufficient evidence of animal carcinogenicity exists for sixty-two of these compounds, while eleven are classified as human carcinogens [7]. The International Agency for Research on Cancer has listed 10 polycyclic aromatic hydrocarbons (PAHs), including benzo[*a*]pyrene (BP), benz[*a*]anthracene, and benzo[*k*]fluoranthene, as probable or possible human carcinogens [7]. Chemically inert, PAHs become potent, locally-acting carcinogens following bioactivation. Bioactivated PAH carcinogens induce tumors in the skin, upper respiratory tract and lung when administered topically, by inhalation, instillation in the trachea, or implantation into the lungs of laboratory animals, respectively [7].

Given the relationship between smoking behavior and the development of cervical precancer and cancer, it follows that carcinogens found in tobacco smoke, including PAHs, could become metabolized and covalently bound to DNA in cervix and other tissues, thus producing the DNA damage that is a necessary event in the early stages of carcinogenesis. Experimental and epidemiological evidence has shown a positive correlation between exposure to carcinogenic PAHs and subsequent tumor formation [8–10]. Moreover, metabolites from cigarette smoke have been found in cervical secretions, suggesting that the cervical epithelium is exposed to the carcinogenic compounds found in cigarette smoke [11;12]. Therefore, detection of PAH-DNA adducts in the cervical epithelium would show that PAH exposure has occurred and that PAHs are metabolized to DNA-damaging species at the target site for tumor development.

Smoking-related DNA damage has been detected in exfoliated cervical cells [13] and cervical biopsies using ³²P-postlabeling [14;15]. Additionally, IHC has been used to visualize PAH-DNA adducts in cervical smears from both smokers and non-smokers [16]. In each of these studies, methods were employed that did not permit the observation of DNA damage in whole tissue. Our laboratory has validated and reported a semi-quantitative method for the immunohistochemical detection of PAH-DNA adducts in archived human tissues [17;18]. In this method, paraffin-embedded tissue sections are stained with primary

antiserum elicited against DNA modified with BPDE, followed by secondary antiserum and Fast Red. Intensity of the resultant nuclear pink color, which localizes the PAH-DNA adducts, is quantified by ACIS. In the present study, we used this approach to evaluate blinded cervical biopsy specimens collected from 75 individuals involved in a prospective study of the natural history of cervical cancer [6;19]. PAH-DNA adducts were detected in a majority of the specimens, and semi-quantitation of these adducts was achieved by comparison with a standard curve. When the samples were decoded, PAH-DNA adduct formation was compared with individual smoking history and cervical cancer status. The resulting data gives insight into the complexity of the relationship between smoking and cervical cancer risk.

2. Materials and methods

2.1. Human subjects and tissue procurement

From April 1, 1989, to November 2, 1990, 23,702 women receiving routine cytologic screening in a prepaid health plan at Northwest Kaiser Permanente in Portland, OR, were recruited for a cohort study of the natural history of HPV infection as described previously [6]. A cohort of 20,810 women with satisfactory baseline cytology was followed prospectively by routine cytology for up to 122 months. Informed consent was obtained under the prevailing institutional review board guidelines at Northwest Kaiser Permanente and the National Institutes of Health. A cervicovaginal lavage was collected from each participant and tested for carcinogenic HPV as previously described [20]. A sub-cohort of carcinogenic HPV-positive women with no evidence of high-grade squamous intraepithelial lesions at enrollment was defined to examine the role of smoking and other cofactors in the development of cervical precancer and cancer. Smoking status (never, former, current) was assessed at baseline by a short, self-administered questionnaire.

We matched cases and controls as previously described [6]. Briefly, controls were matched based on age, screening patterns, and baseline cytology to cases consisting of women with cervical intraepithelial neoplasia grade 3 (CIN3) or cancer. This study comprised cervical tissue taken by biopsy or excisional treatment from a subset of cases and 1–2 matched controls in which corresponding diagnosis was CIN 1 or less (CIN1). In all, 52 clusters of matched cases (n = 52) and controls (n = 86) were initially identified, and each tissue was fixed in formalin and embedded in paraffin. Only 75 individuals, 26 cases and 49 controls, contained the cervical tissue with normal-appearing basal areas that was required for the immunohistochemical analysis. As a consequence, some cases did not have matched controls, and vice versa. We therefore broke the case-control match. However, among the 75 individuals evaluated there were no statistical differences between cases and controls in age, number of lifetime births, marital status, education, or income at the time of enrollment. There were 95 samples immunostained in this analysis because 56 tissues were assayed once, 18 tissues were assayed twice and one tissue was assayed 3 times.

