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Identification of Tobacco-Specific Carcinogen in the Cervical Mucus of Smokers and Nonsmokers

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Background: **In 1996, an estimated 15 700 new cases of cancer of the uterine cervix and 4900 deaths from this disease were expected to occur in the United States. In a recent international study, human papillomavirus DNA was found in more than 90% of cervical tumor specimens examined, irrespective of the nationality of the patients from whom the samples were obtained. Although infection with human papillomavirus is the major known risk factor for the development of cervical cancer, it alone is not sufficient. Other etiologic factors that have been associated with this disease include deficiencies in micronutrients, lower socioeconomic status, oral contraceptive use, and cigarette smoking. Several compounds from cigarette smoke (nicotine and its major metabolite, cotinine) have been identified in cervical mucus, and the occurrence of smoking-related DNA damage in the cervical epithelium has been documented.** ***Purpose:*** **This investigation was conducted to determine for the first time whether carcinogenic tobacco-specific *N*-nitrosamines are present in the cervical mucus of cigarette smokers and of nonsmokers (most likely as a result of environmental exposure).** ***Methods:*** **Cervical mucus specimens from 15 smokers and 10 nonsmokers were subjected to supercritical fluid extraction with the use of carbon dioxide that contained 10% methanol, and the resultant extracts were analyzed for tobacco-specific nitrosamines by use of a very sensitive method that involved gas chromatography and mass spectroscopy analyses.** ***Results:*** **In a total of 16 samples obtained from 15 women who were current smokers (two samples from the same woman), we detected the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) at concentrations that ranged from 11.9 to 115.0 ng/g of mucus. Only one of a total of 10 cervical mucus specimens obtained from 10 women who claimed to be nonsmokers did not contain detectable NNK, and NNK concentrations ranged from 4.1 to 30.8 ng/g of mucus in the specimens from the remaining nine women. The concentrations of NNK in specimens from cigarette smokers were significantly higher than those from nonsmokers (mean ± standard deviation: 46.9 ± 32.5 ng/g of mucus versus 13.0 ± 9.3 ng/g of mucus; two-tailed Student's *t* test, *P* = .004).** ***Conclusion:*** **The cervical mucus of cigarette smokers contains measurable amounts of the potent carcinogen NNK. This compound represents the first tobacco-specific carcinogen identified in this physiologic fluid of women who smoke cigarettes. The presence of NNK in the cervical mucus of nonsmokers is likely due to environmental exposure or to the fact that some of the subjects in this study may not have revealed that they occasionally smoked cigarettes.** ***Implications:*** **The presence of NNK in human cervical mucus further strengthens the association between cervical cancer and tobacco smoking. [J Natl Cancer Inst 1997;89:868-73]**

Cervical cancer is the leading cause of cancer deaths and the most common cancer among women in developing countries (*1*). In the United States, it is the third most common cancer among Hispanic women and the sixth most common cancer among Caucasian women (*2*). In the United States in 1996, there were an estimated 15 700 new cases of cervical cancer and 4900 deaths from this disease (*3*). The incidence and mortality rates of

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See ''Notes'' following ''References.''

cervical cancer among black Americans were about twice those among white Americans (*4*,*5*).

In the past decade, studies (*6*-*8*) have clearly established human papillomaviruses (HPVs) as a necessary agent in the development of nearly all cases of cervical intraepithelial neoplasia and invasive cervical cancer. Indeed, HPV-modified DNA has been detected in up to 93% of cervical tumor specimens (*6*,*8*). However, because HPV infections are widespread in the general population and HPV-immortalized cell lines are generally not tumorigenic, HPV infection likely interacts with one or more cofactors before cancer develops. The cofactors studied to date include deficiency in micronutrients (*9*-*11*), lower socioeconomic status (*12*), and oral contraceptive use (*13*). Cigarette smoking was identified as a possible risk factor by Naguib et al. (*14*), and its adverse effect was also acknowledged in the 1989 report on smoking and health by the Surgeon General of the United States (*15*). The significance of this association, however, was not judged because of ''insufficient data'' (*15*). In 1990, Winkelstein (*16*) reviewed 18 studies of cigarette smoking and cervical cancer; 15 of these studies supported an increased risk (up to 4.3-fold higher) of cervical cancer among smokers, and several of these studies demonstrated a dose–response relationship (*16*,*17*). Environmental exposure to cigarette smoke has also been suggested to increase the risk of cervical cancer (*18*), and a recent report (*19*) stated that smoking cessation may lead to a reduction in the size of minor-grade lesions in the cervix.

