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Carcinogen Biomarkers Related to Smoking and Upper Aerodigestive Tract Cancer

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Abstract Smoking is the major cause of upper aerodigestive tract cancers. Among the many constituents of tobacco smoke, polynuclear aromatic hydrocarbons and tobacco-specific nitrosamines are strongly implicated as causative factors for these cancers. The probability that these compounds will induce cancer in a given individual will depend on that person's ability to metabolically activate or detoxify them. Chronic production of DNA damage by these metabolically activated carcinogens is consistent with current concepts of carcinogenesis in which multiple genetic changes, such as activation of oncogenes or inactivation of tumor suppressor genes, appear to be critical. Chemopreventive agents which decrease the level of DNA damage should therefore decrease the risk for cancer. Biomarkers such as carcinogen-DNA adducts, carcinogen-hemoglobin adducts, and urinary metabolites of carcinogens will indicate the amount of metabolically activated carcinogen which may damage DNA in an individual and can therefore be used as an index of risk. Selected biomarkers are discussed in this paper. These biomarkers of internal dose have great potential for application in chemoprevention trials.

Key words: Carcinogen biomarkers, tobacco smoke carcinogens, polycyclic aromatic hydrocarbons, tobacco-specific nitrosamines, hemoglobin adducts, DNA adducts, chemoprevention by isothiocyanates, phenethyl isothiocyanate

Smoking has been established conclusively as an important cause of upper aerodigestive cancers including squamous cell, small cell, and adenocarcinoma of the lung, as well as oral, oropharyngeal, hypopharyngeal, laryngeal, and esophageal cancers [1]. Shopland *et al.* [2] have estimated the risk of deaths from various cancers which are attributable to smoking. U.S. data for 1991 indicated that 90.3% of deaths from lung cancer, 91.5% from oral cancer, 81.2% from laryngeal cancer, and 78.2% from esophageal cancer in males were attributed to smoking; corresponding values for females were 78.5%, 61.2%, 86.7%, and 74.3%, respectively. Collectively, approximately 165,000 deaths from these cancers were attributed to smoking. It is clear that the best way to prevent upper aerodigestive tract cancer is to avoid smoking. Educational and smoking cessation programs have been partially successful in this respect. Smoking has consequently declined, but there are still approximately 47,000,000 smokers in the U.S. and hundreds of millions worldwide. Apparently, the addictive power of nicotine prevents many individuals from avoiding tobacco products [3]. Chemoprevention represents a viable alternative for preventing upper aerodigestive tract cancer in those people who cannot stop smoking.

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CAUSATIVE AGENTS FOR UPPER AERODIGESTIVE TRACT CANCERS IN TOBACCO PRODUCTS

Tobacco smoke contains approximately 4,000 identified compounds, of which at least 40 are known carcinogens in laboratory animals [1,4]. One can assess the possible contributions of these carcinogens to upper aerodigestive cancers by considering their levels in tobacco smoke, their carcinogenic properties in laboratory animals, as well as biochemical evidence for their uptake and metabolic activation by smokers [1,4–6]. Polycyclic aromatic hydrocarbons (PAHs) are well-characterized respiratory tract carcinogens. Benzo(*a*)pyrene [B(*a*)P, Fig. 1] is by far the most extensively studied of these compounds and has been regarded as a prototype for the PAH class. It is a potent respiratory carcinogen in rodents and occurs in mainstream cigarette smoke in amounts generally ranging from 20–40 ng per cigarette. The presence of B(*a*)P-DNA adducts in the lungs of smokers has been demonstrated and a correlation between induced levels of cytochrome P-4501A1 in human lung and B(*a*)P-DNA adduct levels has been noted [7,8]. In addition to B(*a*)P, other PAHs are present in tobacco smoke; some of these, including the benzofluoranthenes, dibenz(*a,h*)anthracene, and 5-methylchrysene, are well-established potent carcinogens [1,4]. In addition to 5-methylchrysene, numerous other methylated PAHs are also present in tobacco smoke [9]. G to T transversion mutations identified in *ras* and p53 genes isolated from lung tumors could conceivably result from PAH-DNA adducts [10–13]. Extensive studies of tumor promoters and co-carcinogens in tobacco smoke have demonstrated that these constituents enhance the carcinogenicity of PAHs [14,15]. Catechols and their methylated derivatives, well-established co-carcinogens in tobacco smoke, could play a role in lung cancer induction. Collectively, the available data strongly support the potential role of PAHs as an important causative factor for lung cancer induction in smokers.