2.2. Preparation of a standard curve with cultured human cervical keratinocytes

Normal human cervical keratinocytes, immortalized with HPV 16 [21], were cultured and exposed as described [17]. Keratinocytes were exposed to doses of 0, 0.153, and 0.331 μM

BPDE (CAS No. 58917-67-2) for 1 hour to generate the standard curve. After 1 hour of exposure, cells were washed once with PBS and harvested. Cells from 15 identically-treated plates were combined, centrifuged 5 minutes at 1000 rpm and the supernatant removed. The resulting cell pellets were either embedded in agarose, fixed in 10 % buffered formalin (Sigma, St. Louis, Missouri) and embedded in paraffin, or the DNA was extracted using the Intergen DNA extraction kit (Serologicals Corp., Norcross, Georgia) according to the instructions of the manufacturer.

2.3. BPDE-DNA Antiserum

Antiserum elicited against DNA modified with BPDE (rabbit #30 bleed 6/30/78) [22;23] has specificity for the different enantiomers of benzo[a]pyrene (BP) bound to DNA, but no specificity for BP alone, BP metabolites, BP bound to protein or DNA alone [22]. Cross-reactivity of this antiserum for DNA samples modified with a series of carcinogenic PAHs has been reported [23]; therefore, when using human samples, which are likely to contain adducts of multiple PAHs, the values obtained are designated PAH-DNA adducts.

To achieve lower backgrounds in the IHC staining, all antiserum was pre-absorbed with DNA before use. In addition, to prepare immunogen-absorbed BPDE-DNA antiserum, from which the anti-BPDE-DNA antibodies have been removed, 0.05 mL of BPDE-DNA antiserum was absorbed two times with a total of 390 µg BPDE-DNA (1.3% modified, 13.9 nmol BPDE-DNA adducts). Antigen-antibody complexes were pelleted by centrifugation, and the final supernatant was used for staining.

2.4. Immunohistochemistry

Three serial 5 µm sections designated a, b, and c, were cut from each paraffin block and stained within 4 weeks. Section b was stained with hematoxylin and used to obtain an accurate count of all nuclei in each region of interest. Sections a and c were stained with either BPDE-DNA antiserum or with immunogen-absorbed BPDE-DNA antiserum, both diluted 1:1500.

Staining of fresh-cut sections was performed using the Nexes IHC (Ventana, Tucson, AZ) automated slide staining system as described [18]. The system was set for a 20 minute incubation of the primary antiserum or immunogen-absorbed primary antiserum with no counterstaining. Section b for all tissues was treated with normal rabbit serum and counterstained with hematoxylin (Ventana) and bluing reagent (Ventana). Stained slides were rinsed in water containing Dawn dishwashing liquid soap (Proctor and Gamble, Cincinnati, OH) for 1 min, and subsequently rinsed in deionized water to remove the soap. Stained slides were air dried and mounted with Permount mounting media (Fisher Scientific, Pittsburgh, PA).

For the coded replicate samples, all of the sections were cut at the same time but were randomized, along with the other samples, to determine the overall order of staining. This usually, but not always, meant that the replicates were stained on different days. An effort was made to find and count the same areas in the replicate slides, but this was not always possible due to histological variations in the tissue

2.5 Semi-quantitation of nuclear staining intensity by automated cellular imaging system (ACIS)

The intensity of the nuclear PAH-DNA adduct staining (pink color) was measured using the ACIS I (ChromaVision, San Juan Capistrano, CA), for which methodological details have been previously described [17;18]. The nuclei containing PAH-DNA adducts stained pink (hue = 190–250) and hematoxylin nuclear staining was blue (hue = 147–162). The “threshold tool” (ACIS software, version 1.82B, micro-D application), sets thresholds for hue, luminosity, and saturation and therefore eliminates the contribution of background staining. Additionally, the computer was programmed to evaluate only round or oblong shaped groupings of appropriately-colored pixels within the 4–8 micron size range of nuclei found in cervical basal epithelium. The areas to be analyzed within each tissue section were manually selected using the ACIS “region selection” tools.