The mechanisms by which tobacco smoke constituents could induce a genotoxic effect in the cervical epithelium are not known. The presence of nicotine and cotinine in the cervical mucus of smokers (*20*-*22*) may indicate that inhaled tobacco-specific carcinogens could likewise become blood-borne and transported to the cervix, where they may damage cellular DNA. There are at least 60 toxic and/or carcinogenic compounds among the 4000 chemical constituents identified in tobacco smoke; seven of these compounds are known human carcinogens (*23*). Two major classes of carcinogens are the tobacco-specific nitrosamines and polycyclic aromatic hydrocarbons (*24*). One of the tobacco-specific nitrosamines, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), is relatively abundant in tobacco products and is the most active tobacco-specific carcinogen in animal model assays (*25*).

The goal of our study was to determine if NNK could be detected in the cervical mucus of smokers, thus providing biologic plausibility in support of the association between smoking and cervical cancer.

Subjects and Methods

Subjects

Subjects were recruited in a random fashion from among women presenting for Pap smears in an outpatient gynecology clinic at the University of Chicago. The collection of cervical mucus specimens was approved by the University of Chicago's Institutional Review Board, and all patients gave written informed consent before participating in the study. Eligible subjects included women who were between 18 and 45 years of age, who were not receiving oral contraceptives, and who had no evidence of active genital tract infections. Excluded from the study was one patient known to take exceptionally high doses of various medications.

Specimen Collection

Cervical mucus specimens were collected directly from the cervical canal of 32 women during the preovulatory phase of the menstrual period. These 32 women included 15 smokers and 10 nonsmokers whose mucus was analyzed by a capillary gas chromatography-selected reaction monitoring–tandem mass spectrometry technique (GC-SRM-MS/MS), six women whose mucus was used in a preliminary study and was analyzed by capillary gas chromatography interfaced with a thermal energy analyzer, and one woman who was subsequently excluded from the study. Specimens were placed in preweighed collection vials, coded, packed with dry ice, and shipped frozen to the American Health Foundation (Valhalla, NY), where they were stored at −80 °C until they were analyzed (under these conditions NNK is stable). As part of a preliminary study, six mucus specimens were analyzed by capillary gas chromatography interfaced with a thermal energy analyzer; for later analyses, capillary gas chromatography with mass spectrometry detection was developed. GC-SRM-MS/MS was used. Four wet blank control specimens, consisting of two types of cell culture media used (serum-free keratinocyte growth medium and basic E medium; Clometics, Boston, MA), and two serum specimens from nonsmokers were collected and placed in identical collection vials. By use of the same technique, wet blank control specimens, along with mucus specimens, were tested for NNK. These control specimens, however, were analyzed later than most of the mucus specimens.

Chemicals

The reference compounds NNK, CD_3-NNK, and 4-(ethylnitrosamino)-1-(3-pyridyl)-1-butanone (ethyl-NNK) were synthesized according to published methods (*26*,*27*). The purity of the synthesized compounds, verified by capillary gas chromatography and high-performance liquid chromatography analyses, was greater than 99%. Carbon dioxide, supercritical-fluid-extraction grade, was obtained from Cryodyne Technologies Inc., Chester, CT.

Specimen Analysis

A previously developed extraction method for the assessment of tobacco-specific nitrosamines in tobacco was modified for the extraction of nitrosamines from cervical mucus specimens (*27*). As part of a preliminary study, six mucus specimens were analyzed by capillary gas chromatography interfaced with a thermal energy analyzer; for later analyses of cervical mucus of 15 smokers and 10 nonsmokers, the capillary gas chromatography with mass spectrometry detection was developed. In general, cervical mucus specimens (ranging in weight from 31 to 615 mg) were extracted with supercritical carbon dioxide, to which 10% methanol had been added. Specimens were placed inside a filter paper that had been exhaustively prewashed with methanol and *n*-hexane to remove any contaminants, and then 100 μL of a 0.0001% solution of CD_3-NNK (internal standard) was added. The filter paper was then rolled up to fit the extraction vessel. Extractions were performed at 60 °C and 350 atmospheres in a Suprex Prepmaster stand-alone SFE system equipped with a Suprex Model MPA-1 solvent modifier pump and a DuraFlow restrictor (Suprex Corp., Pittsburgh, PA). Each specimen was extracted once for 90 minutes. This period consisted of a 15-minute extraction in a static mode, followed by 30 minutes of dynamic extraction, then another 15-minute static extraction, and finally 30 minutes of dynamic extraction with the flow rate of supercritical carbon dioxide of 1.5 mL/minute. Analytes were collected in a vial containing 2 mL of *n*-hexane. Upon completion of the extraction, most of the organic solvent was removed by a stream of nitrogen to yield an extract of about 50 μL; an equal volume of a 0.0002% solution of ethyl-NNK was added as a chromatographic standard. When applied to tobacco products and human saliva specimens, this extraction method was found to be highly reproducible (*26*).