The nicotine-derived tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK, Figure 1) is also likely to play an important role in lung cancer induction. NNK is a potent respiratory tract carcinogen in rats, mice, and hamsters, inducing tumors—mainly adenocarcinoma—over a wide range of doses; the lowest of which are comparable to human exposure levels, based both on the levels of NNK in cigarette smoke and on levels of its metabolites in smokers' urine [5,6,16,17]. Pyridyloxobutyl-DNA adducts resulting from metabolic activation of NNK, or a related tobacco-specific nitrosamine NNN, have been detected in lung tissue isolated from smokers [18]; 7-methylguanine and O^6-methylguanine, which are formed upon metabolic activation of NNK, have also been detected in lung tissue of smokers [19,20]. Studies in mice have shown that pyridyloxobutyl adducts can induce G → T transversion mutations, as seen in mutated genes from lung tumors (Ronai Z, Peterson L, Hecht SS, unpublished). G to A transition mutations, consistent with the presence of O^6-methylguanine, have also been observed in analyzing p53 genes from lung tumors [21]. Considering the levels of NNK in tobacco smoke, its potent pulmonary carcinogenicity, and available biochemical data, its involvement in lung cancer induction in smokers appears likely.

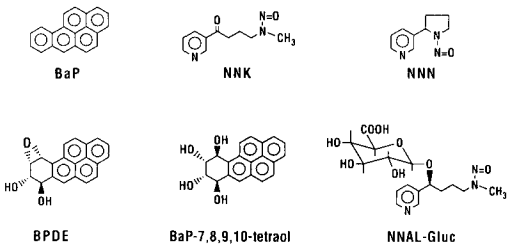

Fig. 1. Structures of some of the compounds discussed in the text. The enantiomer of BPDE illustrated is the most tumorigenic of the four possible enantiomers and most reactive with DNA. The enantiomer of B(a)P-7,8,9,10-tetraol is the predominant one released from DNA upon hydrolysis according to current studies. Two diastereomers of NNAL-Gluc are known metabolites of NNK; only one is illustrated.

A number of other types of carcinogens which may also play a role in lung cancer development are present in tobacco smoke. These include aldehydes such as formaldehyde, acetaldehyde, and acrolein; simple unsaturated compounds such as butadiene; and metals such as chromium, cadmium, and polonium-210 [1,4]. The contribution of these compounds to lung cancer requires further study.

The lack of good animal models makes assessing the role of tobacco constituents in oral cavity cancer somewhat problematic. The compound most widely used to induce oral tumors in hamsters is 7,12-dimethylbenz(*a*)anthracene, a PAH which does not occur in tobacco or tobacco smoke. B(*a*)P is not known to be a strong oral cavity carcinogen, although its metabolic activation by human oral tissue has been established [22]. A mixture of NNK and NNN applied to the rat oral cavity has been shown to induce oral cavity tumors [23]. Human oral tissue metabolically activates these nitrosamines [24], but further studies are required to assess the presence of relevant cytochrome P-450 enzymes in the oral mucosa. Although both PAHs and nitrosamines occur in tobacco smoke, this is not the case for smokeless tobacco, which is not a combustion product and consequently contains only trace amounts of PAHs. Levels of tobacco-specific nitrosamines in smokeless tobacco are unusually high; human exposure to these compounds through snuff-dipping and tobacco chewing is more extensive than exposure to virtually any other carcinogen [5]. Since snuff-dipping is an accepted cause of oral cancer and tobacco-specific nitrosamines are the most prevalent strong carcinogens in snuff, they are considered to play an important role in causing oral cancer in humans [5].

