^{32}P-Postlabeling Analysis of DNA Adducts in Human Sperm Cells from Smokers and Nonsmokers[1]

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Abstract

To determine the feasibility of using human sperm cells for DNA ^{32}P-postlabeling analyses, and to evaluate the baseline level and the possible presence of smoking-related DNA adducts in these cells, sperm DNA was isolated from specimens obtained from 12 heavy smokers, 12 light smokers, and 12 nonsmokers. Background levels of radioactivity were minimized by using magnet transfer of ^{32}P-labeled mononucleotides to new polyethyleneimine cellulose plates. Compared with placental tissues, few adducts were observed. Diffuse radioactivity observed in some of the autoradiograms was minimally above background but the level of radioactivity expressed as putative adducts/nucleotide was not related to smoking status. It was not clear, in some cases, whether this radioactivity was associated with chemically bound adducts or was from nonspecifically bound chemicals, radiolabeled enzymes, or other proteins. One major discrete DNA adduct of unknown chemical structure was detected in three of the 36 samples analyzed (one nonsmoker and two smokers). Based on the level of radioactivity associated with various dilutions of a benzo(*a*)pyrene-derived adduct, our limit of sensitivity was at least 1.2 adducts/ 10^9 nucleotides. Our study emphasizes the need to more clearly define the significance of background radioactivity associated with DNA adduct maps where the measured adduct levels approximate detection limits defined by visual observance of adduct spots. This point is particularly relevant given that the ^{32}P-postlabeling procedures rely, in part, on visual verification of the presence of DNA adducts.

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Introduction

Nicotine and cotinine, a metabolite of nicotine, have been detected in saliva, urine, and blood (1). Because cotinine and nicotine are specifically related to tobacco use, the detection of these markers in human body fluids is important when evaluating risk to humans exposed to tobacco smoke through active smoking or passive exposure to tobacco smoke. The diverse chemicals in tobacco smoke and the recent finding that cotinine is present in the seminal fluid of smokers (2) suggests that cigarette smoke constituents and/or DNA-reactive intermediates may pass through the blood-testis barrier to react directly with germ cells. These events could potentially lead to important heritable germ cell mutations.

Available evidence indicates that cigarette smoking impairs the production of normal sperm as measured by alterations in sperm morphology and decreased sperm motility (3). DNA adducts associated with exposure to cigarette smoke have also been detected in DNA isolated from a variety of tissues including respiratory tract (4–6), placental tissue (7), and blood lymphocytes and monocytes (8, 9).

To our knowledge, no studies have reported DNA adduct levels in human sperm DNA by sensitive ^{32}P-postlabeling methodologies. In this study, human sperm DNA was isolated from 36 men (12 heavy, 12 light, and 12 nonsmokers) and analyzed by ^{32}P-postlabeling analysis for the presence of DNA adducts. Two placental DNA samples isolated from one smoker and one nonsmoker were similarly analyzed and represented a positive and negative control. The detection limits for the ^{32}P-postlabeling conditions used were established by serially diluting a B(*a*)P[3]-modified DNA sample. The data were used both to determine baseline levels of adducts in sperm and to investigate the potential presence of smoking-related adducts.

Materials and Methods

Chemicals. [γ-^{32}P]ATP (3000 Ci/mmol; 10 mCi/ml aqueous solution containing 5 mM 2-mercaptoethanol) was obtained from Amersham, Arlington Heights, IL. PEI cellulose TLC plates were prepared as previously described (10) except that the PEI solution (50% aqueous) was from Aldrich Chemical Co., Milwaukee, WI. Micrococcal endonuclease and nuclease P_1 were from Sigma Chemical Co., St. Louis, MO.; calf spleen phosphodiesterase was from Boehringer Mannheim, Indianapolis, IN; and T_4 polynucleotide kinase was from Pharmacia, Piscataway, NJ. All other chemicals were of analytical grade.

