
Chapter 3

Carcinogenesis

| | | |
|-----------------|---|-----|
| CONTENTS | Oral Carcinoma and Smokeless Tobacco Use: | |
| | A Clinical Profile | |
| | W. Frederick McGuirt and Anna Wray | 91 |
| | Introduction | 91 |
| | Patients | 91 |
| | Field Cancerization | 92 |
| | Discussion | 93 |
| | References | 95 |
| | Chemical Composition of Smokeless Tobacco Products | |
| | Klaus D. Brunnemann and Dietrich Hoffmann | 96 |
| | Introduction | 96 |
| | Chemical Composition | 97 |
| | Carcinogenic Agents in ST | 97 |
| | Carcinogenic N-Nitrosamines | 100 |
| | TSNA | 101 |
| | Control of Carcinogens in ST | 104 |
| | References | 106 |
| | Carcinogenesis of Smokeless Tobacco | |
| | Dietrich Hoffmann, Abraham Rivenson, and Stephen S. Hecht | 109 |
| | Introduction | 109 |
| | Carcinogens in Smokeless Tobacco | 111 |
| | N-Nitrosamines | 113 |
| | TSNA | 113 |
| | Discussion | 115 |
| | References | 116 |
| | Oncogenes in Head and Neck Cancer | |
| | Kenneth D. Somers, Suzanne E. Glickman, Suzanne L. Cartwright, Bryan G. Stoll, Stella L. Maurizio, and Gary L. Schechter | 119 |
| | Introduction | 119 |
| | Materials and Methods | 120 |
| | Results | 120 |
| | Discussion | 123 |
| | References | 126 |

Oncogenes in Tobacco-Induced Oral Cancer

Madhav G. Deo and Dhananjaya Saranath 128

 Introduction 128

 Oncogene Amplification 129

 Expression of Cellular Oncogenes 132

 RFLP Analysis of *L-myc* and *H-ras* Oncogenes 133

H-ras Point Mutations 134

 Identification of a Functional Oncogenes 136

 Conclusions and Future Prospects 137

 References 139

Metabolism and Macromolecular Binding of NNK and NNN, Important Carcinogens in Smokeless Tobacco

Stephen S. Hecht, Steven G. Carmella, and Sharon E. Murphy 141

 Introduction 141

 Metabolism of NNK and NNN 142

 Detection of NNK/NNN-Hemoglobin Adducts 146

 Gaps in Assessing the Role of NNK and NNN 150

 Summary 151

 References 151

Role of Nicotine as a Cofactor in Smokeless Tobacco Carcinogenesis

Christopher A. Squier and Georgia K. Johnson 153

 Introduction 153

 In Vivo Studies with Nicotine 153

 In Vitro Studies with Nicotine 154

 Clinical Implications of Studies with Nicotine 155

 Future Directions 159

 References 159

Perinatal Carcinogenesis by Constituents of Smokeless Tobacco: Animal Models and Potential Human Risk

Lucy M. Anderson and Jerry M. Rice 161

 Introduction 161

 Sensitivity and Susceptibility Factors 161

 Tobacco-Specific Nitrosamines in Animal Models 163

 Other ST Constituents 165

 Potential Risk and Future Research 166

 References 169

Interaction Between Smokeless Tobacco-Related Carcinogens and Human Papillomaviruses in the Pathogenesis of Oral Dysplasia and Cancer

Joel M. Palefsky, John S. Greenspan, Tray E. Daniels, Jennifer Berline, Deborah G. Grady, and Virginia Ernster 175

 Introduction 175

 Methods 177

 Results 178

 Discussion 178

 References 181

| | |
|---|-----|
| Identification of Human Papillomavirus DNA in Smokeless Tobacco Keratosis and Premalignant and Malignant Oral Lesions, by PCR Amplification with Consensus Sequence Primus | |
| Robert O. Greer, Jr., Kenneth R. Shroyer, and Louise Crosby | 183 |
| Introduction | 183 |
| Materials and Methods..... | 184 |
| PCR | 184 |
| Results..... | 185 |
| Discussion..... | 185 |
| References | 189 |
| | |
| Lipids as Factors in the Cell Response to Tobacco Components | |
| George S. Schuster, Scott Lubas, Thomas R. Dirksen, and John F. Erbland | 191 |
| Introduction | 191 |
| Methods..... | 193 |
| PKC Results..... | 194 |
| Discussion..... | 197 |
| References | 200 |
| | |
| Role of Viruses in Oral Carcinogenesis | |
| No-Hee Park, Byung-Moo Min, Sheng-Lin Li, Henry M. Cherrick, and Jay Doniger | 202 |
| Introduction | 202 |
| Methods..... | 203 |
| Results..... | 206 |
| Discussion..... | 211 |
| References | 214 |

Oral Carcinoma and Smokeless Tobacco Use: A Clinical Profile

W. Frederick McGuirt and Anna Wray

ABSTRACT The clinical profile of 116 patients with oral cavity cancer who used smokeless tobacco exclusive of other known carcinogens, such as smoking tobacco and alcohol, is discussed. The patients, whose average age was 78.4 yr and average use 55.5 yr, had a 1:23 male-to-female ratio and a 14.5:1 white-to-black ratio, both different from national rates for patients with oral cavity cancers. The 116 patients' course illustrates the field cancerization concept: In addition to the referral lesion, 55 percent (64/116) had leukoplakia, erythroplasia, dysplasia, or carcinoma in situ previously, at the time of presentation, or after initial therapy. A second malignant oral lesion developed in 18 percent (21/116); 35 percent (41/116) had local recurrence despite predominantly clear margins earlier. Of the 91 patients with documented followup, 45 died of or with cancer. These distressing figures may, unfortunately, be duplicated in another 20 to 40 years in a different population group—current young male smokeless tobacco users.

INTRODUCTION Not only is tobacco an integral part of the local economy in North Carolina, but also its use in all forms is pervasive in the population served by the Wake Forest University Medical Center. Smokeless tobacco use, especially, is endemic to the three or four generations living in our geographic region. Review of our tumor registry files yielded 150 cases of oral cavity cancer in smokeless tobacco users—4.9 percent of our total head and neck cancer caseload. Smokeless tobacco was used exclusive of other known oral carcinogens, such as smoking tobacco and alcohol, by 116 of the 150 patients. These 116 cases form the basis of this report, in which we profile the patients, the nature of the disease process, its treatment, and outcome.

PATIENTS The patient population reported here differed in several ways from the national profile of oral cancer patients. Oral cavity carcinoma in the United States has a 2:1 male-to-female ratio (Boring et al., 1991). This ratio is consistent with our new head and neck cancer patient population (1,393/699) but higher than our oral cancer male-to-female ratio of 483/361 (1.3:1). This oral cavity cancer ratio was strongly influenced (that is, reduced) by our patients who used smokeless tobacco only. This group of patients had a 1:23 male-to-female ratio, representing disease occurrence almost exclusively in females.

A second epidemiological aberration seen in the pool of smokeless tobacco-associated oral cancers relates to the most common tumor sites. The mobile tongue and floor of the mouth are the most common sites of oral cavity carcinoma. In our series of smokeless-tobacco-only users, the cancers occurred most frequently in the buccal and gingival areas, the regions where the quid was held. Ninety-four percent of patients (109/116) had their cancers at these sites.

The average age of these patients was 78.4 yr, with 83 percent (96/116) older than 65 yr and 27 percent (31/116) older than 80 yr. These patients were older than the overall head and neck cancer patients seen in our institution, who averaged 63 yr during the same study period.

The duration of smokeless tobacco use before presentation for treatment averaged 55.5 yr, with 81 percent of patients (86/106) having a history of use > 40 yr.

A further epidemiological aberration occurred in the racial distribution of patients. A 14.5:1 (116:8) ratio of white to black was seen, compared with a 7.5:1 (1,845:247) ratio of all head and neck cancer cases and a population distribution frequency of 3.5:1 in the geographic region served (U.S. Census figures, 1990 to 1991). These figures support the epidemiological study of Winn et al. (1981) from the same geographic region but a different referral base. The use of snuff—and cancer cases attributed to it—was reported by Winn and coworkers to be a phenomenon of white women.

These factors of sex, race, and age are results of sociological trends well recognized and reported previously. Snuff use in the region until the mid-1970's was predominantly a practice of white females, who began to use snuff at an early age in the 1930's and 1940's. This was a period of declining smokeless tobacco use and increased smoking. The social attitudes of that time discouraged women's smoking, but in the rural South, women continued privately to use smokeless tobacco. Dry, powdered snuff (i.e., Scotch snuff) has been the predominant form used by these women, in contrast to the flavored moist strips of tobacco preferred by today's young male users.

Whereas the now elderly women with long-term smokeless tobacco use form the patient population reported here, the obvious concern is that the young males currently using smokeless tobacco may well be the future patients who will be profiled if they continue their chronic use. The addictive qualities of the product would imply this to be the expected outcome.

Oral carcinoma in a high school athlete from Oklahoma who was a regular user of smokeless tobacco has become the focal point for an educational effort spearheaded by the American Academy of Otolaryngology-Head and Neck Surgery (AAO-HNS). Oral carcinoma in the male population under age 30 has not yet been seen by personal experience or become a national problem, according to a questionnaire mailed to members of the American Society of Head and Neck Surgery and selected AAO-HNS members, who are the physicians most likely to be treating such patients. The youngest patient to date in our experience was aged 40; the next youngest, aged 43.

FIELD CAN- CERIZATION Patients with cancers attributed to smokeless tobacco illustrate the field cancerization concept. In addition to the referral lesion, adjacent and distant mucosal lesions exist synchronously or develop metachronously. In the field cancerization model, a diffuse surface area of mucous membrane is bathed by the carcinogens and placed at risk. Logically, the highest risk area for smokeless tobacco use is where the quid is held, but adjacent areas and distal sites are continuously bathed by saliva containing the carcinogenic agents that leach from the quid. Our patients with smokeless tobacco-related oral cancers often exhibited adjacent and distant mucous membrane changes. Varying degrees of abnormality, compatible with the evolutionary transformation to malignancy, were seen.