Among the potential causative agents for esophageal cancer in tobacco smoke, nitrosamines are by far the best established carcinogens in laboratory animals [5]. Numerous nitrosamines produce esophageal tumors in rats. Extensive dose-response studies have established that some, like *N*-nitrosodiethylamine, a constituent of tobacco smoke, are highly carcinogenic [25]. The most prevalent esophageal carcinogen in tobacco smoke is NNN, which typically occurs in concentrations of approximately 200 ng per cigarette in mainstream smoke. Its carcinogenicity for the rat esophagus and relative abundance in smoke support its role as an important agent in the induction of esophageal cancer in smokers [5].

BIOMARKERS OF INTERNAL DOSE OF PAH AND TOBACCO-SPECIFIC NITROSAMINES

PAHs and nitrosamines are procarcinogens, *i.e.*, they must undergo metabolic activation to ultimate carcinogens before they can bind to relevant cellular macromolecules such as DNA. The metabolic activation processes are complex, with competing detoxification pathways. Each person will have a different balance of these activation and detoxification pathways. Therefore, measurement of exposure to a carcinogen will not necessarily produce useful data; what is needed are methods to accurately quantify the amounts of metabolically activated carcinogens which reach target macromolecules in critical cells. Such measures are biomarkers of the internal dose of metabolically activated carcinogens and should be directly relevant to cancer risk. The most commonly used biomarkers of internal dose are carcinogen-DNA adducts and carcinogen-hemoglobin or carcinogen-albumin adducts. The latter are more readily quantified than DNA adducts and have been regarded as surrogates for DNA adducts. Urinary metabolites can also indicate relative amounts of activation and detoxification of a given carcinogen. Several reviews on the topic of biomarkers of internal dose have been published; no attempt will be made here to provide comprehensive coverage of this rapidly developing field [26–28]. Some methods for quantifying internal doses of specific PAHs and nitrosamines will be described.

The best characterized pathway of PAH metabolic activation is the diol epoxide pathway, typified by the formation of *anti*-B(*a*)P-7,8-diol-9,10-epoxide (BPDE, Fig. 1), which has been identified as one ultimate carcinogen of B(*a*)P [29]. Immunoassays and ^{32}P-postlabelling have been widely used to approximate levels of PAH-DNA adducts in human lung tissue, as well as in several other tissues [27,28]. These methods have the advantage of sensitivity, requiring only small amounts of DNA. However, they lack specificity. Other approaches have also been used. Combined gas chromatography-mass spectrometry (GC-MS) as well as synchronous fluorescence spectroscopy (SFS) and high pressure liquid chromatography-fluorescence (HPLC-fluorescence) appear to be more specific and sensitive, at least for B(*a*)P-DNA adducts or B(*a*)P-hemoglobin adducts [7,8,30]. Little information is available about other PAH adducts in humans. The GC-MS and HPLC-fluorescence methods quantify an isomer of B(*a*)P-7,8,9,10-tetraol (Fig. 1), which is released from DNA or

hemoglobin by hydrolysis. GC-MS has been applied for the positive identification of a BPDE-hemoglobin adduct; an *anti*-chrysene-1,2-diol-3,4-epoxide-hemoglobin adduct has also been identified [31]. Quantitative studies using these methodologies have not been published. Immunoaffinity chromatography combined with HPLC and SFS has been used to quantify B(*a*)P-7,8,9,10 tetraol released from human lung DNA, as has HPLC-fluorescence [7,8]. In a recent report, adduct levels were higher in smokers than in non-smokers [7]. These methods appear to be promising for further studies but would only apply to PAH-DNA adducts with fluorescent chromophores. In contrast, GC-MS has potential utility for a broad spectrum of PAH adducts, but its application has been limited to date.