The tobacco-specific nitrosamines were separated and quantified on a Model 3400 gas chromatograph (Varian Instruments, San Fernando, CA) equipped with a 200S autosampler and interfaced with a Finnigan Mat TSQ 700 mass spectrometer (Finnigan Corp., San Jose, CA). Gas chromatography analyses were performed on a DB-5 fused silica capillary column (30 m × 0.32 mm, 0.25-μm film thickness; J & W Scientific, Folsom, CA) by use of GC-SRM-MS/MS. Since on-column injection was used, the DB-5 capillary column was connected to a 90-cm × 0.53-mm deactivated precolumn. The column head pressure was kept at 5 pounds per square inch throughout the analysis. The temperature program was as follows: The initial temperature of 40 °C was kept for 5 minutes, then increased at 30 °C per minute to 140 °C, maintained at this level for 10 minutes, and increased at 1 °C per minute to 145 °C. This temperature was kept for 5 minutes and finally raised at 20 °C per minute to 180 °C, where it was

held for an additional 10 minutes. Manual injections of 2-μL aliquots were done. The mass spectral conditions were as follows: The source temperature was maintained at 70 °C; the instrument was set to monitor the reaction of ion 177.0 (the most abundant ion of NNK fragmentation, resulting from the loss of a nitroso group from the molecular ion 207) to the ion 146 (the daughter ion of 177.0, Fig. 1) with a window of 3 atomic mass units. A time scan of 1 second and a scan rate of 5 atomic mass units per second were used. The collision cell pressure was maintained at 1.2 mtorr of argon, and the collision energy was maintained at −8.0 electron volts to maintain the fragment's transitional energy. NNK was quantified by use of a standard curve of the NNK-to-CD_3-NNK ratio versus the concentration of NNK; the performance of the mass spectrometer was monitored with a chromatographic standard, ethyl-NNK.

Statistical Analysis

Student's *t* test was used to compare the difference in amount of collected cervical mucus specimens and NNK concentration between cigarette smokers and nonsmokers. All reported *P* values were two-tailed. Results were expressed as mean values ± standard deviation.

Results

The initial six specimens had been subjected to capillary gas chromatography with thermal energy analyzer detection. For these analyses, we used thermal desorption, a technique previously developed for sample introduction in gas chromatographic analysis (*28*). Fifty percent of the supercritical fluid extraction concentrate obtained after evaporating the organic solvent was applied to the thermal desorption glass cartridge filled with Tenax GR. Tenax is an adsorbent consist-

