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Biochemical Epidemiology of Cervical Neoplasia: Measuring Cigarette Smoke Constituents in the Cervix[1]

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ABSTRACT

In preparation for an epidemiological investigation of cigarette smoking and cervical neoplasia, we studied methods of measuring cervical exposure to tobacco smoke. The measurement of cotinine in cervical flushes by radioimmunoassay proved to be highly accurate in distinguishing smokers from nonsmokers, achieving 100% sensitivity and 97% specificity. In most subjects, quantitative levels of cervical cotinine and nicotine mirrored recent smoking intensity. Some of the apparent exceptions may have resulted from metabolic/secretory traits of the subjects. If so, the biochemical measurement of smoke constituents in the cervix might prove more valuable for epidemiological studies of cervical neoplasia than data on current smoking behavior collected by interview.

INTRODUCTION

Cigarette smoking has been associated with an increased risk of cervical neoplasia in numerous epidemiological investigations (1–4). The association appears to be independent of other well-known demographic and behavioral risk factors for cervical cancer, such as sexual history, Pap smear screening frequency, socioeconomic status, and contraceptive practices (2, 4). However, the recent emergence of HPV[2] infection as a possibly direct cause of cervical neoplasia demands a reconsideration of the presumed independent role of cigarette smoking (5). Smoking might act only as a cofactor, increasing the oncogenic potential of genital HPV infections, or it could be a correlate of infection with no causal role. Consequently, we are planning an epidemiological study of smoking, HPV infection, and cervical neoplasia. To supplement the self-reported smoking data, we wish to obtain a biochemical measurement of cigarette smoke constituents directly in contact with the cervix.

Accordingly, we have field-tested potential methods of conducting epidemiological surveys of cervical exposure to tobacco smoke. The first method that we evaluated was the measurement of mutagenic activity in cervical flushes, using the *Salmonella*/mammalian microsomal assay (6). This approach proved unsuccessful due to frequent, mixed-bacterial contamination of the cervical flushes, a phenomenon which may itself be linked to cigarette smoking (7).

In the present investigation, we evaluated an alternative approach suggested by Sasson *et al.*, who detected increased levels of the smoke constituents, nicotine and cotinine, in the cervical mucus of smokers collected by direct aspiration from the endocervix and assayed by RIA (8). In ten smokers they found mean nicotine and cotinine levels of 740 ng/ml and 316 ng/ml, respectively, compared to mean values of 16 ng/ml nicotine and 3 ng/ml cotinine in eight nonsmokers. Since endocervical aspiration is not practical for large-scale epidemiological studies, we restricted our investigation of collection methods to cervical swabs and saline flushes, reasoning that an ideal method of sample collection should be fast, simple, and already familiar to clinicians. We report here that the measurement of smoke constituents in cervical flushes is a practical and informative technique that merits incorporation into future epidemiological research.

PATIENTS AND METHODS

All participants in the study were women scheduled for Pap smears in the Obstetrics and Gynecology Clinics of Georgetown University Hospital. Prior informed consent was obtained from each subject, and a brief, standardized questionnaire concerning smoking practices was administered. A routine Pap smear consisting of an endocervical swab and exocervical scraping was obtained from all women. Samples for this study were then collected by an additional swab or flush. The overall participation rate exceeded 90% for both sample collecting and interviewing.

During the interview, women were questioned regarding their current smoking status (yes/no). Smokers were asked how many cigarettes they usually smoked per day and how many cigarettes they had smoked in the past 24 h. No women reported current pipe, cigar, snuff, or chewing tobacco use. Subjects were also asked about ETS exposure within the past 24 h, both in the home and outside the home. ETS exposure (passive smoking) in the home was categorized by number of active smokers and usual smoking intensity of those individuals. Exposure outside the home was ascertained using a single yes/no question. The ETS exposure histories were then summarized in a crude, trichotomous score: none; light; or heavy.

