Metabolic Activation and Carcinogen-DNA Adduct Detection in Human Larynx

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ABSTRACT

Putative carcinogen-DNA adducts in human larynx tissues (n = 25) from smoker and non/ex-smoker patients were examined by 32P-postlabeling and compared with the metabolic activation capacity of larynx microsomes and cytosols from the same tissues. Hydrophobic DNA adducts were evident only in smokers, and chromatographic profiles of the adducts were similar using either the butanol extraction or nuclelease P1 enhancement method, which suggested that the adducts may be derived from polycyclic aromatic hydrocarbons but not aromatic amines. Immunoblots of larynx microsomes using anti-cytochrome P450 450 1A1/1A2, 2C, 3A4, 2E1, and 2A6 antibodies showed intensities ranging from 1–10% of that typically observed with human liver microsomes. Enzymatic assays of larynx microsomes showed appreciable activity for benzo(a)pyrene hydroxylase (P450 1A1 and 2C) but not for 4-aminobiphenyl N-oxidation (P450 1A2), which indicated that the observed immunoreactivity was for P450 1A1; this represents the highest level of this P450 yet detected in human extrahapatic tissues. Accordingly, total DNA adduct levels in the larynx correlated strongly with levels of P450 2C, 1A1, and 3A4 but not with P450 2E1 or 2A6.

Larynx cytosols also showed appreciable aromatic amine N-acetyltransferase activity for p-aminobenzoic acid (NAT-1) but not for sulfamethazine (NAT-2); however, NAT-1 activity was not correlated with total DNA adducts, which is again consistent with the lack of aromatic amine-DNA adducts detected by 32P-postlabeling. Thus, these results suggest that the DNA adducts detected in human larynx are largely derived from metabolic activation of polycyclic aromatic hydrocarbons in cigarette smoke by P450 2C, 3A4, and/or 1A1.

INTRODUCTION

Chemical carcinogenesis in the laryngeal mucosa of humans is not well understood. Even though epidemiological studies have strongly implicated cigarette smoking as a causative factor for squamous cell carcinomas of the larynx (1), information on carcinogen metabolism, on the identification of carcinogen-DNA adducts, and on concomitant genotypic and phenotypic changes in the pathogenesis of laryngeal cancer is very limited (2).

PAHs4 initially were implicated in the etiology of laryngeal cancer from studies that showed a strong correlation between aryl hydrocarbon hydroxylase inducibility in lymphocytes and individual susceptibility to laryngeal cancer (3–5). However, recent studies have been performed to reexpose the larynx mucosa; and alcohol consumption is known to increase substantially the relative risk of smokers to laryngeal cancer (13). Conceivably, the surface of the larynx may only be susceptible to carcinogens in cigarette smoke, with alcohol serving as a cocarcinogenic stimulus; thus, studies on DNA adduct detection and on the metabolic activation capacity of the laryngeal mucosa of cigarette smokers may provide a useful model for carcinogenesis in the upper aerodigestive tract.

Cigarette smoke is a complex mixture of compounds including carcinogenic PAHs, nitrosamines, and aromatic amines, all of which are known to form DNA adducts after undergoing metabolic activation. Given the role of such DNA modification in the initiation of carcinogenesis, we sought to examine human tissues for the presence of carcinogen-DNA adducts in order to determine possible correlations with specific metabolic activation pathways leading to cancer induction. In this study, we investigated smoking-related DNA adduct levels in the larynx tissues of individuals with laryngeal cancer using the 32P-postlabeling method with either butanol extraction or nuclelease P1 enhancement. We further examined the correlation between the DNA adduct levels and the levels of carcinogen-activating enzymes such as cytochromes P450 and acetyltransferases.

MATERIALS AND METHODS

Materials. [3H]BP (350–550 mCi/mmol) was obtained from Chem-Syn Science Laboratories (Lenexa, KS), re-purified just prior to use, and then judged to be >99% pure by high performance liquid chromatography (14). Other reagents used in this study were from the same sources as reported earlier (15–18). Human larynx tissues were obtained surgically from 33 (numbered consecutively) laryngeal cancer patients, which included 29 current smokers and 4 non/ex-smokers (>1 year smoking cessation). After informed consent was obtained, normal laryngeal mucosa of approximately 0.5–2 cm2 was excised from a nonadjacent site, placed in saline, and then on ice; and the tissue was stored at —80°C until ready for analysis. For the postlabeling analyses, samples from 21 smokers and the 4 non/ex-smokers were used; of these, immunoblotting analyses and aromatic amine metabolism studies were carried out only on larynx microsomes from nine smokers and two non/ex-smokers, due to the limited amount of tissue available. The additional eight samples obtained were used only for the BP metabolism studies.