These abnormal changes included clinical leukoplakia and erythroplasia as well as histologically varying degrees of dysplasia and carcinoma in situ. Fifty-five percent of patients in our series (64/116) had had these types of lesions excised previously, had them at the time of their presentation, or developed them subsequent to therapy. Further evidence for an evolutionary transformation of a condemned mucosa was the high incidence (18 percent) of second or metachronous malignant oral lesions that developed in these patients. A third indicator of this evolutionary transformation was the 35 percent incidence (41/116) of local cancer recurrence, despite predominantly clear margins on histological examination (28 of 33 surgical specimens, 85 percent). These lesions were most likely not recurrences of the original cancer but, rather, malignant transformations of previously exposed mucous membranes at risk for new cancers in the same region.

DISCUSSION The disease course is most often silently and indolently progressive. Patients may note a change or abnormality within the mouth, but the symptoms are usually minimal and a delay in seeking evaluation occurs. Forty-three percent of patients (48/111) had symptoms for 3 mo or longer before presentation. The silent but progressive nature of the disease is further illustrated by the 53 percent (62/116) incidence of advanced stage III and stage IV lesions at the time of diagnosis, of which one-third (34 percent, 21/62) had been symptomatic for less than 2 mo.

Although the premalignant changes of leukoplakia and erythroplasia have a slow transformation and progression, with only minimal symptoms, once the smokeless tobacco-related cancers are established, they act in aggressive fashion, similar to other oral cavity cancers. This is confirmed by the high incidence of stage III and stage IV disease. The advanced stages were attributable to regional metastases in 27 percent of the cases, to locally advanced disease (T3-T4) in 43 percent of the cases, and in many cases to both characteristics.

Although many of these smokeless tobacco-related tumors have a verruciform appearance, they should never be considered, on clinical grounds alone, to be the less aggressive verrucous carcinoma described histologically by Ackerman (1948). Although 24 percent (28/116) of our patients had verruciform lesions, less than one-fifth of those, or 4.3 percent overall (5/116), had verrucous carcinomas by the histological criteria of Ackerman.

Localized stage I and stage II carcinomas may be treated equally well with either surgery or irradiation. Therapy of advanced stage III and stage IV cases usually required both surgery and irradiation. The surgical therapy in advanced stage lesions may result in a significant alteration in form and function of the oral cavity. The major problems of postsurgical resection relate to the loss of the mandibular-dental function of mastication and the oral-buccal-lingual function of deglutition. The addition of irradiation to surgery in these advanced cases or as the primary modality of therapy for early lesions causes morbidity related to the resulting xerostomia and the lifelong need for frequent dental care and rehabilitation. The use of irradiation and the advanced age of the patients reduce the success rate of mandibular reconstruction.

Mandibular resection results in cosmetic disfigurement, the degree usually being directly proportional to the amount of bone resected and how far anteriorly the resection proceeds. In general, lateral mandibular defects are usually left unreconstructed. Anterior defects, though, require reconstruction, not only to correct severe cosmetic problems, but also to correct basic functional problems. Various reconstructive methods and synthetic replacement prostheses have been used. Our current preference is to use microvascular anastomotic techniques for restoration by a composite free flap. This provides soft-tissue support and bone with its own direct blood supply.

There is a general lack of success in fitting dentures that allow adequate mastication for solid food, even when the mandibular defect is restored by bone grafting or a metal bar. The future use of implanted prosthetic devices holds promise for nonirradiated cases, but such devices are still contraindicated in irradiated bone.

Intraoral soft tissue loss has been handled in various ways. In the majority of our cases, primary closure of the defect is performed, because patients have better function if sensate, normally lubricated, moist membranes are present. Tethering of the tongue is to be avoided if possible. The tongue dysfunction is more often seen secondary to reconstruction of the floor of the mouth. Lateral tongue flaps and skin grafts used to close the anterior and lateral floor-of-mouth defects frequently contract and form scar tissue that restricts movement of the oral structures. When oral tissue must be replaced, use of the platysma myocutaneous flap is our most effective method because of the pliability and thinness of the flap tissue. Unless the resection has been quite extensive, other myocutaneous flaps often result in an adynamic and obstructive tissue mass in the oral cavity.

Long-term followup was available for 91 patients; 49.5 percent of these (45/91) have died of or with cancer. There was a linear survival rate correlating to the stage of lesion, and survival was better for patients with buccal lesions than for those with alveolar lesions.

In summary, oral cavity carcinoma associated with smokeless tobacco use has been a disease of elderly, white women with histories of long-term (> 40 yr) snuff use. The disease is indolent and progressive, and it manifests a high rate of associated leukoplakia, metachronous second primary cancers of the oral cavity, and a high local recurrence rate, all manifestations of the field cancerization phenomenon. The tumor is aggressive, with a high incidence of advanced local disease, bony mandibular involvement, and regional metastases. A midrange (43 percent) 3-yr cure rate results. Therapy should be aggressive, but it is associated with significant morbidity related to cosmetic effects, mastication, deglutition, xerostomia, and dental complications. Prevention through education of young potential users of smokeless tobacco promises to be the most effective therapeutic measure.

REFERENCES

Ackerman, L.V. Verrucous carcinoma of the oral cavity. *Surgery* 23: 670-678, 1948.

Boring C.C., Squires T.S., Tung T. Cancer statistics, 1991. *CA—A Journal for Clinicians* 41: 19-36, 1991.

Winn, D.M., Blot, W.J., Shy, C.M., Pickle, L.W., Toledo, A., Fraumani, J.F., Jr. Snuff dipping and oral cancer among women in the southern United States. *New England Journal of Medicine* 304: 745-749, 1981.

Chemical Composition of Smokeless Tobacco Products¹

Klaus D. Brunnemann and Dietrich Hoffmann

ABSTRACT To date, 28 carcinogens have been identified in smokeless tobacco. In addition to certain volatile aldehydes, traces of benzo[*a*]pyrene, certain lactones, urethan, hydrazine, metals, polonium-210, and uranium-235 and -238 can be found in ST. However, the major contributors to the carcinogenicity of chewing tobacco and of snuff are the *N*-nitrosamines, especially the tobacco-specific *N*-nitrosamines. The latter are formed from the *Nicotiana* alkaloids during tobacco processing. In the United States, daily exposure to carcinogenic nitrosamines for snuff users is at least 250 times higher than for those who do not use tobacco. Although there has been a decline in the concentrations of nitrosamines in U.S. and Swedish ST products during the past decade, this trend is not evident for all snuff brands. One new snuff brand contains extremely high concentrations of carcinogenic nitrosamines. This observation adds to the urgency of the recommendation of the World Health Organization to regulate harmful substances in chewing tobacco and snuff. Similarly, flavorants and additives to tobacco should be controlled.

INTRODUCTION In the United States, we differentiate between four primary types of smokeless tobacco: Three are chewing tobaccos, namely loose leaf (scrap leaf), plug, and twist or roll; the fourth is oral snuff. Loose leaf chewing tobacco accounted for 52.7 percent of the U.S. output of total ST products in 1988 (124.5 million lb) (USDA, 1990). Loose leaf chewing tobacco consists primarily of air-cured tobacco and, in most cases, is heavily treated with licorice and sugars. Plug tobacco (7.2 percent of 1988 ST production) is the oldest form of chewing tobacco. Plug tobacco is produced from the heavier grades of leaves harvested from the top of the plant, freed from stems, immersed in a mixture of licorice and sugar, pressed into a plug, covered by a wrapper leaf, and reshaped. Plug tobacco is kept between cheek and gum and is chewed in bites. Twist or roll tobacco is less important (1.1 percent of 1988 U.S. production). Twist tobacco is made from cured burley, and air- and fire-cured leaves, which are flavored and twisted to resemble a decorative rope or pigtail.

The only U.S. tobacco product with increasing consumption is oral snuff (39.0 percent of the U.S. smokeless tobacco production in 1988). Dry snuff is made primarily from Kentucky and Tennessee fire-cured tobaccos. The initial curing requires several weeks and goes through multiple phases. In contrast to most other tobacco products, snuff undergoes an additional fermentation process. Dry snuff is processed into a powdered substance that may contain flavor and aroma additives, including spices. U.S. dry snuff, which is taken orally, is similar to European nasal snuff.

Moist snuff consists primarily of air- and fire-cured tobaccos and contains tobacco stems as well as leaves that are powdered into fine particles (containing between 20 and 55 percent moisture). Many brands of moist

¹ Supported by National Cancer Institute grant no. CA-29580.