As illustrated in Figure 2, NNK and NNN undergo metabolic activation to a common intermediate, 4-(3-pyridyl)-4-oxobutanediazohydroxide (structure 7). This intermediate reacts with hemoglobin to give pyridyloxobutyl ester adducts and with DNA to give adducts of unknown structure [32,33]. Base hydrolysis of the hemoglobin adducts or acid hydrolysis of the DNA adducts produces 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB), which can be quantified as its pentafluorobenzoate derivative by GC-MS [18,34]. This methodology has detected pyridyloxobutylated DNA in the lung and trachea of smokers at levels higher than in non-smokers. It has also been used to analyze hemoglobin adducts in smokers and snuff-dippers. Typical results of these studies are illustrated in Figure 3. In studies carried out to date, approximately 20% of smokers have hemoglobin adduct levels elevated above those seen in non-smokers. Adduct levels in snuff-dippers were generally higher than in smokers. The results of these studies suggest that some individuals can metabolically activate NNK or NNN more extensively than others and may therefore be at higher risk for upper aerodigestive tract cancers.

NNK is metabolized rapidly to its carbonyl reduction product NNAL in virtually all systems examined to date. NNAL, like NNK, is a strong pulmonary carcinogen [35]. NNAL is in turn metabolized to diastereomeric glucuronides, NNAL-Gluc (one diastereomer is shown in Fig. 1). NNAL and NNAL-Gluc have been quantified in the urine of smokers [36] in quantities

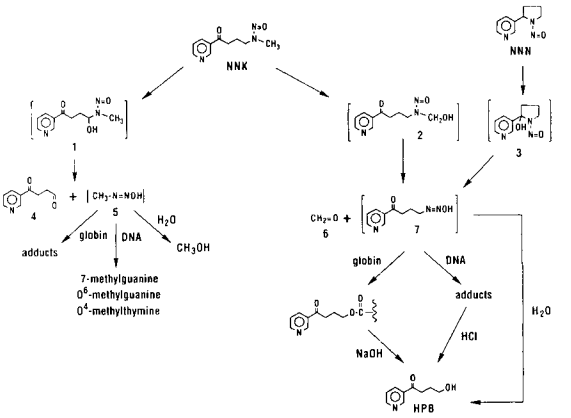

Fig. 2. Metabolic activation pathways of NNK and NNN known to produce DNA or hemoglobin adducts *in vivo*. With permission of the National Institute of Environmental Health Sciences.

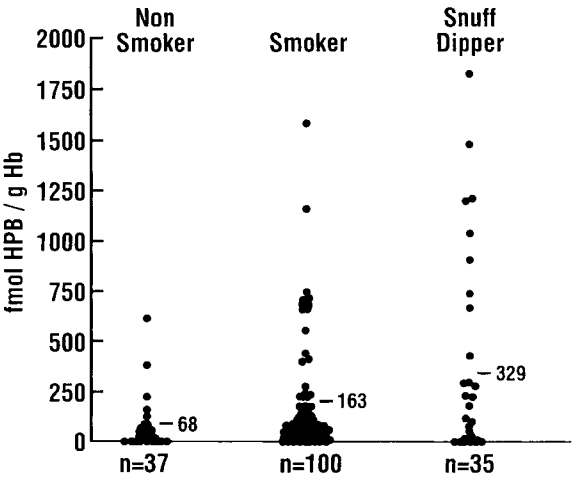

Fig. 3. Data from ongoing studies on levels of HPB released from hemoglobin of non-smokers, smokers, and snuff-dippers. With permission of the National Institute of Environmental Health Sciences.

consistent with exposure to NNK in cigarette smoke, but not in the urine of non-smokers. Thus, NNAL and NNAL-Gluc are good biomarkers for the uptake of NNK by smokers.

Metabolism of NNK also produces methanediazohydroxide, a methylating agent which may react with DNA to produce 7-methylguanine, O^6-methylguanine, and other adducts (see Fig. 2). Several studies have implicated O^6-methylguanine as an important adduct in NNK carcinogenesis in mice and rats [37,38]. In hu-

mans, levels of O^6-methyldeoxyguanosine do not differ between smokers and non-smokers in analyses of DNA samples obtained from peripheral lung and placenta [19,39]. However, a recent study demonstrated higher levels of 7-methyldeoxyguanosine in bronchial DNA samples from smokers compared to non-smokers [20].

Although exposure to PAHs derives from many sources, most notably food, exposure to NNK and NNN comes exclusively from tobacco products. Therefore, biomarkers of NNK and NNN uptake and metabolic activation may be particularly useful in assessing the relationship between cancer induction and tobacco products. Among these biomarkers, the adducts and metabolites containing pyridyloxobutyl or pyridylhydroxybutyl groups are potentially more valuable than biomarkers of DNA methylation because of their specific structural relationship to tobacco-specific nitrosamines.