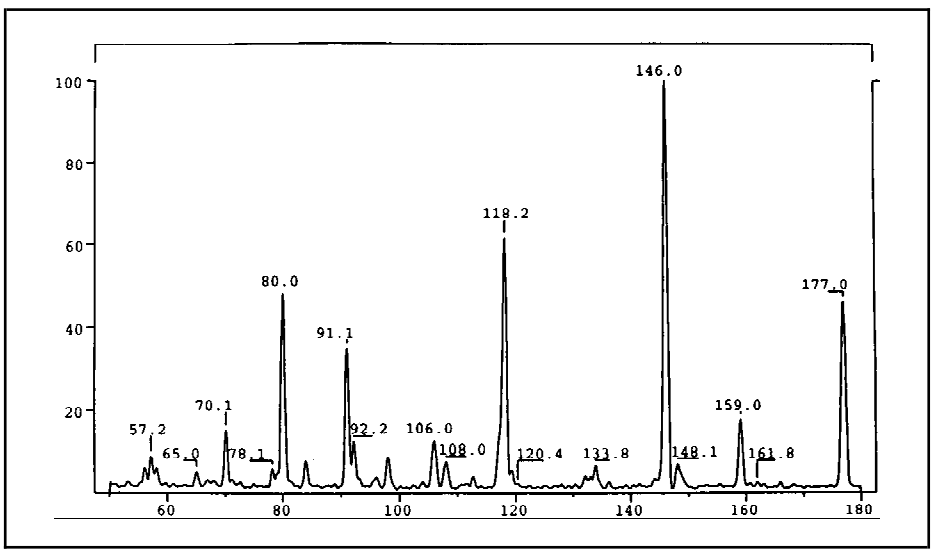

Fig. 1. Full daughter ion spectrum of the parent ion 177.0 of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. *x* axis = daughter ions detected; *y* axis = relative ion intensity.

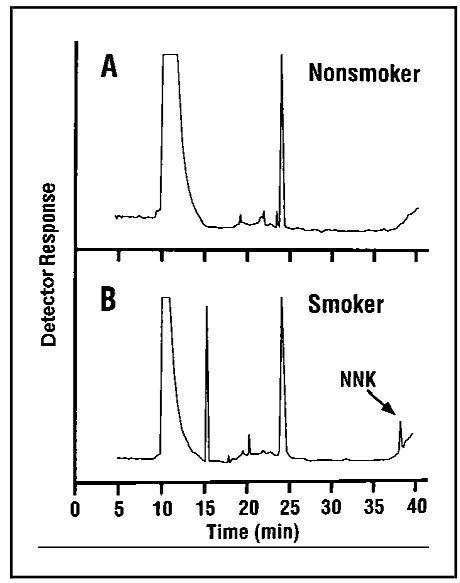

Fig. 2. Capillary gas chromatography-thermal energy analysis of *N*-nitrosamines from an extract of cervical mucus. NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