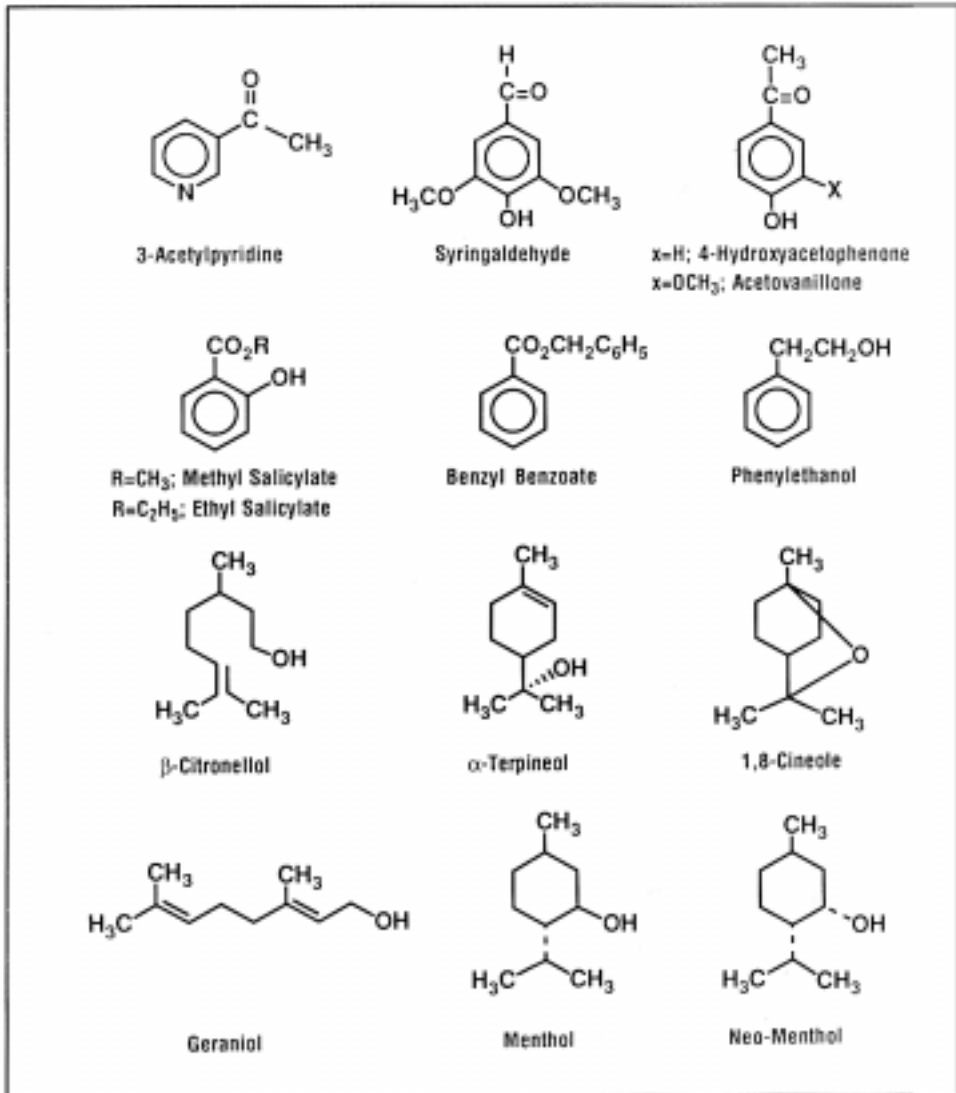
snuff are flavored with wintergreen, but mint and raspberry also are popular. Since about 1975, the consumption of moist snuff has been steadily growing in all parts of the United States, except for a temporary decline immediately after the Surgeon General's report on smokeless tobacco in 1986 (US DHHS, 1986). During the past 3 yr, the manufacture of moist snuff has again steadily risen by more than 13 percent (Smyth, 1989; USDA, 1990). Oral use of snuff, also termed "snuff dipping," means placing a pinch of the tobacco between the cheek or lip and the gums or beneath the tongue.

CHEMICAL COMPOSITION Extensive literature on the chemistry of tobacco, beginning with Brückner (1936), has led to our current knowledge that natural tobacco contains at least 3,050 different components (Robert, 1988). The quantitative composition of tobacco undergoes substantial changes during processing for smokeless tobaccos. In curing, the starch content of the leaves declines drastically, and the reducing sugars increase by 100 percent. Protein and nicotine decrease slightly. Fermentation of cured tobacco causes the contents of carbohydrates and polyphenols in the leaves to diminish. The bulk of the processed tobacco leaf before fermentation consists of carbohydrates (about 50 percent) and proteins. Other major components are alkaloids (0.5 to 5.0 percent) with nicotine as the predominant compound (85 to 95 percent of total alkaloids), terpenes (0.1 to 3.0 percent), polyphenols (0.5 to 4.5 percent), phytosterols (0.1 to 2.5 percent), carboxylic acids (0.1 to 0.7 percent), alkanes (0.1 to 0.4 percent), aromatic hydrocarbons, aldehydes, ketones, amines, nitriles, *N*- and *O*-heterocyclic hydrocarbons, pesticides, alkali nitrates (0.01 to 5.00 percent), and at least 30 metallic compounds (International Agency for Research on Cancer, 1985; Wynder and Hoffman, 1967). The given percentages apply to the *Nicotiana tabacum* species, which is grown in North America and throughout the world, but not to *N. rustica*, which is cultivated in parts of Eastern Europe and Asia Minor. The leaves of *N. rustica* may contain up to 12 percent nicotine (McMurtrey et al., 1942). Many ST formulations use plant extracts or chemicals as flavoring agents (LaVoie et al., 1989; Mookherjee, 1988; Robert, 1988; Sharma et al., 1991). Such additives may include methyl or ethyl salicylate, β -citronellol, 1,8-cineole, menthol, benzyl benzoate, and possibly coumarin (Figure 1) (LaVoie et al., 1989; Sharma et al., 1991). However, most of the flavor additives are present in only small amounts; their formulations remain trade secrets.

CARCINOGENIC AGENTS IN ST Until now, 28 tumorigenic agents have been isolated and identified in smokeless tobacco products (Table 1). These include some carcinogenic polynuclear aromatic hydrocarbons (PAH), especially benzo[*a*]pyrene (B[*a*]P). PAH originate primarily from polluted air (Campbell and Lindsay, 1956 and 1957; Wynder and Hoffmann, 1967) and, in the case of plug tobacco and snuff, probably also from fire-curing. In fact, the highest reported values for B[*a*]P were found in snuff at levels of up to 90 ppb (Ough, 1976).

The α - and β -angelica lactones have been reported in natural tobacco (Robert, 1988). These tumorigenic agents may also be added to ST as part of the flavoring mixtures made from plant extracts. A minor group of polyphenols in tobacco are the coumarins, of which scopoletin is the major

Figure 1
 Flavor compounds identified in snuff tobacco



representative (Figure 2) (Wynder and Hoffmann, 1967). Thus, it is not surprising that tobacco also contains the parent compound, coumarin (≤ 600 ppm). It is known that the fermentation of food and beverages leads to the formation of urethan (Ough, 1976). Therefore, it is not unexpected that burley tobacco, which is fermented, contains up to 400 ppm of urethan (Schmoltz et al., 1978).

The most abundant carcinogens in smokeless tobacco are some volatile aldehydes (Table 1). Although formaldehyde, acetaldehyde, and croton aldehyde are weakly carcinogenic, they contribute most likely to the carcinogenic potential of smokeless tobacco (Weybrew and Stephens, 1962). It is

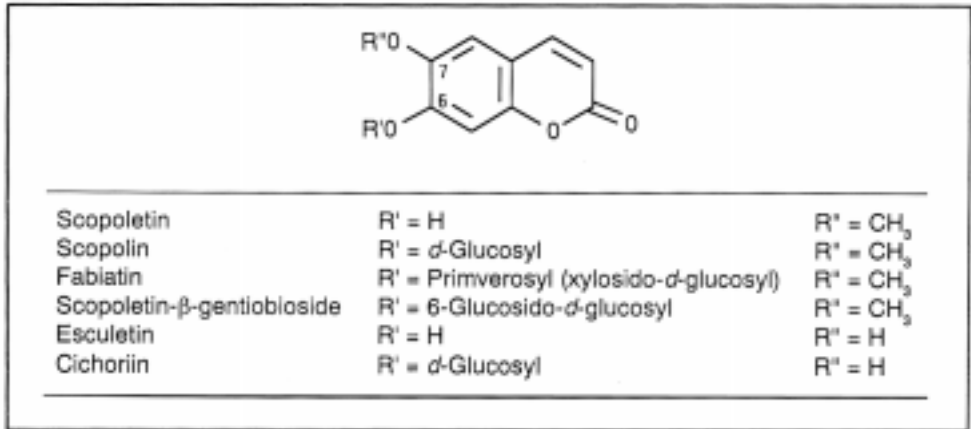
Table 1
Carcinogenic agents in tobacco

| | Type of Tobacco ^a | Concentration ^b (ng/g) |
|--|------------------------------|-----------------------------------|
| Benzo[a]pyrene | NT, S | > 0.1 - 90.0 |
| α-Angelica Lactone | NT | present |
| β-Angelica Lactone | NT | present |
| Coumarin | NT | 600 |
| Ethylcarbamate | CT | 310 - 375 |
| Volatile Aldehydes | | |
| Formaldehyde | NT, S | 1,600 - 7,400 |
| Acetaldehyde | NT, S | 1,400 - 27,400 |
| Croton aldehyde | S | 200 - 2,400 |
| Nitrosamines | | |
| Nitrosodimethylamine | CT, S | ND - 270 |
| Nitrosopyrrolidine | CT, S | ND - 760 |
| Nitrosopiperidine | CT, S | ND - 110 |
| Nitrosomorpholine | CT, S | ND - 690 |
| Nitrosodiethanolamine | CT, S | 40 - 6,800 |
| Nitrosamino Acids | | |
| Nitrososarcosine | S | ND - 2,500 |
| 3-(Methylnitrosamino)-propionic acid | CT, S | 200 - 65,700 |
| 4-(Methylnitrosamino)-butyric acid | CT, S | ND - 9,100 |
| Nitrosoazetadine-2-carboxylic acid | CT | 4 - 140 |
| Tobacco-Specific Nitrosamines | | |
| N'-Nitrosornicotine | CT, S | 400 - 147,000 |
| 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone | CT, S | ND - 18,000 |
| 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol | S | present |
| N'-Nitrosoanabasine | SM, S | present - 560 |
| Inorganic Compounds | | |
| Hydrazine | SM | 14 - 51 |
| Arsenic | NT | 500 - 900 |
| Nickel | SM, S | 180 - 2,700 |
| Cadmium | SM | 700 - 790 |
| | | (pCi/g) |
| Polonium-210 | NT, S | 0.16 - 1.22 |
| Uranium-235 | S | 2.4 |
| Uranium-238 | S | 1.91 |

^a NT, natural tobacco; SM, smoking tobacco; S, snuff; CT, chewing tobacco.