We obtained cervical swabs from 41 unselected women, including 16 smokers and 25 nonsmokers. The women ranged in age from 18 to 79 yr. The racial composition was as follows: 22% white; 72% black; 6% Hispanic. Four women were found to have mild dysplasia.

In a separate group of 12 smokers and 29 nonsmokers, we flushed the cervix with 2 ml of sterile normal saline, recollected the fluid in the vaginal cul-de-sac, and flushed the cervix again. The women in this group ranged in age from 16 to 76 yr. Sixty-six % were black, 22% were white, and 12% were Hispanic. Four women had mild dysplasia on Pap smear.

The swabs and flushes were stored at −20°C or colder until testing. Since the amount of sample collected on the cotton tip of the swab was very small and sample was in some cases adherent to the wooden stick, the entire swab was extracted. One ml of a Tris isogel buffer (pH 7.48 containing 1.21 g TRIZMA base, 8.26 g NaCl, 3.72 g EDTA, and 1 g gelatin powder in 1 liter distilled H_2O) was added to each swab in its polyethylene tube. The tubes were vortexed for approximately 30 s and then sonicated at room temperature in an ultrasonic bath for 25 min. Liquid was repeatedly squeezed from the cotton before the swab was discarded.

For biochemical analyses of the flushes, samples were defrosted and vortexed for 30 s, then allowed to sit at room temperature for about 1 h, and vortexed again before duplicate aliquots were removed for analysis. Flushes were assayed directly and not extracted.

Aliquots of the flushes and swab extracts were analyzed for nicotine and cotinine by RIA according to the procedure described by Haley *et*

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[2] The abbreviations used are: HPV, human papillomavirus; RIA, radioimmunoassay; ETS, environmental tobacco smoke.

al., using antibodies developed according to the method of Langone *et al.* (9, 10). The limits of detection for these quantitative assays are below 1 ng/ml of biological fluid, and coefficients of variation in the laboratory are maintained at 6 to 7% for both nicotine and cotinine measurements.

To compare the number of samples positive in smokers to the number of samples positive to nonsmokers, we used Fisher's exact test (11). To correlate smoking characteristics with cotinine or nicotine measurements, we chose the nonparametric Spearman's rank correlation test (11).

RESULTS

In Table 1, subjects are shown classified by current smoking status and by presence of nicotine or cotinine in their samples. When the cervical samples were collected by swab, nicotine and cotinine were each detected in 14 (88%) of 16 self-reported smokers. Interestingly, the two smokers found not to have nicotine in their swabs were different from the two found not to have cotinine. In regard to specificity, nicotine was found in 5 of the 25 self-reported nonsmokers, compared to only 2 (of the same 5) for the cotinine assay.

The flushes provided more cervical material and increased sensitivity compared to the swabs. The cotinine assay of flushes was almost perfectly accurate. All current smokers were correctly classified, and only one nonsmoker, who reported ETS exposure that day, was judged positive. The nicotine assay also detected all smokers, but was only 28% specific (21 of 29 nonsmokers had some nicotine present in their samples). This nonspecificity could not be explained by reported ETS exposure which was similar in the nonsmokers with or without detectable nicotine levels (data not shown).

With regard to the quantified levels of nicotine or cotinine, flushes provided higher values than the swabs. The mean levels of cervical nicotine in the 2-ml flushes from smokers were 125 ng/ml and 22 ng/ml, compared to 18 ng/ml and 4 ng/ml in the 1-ml swab extracts. This difference does not result from group differences in smoking intensity, since the usual numbers of cigarettes smoked per day were similar in smokers from the flush and swab groups (means of 11 *versus* 14 cigarettes per day).