To quantify the PAH-DNA adduct staining, the “histological reconstruction” feature of the ACIS software was used. Each section was first visualized as a whole, and then epithelial basal areas between 300 and 400 microns in length and no more than 40 microns (3–4 cell layers) deep were selected for analysis using the ACIS region selection tools. The depth of the regions was limited to those cells visually having a nuclear diameter greater than half of the overall cell diameter. For staining with specific antiserum, up to four regions per section were selected, and, for each region, the number of pink nuclei and an average AU/pink nucleus value were obtained. On the corresponding hematoxylin stained sections, regions in the same location and of the same size and shape were selected to determine the total number of nuclei. On average, 415 nuclei were evaluated per section (range: 72 to just over 2200). For each region a total intensity value was obtained by multiplying the average AU/pink nucleus value by the total number of pink nuclei. A mean AU/nucleus for the whole region was calculated by dividing the total intensity value by the number of blue nuclei in the corresponding region of the hematoxylin stained section. For sections having more than one counted region, the means for each region were averaged and reported.

2.6 BPDE-DNA CIA

The microtiter plate immunoassay methodology for quantifying BPDE-DNA adducts by CIA has been reported in detail [24]. For this study the standard curve 50% inhibition was 1.33 ± 0.17 (mean $\pm$ SD) fmol BPdG adducts/well ($n = 3$), and using 10 μ g DNA per well, the lower limit of detection was 1.28 ± 0.43 (mean $\pm$ SD) BPdG adducts/ 10^8 nucleotides.

2.7 Statistical methods

The mathematical relationship and correlation between the IHC/ACIS and CIA evaluations of the human cervical keratinocytes was determined using the method of least squares. We tested for statistical differences in median PAH-DNA adduct levels between current smokers, former smokers, and never smokers using the Kruskal-Wallis test and a non-parametric test for trend (current smoker, 10 or more cigarettes/day > current smoker, <10 cigarettes/day > former smoker > never smoker). We tested for statistical differences in median PAH-DNA adduct levels between cases and controls, un-stratified and stratified for smoking status using the Kruskal-Wallis test. For blinded repeat slides from the same tissue block, a Spearman correlation coefficient was calculated as a measure of reproducibility.

3. Results

3.1 Keratinocyte standard curve

A standard curve for semiquantifying PAH-DNA adducts in human tissues was prepared by exposing cultured human cervical keratinocytes to 0, 0.153, or 0.331 μM BPDE. A portion of the cells was subjected to DNA extraction and quantification of BPdG adducts by BPDE-DNA CIA, while the remaining cells were paraffin embedded, and fresh-cut sections were stained with specific BPDE-DNA antiserum to reveal adducted nuclei. Figure 1A shows a representative dose-response for BPDE-DNA adduct formation (pink staining) in the keratinocyte nuclei, the doses of BPDE used, and the resulting ACIS-derived intensity values. The ordinate of Figure 2 shows a composite IHC/ACIS standard curve that includes mean $\pm$ SD for the unexposed cells (6.3 ± 3.2 AU/nucleus, $n = 13$), cells exposed to 0.153 μM BPDE (37.6 ± 5.1 AU/nucleus, $n = 5$) and cells exposed to 0.331 μM BPDE (73.3 ± 14.9 AU/nucleus, $n = 11$). The abscissa of Figure 2 shows BPdG adducts/ 10^8 nucleotides determined by BPDE-DNA CIA, for the 0.153 μM and 0.331 μM BPDE doses, which were 135.0 ± 26.5 and 287.2 ± 12.7 (mean $\pm$ SD, $n = 3/\text{group}$), respectively. A linear relationship was obtained between the BPdG values determined by IHC/ACIS and those determined by BPDE-DNA CIA ($R^2 = 0.9997$). This standard curve was used with human cervix samples subjected to IHC/ACIS to estimate the quantity of PAH-DNA adducts.