^b ND, not detected.

Figure 2
Coumarins in tobacco



known that tobacco contains a sizeable spectrum of alkyl aldehydes, which contribute to its scent. In commercial U.S. snuff brands, formaldehyde and acetaldehyde were each found up to 7,400 ppb, and croton aldehyde up to 2,400 ppb (Sharma et al., 1991).

Both air- and fire-cured tobaccos contain hydrazine. In burley leaves treated with the sucker growth inhibitor maleic hydrazide, the hydrazine content was significantly higher (Liu et al., 1974). Like other plant products, tobacco contains trace amounts of nickel, cadmium, and arsenic. These animal carcinogens were found in concentrations up to 2,700 ppb. Uranium-235 and -238 were reported only in Indian snuff, each at about 2 pCi/g tobacco (Sharma et al., 1985). The radioactive polonium-210, which decays to yield the human carcinogen radon, originates in U.S. tobacco from soil that is fertilized with phosphates rich in radium-226 (Tso et al., 1986), or from airborne particles that were taken up by the glandular hair (trichomes) of the tobacco leaf (Martell, 1974). In U.S. commercial snuff, we found between 0.16 and 1.22 pCi/g of polonium-210 (Hoffmann et al., 1987).

CARCINOGENIC N-NITROSAMINES The most detailed studies on carcinogens in smokeless tobacco have been reported for *N*-nitrosamines. These agents are present in fresh green leaf in only minute amounts and are primarily formed during curing, fermentation, and aging from secondary or tertiary amines and nitrite or nitrogen oxides. Basically, in smokeless tobacco there are three types of nitroso compounds: volatile nitrosamines, nitrosamino acids, and tobacco-specific *N*-nitrosamines (TSNA). In addition, smokeless tobacco contains *N*-nitrosodiethanolamine (NDELA), which is formed from diethanolamine, a contamination product in tobacco. Table 2 presents data on carcinogenic volatile *N*-nitrosamines (VNA) in various smokeless tobacco types from the United States, Sweden, and other European countries (Andersen et al., 1989; Brunnemann et al., 1985; Chamberlain et al., 1988; Hoffman et al., 1987; International Agency for Research on Cancer, 1985;

Table 2
Major volatile *N*-nitrosamines in smokeless tobacco, 1981 to 1990

| Country | Tobacco Type | Samples (n) | NDMA ^a (µg/kg) | NPYR ^a (µg/kg) | NMOR ^a (µg/kg) |
|----------------|-----------------|-------------|---------------------------|---------------------------|---------------------------|
| United States | Moist snuff | 32 | 3.8 - 215.0 | 7.4 - 360.0 | ND - 690.0 |
| | Dry snuff | 3 | ND - 19.0 | 72.0 - 148.0 | ND - 39.0 |
| | Chewing tobacco | 6 | 64.0 | 0.8 | 0.6 |
| Sweden | Moist snuff | 98 | 0.1 - 50.0 | ND - 95.0 | ND - 44.0 |
| | Chewing tobacco | 4 | 0.2 | 0.8 | 0.4 |
| Norway | Moist snuff | 2 | 130.0 | 8.9 | 32.0 |
| Denmark | Chewing tobacco | 8 | 5.5 | 16.0 | ND |
| United Kingdom | Nasal snuff | 5 | 4.5 - 82.0 | 1.5 - 130.0 | ND |
| | Moist snuff | 7 | 6.0 - 82.0 | 64.0 - 860.0 | ND - 1.5 |
| Germany | Nasal snuff | 7 | 2.0 - 42.0 | 5.0 - 75.0 | ND |

^a NDMA, nitrosodimethylamine; NPYR, nitrosopyrrolidine; NMOR, nitrosomorpholine; ND, not detected. Single numbers represent mean of all samples.

Tricker and Preussmann, 1989). In general, the highest amounts of VNA are found in moist and dry snuff, *N*-nitrosodimethylamine up to 265 ppb and *N*-nitrosopyrrolidine up to 760 ppb. *N*-Nitrosomorpholine (NMOR), a strong animal carcinogen, has been detected only in those U.S. snuff brands that were packed in containers lined with a morpholine-containing wax coating (Brunnemann et al., 1982).

Like volatile amines, the amino acids in tobacco, and probably also the proteins with secondary amino groups, are amenable to *N*-nitrosation. Since 1983, numerous studies have reported the presence of nitrosamino acids in smokeless tobacco (Brunnemann et al., 1983; Djordjevic, 1989; Ohshima, 1985; Tricker and Preussmann, 1989 and 1990). Until now, 10 nitrosamino acids have been identified in smokeless tobacco. Of these, nitrosoproline, nitrosothiopropine, and iso-NNAC are not carcinogenic; nitrososarcosine, 3-(methylnitrosamino)propionic acid, 4-(methylnitrosamino)butyric acid, and *N*-nitrosoazetadine-2-carboxylic acid are known carcinogens; and the remainder of the identified nitrosamino acids have so far not been bioassayed. (See Table 3.) The concentration of the nitrosamino acids depends on the nitrate or nitrite content of the tobacco as well as on the processing and aging of the tobacco.

TSNA

The most powerful carcinogens in smokeless tobacco derive from the *N*-nitrosation of the *Nicotiana* alkaloids, especially from nicotine and nornico-tine. They are formed during the curing, fermentation, and aging of tobacco. These carcinogens are present in tobacco, tobacco smoke, and

Table 3
Major *N*-nitrosamino acids in smokeless tobacco, 1989 to 1991^a

| Country and Tobacco Type | Samples (n) | NSAR ^b (µg/g) | MNPA ^b (µg/g) | MNBA ^b (µg/g) | NPRO ^b (µg/g) | iso-NNAC ^b (µg/g) |
|--------------------------|-------------|--------------------------|--------------------------|--------------------------|--------------------------|------------------------------|
| United States | | | | | | |
| Moist snuff | 10 | ND - 2.5 | 2.2 - 66.0 | 0.09 - 9.10 | 1.3 - 60.0 | 0.05 - 21.00 |
| Chewing tobacco | 1 | nd | 0.6 | 0.03 | 0.2 | 0.01 |
| Dry snuff | 3 | nd | 1.2 - 4.5 | 0.14 - 0.46 | 3.0 - 8.1 | 0.05 - 0.21 |
| Sweden | | | | | | |
| Moist snuff | 8 | 0.01 - 0.68 | 1.0 - 3.3 | 0.05 - 0.23 | 0.63 - 8.30 | 0.04 - 0.11 |
| United Kingdom | | | | | | |
| Moist snuff | 7 | 0.03 - 1.10 | 1.4 - 19.0 | 0.06 - 8.00 | 0.33 - 5.00 | nd |
| Nasal snuff | 5 | ND - 0.04 | 1.0 - 2.8 | 0.10 - 0.28 | 2.7 - 8.7 | nd |
| Germany | | | | | | |
| Nasal snuff | 7 | ND - 0.09 | 0.49 - 4.30 | 0.08 - 0.41 | 0.77 - 7.50 | nd |

^a Adapted from Djordjevic et al., 1989; Hoffmann et al., 1991; Tricker and Preussmann, 1989.

^b NSAR, *N*-nitrososarcosine; MNPA, 3-(methylnitrosamino)propionic acid; MNBA, 4-(methylnitrosamino)butyric acid; NPRO, *N*-nitrosoproline; iso-NNAC, 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid; ND, not detected; nd, not determined.

in environmental tobacco smoke. Of the seven TSNA identified in ST (Figure 3), *N*'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are the only known carcinogens in tobacco that induce oral tumors in laboratory animals. *N*'-nitrosoanabasine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol, and 4-(methylnitrosamino)-4-(3-pyridyl)butanol are carcinogenic in mice or rats (Hoffmann et al., this volume). The high carcinogenic potency and high levels of TSNA have prompted in-depth investigations on the formation and concentration of the alkaloid-derived nitrosamines in the various tobacco products (Table 4). As for the other nitrosamines, the nitrate or nitrite content and the various steps of processing are the determining factors for the yields of carcinogenic TSNA in ST products. According to analytical studies, NNN, nitrosoanabasine, and nitrosoanatabine are formed primarily from the corresponding secondary amines at the early stages of the tobacco processing, whereas TSNA such as NNK are formed from the tertiary amine nicotine (Figure 3) and occur at the later stage of tobacco curing and fermentation (Spiegelhalter and Fisher, 1991). This observation provides a partial explanation of the abundance of TSNA in snuff.

The carcinogenic risk associated with oral ST use and the major contributions of TSNA to this risk are underscored by a number of analytical data. In 1981, the National Research Council estimated the daily exposure of U.S. residents to carcinogenic nitrosamines and found the average nonsmoker is exposed to about 1 µg and the smoker of 20 cigarettes per day to about 11 to 12 µg of carcinogenic nitrosamines (U.S. National Research Council, 1981).