Subjects and Specimens. Participants eligible for inclusion in the study were healthy white males aged 18 to 35 years

[3] The abbreviations used are: B(*a*)P, benzo(*a*)pyrene; TLC, thin layer chromatography; PEI, polyethyleneimine; RAL(s), relative adduct labeling value(s).

who enrolled by responding to advertisements in local media. Sperm cells were obtained from male volunteers via masturbation into sterile polypropylene containers. Participants were excluded if they had a sperm count of less than 20 million sperm cells/ml or if their total sperm count was less than 40 million cells because adequate amounts of DNA could not be extracted to conduct replicate assays. Men included in the current investigation were matched on smoking status (nonsmoker, light smoker, and heavy smoker) and age category (18–21, 22–25, 26–29, 30+ years). Nonsmokers were defined as men who smoked fewer than 100 cigarettes in their lifetimes. Light smokers included men who smoked between 1 and 19 cigarettes/day on average and heavy smokers were defined as men who currently smoked 20 or more cigarettes/day on average. All smokers had smoked cigarettes for at least 1 year.

Isolation of DNA. Human sperm cells were washed in phosphate buffered saline at 4°C and stored in saline-Tris-EDTA (150 mM NaCl-10 mM Tris-1 mM-EDTA, pH 7.4) at −70°C. Sperm cells (4×10^7) were thawed, resuspended in equal volumes (1.5 ml) of saline-Tris-EDTA, and lysis buffer reagent (2× stock; Applied Biosystems, Foster City, CA), and β-mercaptoethanol was added to a final concentration of 2.5%. After a 15-min incubation, 100 units of RNase T_1 and 20 units RNase A were added and samples were digested for 1 h at 37°C. Forty units of proteinase K (4× stock from Applied Biosystems) and an additional 1.5 ml of lysis buffer reagent were then added and samples were digested overnight at 37°C. Two 70% phenol/water/chloroform reagent extractions, one chloroform extraction, and an isopropanol precipitation were performed on the Applied Biosystems Model 340A Nucleic Acid extractor using the manufacturer's reagents. DNA was redissolved in high performance liquid chromatography grade water, quantitated spectrophotometrically, and stored at −70°C until the samples were analyzed. DNA from placental samples were isolated according to the methods of Gupta (10). β-mercaptoethanol was not added to the placental DNA isolates.

^{32}P-Postlabeling Analysis. Ten μg of DNA were enzymatically treated for 3.5 h in the presence of spleen phosphodiesterase and micrococcal endonuclease according to the method of Gupta *et al.* (11). DNA digests were then nuclease P_1 treated according to the methods of Reddy and Randerath (12) except the stock concentrations of nuclease P_1 sodium acetate, zinc chloride buffers, and Tris-base were doubled because 10 μg of DNA rather than the standard 5-μg amounts were enzymatically digested. These concentrations minimized the volume prior to the subsequent ^{32}P-postlabeling step. The digest was then labeled for 30 min with approximately 50 μCi [γ^{32}P]ATP (3000 Ci/mmol) and 3.5 units T_4 polynucleotide kinase. Potato apyrase (40 milliunits) was added to terminate the reaction. The radiolabeled digest was applied to a PEI-cellulose sheet and developed in the first dimension with 1 M sodium phosphate, pH 6.8. The origin area containing aromatic adducts were excised, attached with the aid of magnets to fresh PEI-cellulose sheets, and developed using the following solvents and directions: D-2 as a predevelopment to 1 cm with 2.5 M ammonium formate, pH 3.5; D-3 4 M lithium formate, 7 M urea, (pH 3.45) with (D-4) 0.8 M lithium chloride; 7 M urea-0.5 M Tris-HCL, pH 8.0, with predevelopment to 1.0 cm with 0.5 M Tris-HCl, pH 8.0; (D-5) same direction as D-4 using 0.5 M magnesium chloride.

To measure the total number of nucleotides, an aliquot (1.25 μl of enzymatic digest) was diluted approximately 700-fold and 2.5 μl of this dilution was added to an equivalent amount of radiolabeled mix. A further 40-fold dilution was made, and 5 μl were spotted on a PEI-cellulose plate previously pretreated with 100 mM ammonium formate, pH 3.5, and developed in 1 dimension with 4.5 M ammonium formate, pH 3.5.