3.2. PAH-DNA adduct staining of human cervix

Cervical biopsy samples were collected from 114 HPV-infected women. Serial sections from each paraffin-embedded tissue were stained with specific BPDE-DNA antiserum, immunogen-absorbed BPDE-DNA antiserum, or hematoxylin. Intact basal epithelium suitable for evaluation was found in 75 of the 114 individuals. For each of these individuals, up to four separate regions of basal epithelium were analyzed by ACIS and the data expressed as arbitrary units (AU)/nucleus. On average, staining was evaluated in 415 nuclei per section (range: 72 to 2200).

Figure 1B shows two representative samples (#43196 and #42467), in which the region of basal cells chosen for ACIS analysis is outlined. In Figure 1B, panels (i) and (iv) show blue hematoxylin staining indicating the localization of nuclei, and panels (ii) and (v), show identical, outlined areas stained with specific anti-BPDE-DNA. The pink nuclei in the latter panels indicate localization of PAH-DNA adducts, and the observed color intensity (AU/nucleus) is shown.

Figure 1B, panels (iii) and (vi), show staining with the immunogen-absorbed BPDE-DNA antiserum, where a virtually-complete loss of pink nuclear intensity confirms that the PAH-DNA staining is specific. About 15% of the human cervix samples were stained with immunogen-absorbed serum, and Figure 3 shows color intensity values for thirteen paired sections stained with specific and absorbed serum. The overall mean value for the sections stained with absorbed serum was 6.3 AU/nucleus. Because the BPDE-DNA antiserum has cross-reactivity for DNA modified with several PAHs [23], a positive signal in these human samples is interpreted as indicative of PAH-DNA adduct formation, and comparison with a standard curve of cells exposed to BPDE semi-quantifies the PAH-DNA adducts.

In addition to the human cervix samples, Figure 3 also shows specific and absorbed BPDE-DNA antiserum staining for a single sample from the BPDE-exposed human keratinocytes at the highest dose (0.331 μ M), used as a positive control (designated “Control” in Figure 3). The substantial reduction of pink staining in all samples treated with absorbed serum, relative to that of the samples treated with the specific antiserum, indicates specific staining for PAH-DNA adducts in the cervix samples and BPdG adducts in the keratinocytes. Due to the variation in staining intensity among the sections stained with immunogen-absorbed antiserum, and because not all specimens were evaluated with immunogen-absorbed serum, no background correction was performed.

3.3 ACIS evaluation and semi-quantitation using the keratinocyte standard curve

Using the human cervical keratinocyte standard curve to estimate the quantity of PAH-DNA adducts in the human cervical biopsy specimens, the detectable PAH-DNA adduct levels ranged from 25 to 191 PAH-DNA adducts/ 10^8 nucleotides. The adduct levels for three samples fell below the lower limit of detection of 20 adducts/ 10^8 nucleotides. For quality control purposes 18 samples were assayed in duplicate and 1 sample was assayed in triplicate, and the data are shown in Figure 4. The Spearman correlation for the repeats was 0.44 ($P = 0.06$).

Figure 5A shows the variation in PAH-DNA adduct levels for the entire set of human samples. One aspect of the variability of the assay is reflected in the positive controls, which were stained on different days and gave a mean ($\pm$ SE) BPdG level of 283.3 ± 17.6 adducts/ 10^8 nucleotides ($n = 11$). Data for PAH-DNA adducts in the human samples, grouped in increments of 75 adducts/ 10^8 nucleotides, are shown in Figure 5B. The largest group, 36 individuals, had adduct values of 25 – 75/ 10^8 nucleotides, and 8 individuals had adduct values 150/ 10^8 nucleotides.