Table 4
TSNA in smokeless tobacco, 1981 to 1989

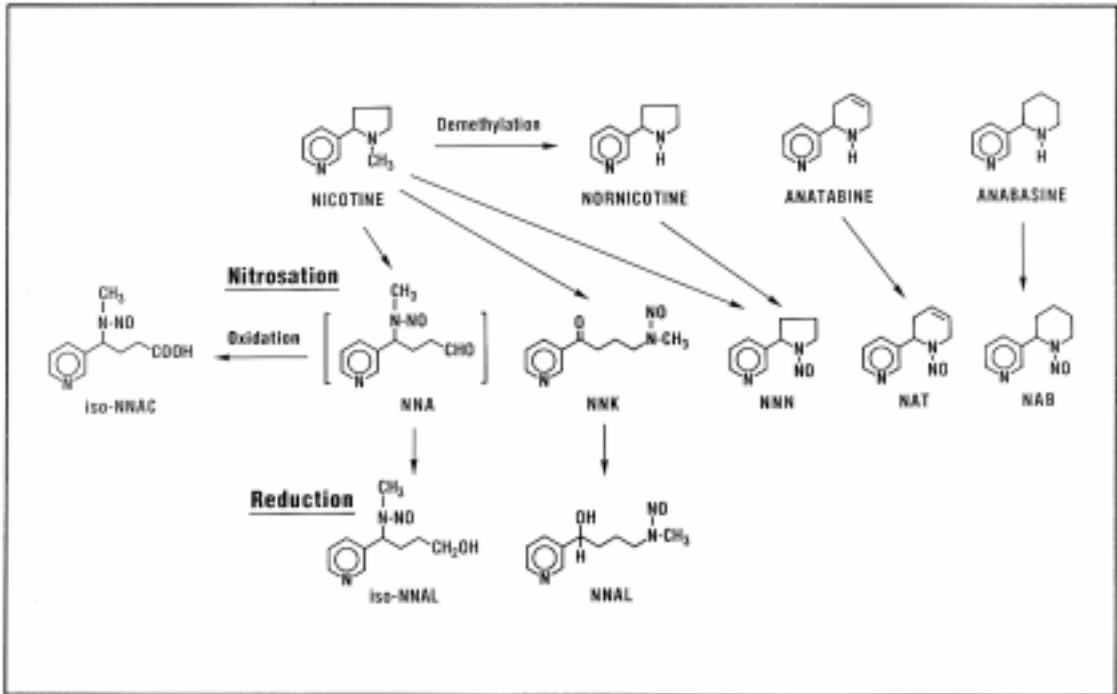
| Country and Tobacco Type | Samples (n) | NNN ^a (µg/g) | NAT ^a (µg/g) | NAB ^a (µg/g) | NNK ^a (µg/g) |
|--------------------------|-------------|-------------------------|--------------------------|-------------------------|-------------------------|
| United States | | | | | |
| Moist snuff | 16 | 0.83 - 64.00 | 0.24 - 215.00 | 0.01 - 6.70 | 0.08 - 8.30 |
| Chewing tobacco | 2 | 0.67 - 1.50 | 0.7 - 2.4 ^b | | 0.11 - 0.38 |
| Dry snuff | 6 | 9.4 - 55.0 | 11 - 40 | 0.5 - 1.2 | 0.88 - 14.00 |
| Sweden | | | | | |
| Moist snuff | 8 | 2.0 - 6.1 | 0.9 - 2.4 | 0.04 - 0.14 | 0.61 - 1.70 |
| Canada | | | | | |
| Moist snuff | 2 | 50 - 79 | 152 - 170 | 4.0 - 4.8 | 3.2 - 5.8 |
| Plug | 1 | 2.1 | 1.7 ^b | | 0.24 |
| Germany | | | | | |
| Plug | 2 | 1.4 - 2.1 | 0.36 - 0.55 ^b | | 0.03 - 0.04 |
| Nasal snuff | 7 | 2.8 - 19 | 1.0 - 5.8 ^a | | 0.58 - 6.40 |
| India | | | | | |
| Chewing tobacco | 4 | 0.47 - 0.85 | 0.40 - 0.50 ^b | | 0.13 - 0.23 |
| Zarda | 11 | 0.40 - 79.00 | 0.78 - 99 ^b | | 0.22 - 24.00 |
| USSR | | | | | |
| Nass | 4 | 0.12 - 0.52 | 0.04 - 0.33 ^b | | 0.02 - 0.11 |
| United Kingdom | | | | | |
| Moist snuff | 7 | 1.1 - 52.0 | 2.0 - 65.0 ^b | | 0.4 - 13.0 |
| Nasal snuff | 5 | 3.0 - 16.0 | 1.8 - 2.5 ^b | | 0.97 - 4.30 |

^a NNN, N-nitrosornnicotine; NAT, N-nitrosoanatabine; NAB, N-nitrosoanabasine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

^b Contains NAB.

On the basis of 1986 data for the two leading U.S. snuff brands, which had about 90 percent of the market share, the average snuff dipper, who consumes snuff at 10 g/d, is exposed to an additional 270 to 280 µg of carcinogenic nitrosamines (Hoffmann et al., 1987). Most of the nitrosamines are extracted from the tobacco during snuff dipping, as is reflected in data from saliva analysis (Hoffmann and Adams, 1981; Nair et al., 1985; Oesterdahl and Slorach, 1988; Paladino et al., 1986; Sipahimalani et al., 1984). In addition, it is strongly indicated that additional amounts of TSNA are endogenously formed during chewing (Nair et al., 1985). Recently we estimated that the average snuff dipper has a lifetime exposure to about 0.70 mmol/kg body weight of NNN and 0.03 mmol/kg body weight of NNK. These levels compare with 1.6 mmol/kg body weight of a mixture of NNN and NNK that induced tumors in the mouths of rats after oral swabbing (Hecht et al., 1986; Hoffmann et al., 1990).

Figure 3

Formation of tobacco-specific N-nitrosamines**CONTROL OF CARCINOGENS IN ST**

The chemical-analytic data and the results from bioassays and epidemiological studies (Hoffmann et al., 1992; Preston-Martin, 1991; Winn et al., 1981) strongly support the World Health

Organization's recommendation that, short of getting people to cease using tobacco, the harmful agents in chewing tobacco and snuff must be reduced (WHO, 1988). The history of the snuff analyses in the United States and Sweden has shown that a drastic reduction of the major carcinogens in ST products is feasible.

In 1981, the U.S. Environmental Protection Agency mandated a ban of maleic hydrazide diethanolamine (MH-30) for use on tobacco (US EPA, 1981). The diethanolamine part of this sucker growth inhibitor gives rise to the carcinogen NDELA (Brunnemann and Hoffmann, 1981). Following the ban of MH-30, the NDELA concentrations in smokeless tobacco declined, as shown by our monitoring of leading brands of snuff and chewing tobaccos. The reduction of NDELA values occurred gradually between 1981 and 1990, from 6,840 ppb to 94 ppb in snuff and from 224 ppb to 74 ppb in chewing tobacco (Brunnemann and Hoffmann, 1991). The concentration of the strongly carcinogenic NMOR in a snuff brand fell from 690 ppb in 1981 to a nondetectable level (< 2 ppb) in 1990 with the elimination of traces of morpholine in the packaging (Brunnemann et al., 1982; Brunnemann and Hoffmann, 1991).

While the reduction or disappearance of NDELA and NMOR was possible through the elimination of their precursors, this approach is not feasible for the reduction of nitrosamino acid and TSNA levels, because proteins and alkaloids, the precursors for these carcinogens, are integral parts of the tobacco. Nevertheless, elimination of nitrate-rich ribs and stems of certain tobacco varieties and changes in ST processing, especially of snuff, can lead to a major reduction of nitrosamines. Using NNN as an indicator for levels of TSNA, we have confirmed its gradual decrease in the two U.S. moist snuff brands that account for more than 85 percent of the current market share. In 1980, we reported 26.5 ppm and 39 ppm of NNN for brands A and B, respectively; in 1990, these levels had decreased to 10.4 and 9.6 ppm, respectively. In Sweden, the average NNN value for the leading five snuff brands in 1980 amounted to 11.4 ppm and in 1990 for three leading brands to 5.4 ppm. Two new snuff brands introduced in 1989 and 1990 on the U.S. market had NNN values of 4.1 and 3.2 ppm, respectively. Because the volatile nitrosamines and the nitrosamino acids are formed during the preparation of snuff by mechanisms similar to those leading to TSNA, their concentrations also have been reduced.

These observations strongly support the concept that product modifications can lead to a significant reduction of nitrosamines in smokeless tobacco (Table 5). Therefore, it was rather surprising that another snuff brand introduced in the United States in 1989 and 1990 contained extremely high concentrations of TSNA and other carcinogenic nitrosamines, in fact the highest ever reported (Table 5; see brand D). The increased pH of this snuff (7.7 to 8.2), compared with other U.S. brands (5.6 to 7.3), suggests that changes in manufacturing were possibly intended to facilitate the absorption of nicotine through the oral mucosa. Unprotonated nicotine, which increases steadily with increased pH above 6.2, is absorbed more rapidly than protonated nicotine (Brunnemann and Hoffmann, 1974; US DHHS, 1988).

The latter finding underscores the WHO recommendation to have the harmful substances in ST subject to governmental control (WHO, 1988), at least as it concerns the United States. Regulating agencies should be encouraged also to evaluate the flavor components and other chemical additives that are used in the manufacture of smokeless tobacco products. Any agents that are teratogenic or genotoxic should be banned.

Table 5

Alkaloids and N-nitroso compounds in moist snuff brands, 1990 to 1991^a

| | United States | | | | | Sweden |
|--|-----------------|-----------------|-----------------|----------------------|----------------------|-----------------------|
| | Brand A | Brand B | Brand C | Brand D ^b | Brand E ^c | Three Brands |
| Moisture, % | 56.0 | 57.8 | 51.8 | 50.00 - 57.8 | 51.9 | 46.6 - 54.2 |
| pH | 7.11 | 7.30 | 5.61 | 7.72 - 8.17 | 7.36 | 7.67 - 7.90 |
| Nicotine, % | 2.04 | 2.17 | 2.15 | 1.22 - 2.21 | 1.47 | 1.13 - 1.25 |
| Total Alkaloids, % | 2.18 | 2.32 | 2.32 | 1.32 - 2.38 | 1.59 | 1.24 - 1.41 |
| Volatile Nitrosamines, ^d ng/g | | | | | | |
| NDMA | ND ^g | ND ^g | ND ^g | 147 - 265 | ND ^g | 51 - 63 |
| NPYR | 44 | 59 | 120 | 245 - 757 | ND ^g | ND ^g - 155 |
| Nitrosamino Acids, ^e µg/g | | | | | | |
| NSAR | 0.06 | 0.06 | ND ^h | 0.4 - 2.5 | 0.10 | 0.03 - 0.68 |
| MNPA | 5.13 | 3.62 | 2.72 | 8.9 - 65.7 | 2.20 | 3.10 - 3.28 |
| MNBA | 0.47 | 0.26 | 0.09 | 1.9 - 9.1 | 0.20 | 0.19 - 0.23 |
| Total | 5.70 | 3.90 | 2.80 | 11.2 - 77.3 | 2.50 | 3.30 - 4.20 |
| TSNA, ^f µg/g | | | | | | |
| NNN | 10.40 | 9.57 | 4.14 | 21 - 147 | 3.20 | 5.24 - 5.67 |
| NNK | 2.19 | 3.14 | 1.24 | 6 - 18 | 0.70 | 1.37 - 2.08 |
| NAT + NAB | 9.76 | 7.90 | 2.97 | 22 - 115 | 2.00 | 2.58 - 3.47 |
| Total | 22.30 | 20.60 | 8.30 | 48 - 280 | 5.90 | 9.20 - 11.20 |

^a All values are based on dry weight. Total alkaloids include nicotine, nor nicotine, mysomine, anatabine, anabasine, 2,3'-dipyridyl, and cotinine.