32P-Postlabeling Methods. DNA was isolated from thawed specimens by a solvent extraction method (15), which yielded 200–500 µg of DNA/g tissue. 32P-Postlabeling analyses were performed according to the methods described previously (16, 17). In brief, the DNA samples (7.5 µg) were hydrolyzed to 3′-nucleotides with 0.25 units of micrococcal endonuclease and 2.5 µg of spleen phosphodiesterase by incubation at 37°C for 3 h. Adducted nucleotides were enriched either by a butanol extraction (19) or by a 40-min digestion at 37°C with 6 µg of nuclease P1 (20). Samples were then 5′-32P-Phosphorylated at 37°C for 40 min using 2 units of polynucleotide kinase and 300 mCi of

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4 The abbreviations used are: PAHs, polycyclic aromatic hydrocarbons; BP, benzo(a)pyrene; ADP, 4-aminobiphenyl; NAT-1, monomorph acetyltransferase; NAT-2, polymorphic acetyltransferase; PABA, p-aminobenzoic acid; SMZ, sulfamethazine.

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within a radioactive zone, the adduct spots were excised and quantitated separately. However, because of sample-to-sample variation, adduct levels were summed for correlational analyses.

**Metabolism Studies and Immunoblotting Analyses.** Larynx tissues were homogenized with 3 volumes (v/w) of 20 mM Tris-HCl buffer (pH 7.8) containing 0.25 mM sucrose, 0.5 mM EDTA, 0.1 mM dithiothreitol, and 20 μM butylated hydroxytoluene. Microsomal and cytosolic fractions were prepared by differential centrifugation as described previously (21). Immunoreactive levels of P450 enzymes were determined on microsomal preparations by means of immunoblotting with anti-P450 1A1/1A2, 2A6, 2C, 2E1, and 3A4 antibodies; and the levels of each P450 isozyme were estimated by densitometry, as described previously (22, 23). Microsomal BP hydroxylation (14, 24) and ABP N-oxidation (25) were measured by published methods.

N-Acetyltransferase assays for the monomorphic (NAT-1) and polymorphic (NAT-2) enzymes were carried out using larynx cytosol preparations. PABA and SMZ were used as selective substrates for NAT-1 and NAT-2, respectively, and were assayed as described previously (26).

**RESULTS**

Putative carcinogen-DNA adduct levels were examined by 32P-postlabeling of larynx mucosa obtained from smoker (n = 21) and non/ex-smoker (n = 4) patients with laryngeal cancers. Representative profiles of DNA adducts in smoker and non-smoker samples are shown in Fig. 1. DNA adducts visualized by 32P-postlabeling with either butanol extraction or nuclease P1 enhancement consistently showed discrete radioactive spots and diagonal zones in all smoker samples but not in non/ex-smokers. DNA adduct profiles obtained by butanol extraction and nuclease P1 enhancement methods were very similar with the exception of two adducts (spots designated 1 and 2), which were observed only by the butanol extraction method. In each sample, total DNA adduct levels obtained by the butanol extraction method were usually higher than those obtained by the nuclease P1 enhancement method (Fig. 2).

Since cytochromes P450 are known to catalyze the metabolic activation of carcinogens, we initially examined the P450 enzyme patterns by means of immunoblot analysis in larynx microsomes (Fig. 3)
and then determined the correlation between specific P450 immunoreactive intensity and total DNA adduct levels in smoker samples. Immunoblots of the microsomes from smoker and non/ex-smoker larynx tissues using anti-P450 1A1/1A2, 2C, 3A4, 2E1, and 2A6 antibodies demonstrated levels ranging from 1–10% that of human liver (expressed per mg protein) for each P450 enzyme examined. Subsequent metabolism studies showed that larynx microsomes contained appreciable BP hydroxylase (24) activity (30 ± 28 pmol/min/mg protein; n = 8; range, 4–64 pmol/30 min/mg protein), which is known to be catalyzed by cytochromes P450 1A1 and 2C (27, 28). Moreover, high performance liquid chromatographic analysis (14) showed 9-hydroxy-BP and 3-hydroxy-BP as major metabolites (data not shown). In contrast, no cytochrome P450 1A2-dependent ABP N-oxidation activity (<1 pmol/min/mg protein; <0.2% of mean liver activity) could be detected. Thus, the component detected by immunoblotting using an anti-P450 1A1/1A2 antibody appears to be predominantly cytochrome P450 1A1. This level of P450 1A1 was about 10% that of the liver P450 1A2 intensity and was about 10 times higher that that observed previously with human lung microsomes (29).

Significant correlations were observed between total DNA adduct level and the immunoreactive amounts of P450 1A, 2C, and 3A4 in the smoker samples (Fig. 2). In contrast, no correlation between total DNA adduct level and other P450 enzymes examined, P450 2E1 and P450 2A6, was found.

NAT-1 and NAT-2, which catalyze the N-acetylation of aromatic amines and the O-acetylation of their N-hydroxy metabolites (30), were assayed in larynx cytosols from the same individuals using PABA and SMZ, respectively, as selective substrates. As summarized in Table 1, cytosols from all larynx samples showed appreciable activity for PABA (NAT-1) but not for SMZ (NAT-2). However, NAT-1 activity did not correlate with total DNA adduct levels (Fig. 4).