3.4. PAH-DNA adduct formation in human cervix: relationship to smoking and cervical cancer status

Once the codes were revealed, PAH-DNA adduct levels were evaluated with respect to smoking and cervical cancer status (CIN3 versus CIN1) and no correlations were observed between any of the parameters investigated. Figure 6A shows that there was no significant difference in median PAH-DNA adduct levels for participants grouped as never, former, and current smokers of either <10 or ≥ 10 cigarettes per day among cases (CIN3) ($P = 0.3$; $P_{\text{trend}} = 0.3$) or among controls (CIN1) ($P = 0.3$; $P_{\text{trend}} = 0.2$). Figure 6B shows that when CIN3 cases and controls were combined the difference in PAH-DNA adduct level between smoking and non-smoking groups was marginal ($P = 0.05$; $P_{\text{trend}} = 0.6$). Combining all smoking classifications and comparing individuals with and without cervical cancer, the median levels of PAH-DNA adducts were not significantly different (Figure 6C), showing no correlation between formation of PAH-DNA adducts and the development of CIN3 in these individuals ($P = 0.05$).

4. Discussion

PAH-DNA adducts were detected by staining paraffin-embedded human cervical biopsy specimens with BPDE-DNA antiserum. The biopsy donors were participating in a 10-year prospective epidemiological study and were chosen because they were found to be infected with carcinogenic HPV types at baseline [3]. We selected as cases women who were diagnosed with cervical precancer (CIN3) or cervical cancer during follow-up and used the diagnostic biopsy collected at follow-up visit. The controls were women whose biopsies were diagnosed as either normal or CIN1, a histopathologic manifestation of HPV infection, at approximately the same follow-up time as cases. Of those having usable tissue, all but 3 of the individuals had measurable PAH-DNA adducts. Consistent with our earlier observations in human esophagus [17;18], the highest concentration of PAH-DNA adducts in the human cervix was localized in nuclei of the basal epithelial layer, where carcinogenic HPV infection is established and where progression of a persistent carcinogenic HPV infection to precancer occurs. When quantified by ACIS and calculated against a standard curve, PAH-DNA adduct values ranged from background levels to 191 adducts/10⁸ nucleotides. Whereas these numbers appear high when compared with values obtained by ³²P-postlabeling, immunoassays, or mass-spectrometry-based methods, they are plausible if one considers that we are localizing adducts in specific nuclei of the basal epithelium. The more common approach of extracting DNA from a whole tissue (containing epithelium and stroma) will likely result in a dilution of DNA adducts and lowered values (concentrations). In addition, the nature of the IHC/ACIS approach does not support determination of a single chemically-specific DNA adduct because the antiserum has broad specificity for multiple PAHs bound to DNA [23].

In the BP-DNA standard curve, which was generated by treating cultured cells with BPDE, the predominant adduct is BPdG. This is still the predominant adduct, in addition to some minor adducts formed in smaller amounts, when BP is used to modify the DNA. The metabolic competency of cervical epithelial cells in culture has been demonstrated elsewhere by the detection of CYP1A1 and 1A2 mRNA by RT/PCR and CYP1A1 and 2E1 proteins by western blot along with induction by PAH exposure [25]. The potential for metabolic activation of numerous PAHs in cervical tissue therefore exists and the formation of PAH-DNA adducts by many of these bioactivated PAHs would be detectable using this antiserum and the IHC/ACIS approach.

Smoking-related DNA adduct formation has previously been reported in DNA extracted from both exfoliated cervical cells and cervical biopsies using ³²P-postlabeling, or by using IHC to evaluate cervical smears [13-15;15;16]. The present study is the first to report the semiquantitation of PAH-DNA adducts in tissue sections where the tissue architecture is intact. The use of the standard curve composed of cultured human keratinocytes exposed to BPDE resulted in semiquantitative estimates of the PAH-DNA adducts in these cervical biopsy samples. The methodology described here for PAH-DNA adduct estimation is still only semi-quantitative, although the adduct visualization capability is a potentially very powerful tool.