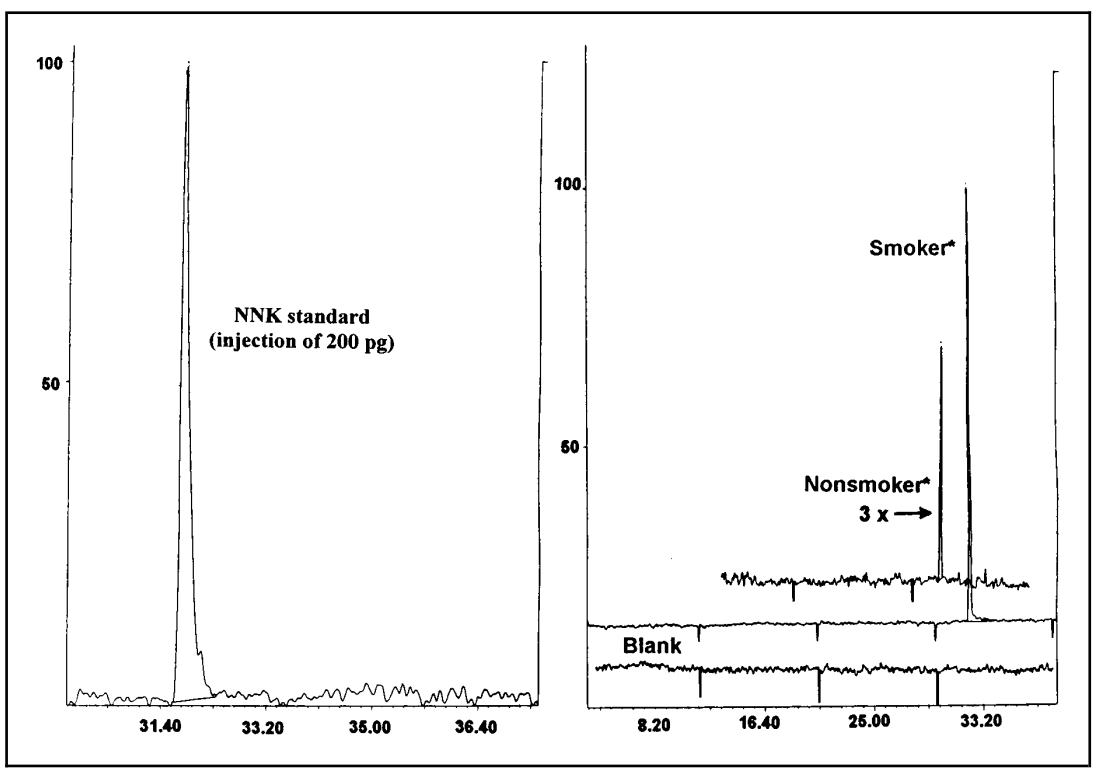

Fig. 3. Capillary gas chromatography–selected reaction monitoring–tandem mass spectrometry analysis of human cervical mucus for 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). The peaks observed result from monitoring the major daughter ion of 146. *Self-reported smoking status. NNK peak in nonsmoker mucus has an identical retention time with that in smoker mucus but has been shifted for illustration clarity; its intensity has been increased three times [3×]. *x* axis = time (minutes); *y* axis = relative ion intensity.

ing of a low-bleed polymer mixed with 23% of graphite (Tenax GR80/100 mesh). It was purchased from Alltech, Deerfield, IL. After the specimen was introduced, the cartridge was placed in the thermal desorption unit and heated to 220 °C over a 12-minute period. During that time, the temperature of the thermal desorption unit was maintained at 0 °C, while the flow rate of helium was kept at 10 mL/minute. Afterward, the gas flow was changed to 1 mL/minute, and we transferred the sample to the first part of the capillary column by rapidly heating that part of the thermal desorption unit where the analytes were initially concentrated (15 °C/second) to 220 °C, while maintaining the temperature of the oven of the gas chromatograph at 40 °C.

Two cervical mucus specimens analyzed for tobacco-specific nitrosamines by this method demonstrated a well-defined, thermal energy analyzer-positive peak eluting at the retention time of NNK (Fig. 2). These specimens had been collected from cigarette smokers. The levels of NNK in these specimens were 65.0 and 87.0 ng/g of mucus, respectively. Two other specimens, one from a smoker and one from a nonsmoker, indicated the presence of NNK at a very low level, which could not be quantified by this method.

Capillary gas chromatography combined with thermal energy analyzer detection has been regarded as the most sensitive and selective method for nitrosamine analysis (*25*). However, it does not provide spectral characterization of the analytes. Therefore, to confirm that the peak detected by the thermal energy analyzer was indeed NNK, we analyzed the remaining 26 samples by GC-SRM-MS/MS. This technique proved to be at least 60 times more sensitive (detection limit of 5 pg per injection, Fig. 3) than gas chromatography combined with thermal energy analysis. Since this method proved to be very sensitive, it was important to perform a blank extraction after each extraction of cervical mucus to avoid sample cross-contamination. For this, the entire analytic procedure was repeated as a sham procedure. Carry-over effects were not observed (Fig. 3). NNK was detected in all samples from smokers (11.9-115.0 ng of NNK per gram of mucus; Table 1) and in nine of 10 samples from nonsmokers (4.1-30.8 ng of NNK per gram of mucus; Table 1). The concentration of NNK in cervical mucus specimens from cigarette smokers was significantly higher than that in specimens from nonsmokers (mean ± standard deviation: 46.9 ± 32.5 ng of NNK per gram of mucus versus 13.0 ± 9.3 ng of NNK per gram of mucus; $P = .004$, Student's t test; Table 1). Two specimens collected from the same smoker (5a and 5b) on two occasions 4 months apart showed relatively close levels (25.9 ng and 39.4 ng of NNK per gram of mucus; Table 1).

NNK was not detected in the four wet blank control specimens carried through the entire procedure and analyzed by GC-SRM-MS/MS.

The amount of mucus extracted for analysis did not differ significantly between smokers and nonsmokers (mean ± standard deviation: 167.2 ± 127.8 mg [smokers] versus 218.2 ± 146.2 mg [nonsmokers]; $P = .36$; Table 1).