Intensifying screen enhanced autoradioagraphy at −70°C was used to detect the presence of radiolabeled adducts on the TLC plates. Putative placental DNA adducts that migrated along a diagonal zone of radioactivity were carefully excised with the aid of a template which outlined the boundary of radioactivity. The same template was used to excise the identical area on the chromatogram for all 36 sperm DNA samples regardless of whether radioactive spots (putative adducts) were observed. Discrete adducts detected within or outside this templated area were separately excised. RALs for discrete adducts or those which migrated along the diagonal zone of radioactivity were calculated by dividing the cpm/adduct (or adducts) by the cpm in the adducts and the normal nucleotides, correcting for dilution factors and volumes spotted.

To determine the limits of detection for our ^{32}P-postlabeling conditions, DNA was isolated from rat liver following i.p. injection of 50 μg/kg B(*a*)P. The B(*a*)P-modified DNA sample was then diluted with high performance liquid chromatography grade water following digestion with spleen phophodiesterase and micrococcal endonuclease. This B(*a*)P-modified DNA sample containing 0.5, 0.05, and 0.01 μg DNA was radiolabeled with the [γ-^{32}P]ATP cocktail described above and autoradiography performed for 24–72 h at −70°C.

DNA Adduct Analysis. Thirty-six DNA samples (12 heavy smokers, 12 light smokers, and 12 nonsmokers) were analyzed in duplicate in two separate experiments blinded as to smoking status. Duplicate analyses of the same sample

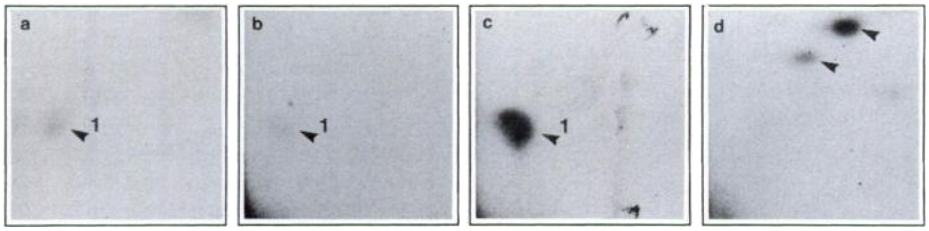

Fig. 1. ^{32}P-postlabeling autoradiograms of DNA isolated representing each of the three exposure groups (*a*, non smoker; *b*, light smoker; *c*, heavy smoker). The discrete radioactive spot (putative adduct) was reproducibly detected in three sperm DNA samples only (spot 1). This adduct was not related to smoking status and was reproducibly detected in two separate ^{32}P-postlabeling experiments. The chemical nature of this putative adduct remains unknown. Several background spots of unknown origin (*d, arrowheads*) were present in many DNA samples regardless of smoking status. Exposure of the film was for 24 h at −70°C.

Table 1 Putative DNA adducts (DNA adducts/10^8 nucleotides)[a] in sperm DNA isolated from heavy, light, and nonsmokers as determined by ^{32}P-postlabeling analysis

	RAL[a]	RAL	Mean	SD
Nonsmokers				
1	1.75	0.88	1.32	0.62
2	1.25	1.00	1.13	0.18
3	0.85	1.00	0.93	0.11
4	1.25	0.75	1.00	0.35
5	1.00	0.75	0.88	0.18
6	0.75	0.75	0.75	0.00
7	0.75	0.75	0.75	0.00
8	1.50	1.25	1.38	0.18
9	0.75	0.50	0.63	0.18
n = 9			0.97 (group $\bar{x}$)	0.20 (Mean SD)
n = 1 (placental DNA)	1.59	ND	1.59	
Light smokers				
10	0.75	0.75	0.75	0.00
11	0.75	0.50	0.63	0.18
12	0.75	0.75	0.75	0.00
13	1.00	0.75	0.88	0.18
14	0.75	0.50	0.63	0.18
15	0.75	0.50	0.63	0.18
16	0.75	0.75	0.75	0.00
17	1.00	0.75	0.88	0.18
18	1.25	1.13	1.19	0.08
19	0.50	0.50	0.50	0.00
n = 10			0.76 (group $\bar{x}$)	0.10 (Mean SD)
n = 1 (placental DNA)	6.62	4.15	5.38	
Heavy smokers				
20	2.75	2.70	2.73	0.04
21[a]	1.00	1.08	1.04	0.06
22	1.00	1.05	1.03	0.04
23	1.00	0.50	0.75	0.35
24	0.75	1.00	0.88	0.18
25	0.75	0.75	0.75	0.00
26	0.75	1.00	0.88	0.18
27	1.00	0.50	0.75	0.35
28	0.50	0.75	0.63	0.18
29	0.75	0.50	0.63	0.18
n = 10			1.0 (group $\bar{x}$)	0.15 (Mean SD)