The concentrations of nicotine and cotinine found in the cervical flushes seemed to reflect recent smoking behavior. In Table 2, the nicotine and cotinine levels from the 12 smokers in the flush group are shown, along with selected smoking characteristics. The nicotine and cotinine measurements themselves were significantly correlated (Spearman's rank correlation coefficient = 0.60, $P = 0.04$), as were the two measures of smoking intensity, "number of cigarettes smoked in the past 24 h" and "usual number of cigarettes smoked per day" (Spearman's $r = 0.69$, $P = 0.02$). Of the two intensity measures, smoking in the past 24 h was more closely associated with both nicotine levels ($r = 0.58$, $P = 0.05$) and cotinine levels ($r = 0.57$, $P = 0.06$) than was usual number of cigarettes ($r = 0.35$ for nicotine and $r = 0.38$ for cotinine, both nonsignificant). Heavy ETS exposure may help to explain the relatively high test values of Subjects 7 and 12. Interestingly, Subject 10, who had an exceptionally high nicotine level, is diabetic.

Table 1 *Nicotine and cotinine in cervical samples from 82 current smokers and nonsmokers*

	Smokers	Nonsmokers	*P* (smokers *vs.* nonsmokers)[a]
Swabs ($n = 41$ women)	16	25	
Nicotine detected	14 (88)[b]	5 (20)	<0.001
Cotinine detected	14 (88)	2 (8)	<0.001
Flushes ($n = 41$ women)	12	29	
Nicotine detected	12 (100)	21 (72)	0.08
Cotinine detected	12 (100)	1 (3)	<0.001

[a] *P* values by Fisher's exact test, 2-tailed.
[b] Numbers in parentheses, percentage.

Table 2 *Nicotine and cotinine in cervical flushes related to smoking intensity for twelve smokers*

Subject	Nicotine (ng/ml)	Cotinine (ng/ml)	Usual smoking intensity (no. of cigarettes/ day)	No. of cigarettes in past 24 h	ETS exposure in past 24 h
1	108	12	10	10	Light
2	42	5	10	7	None
3	298	29	18	18	None
4	161	59	20	15	Light
5	12	10	5	5	Light
6	71	7	10	10	Light
7	81	63	8	9	Heavy
8	28	2	4	4	None
9	83	8	0	10	None
10	512	58	20	10	Light
11	15	6	20	10	Heavy
12	85	6	3	2	Heavy

DISCUSSION

In the epidemiological study of cigarette smoking and cervical neoplasia, an ideal biochemical assay would: (*a*) be practical for large-scale population studies; (*b*) classify as positive all true smokers, including heavy passive smokers as well as those women who incorrectly claim to be nonsmokers upon interview (high sensitivity); (*c*) classify as negative all true nonsmokers (high specificity); (*d*) permit the quantification of smoke constituents reaching the cervix, allowing for smoking practices as well as for possible metabolic and secretory variability among women of similar smoking behavior; (*e*) correlate with cervical levels of carcinogens found in cigarette smoke, such as polycyclic aromatic hydrocarbons, aromatic amines, or nicotine-specific nitrosamines; and (*f*) provide a historical view of cumulative smoking exposure over time, relevant to the years over which cervical pathology develops.

For our investigations of smoking, HPV infection, and cervical neoplasia, we hoped initially to use the method described by Holly *et al.*, measuring mutagenicity in cervical flushes by the *Salmonella*/mammalian microsomal assay. However, the unexpected finding of increased bacterial contamination of cervical flushes from smokers greatly complicated the assessment of mutagenicity, limiting the current value of this approach. The contamination, which included *Staphylococcus, Streptococcus*, diphtheroid, and micrococcus species, as well as non-*Salmonella* Gram-negative rods, could be reduced by overnight incubation in 50% dimethyl sulfoxide but, as reported earlier, we still found no difference between smokers and nonsmokers in the number of mutagenic samples obtained (7).

Similarly, mutagenicity testing of cervical scrapes cannot be recommended for large-scale studies. In an unpublished series of 34 smokers, we found that less than 10% of the women gave positive results when scrapes were tested, using either an extract of the cell pellet or the supernatant. Thus, the sensitivity is unacceptably low, and bacterial contamination remains a potential problem.