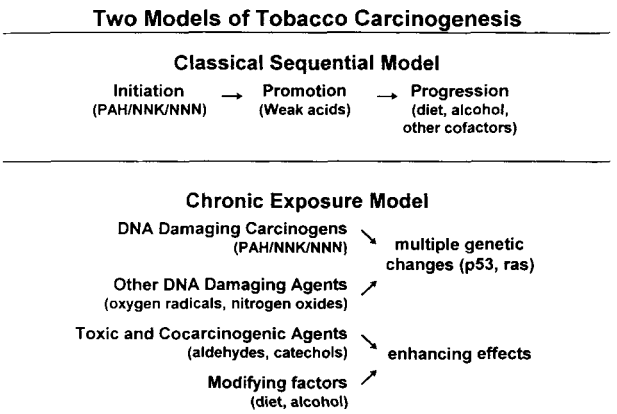

Fig. 4. Two models of tobacco carcinogenesis. In the classical sequential model, exposure to a DNA damaging initiator is followed by exposure to agents which cause promotion and progression. In the chronic exposure model, continual simultaneous exposure to all compounds in tobacco smoke leads to multiple genetic changes and other effects associated with the multistage carcinogenic process.

BIOMARKERS OF INTERNAL DOSE: POTENTIAL APPLICATION IN CHEMOPREVENTION STUDIES

Classically, the genesis of upper aerodigestive cancers has been described by the three-stage model of carcinogenesis; initiation, promotion, and progression [40]. Laboratory and epidemiologic studies support the hypothesis that tobacco smoke has tumor promoting activity [1,4]. However, it is unrealistic to consider tobacco carcinogenesis only in this framework; people who use tobacco products are continually and simultaneously exposed to all constituents of the mixture, which include both "initiating" and "promoting" agents. Two models of tobacco carcinogenesis, the "classical sequential model" and the "chronic exposure model" are outlined in Figure 4. The latter model is consistent with presently evolving data demonstrating that multiple genetic changes occur during the process of upper aerodigestive tract carcinogenesis, including the activation of oncogenes such as those in the *ras* family and the inactivation of tumor suppressor genes such as p53 [10–13]. These multiple genetic changes are most likely involved at different stages of the multistage carcinogenic process. They probably result, at least in part, from direct interaction of metabolically activated carcinogens such as B(*a*)P and NNK with critical target genes. Thus, chemopreventive agents which would decrease the extent of these interactions would potentially be effective in delaying or blocking cancer induction by tobacco products. Experimental studies have led to the discovery of many agents, both naturally occurring and synthetic, which inhibit lung tumorigenesis by B(*a*)P and NNK, through mechanisms that result partially or completely from inhibition of metabolic activation or enhancement of detoxification. These include arylalkyl isothiocyanates [41], antioxidants such as butylated hydroxyanisole [42], constituents of citrus oils such as D-limonene [43,44], analogues of experimental chemotherapeutic drugs such as ipomeanol [45], ellagic acid [46], and constituents of tea [47] to name just a few. The use of suppressing agents, *i.e.*, compounds which inhibit or reverse the promotion or progression stages of carcinogenesis has, to date, been relatively unsuccessful in experimental models of lung cancer [48]; however, retinoids and perhaps other agents are effective in chemoprevention of other upper aerodigestive tract cancers. The potential application of biomarkers of internal dose in chemoprevention studies can be illustrated using our own studies on chemoprevention of NNK-induced lung carcinogenesis by arylalkyl isothiocyanates.