[a] Putative DNA adduct levels equivalent to the amount of radioactivity migrating along a radioactive diagonal zone in sperm DNA isolated from heavy smokers, light smokers and non-smokers. Data are expressed as adducts/10^8 nucleotides, mean ($\bar{x}$) ± SD, and are based on two determinations where DNA was analyzed twice (i.e., once in two separate experiments from the same DNA isolate). Adduct levels as reported may actually represent background levels of radioactivity and not measurements of DNA adducts themselves. RALs, relative adduct labeling values. ND, not determined.

which showed greater than 20% variability were eliminated from the analysis. Variability, as defined in these experiments, was the SD between duplicate analyses of the same sample divided by the average value for the two samples ×100. All data points were considered initially to ascertain whether a statistical association between smoking status and adduct level existed. If the variability between RALs as determined in the two separate analyses, exceeded 20%, these values were omitted from the analysis. Generally, this variability was attributed in these cases to high background levels of radioactivity in one of the two DNA samples. Seven of the 36 samples analyzed fell into this category although inclusion of them in the data base had no influence on the association between smoking and putative adduct level.

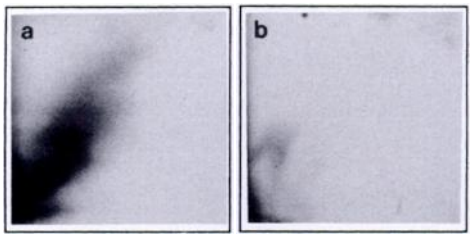

Fig. 2. Representative DNA adduct maps of placental DNA isolated from a smoker (*a*) and non smoker (*b*) following 72 h of screen enhanced radiography at −70°C.

Results

No smoking-related adducts were detected in the DNA isolated from the sperm cell DNA. Three specimens however, reproducibly showed a single discrete spot (spot 1) corresponding to adduct levels of 0.45, 0.26, and 0.80 adducts/10^8 nucleotides, respectively (Fig. 1, *a–c*). The three DNA samples which showed this adduct were isolated from a nonsmoker (26 years old), a light smoker (21 years old), and a heavy smoker (27 years old) (Fig. 1, *a–c*, spot 1, respectively). Thus, no apparent relationship between this adduct and age was observed. Based on the similarity in chromatography patterns and lack of association between adduct levels and exposure status, the chemical basis for this adduct is unclear.

Several DNA samples showed extraneous spots in the upper right corner of the autoradiogram (Fig. 1*d*, *arrowheads*). These spots were not reproducible in replicate assays of the same specimens and were not quantitated. The presence of these spots was not related to exposure status or age of the individuals analyzed. The chemical nature of these spots is currently unknown but may represent partially digested modified or unmodified dinucleotides or mononucleotides which were not completely removed in the final wash step.[4]

Putative DNA adduct levels as detected in the diagonal radioactive zone were determined for all of the DNA samples that were analyzed regardless of whether radioactivity was observed visually. Thus, the adduct levels as reported in Table 1 may actually represent background levels of radioactivity and not measurements of DNA adducts. The radioactivity present in the diagonal radioactive zone was equivalent to a mean putative adduct level for nonsmokers of 0.97 adducts/10^8 (range, 0.63–1.38; n = 9); 0.76 adducts/10^8 for light smokers (range, 0.50–1.19; n = 10), and 1.0 adducts/10^8 for heavy smokers (range, 0.63–2.73; n = 10) (Table 1). Levels of radioactivity in this diagonal radioactive zone were not statistically different by t test analysis comparing nonsmokers and light smokers ($P < 0.10$) or nonsmokers and heavy smokers ($P < 0.50$). There was an association of borderline statistical significance between age and mean putative adduct level for nonsmokers only (Pearson correlation coefficient r = 0.65; P = 0.057).