^b Range of five samples bought in different stores in Texas.

^c Snuff in sachets imported from Sweden.

^d NDMA, N-nitrosodimethylamine; NPYR, N-nitrosopyrrolidine.

^e ND, not detected < 0.005 µg/g.

^f NSAR, N-nitrososarcosine; MNPA, 3-(methylnitrosamino)propionic acid; MNBA, 4-(methylnitrosamino)butyric acid.

^g ND, not detected < 0.01 µg/g.

^h TSNA, tobacco-specific N-nitrosamines; NNN, N'-nitrosanornicotine; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; NAT, N'-nitrosoanatabine; NAB, N'-nitrosoanabasine.

ACKNOWLEDGMENT

We thank Ilse Hoffmann and Jennifer Johnting for their editorial assistance. Mookherjee, B.D., Wilson, R.A. Tobacco constituents: Their importance in

REFERENCES

- Andersen, R.A., Burton, H.R., Fleming, P.D., et al. Effect of storage conditions on nitrosated, acetylated and oxidized pyridine alkaloid derivatives in smokeless tobacco products. *Cancer Research* 49: 5895-5900, 1989.
- Brückner, H. *Die Biochemie des Tabaks und der Tabakverarbeitung*. Berlin: Verlagsbuchhandlung Paul Parey, 1936.
- Brunnemann, K.D., Genoble, L., Hoffmann, D. N-nitrosamines in chewing tobacco: An international comparison. *Journal of Agricultural and Food Chemistry* 33: 1178-1181, 1985.
- Brunnemann, K.D., Hoffmann, D. The pH of tobacco smoke. *Food and Cosmetics Toxicology* 12: 115-124, 1974.
- Brunnemann, K.D., Hoffmann, D. Assessment of the carcinogenic N-nitrosodiethanolamine in tobacco products and tobacco smoke. *Carcinogenesis* 2: 1123-1127, 1981.

- Brunnemann, K.D., Hoffmann, D. Decreased concentrations of *N*-nitrosodiethanolamine and *N*-nitrosomorpholine in commercial tobacco products. *Journal of Agricultural and Food Chemistry* 39: 207-208, 1991.
- Brunnemann, K.D., Scott, J.C., Hoffmann, D. *N*-Nitrosomorpholine and other volatile *N*-nitrosamines in snuff tobacco. *Carcinogenesis* 3: 693-696, 1982.
- Brunnemann, K.D., Scott, J.C., Hoffmann, D. *N*-Nitrosoproline, an indicator for *N*-nitrosation of amines in tobacco. *Journal of Agricultural and Food Chemistry* 31: 905-909, 1983.
- Campbell, J.M., Lindsey, A.J. Polycyclic hydrocarbons extracted from tobacco: The effect upon total quantities found in smoking. *British Journal of Cancer* 10: 649-652, 1956.
- Campbell, J.M., Lindsey, A.J. Polycyclic aromatic hydrocarbons in snuff. *Chemical Industry* July 6, 1957, p. 951.
- Chamberlain, W.J., Schlotzhauer, W.S., Chortyk, O.T. Chemical composition of commercial tobacco products. *Journal of Agricultural and Food Chemistry* 36: 48-50, 1988.
- Djordjevic, M.V., Brunnemann, K.D., Hoffmann, D. Identification and analysis of a nicotine-derived *N*-nitrosamino acid and other nitrosamino acids in tobacco. *Carcinogenesis* 10: 1725-1731, 1989.
- Hecht, S.S., Rivenson, A., Braley, J., et al. Induction of oral cavity tumors in F-344 rats by tobacco-specific nitrosamines in snuff. *Cancer Research* 49: 3063-3069, 1986.
- Hoffmann, D., Adams, J.D. Carcinogenic tobacco-specific *N*-nitrosamines in snuff and in the saliva of snuff dippers. *Cancer Research* 41: 4305-4308, 1981.
- Hoffmann, D., Adams, J.D., Lisk, D., et al. Toxic and carcinogenic agents in dry and moist snuff. *Journal of the National Cancer Institute* 79: 1281-1286, 1987.
- Hoffmann, D., Brunnemann, K.D., Venitt, S. Carcinogenic nitrosamines in oral snuff. *Lancet* 1988, p. 1232.
- Hoffmann, D., Djordjevic, M.V., Brunnemann, K.D. On the control of toxic substances in smokeless tobacco. *Journal of Smoking-Related Disease* 2: 165-172, 1991.
- Hoffmann, D., Rivenson, A., Chung, F.L., et al. Relevance of nicotine-derived *N*-nitrosamines in tobacco carcinogenesis. In: *Effects of Nicotine on Biological Systems*, F. Adlkofer and K. Thureau (Editors). Boston: Birkhäuser, 1990, pp. 89-101.
- International Agency for Research on Cancer. *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Tobacco Habits Other Than Smoking; Betel Quid and Areca-Nut Chewing; and Some Related Nitrosamines* (volume 37). Lyon: IARC, 1985.
- LaVoie, E.J., Tucciarone, P., Kagan, M., et al. Quantitative analysis of steam distillates and aqueous extracts of smokeless tobacco. *Journal of Agricultural and Food Chemistry* 37: 154-157, 1989.
- Liu, Y.Y., Schmeltz, I., Hoffmann, D. Quantitative analysis of hydrazine in tobacco and cigarette smoke. *Analytical Chemistry* 46: 885-889, 1974.
- Martell, E.A. Radioactivity of tobacco trichomes and insoluble cigarette smoke particles. *Nature* 249: 215-217, 1974.
- McMurtrey, J.E., Jr., Bacon, C.W., Ready, D. Growing tobacco as a source of nicotine. *USDA Technical Bulletin* 820: 1-38, 1942.
- Mookherjee, B.D., Wilson, R.A. Tobacco constituents: Their importance in flavor and fragrance chemistry. *Recent Advances in Tobacco Science* 14: 114-168, 1988.
- Nair, J., Ohshima, H., Friesen, M., et al. Tobacco-specific and betel nut-specific *N*-nitroso compounds: Occurrence in saliva and urine of betel quid chewers and formation in vitro by nitrosation of betel quid. *Carcinogenesis* 6: 295-303, 1985.
- Oesterdahl, B.G., Slorach S. Tobacco-specific *N*-nitrosamines in the saliva of habitual snuff dippers. *Food Additives and Contamination* 57: 581-586, 1988.
- Ohshima, H., Nair, J., Bourgarde, M.-C., et al. Identification and occurrence of two new *N*-nitrosamino acids in tobacco products: 3-(*N*-nitroso-*N*-methylamino)propionic acid and 4-(*N*-nitroso-*N*-methylamino)butyric acid. *Cancer Letters* 26: 153-162, 1985.
- Ough, C.S. Ethyl carbamate in fermented beverages and foods: I. Naturally occurring ethyl carbamate. *Journal of Agricultural and Food Chemistry* 24: 323-328, 1976.
- Palladino, G., Adams, J.D., Brunnemann, K.D., et al. Snuff dipping in college students: A clinical profile. *Military Medicine* 151: 342-346, 1986.
- Preston-Martin, S. Evaluation of the evidence that tobacco-specific nitrosamines (TSNA) cause cancer in humans. *Critical Review of Toxicology* 21: 295-298, 1991.
- Robert, N.L. Natural tobacco flavor. *Recent Advances in Tobacco Science* 14: 49-81, 1988.
- Schmeltz, I., Chiang, K.G., Hoffmann, D. Formation and determination of ethyl carbamate in tobacco and tobacco smoke. *Journal of Analytical Toxicology* 2: 265-268, 1978.
- Sharma, A.K., Prokopczyk, B., Hoffmann, D. Supercritical fluid extraction of moist snuff. *Journal of Agricultural and Food Chemistry* 39: 508-510, 1991.
- Sharma, P.K., Lal, N., Nagpaul, K.K. Study of trace amounts of U in snuff. *Health Physics* 48: 811-812, 1985.