No correlation was found between the level of PAH-DNA adducts in human cervix and either the smoking or cervical cancer status of the individual study subjects. However, the study was compromised by a number of factors including the small number of individuals examined. Although we started with tissue blocks from 114 individuals, after staining we found that only 75 individuals had discernable basal epithelial areas and could therefore be included in the analysis. Stratification by case/control and smoking status further fragmented group sizes leaving smaller numbers of individuals in each group. Another confounding issue is the fact that human carcinogens other than PAHs are present in cigarette smoke; of the 11 known human carcinogens in tobacco smoke [7], many, including 4-aminobiphenyl and tobacco-specific nitrosamines, would not induce DNA damage measurable by the methods used here. Finally, smoking is not the only source of human PAH exposures, as ingestion of char-broiled red meat has been documented to provide higher exposures than inhalation [26–28]. Therefore, it appears likely that PAH-DNA adduct formation due to smoking may not be a major component in the progression of carcinogenic HPV infections to cervical precancer and cancer. In a *post-hoc* analysis, given the final sample size, PAH-adduct levels in controls, and a 50% standard deviation in the measurements, there was 89% power ($\alpha = 0.05$) to detect a 50% increase in PAH-adduct levels in cases versus controls. This does not, however, preclude the possibility that PAH-DNA damage from smoking, topical exposure or diet may act in concert with other factors or in an indirect manner to promote neoplasia.

PAH exposure from sources other than cigarette smoking potentially confounds the data reported here. Several published studies suggest that ambient environmental exposures and dietary practices have a greater influence than personal smoking status on the formation of PAH-DNA adducts measured in human peripheral blood leukocytes. For example, in US Army soldiers stationed in Germany and deployed to Kuwait, the level of leukocyte PAH-DNA adducts was highest in Germany, where the ambient PAH levels were also highest, compared to lower PAH-DNA adduct levels found for the same soldiers after 8 weeks in Kuwait, where the ambient PAH levels were lower [29]. Interestingly, in Kuwait, in addition to low ambient PAH levels, there was no grilled meat available, and both factors may have contributed to the low PAH-DNA adduct levels. In other studies a correlation has been reported between the consumption of heavily-grilled meats and the formation of PAH-DNA adducts in peripheral white blood cells [26–28]. In Rothman *et al.* [27], volunteers abstained from meat for 4 weeks, ate heavily-grilled hamburger for 1 week, and abstained again for 4 weeks. As expected, leukocyte PAH-DNA adduct levels spiked during the week of grilled hamburger consumption, demonstrating the importance of dietary PAH exposure to leukocyte PAH-DNA adduct formation.

In this study sociodemographic characteristics of the cervical tissue donors were available; however, information regarding their exposures to second hand tobacco smoke, home and occupational environments, and dietary habits was lacking. Oral contraceptive use was found to be unrelated to any other parameter. In retrospect, we had hypothesized that PAH-DNA adducts might be related to smoking exposure and or cervical cancer status, and this preliminary study with relatively small numbers of individuals in each group suggests that there may be other contributory factors. One such possibility is immune response. For

example, Szarewski *et al.* [30] followed a group of women who smoked heavily and then quit. This study showed that, while smoking, these women had significantly higher numbers of Langerhans cells, CD8 T lymphocytes and total T lymphocytes in the cervix, compared to 6 months after quitting smoking. Therefore, smoking appears to trigger an immune response in the cervix that may potentially impact cancer risk by permitting the HPV infection to persist.

Here we have shown semi-quantification of PAH-DNA adducts in archived, paraffin-embedded human cervix using a combination of immunohistochemical staining and nuclear color intensity quantification. This study demonstrates the feasibility of using the IHC/ACIS approach for determination of carcinogen exposure and target tissue biologically-effective dose in archived paraffin-embedded human tissues within the context of large epidemiological study designs.

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Abbreviations

ACIS	Automated Cellular Imaging System
AU	arbitrary units, used to express color intensity
BP	benzo[<i>a</i>]pyrene (CAS No. 50-32-8)
BPDE	r7, t8-dihydroxy-t-9, 10-oxy-7, 8, 9, 10-tetrahydrobenzo[<i>a</i>]pyrene (CAS No. 58917-67-2)
BPdG	r7, t8, t9-trihydroxy-c-10-(N ² deoxyguanosyl)-7, 8, 9, 10-tetrahydro-benzo[<i>a</i>]pyrene
CIA	chemiluminescence immunoassay
CIN3	Cervical Intraepithelial Neoplasia grade 3
CIN3	Patients having either CIN3 or cancer of the cervix were grouped together and included as the “cases” in this study
CIN1	Patients having either CIN1 or normal-appearing epithelium were grouped together and included as “controls” in this study
HPV	Human Papillomavirus
IHC	immunohistochemistry
PAH	polycyclic aromatic hydrocarbon

Figure 1.