For comparison, mean adduct levels for DNA isolated from placental tissue were 5.38 and 1.59 adducts/10^8 for a smoker and nonsmoker, respectively (Fig. 2, *a* and *b*; Table 1). Adduct values for placental DNA isolated from one

[4] M. V. Reddy, personal communication.

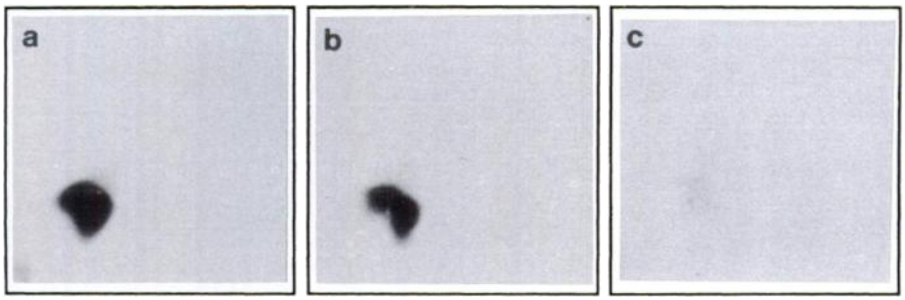

Fig. 3. ^{32}P-postlabeling autoradiograms of B(*a*)P-modified DNA sample serially diluted to establish a limit of detection based on the visual detection of the major B(*a*)P-derived adduct. *a* represents the equivalent of 5 μg of radiolabeled mononucleotides; *b*, 0.5 μg; *c*, 0.05 μg. The major adduct shown in (*c*) was detectable following film exposure for 72 h. The RAL corresponding to the visual detection of the major B(*a*)P-derived DNA adduct was 1.18 adducts/10^9.

smoker was 5.5-fold higher than the mean adduct value determined for sperm DNA isolated from the nonsmokers (Table 1).

To determine the lower limit of sensitivity for the ^{32}P-postlabeling conditions, various dilutions were made of a stock concentration of B(*a*)P-modified DNA sample such that 0.01, 0.5, and 5.0 μg of B(*a*)P-modified DNA were digested to mononucleotides and labeled with [γ-^{32}P]-ATP as outlined in "Materials and Methods." Autoradiograms representing DNA adduct profiles for these various dilutions are shown in Fig. 3, *a* (5 μg of DNA), *b* (0.5 μg), and *c* (0.01 μg). Based on the radioactivity determined for the major DNA adduct and the amount of DNA which was radiolabeled, the detection limit based on visual substantiation was approximately 1.2 adducts/10^9 nucleotides.

Discussion

Previous reports have established that individuals exposed to tobacco smoke have elevated cotinine levels in their urine and blood (1). More recently, specimens from subjects included in this study (as well as additional subjects) were analyzed for cotinine in seminal fluid, urine, and blood. Cotinine levels were similar in blood and seminal fluid with higher levels detected in urine (2).

In this study, DNA was isolated from human sperm from 12 nonsmokers, 12 light smokers, and 12 heavy smokers and analyzed using the nuclease P_1 version of the ^{32}P-postlabeling methodology. Discrete areas of radioactivity were reproducibly detected in sperm DNA samples isolated from three individuals (one heavy, one light, and one nonsmoker). Whether these putative adducts were truly modifications to sperm DNA related perhaps to diet, or exposure to an environmental agent, can not be determined from our data. Replicability of the finding does not suggest any artifact related to DNA isolation or ^{32}P-postlabeling procedures. Several samples showed extraneous spots detected in the upper right corner of the autoradiogram (Fig. 1*d*). The chemical nature of these spots is currently unknown, but based on chromatographic properties, they may represent partially digested modified, unmodified dinucleotides, or traces of monnucleotides which were incompletely removed during the final wash step. These spots, therefore, were not quantitated or studied further. Although exact comparisons are difficult due to differences in solvents used in the chromatography steps, DNA adducts with similar chromatographic properties have been observed in control placental DNA samples isolated from nonsmoking women (13).