**Table 1. Acetylttransferase activity of human larynx cytosol**

<table>
<thead>
<tr>
<th>Samples (n)</th>
<th>PABA (NAT-1)</th>
<th>SMZ (NAT-2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smokers (13)</td>
<td>3.09 ± 1.39</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Nonsmokers (3)</td>
<td>3.06 ± 0.46</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Assays were carried out as described in "Materials and Methods," and the results are expressed as the mean rates ± SD.

**DISCUSSION**

Cigarette smoke contains carcinogenic PAHs, nitrosamines, and aromatic amines, as well as a variety of lower molecular weight carcinogens. BP-, ABP-, and nitrosamine-derived DNA adducts have already been identified as smoking-related DNA adducts in human lung (31–35) or urinary bladder tissues (15). These reports suggested to us that DNA adducts in the larynx of smokers may also be derived from carcinogenic PAHs, aromatic amines, and/or nitrosamines. Insight into the nature of the adducts detected in human tissues has been gained by comparing adduct profiles obtained following the butanol extraction and nuclease P1 enhancement procedures of the 32P-postlabeling method. Gupta and Earley (19), Gallagher et al. (36, 37), and Talaska et al. (15) have demonstrated that most aromatic amine-derived DNA adducts are very poorly recovered using nucleic P1 enrichment but are preferentially enhanced by the butanol extraction procedure. On the other hand, aromatic hydrocarbon-derived DNA adducts generally exhibit similar recoveries in both assays (19, 36–38), whereas DNA adducts of the tobacco-specific nitrosamines are not readily detectable by either method (2). Recently, Jones et al. (12) used 32P-postlabeling assays with butanol extraction and nuclease P1 enhancement and suggested that aromatic amines and nitroaromatics may be important sources of the smoking-related-DNA adducts detected in human oral tissues such as the floor of mouth, buccal mucosa, and tongue. Earlier, Chacko and Gupta (10) reported the presence of hydrophobic DNA adducts and two putative aromatic amine-DNA adducts in oral mucosal cells of tobacco chewers and smokers.

When we compared the hydrophobic DNA adduct profiles in the larynx of smokers using butanol extraction and nuclease P1 enhancement methods, both procedures gave similar results with the exception of two putative adducts (spots designated nos. 1 and 2) that were observed only with the butanol extraction method. Accordingly, total relative adduct levels were only slightly greater with butanol extraction than with nuclease P1 enhancement. Efforts were also made to detect pyridyloxobutyl-DNA adducts derived from tobacco-specific nitrosamines in our samples by mass spectrometric means (33); however, these were unsuccessful due to limited sample size, i.e., below
the limits of detection.\textsuperscript{5} Thus, these results suggested that the majority of hydrophobic DNA adducts detected in the larynx of smokers are probably derived from PAHs but not from aromatic amines or nitrosamines.

Carcinogenic PAHs, nitrosamines, and aromatic amines are known to express their carcinogenicity after undergoing metabolic activation via catalysis of drug metabolizing enzymes such as P450s and acetyltransferases. In general, metabolic activation of these carcinogens is mediated by different enzymes: P450 1A1, 2C, and 3A4 enzymes for PAHs (27–29); P450 2E1 and 450 2A6 for nitrosamines (22, 23); and P450 1A2 (39, 40) and acetyltransferases (NAT-1 and NAT-2) for aromatic amines (26). To clarify further the nature of smoking related-DNA adducts in the larynx, we compared the immunoreactive levels of these P450 enzymes and the enzymatic activity of the acetyltransferases, NAT-1 and NAT-2, in this tissue. P450s 1A1, 2C, 2E1, 3A4, and 2A6 were each detected in all larynx microsomes, along with NAT-1 but not NAT-2 in the larynx cytosols. Moreover, the known metabolic activation pathways for BP by cytochromes P450 1A1, 2C, and 3A4, shown in Fig. 5, were consistent with the high correlations that we observed between total DNA adduct level and these P450 isozyme levels in larynx microsomes. A correlation between a carcinogen-activating enzyme and carcinogen-DNA adduct level in human tissues had not been reported previously except for a recent study that we observed between total DNA adduct level and these P450 iAl activity and BP-DNA adduct level in human lung tissue (30, 31). It has long been hypothesized that carcinogen-activating enzymes are at least one of the factors determining the susceptibility of animal species, including humans, and their organs to a carcinogen and that chemical carcinogenesis is initiated by carcinogen-DNA adduct formation in a target tissue (41–45). Therefore, studies on DNA adducts together with carcinogen-metabolizing enzymes in the upper aerodigestive tract and other human tissues should be very useful in determining the carcinogen class for each tissue and in providing better approaches for cancer prevention.

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