In contrast, the assay for nicotine and cotinine that we report here appears to be quite promising. Sasson *et al.* aspirated mucus from the endocervix in women during the follicular

phase of their menstrual cycles. However, endocervical aspiration is impractical for epidemiological studies of large populations, since the technique is cumbersome, and the population samples include many women for whom mucus is less copious (luteal phase).

Using a swab, we found that the cotinine or nicotine assays were fairly accurate in distinguishing smokers from nonsmokers. Either method detected 88% of self-reported smokers, but the amounts of cervical material provided by a swab were too little to permit reliable quantification of nicotine or cotinine levels. Improved swabs or a better elution method could be devised, but the flush worked well enough to consider it the method of choice.

By collecting a cervical flush and testing it for cotinine, we achieved 100% sensitivity and 97% specificity in distinguishing smokers and nonsmokers. Therefore, if the smoking histories are deemed accurate, then the predictive value of the cotinine assay (the percentage of subjects judged positive by the assay who are true smokers) was excellent. The predictive value of the nicotine assay was poor due to nonspecificity. In regard to the one nonsmoker found to have cotinine in her flush, incorrect reporting of smoking status is a possibility, since we did not validate smoking histories. Alternatively, ETS exposure could possibly explain the results. We are currently evaluating in a larger series the predictive value of the cervical cotinine assay for exposure to environmental tobacco smoke.

In addition to the validation of smoking histories, the cotinine and nicotine assays also permitted the quantification of smoke constituents found in contact with the cervix. The cervical levels of nicotine and cotinine seemed in our investigation to relate to current smoking intensity. The apparent exceptions (such as the diabetic subject's very high level) may have resulted from chance laboratory variability, from error in subject recall, or from differences in the quantities of mucus relative to saline recovered by the flushes. Perhaps, however, ETS exposure or metabolic/secretory traits of the subjects could also play a role in establishing the measured cervical level. If so, the measurement of smoke constituents in the cervix might prove more valuable for epidemiological studies of cervical neoplasia than data on current smoking behavior collected by interview, or cotinine levels in blood or urine.

Since the cotinine and nicotine assay results appear to be correlated, the advantages of each assay should be considered. Cotinine is a nicotine metabolite not found in tobacco smoke itself and, as such, it is quite specific for inhaled as opposed to ambient smoke. This is important since ambient smoke in the clinic could, theoretically, affect the nicotine assay and produce some of the nonspecificity seen in the nicotine results.

Nicotine measurements offer one important advantage, related to the correlation of the assay with actual carcinogens. Although cotinine is considered a harmless metabolite, nicotine is known to also give rise to nicotine-specific nitrosamines which could directly contribute to the development of cervical neoplasia (12). Nitrosamines are, at present, difficult to detect in cervical mucus. The measurement of nicotine may suggest which women are potential "high secretors" of nicotine-specific nitrosamines who could be further studied.

One intrinsic limitation of both the cotinine and nicotine assays is their time dimension. As is the case for cervical HPV testing by DNA-DNA hybridization, the smoking assays represent a current measurement of a possibly carcinogenic exposure, not a cumulative or "usual" measurement. We cannot yet biochemically assess cumulative dose of smoke to the cervix. The smoking assays described here will undoubtedly misclassify the lifetime exposures of some people who have recently changed their smoking behaviors. However, this temporal bias may be advantageous if current smoking practices influence current cervical cytology, which can sometimes show progression or regression over relatively short periods of time.

In summary, we believe that the measurement of tobacco smoke constituents in cervical flushes will provide a useful tool in epidemiological studies of smoking and cervical neoplasia. A recent report indicates that a flush can be used for HPV testing, as well (13). As a result, it appears that a single cervical flush can be used to accurately examine both of the leading putative risk factors for cervical cancer. Thus, large-scale epidemiological studies can be undertaken to determine if some women, because of type-specific HPV infection and high levels of cervical smoke constituents, are at especially elevated risk of incident or progressive cervical neoplasia.

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