- Sipahimalani, A.T., Chadka, M.S., Bhide, S.V., et al. Detection of *N*-nitrosamines in the saliva of habitual chewers of tobacco. *Food Chemistry and Toxicology* 22: 261-264, 1984.
- Smyth, J.H. Moist snuff sales gain. *Tobacco Reporter* 116 (8): 30-31, 1989.
- Spiegelhalder, B., Fischer, S. Formation of tobacco-specific nitrosamines. *Critical Reviews of Toxicology* 21: 241, 1991.
- Tricker, A.R., Preussmann, R. Preformed nitrosamines in smokeless tobacco. In: *Tobacco and Cancer: Perspectives in Preventive Research*, A.P. Maskens, R. Molimard, R. Preussmann, and J.W. Wilmer (Editors). Amsterdam: Excerpta Medica, 1989, pp. 35-47.
- Tricker, A.R., Preussmann, R. Exposure to nicotine-derived *N*-nitrosamines from smokeless tobacco and evidence against endogenous formation. In: *Effects of Nicotine on Biological Systems*, F. Adlkofer and K. Thureau (Editors). Boston: Birkhäuser, 1990, pp. 109-113.
- Tso, T.C., Harley, N., Alexander, L.T. Source of lead-210 and polonium-210 in tobacco. *Science* 153: 880-882, 1966.
- U.S. Department of Agriculture. *Tobacco Situation and Outlook* 213: 18, 1990.
- U.S. Department of Health and Human Services. *The Health Consequences of Using Smokeless Tobacco: A Report of the Advisory Committee to the Surgeon General*. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, National Cancer Institute. DHHS Publication No. 86-2874, 1986.
- U.S. Department of Health and Human Services. *The Health Consequences of Smoking: Nicotine Addiction. A Report of the Surgeon General*. U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control, Center for Health Promotion and Education, Office on Smoking and Health. DHHS Publication No. (CDC) 88-8406, 1988.
- U.S. Environmental Protection Agency. Maleic hydrazine: Notification of issuance of intent to suspend pesticide registrations. *Federal Register* 46(179): 45999-46000, 1981.
- U.S. National Research Council. *The Health Effects of Nitrate, Nitrite and N-nitroso Compounds*. Washington, DC: U.S. National Research Council, 1981, p. 51.
- Weybrew, J.A., Stephens, R.L. Survey of the carbonyl contents of tobacco. *Tobacco Science* 6: 53-57, 1962.
- Winn, D.M., Blot, W.J., Shy, C.M., Picke, L.W., Toledo, A., Fraumeni, J.F. Snuff dipping and oral cancer among women in the southern United States. *New England Journal of Medicine* 304: 745-749, 1981.
- World Health Organization. *Smokeless Tobacco Control*. Technical Report Series 773. Geneva: WHO, 1988.
- Wynder, E.L., Hoffmann, D. *Tobacco and Tobacco Smoke: Studies in Experimental Carcinogenesis*. New York: Academic Press, 1967.

Carcinogenesis of Smokeless Tobacco¹

Dietrich Hoffmann, Abraham Rivenson, and Stephen S. Hecht

ABSTRACT Smokeless tobacco induces tumors in the oral cavity of rats. Of the 28 known carcinogens in tobacco, the major contributors to the carcinogenic activity of ST are the nitrosamines, especially the tobacco-specific nitrosamines (TSNA). Among seven TSNA that have been identified in ST, *N*'-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are the most potent carcinogens. A total dose of 450 µg NNK is sufficient to induce tumors in rats, and 420 µg NNK suffice to elicit tumors in mice. A mixture of NNN and NNK causes oral tumors in rats at a dose comparable to that ingested by a lifelong snuff dipper. In accordance with the recommendations of the World Health Organization, harmful substances in ST should be reduced and should be subject to governmental control and regulations.

INTRODUCTION Epidemiological investigations have revealed that tobacco chewers and snuff dippers face an increased risk for cancer of the oral cavity and pharynx. Chewing of smokeless tobacco has also been linked with cancer of the nasal cavity, esophagus, pancreas, and urinary bladder (International Agency for Research on Cancer, 1985; Kabat et al., 1986; Winn, 1992). Topical application of extracts from tobacco and from snuff has induced tumors in mouse skin. Such extracts have also exhibited cocarcinogenic activity (IARC, 1985; US DHHS, 1986). In some bioassays, ST products were tested for tumorigenicity by painting the oral cavity with extracts, by implantation, or by repeated insertion of chewing tobacco or snuff into the cheek pouch (Table 1) (Gothoskar et al., 1975; Hecht et al., 1986; Homburger, 1971; Homburger et al., 1976; Kandarkar et al., 1981; Peacock et al., 1960). Although most of these bioassays have led to epithelial hyperplasia in the mouth or cheek pouches, none of them actually induced oral tumors.

Hirsch and Thilander (1981) developed a new method for the bioassay of ST in the oral cavity of laboratory animals. A canal is created in the lower lip of rats by surgery, and snuff is inserted and exchanged twice daily (Hirsch and Thilander, 1981). The first assays with this technique led to hyperplasia, dysplasia, and other lesions in the lip canal and oral cavity but not to oral tumors (Hirsch and Johansson, 1983; Hirsch and Thilander, 1981). Subsequently, other investigators modified the lip canal methodology and assayed commercial snuff. In one case, 2 of 32 rats treated with snuff developed epithelial tumors in the lip canal and 1 rat had a papilloma of the palate (Hecht et al., 1986). Johansson and colleagues (1989) induced five oral tumors and two nasal tumors in a group of 29 rats in a long-term assay with snuff. Together with experimentally induced *herpes simplex* virus type 1 (HSV-1) infection, snuff treatment caused squamous cell carcinoma of the oral cavity in two of seven rats (Hirsch et al., 1984). Park and associates (1986) repeatedly infected the buccal pouches of hamsters with either

¹ Supported by National Cancer Institute grant no. CA-29580.

Table 1
Bioassays for the induction of oral tumors with ST

| Material ^a | Animal ^b | Method | Result | Reference |
|-----------------------|---------------------|---|--|----------------------------|
| CT | Hamster | Implantation into cheek pouch | No oral tumors | Peacock et al., 1960 |
| CT | Hamster | Thrice weekly insertion of CT with lime into cheek pouch | No neoplastic changes | Kandarkar et al., 1981 |
| CT | Rat | Painting of oral cavity with extract twice weekly | No oral tumors | Gothoskar et al., 1975 |
| S | Hamster, M | Feeding of S with diet (20%) | No oral tumors | Homburger et al., 1976 |
| S | Hamster | Insertion in cheek pouch | No tumors | Peacock et al., 1960 |
| S | Hamster | Daily attachment of cartridge containing 0.5 g S to lower incisors for 30 min; 1 yr | No oral tumors | Homburger, 1971 |
| S | Rat, M | Twice daily painting of oral cavity and lips with concentrated S extract | No oral tumors | Hecht et al., 1986 |
| S | Rat | Lip canal; twice daily insertion of 0.2 g S; up to 18 to 22 mo | Epithelial hyperplasia and dysplasia, no oral tumors | Hirsch and Thilander, 1981 |
| S | Rat, M | Lip canal; one daily insertion of 0.05 g S; up to 25 mo | 3/32 oral tumors | Hecht et al., 1986 |
| S | Rat | Lip canal; twice daily insertion of 0.2 g S; up to 18 mo | 0/10 oral tumors | Hirsch et al., 1984 |
| | | As above plus HSV-1 infection | 2/7 oral tumors | |
| S | Rat | Lip canal; twice daily insertion of 0.1 g; up to 20 mo | 5/29 oral and lip tumors 2/29 nasal tumors | Johansson et al., 1989 |
| S | Hamster | Insertion twice daily of 0.15 g S into buccal pouch; up to 6 mo | 0/15 oral tumors | Park et al., 1986 |
| | | As above plus HSV-1 infection | 10/20 oral carcinoma | |
| | | As above plus HSV-2 infection | 11/20 oral carcinoma | |

^a CT, chewing tobacco; S, snuff.

^b M, males.

HSV-1 or HSV-2 and inserted 150 mg of commercial snuff into the infected pouches twice daily. After 6 mo, 10 of 20 hamsters inoculated with HSV-1 and exposed to snuff developed invasive squamous cell carcinoma in the buccal pouch; in the group treated with HSV-2 and snuff, 11 of 20 hamsters developed carcinoma in the buccal pouch. None of the animals treated with either virus type or with snuff alone developed oral tumors within 6 mo (Park et al., 1986).

Thus, bioassays support the epidemiological observation that the long-term use of ST leads to cancer in the oral cavity. The next goal in explaining the causes of oral cancer by tobacco is the identification of those agents among the more than 3,050 known tobacco constituents (Roberts, 1988) that make smokeless tobacco carcinogenic. Until now, 28 known carcinogens have been reported in processed tobacco (Table 2) (Brunnemann and Hoffmann, 1992; Hoffmann et al., 1991; IARC, 1987 and 1988).

CARCINOGENS IN SMOKELESS TOBACCO The contamination of processed tobacco with benzo[*a*]pyrene and other carcinogenic polynuclear aromatic hydrocarbons (PAH) stems mostly from polluted air (Campbell and Lindsey, 1956 and 1957; Wynder and Hoffmann, 1967). However, the levels of PAH appear to be too low to make a significant contribution to the carcinogenicity of snuff in the oral cavity (IARC, 1973). The possible carcinogenic effect of α - and β -angelica lactones cannot be evaluated at this time, because these tobacco constituents have not yet been assayed by oral application. Coumarin applied to rats as a dietary component induced tumors in the bile duct but not in the upper digestive tract (IARC, 1976). Urethan (ethyl carbamate), when given in the drinking water to mice, induces primarily lung tumors; in rats, urethan causes tumors at multiple sites, but predominantly hepatomas. In hamsters, urethan causes tumors of the forestomach and melanotic tumors of the skin (IARC, 1975).

Three carcinogenic volatile aldehydes have been detected in smokeless tobacco; their concentrations in oral snuff are lower than in other processed tobacco types and products (Brunnemann and Hoffmann, 1992; Wynder and Hoffmann, 1967). Acetaldehyde, for example, is found in the major tobacco types in amounts up to 270,000 ng/g (270 ppm) (Weybrew and Stephens, 1962), yet the data reported for U.S. commercial snuff showed about 1/10 of those concentrations (Table 2). The International Agency for Research on Cancer (1987) regards formaldehyde as an animal carcinogen and as probably carcinogenic to humans. After inhalation of formaldehyde (14.3 ppm), rats developed squamous cell carcinoma of the nasal cavity (Albert et al., 1982; IARC, 1982 and 1987). Inhalation of acetaldehyde produced adenocarcinoma and squamous cell carcinoma of the nasal mucosa in rats and laryngeal carcinoma in hamsters (IARC, 1987). Acetaldehyde is also known to inhibit the repair of DNA lesions (Grafström et al., 1986). Croton aldehyde, fed to rats in the drinking water (0.6 mM), induced benign and malignant tumors of the liver (Chung et al., 1986). Data are lacking for the carcinogenicity of volatile aldehydes in the upper digestive tract, but one would assume that these components contribute to the carcinogenicity of ST.