A. Cultured human cervical keratinocytes exposed to 0, 0.153 or 0.331 μ M BPDE for 1 hr were stained with rabbit anti-BPDE-DNA, a secondary antibody and Fast Red. Pink nuclei indicate the presence of BPdG adducts, and color intensity values (AU/nucleus) for ACIS scoring of 607 – 1223 cells per sample are shown below each picture. Nuclear counts were obtained from hematoxylin-stained adjacent sections (not shown). The dosage of BPDE is shown above and the ACIS-derived intensity values are shown below each photograph. **B.** Immunohistochemical staining of human cervix samples from two different individuals (Identification numbers 43196 and 42467) with ACIS scoring. Serial sections from each sample were stained with either hematoxylin, BPDE-DNA antiserum, or immunogen-absorbed BPDE-DNA antiserum. Regions from the same location and of the same shape and size were evaluated for each of the stained sections. Panels **i** and **iv** show hematoxylin staining of nuclei used to obtain a nuclear count. Panels **ii** and **v** show nuclear staining for PAH-DNA adducts with the specific BPDE-DNA antiserum; AU/nucleus values determined by IHC/ACIS are shown. Panels **iii** and **vi** show the absorbed antiserum control. Gridlines show 10 μ m increments.

Figure 2.

Cultured human cervical keratinocytes were exposed to BPDE as in Figure 1, and BPdG adducts quantified by IHC/ACIS (AU/nucleus, ordinate) are plotted against BPdG adducts measured by quantitative BPDE-DNA CIA (BPdG adducts/10⁸ nucleotides, abscissa), with a correlation coefficient, R^2 , of 0.9997. Values are mean $\pm$ SD for 3 assays per point for BPDE-DNA CIA and mean $\pm$ SD for 5 stainings per point for the IHC/ACIS.

Figure 3.

Human cervix samples ($n = 13$) were stained with immunogen-absorbed antiserum, and AU/nucleus values for paired specific (■) and absorbed (□) serum are shown; the average of the absorbed serum values was 6.3 AU/nucleus. The graph also shows mean values for human keratinocytes exposed to 0.331 μM BPDE and stained with specific (positive control, $n = 13$, ■) or absorbed ($n = 1$, ▨) BPDE-DNA antiserum.

Figure 4.

Replicate sections were stained in random order and usually on different days throughout the experiment. ACIS analysis was confounded by the histological changes in tissue morphology occurring throughout the series of slides, making it difficult to identify and re-score the corresponding area. Therefore the variability shown here is a composite of the variability introduced by staining on different occasions and reading different areas of the slide. The Spearman correlation for the replicates was 0.44 ($P = 0.06$).

Figure 5.

A. The range of sample PAH-DNA adduct values/ 10^8 nucleotides found in human cervix samples from 75 individuals and obtained by comparison with the standard curve shown in Figure 2. The open symbols show all the human cervix biopsies stained with either anti-BPDE-DNA (Δ), ordered by increasing adduct levels, or immunogen-absorbed serum ($\diamond$). The filled symbols ($\blacktriangle$) represent the positive control, human keratinocytes exposed to 0.331 μ M BPDE and stained with specific antiserum, which were assayed with each set of samples. Use of immunogen-absorbed antiserum with a positive control sample is indicated

by (◆). **B.** Distribution of PAH-DNA adduct values, expressed as PAH-DNA adducts/ 10^8 nucleotides, in human cervix samples from 75 individuals. The number of individuals in each grouping is shown at the top of each bar. ND = not detectable.

Figure 6.

Assessment of the relationship between PAH-DNA adducts, smoking status, and CIN3/ cervical cancer development; n = number of individuals in each category. Box and whisker plots show median $\pm$ 25 percentile for adducts/ 10^8 nucleotides with bars representing the most extreme outliers. **A.** Relationship between PAH-DNA adduct levels by smoking status (never, former, and current) nested within cancer status (controls vs. cases). Relationship between PAH-DNA adduct levels by **B.** smoking status (never, former, and current) without regard for case status and **C.** cancer (controls vs. cases) status without regard for smoking status.