Table 1. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in cervical mucus of cigarette smokers and nonsmokers*

Patient No.	Age, y	No. of cigarettes smoked/day	Amount of mucus extracted, mg	NNK, ng/g cervical mucus
		Cigarette smokers		
1	34	5	48.8	30.8
2	31	5	496.0	19.7
3	28	10	237.0	84.9
4	30	3-5	31.0	115.0
5[a]†	20	7-8	155.0	25.9
5[b]†	20	7-8	85.0	39.4
6	n.a.	8-10	121.0	48.8
7	35	20	43.2	64.2
8	18	1-2	356.0	17.9
9	43	20	265.0	43.0
10	32	10	161.0	42.7
11	41	10	56.0	58.0
12	28	10	163.0	11.9
13	24	10	203.0	14.9
14	n.a.	n.a.	207.0	22.7
15	18	n.a.	47.3	111.0
Mean ± standard deviation		9.25 ± 5.29	167.2 ± 127.8‡	46.9 ± 32.5§
		Nonsmokers		
1	49	—	234.0	30.8
2	30	—	194.0	19.1
3	26	—	218.0	6.7
4	32	—	195.0	6.0
5	43	—	165.0	10.6
6	42	—	107.0	4.1
7	40	—	144.0	18.3
8	25	—	106.0	n.d.
9	21	—	204.0	9.7
10	51	—	615.0	22.7
Mean ± standard deviation			218.2 ± 146.2‡	13.0 ± 9.3§

*All samples were analyzed by the capillary gas chromatography–selected reaction monitoring–tandem mass spectroscopy method. For statistical calculations, the not-detected (n.d.) value was assigned as 2 ng of NNK per gram of mucus (50% of the lowest detected amount). n.a. = not available.

†Same smoker; samples collected at different times.

‡Mucus levels subjected to supercritical fluid extraction did not differ significantly between smokers and nonsmokers (two-sided $P = .36$).

§NNK levels in mucus specimens from smokers differ significantly from those from nonsmokers (two-sided $P = .004$).

Discussion

Our findings provide the first unequivocal evidence of the presence of a tobacco-specific carcinogen in the cervical mucus of women who smoke cigarettes. The lower levels of NNK identified in cervical mucus specimens from nonsmokers may relate to exposure to environmental tobacco smoke, which also has been reported to be a risk factor for cervical cancer (*18*). Since NNK was not detected in wet blank control specimens and blank analyses, it is unlikely that sample contamination during collection or sample carry-over throughout the assay occurred. Significant differences in the amount of mucus collected between the two groups were not observed and thus cannot account for the variation between smokers and nonsmokers in levels of NNK in specimens of cervical mucus.

One cannot be certain whether one or another of the ''nonsmokers'' with a relatively high level of NNK in the cervical mucus (>20 ng/g) was not actually a cigarette smoker. Validation of smoking behavior with urine or serum cotinine assays was not performed in this study, although, in general, there is good agreement between reported daily cigarette consumption and concentration of cotinine in serum (*29*). Second-hand smoke exposure per se was not evaluated in this pilot study, although it may be relevant, inasmuch as detectable levels of cotinine can be identified in the cervical mucus of women exposed to environmental tobacco smoke (*22*).

NNK is metabolized in vivo mainly by α-hydroxylation. In rodents, α-hydroxylation of NNK contributes at least 50% to its total metabolites (*30*). This key metabolic process leads to formation of electrophiles that readily react with various macromolecules. Cytochrome P450 isozymes, including CYP1A1, CYP1A2, CYP2A3, CYP2A6, CYP2B1, CYP2B2, CYP2B4, CYP2D6, and CYP2E1, have been shown to catalyze the metabolic activation of NNK in rodent and human lung and liver tissues (*31*). Since CYP1A1, CYP1A2, CYP2D6, and CYP2E1 have been expressed in human cervical cells (*32*,*33*), it is quite possible that human cervical epithelium is also capable of metabolically activating NNK. Thus, it is feasible that NNK, detected in cervical mucus, can be metabolically activated by enzymes in the cervical epithelium and can cause damage to cervical DNA. Indeed, smoking-related DNA damage has been demonstrated by several studies (*34-38*) through ^{32}P-postlabeling techniques; however, structures of these putative adducts remain unknown.

NNK induces tumors in the lung, liver, and nasal mucosa of rodents independent of route of administration (i.e., oral swabbing or intraperitoneal, subcutaneous, or intravesical injection) (*39-43*). When administered in drinking water, NNK also induces tumors of the exocrine pancreas in rats (*40*). Animal models to study the induction of cervical carcinoma by NNK or other tobacco-specific nitrosamines have not yet been developed and suggest an important area for future research.

In summary, our findings support existing epidemiologic observations regarding the increased risk for cervical cancer among smokers and offer biologic plausibility in support of the association between cigarette smoking and this disease.

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Notes

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