Unlike placenta and several other tissues from smokers, radioactivity was not detected in a diagonal zone across the TLC plates in DNA isolated from sperm cells. Nonetheless, the low levels of radioactivity present in this area were quantitated. No association was found between smoking status and levels of radioactivity in this area. Whether the low levels obtained are DNA adducts or background radioactivity can not be determined from our data. An association of borderline statistical significance between age and mean putative adduct level for nonsmokers but not smokers was observed.

Small or polar DNA adducts would go undetected using the nuclease P_1 version of the ^{32}P-postlabeling assay. Unstable DNA adducts such as those derived from nitrosamines present in tobacco smoke would not be detectable by ^{32}P-postlabeling analysis (14). Thus, alternate chromatographical systems to resolve more polar or low molecular weight polycyclic aromatic hydrocarbon-derived DNA adducts should be considered. DNA lesions occurring in early spermatogenesis may be repaired, selected against, and/or diluted as the cells enter the meiotic phases. During final stages of differentiation spermatid cells have minimal DNA repair capacity and do not repair DNA damage once their chromatin has condensed (15). Once chromatin is condensed, its compact structure may preclude adduct formation. Finally, even though cotinine has been shown to be present in seminal fluid of smokers, any DNA reactive intermediates available for spermatid DNA binding may not cross the blood testis barrier.

The detection limit for the ^{32}P-postlabeling conditions was approximately 1.2 adducts/10^9 nucleotides, based on the visual detection of serially diluted B(*a*)P-modified DNA samples. It is possible, however, that cigarette smoke-derived adducts may label with higher or lower efficiency compared to the B(*a*)P-derived DNA adducts. Adduct levels for the placental DNA sample isolated from a smoker was 3.3-fold higher compared to placental DNA from a nonsmoker and approximately 5-fold higher than sperm DNA isolated from non-smokers. The higher placental DNA adduct levels associated with smokers is consistent with previous studies conducted using a different modification of the ^{32}P-postlabeling procedure in which a smoking-related adduct was detected in DNA isolated from smokers (7).

This is the first study that attempts to evaluate DNA adduct formation in germ cells using ^{32}P-postlabeling procedures which, in part, rely on visual verification for the presence of DNA adducts. Thus, speculation as to possible

factors contributing to the variability and/or interpretation of the putative adduct levels for the three exposure groups seems warranted. One technical difficulty encountered with the analyses was the ability to recover sperm DNA. Since sperm chromatin is stabilized by inter- and intramolecular protamine disulfide bonds, isolation of sperm DNA requires the inclusion of disulfide-reducing agents such as β-mercaptoethanol or dithiothreitol to facilitate the lysis of the cells and increase the yield of DNA. Whether betamercaptoethanol contributed to the background level of radioactivity or lack of adducts is not known.

Additional experiments are required to establish unequivocally the ability of testicular cells to metabolize cigarette smoke constituents to DNA-reactive intermediates or effectively bind circulating DNA-reactive intermediates which might be present in the blood. The methods used in this study would have detected only stable covalently bound bulky DNA adducts such as those formed from the genotoxic polycyclic aromatic hydrocarbons, i.e., B(*a*)P found in tobacco smoke. It should be noted that although tobacco smoke contains B(*a*)P and other polycyclic aromatic hydrocarbons, their relative concentration is lower in tobacco smoke than in several occupational and environmental exposures from coal tar-derived emissions that give rise to DNA adducts in blood (16). It is not known whether these coal tar exposures result in DNA adducts in sperm cells.

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