We have shown that a non-toxic dose of phenethyl isothiocyanate (PEITC), administered in the diet of F344 rats before and during treatment with NNK by subcutaneous (sc) injection, causes a 50% decrease in adenocarcinoma of the lung; there was no effect on tumors induced in the liver and nasal cavity, which are subsidiary target tissues of NNK carcinogenesis [49]. The molar ratio of PEITC dose to NNK dose per kg body weight in this study was approximately 30; this ratio can be achieved in humans with a daily PEITC dose of less than 0.2 mg. In parallel studies of NNK-DNA binding at similar doses, there was approximately a 50% decrease in both DNA methylation and pyridyloxobutylation in the lungs of rats treated with NNK and PEITC compared to those treated with NNK alone. In liver and nasal mucosa, simultaneous inhibition of both pathways of DNA alkylation was not achieved [49].

These results indicate that the chemoprevention mechanism of PEITC in rats involved inhibiting both DNA methylation and pyridyloxobutylation pathways in the lung. These results are consistent with ongoing studies that indicate the importance of both pathways in lung cancer induction by NNK in rats. Inhibition of DNA adduct formation results in part from the ability of PEITC to inhibit cytochrome P-450 isozymes involved in the metabolic activation of NNK [50,51]. We have also shown that the formation of DNA adducts by NNK in the lung and liver increases in proportion to the formation of hemoglobin adducts over a wide dose range, although the relationship between these two parameters is complex [52]. In unpublished studies, we have examined the effect of dietary PEITC on the formation of pyridyloxobutyl ester adducts in the hemoglobin of rats treated with NNK and found that formation of these adducts was inhibited. Collectively, these data suggest that hemoglobin adduct formation could be used as a biomarker to assess the efficacy of PEITC as a chemopreventive agent in upper aerodigestive tract cancer in smokers. Presumably, hemoglobin adduct levels in smokers who consumed PEITC regularly would decrease, as would the extent of DNA damage by NNK. Based on data from rodent studies, such decreases would signal a decrease in cancer risk.

The mechanism of NNK-induced pulmonary carcinogenesis has been extensively studied in strain A/J mice using a single dose model which rapidly induces lung tumors. These studies have shown that the formation and persistence of O^6-methylguanine in pulmonary DNA is a critical event in NNK carcinogenesis, and that pyridyloxobutylation of DNA acts as a co-carcinogen by inhibiting the repair of O^6-methylguanine [37]. Analyses of lung tumors from NNK-treated mice revealed the presence of mutations in codon 12 of the K-*ras* gene in a high percentage of the tumors; the predominant mutation detected in these studies was a G to A transition, consistent with the presence of O^6-methylguanine [53]. We have shown that treatment of strain A/J mice with arylalkyl isothiocyanates prior to injection of NNK causes a decrease in the levels of O^6-methylguanine in the lung, with a corresponding decrease in lung tumor formation [41]. As in the rat, this is at least partially due to inhibition of cytochrome P-450 enzymes which metabolize NNK [54]. The efficacy of inhibition of lung tumor formation increases with increasing alkyl chain length; 6-phenylhexyl isothiocyanate is a highly effective inhibitor with activity at least ten times greater than that of PEITC. Although the use of NNK-hemoglobin adducts as a surrogate for DNA alkylation has not been explored in the mouse, the same principles apply and results analogous to those obtained in the rat could be expected.

CONCLUSIONS

PAHs and nitrosamines are likely to play a major role in the induction of upper aerodigestive tract cancers in smokers. Chemopreventive agents which inhibit the metabolic activation of these carcinogens or enhance their detoxification have great potential in inhibiting or delaying the development of upper aerodigestive tract cancers in smokers. Such agents would decrease the extent to which these carcinogens interact with critical target genes involved in the multistage cancer development process. Therefore, biomarkers of internal dose of these carcinogens would be useful in chemoprevention trials. These biomarkers, which include quantitative measurements of DNA adducts, hemoglobin adducts, or urinary metabolites can be applied in chemoprevention trials to indicate the potential efficacy in humans of compounds which

have demonstrated chemopreventive activity in laboratory animals.

While it is true that the great majority of upper aerodigestive tract cancers could be prevented by abstinence from tobacco, a large population still uses tobacco products and is therefore at high risk. The use of tobacco products will not cease in the near future. For those who are addicted, chemoprevention is one way to decrease risk. Beyond this, tobacco users, with their known exposure to carcinogens and quantifiable risk for cancer, represent an important target population for testing chemoprevention strategies.

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