Table 2
Carcinogenic agents in tobacco

| | Tobacco Type ^b | Concentration, ng/g ^c | IARC Evaluation of Evidence of Carcinogenicity ^a | |
|--|---------------------------|----------------------------------|---|------------|
| | | | In Laboratory Animals | In Humans |
| Benzo[a]pyrene | NT, S | > 0.1 - 90.0 | Sufficient | Probable |
| α-Angelica Lactone | NT | Present | | |
| β-Angelica Lactone | NT | Present | | |
| Coumarin | NT | 600 | Limited | |
| Ethyl Carbamate | CT | 310 - 375 | Sufficient | |
| Volatile Aldehydes | | | | |
| Formaldehyde | NT, S | 1,600 - 7,400 | Sufficient | Probable |
| Acetaldehyde | NT, S | 1,400 - 27,400 | Sufficient | |
| Crotonaldehyde | S | 200 - 2,400 | | |
| Nitrosamines | | | | |
| Nitrosodimethylamine | CT, S | ND - 270 | Sufficient | Probable |
| Nitrosopyrrolidine | CT, S | ND - 760 | Sufficient | |
| Nitrosopiperidine | CT, S | ND - 110 | Sufficient | |
| Nitrosomorpholine | CT, S | ND - 690 | Sufficient | |
| Nitrosodiethanolamine | CT, S | 40 - 6,800 | Sufficient | |
| Nitrosamino Acids | | | | |
| Nitrososarcosine | S | ND - 2,500 | Sufficient | |
| 3-(Methylnitrosamino)-propionic acid | CT, S | 200 - 65,700 | | |
| 4-(Methylnitrosamino)-butyric acid | CT, S | ND - 9,100 | | |
| Nitrosoazetadine-2-carboxylic acid | CT | 4 - 140 | | |
| Tobacco-Specific Nitrosamines | | | | |
| N'-Nitrosonornicotine | CT, S | 400 - 147,000 | Sufficient | |
| 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone | CT, S | ND - 18,000 | Sufficient | |
| 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol | S | Present | | |
| N'-Nitrosoanabasine | SM, S | Present - 560 | Limited | |
| Inorganic compounds | | | | |
| Hydrazine | SM | 14 - 51 | Sufficient | Inadequate |
| Arsenic | NT | 500 - 900 | Inadequate | Sufficient |
| Nickel | SM, S | 180 - 2,700 | Sufficient | Sufficient |
| Cadmium | SM | 700 - 790 | Sufficient | Probable |
| | | p Ci/g | | |
| Polonium-210 | NT, S | 0.16 - 1.22 | Sufficient | Sufficient |
| Uranium-235 and -238 | S | 2.4, 1.91 | | |

^a Absence of a designation indicates that IARC has not evaluated.

^b NT, natural tobacco; SM, smoking tobacco; S, snuff; CT, chewing tobacco.

^c ND, not determined.

There is no information on the possible contribution of inorganic carcinogenic ST constituents to the increased oral cancer risk of chewers and snuff dippers. However, tobacco chewers have a higher level of trace amounts of some metals in the oral mucosa than do nontobacco users (Robertson and Bray, 1988). Of special concern is the human carcinogen polonium-210, which is a decay product of radon (IARC, 1988). Data on the polonium-210 content of oral tissues are needed before one can consider the carcinogenic effect of polonium-210 on the oral cavity of chewers.

N-NITROSAMINES Processing of tobacco to chewing tobacco or snuff yields several types of nitrosamines. Precursors to such carcinogens are nitrate or nitrite, the amino acids and proteins of the tobacco, and the *Nicotiana* alkaloids. These constituents form volatile nitrosamines (VNA), nitrosamino acids (NNA), and tobacco-specific nitrosamines (TSNA), respectively. In addition, residues of morpholine and diethanolamine from tobacco contaminants can serve as precursors for the corresponding nitrosamines.

Nitrosamines are generally organ-specific carcinogens: they induce benign and malignant tumors in specific organs, independent of site and mode of application (Table 3). The VNA are powerful animal carcinogens. For example, a daily dose of 40 µg of nitrosodimethylamine in the drinking water during the lifetime of rats induces liver tumors in 50 percent of the animals (Peto et al., 1984). None of the nitrosamines listed in Table 3, except nitrosodiethanolamine, are known to induce oral cavity tumors. Nitrosodiethanolamine causes tumors of the upper aerodigestive tract in hamsters in addition to tracheal tumors after swabbing of the oral cavity with an aqueous solution (Hoffmann et al., 1983).

So far, 10 nitrosamino acids have been identified in ST (Brunnemann and Hoffmann, 1991). Only six of these have been assayed for carcinogenicity. Nitrosoproline and nitrosothiopropine are inactive in mice and rats. Nitrososarcosine and 3-(methylnitrosamino)propionic acid induce liver tumors in mice or rats, whereas 4-(methylnitrosamino)butyric acid induces bladder cancer in rats (Preussmann and Stewart, 1984; Rivenson et al., 1989). None of the nitrosamino acids have been assayed by topical application to the oral cavity, although their relatively high concentrations in snuff (up to 65 ppm) make such tests highly desirable.

TSNA The most important carcinogens in ST are TSNA. They are formed by nitrosation of the *Nicotiana* alkaloids during curing, fermenting, and aging of the leaves. Seven TSNA have been identified in ST (Brunnemann and Hoffmann, this volume). Two of these, *N'*-nitrososornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridine)-1-butanol (NNAL), are powerful carcinogens in mice, rats, and hamsters, inducing tumors in the lung, upper aerodigestive tract, or pancreas (Table 4). *N'*-Nitrosoanabasine is a weak esophageal carcinogen in rats. *N'*-Nitrosoanatabine (NAT) and 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid (iso-NNAC) are not carcinogenic and 4-(methylnitrosamino)-4-(3-pyridyl)-1-butanol (iso-NNAL) has not been bioassayed.

Table 3
Organ-specific carcinogenicity of nitrosamines in mice, rats, and hamsters

| | Major Target Organs for Carcinogenicity | | |
|-----------------------|---|---------------------|------------------------|
| | Mice | Rats | Syrian Golden Hamsters |
| Nitrosodimethylamine | Liver, lung | Liver, kidney | Liver |
| Nitrosopyrrolidine | Lung | Liver | Lung, trachea |
| Nitrosopiperidine | Lung, forestomach | Esophagus, liver | Trachea, nasal cavity |
| Nitrosomorpholine | Liver, lung | Liver, nasal cavity | Trachea, nasal cavity |
| Nitrosodiethanolamine | | Liver | Trachea, nasal cavity |

Table 4
Carcinogenicity of TSNA

| | Animal (Strain) | Route of Application | Principal Target Organ | Dose, mmol/Animal |
|-------------------|-----------------------|---------------------------|-------------------------|-------------------|
| TSNA ^a | | | | |
| NNN | Mouse | Topical (TI) ^b | None | 0.028 |
| | Mouse (A/J) | Intraperitoneal | Lung | 0.1 |
| | Rat (F-344) | Subcutaneous | Nasal cavity, esophagus | 0.2 - 3.4 |
| | Rat (F-344) | Oral | Esophagus, nasal cavity | 1.0 - 3.6 |
| | Rat (Sprague-Dawley) | Oral | Nasal cavity | 8.8 |
| | Syrian golden hamster | Subcutaneous | Trachea, nasal cavity | 0.9 - 2.1 |
| NNAL | Mouse (A/J) | Intraperitoneal | Lung | 0.12 |
| | Rat (F-344) | Subcutaneous | Lung, pancreas | 0.32 |
| NAB | Rat (F-344) | Oral | Esophagus | 3 - 12 |
| | Syrian golden hamster | Subcutaneous | None | 2 |
| NAT | Rat (F-344) | Subcutaneous | None | 2.8 |
| iso-NNAC | Mouse (A/J) | Intraperitoneal | None | 0.2 |

Source: Hecht and Hoffmann (1989), except iso-NNAC, Rivenson et al. (1989).

^a For data on NNK, see Table 20-5.

^b TI, tumor-initiating assay with TPA as promoter; NNN, N'-nitrosomnicotine; NNAL, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol; NAB, N'-nitrosoanabasine; NAT, N'-nitrosoanatabine; iso-NNAC, 4-(methylnitrosamino)-4-(3-pyridyl)butyric acid.

The most powerful carcinogen in ST is 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) (Table 5). NNK induces lung cancer and tumors of the nasal cavity and liver in rats. When NNK is administered in drinking water to rats, it also induces malignant tumors of the exocrine pancreas. In fact, NNK and its enzymatic reduction product NNAL are the only pancreas carcinogens known to occur in ST and in tobacco smoke. This finding is significant because both tobacco smokers and ST users have an increased risk for cancer of the exocrine pancreas (IARC, 1985; US DHHS, 1986 and 1989). NNK is also a weak transplacental carcinogen in mice and hamsters, resulting in lung tumors in offspring (Anderson et al., 1989; Anderson and Rice, this volume; Correa et al., 1990). Perhaps the most important aspect of NNK is its high carcinogenic potency; 450 µg induce tumors in rats (Belinsky et al., 1990).

NNN and NNK as an admixture have induced oral tumors in laboratory animals, when an aqueous solution of these TSNA was used to swab the oral cavity of rats twice daily for up to 131 wk (Hecht et al., 1986). The total dose applied amounted to about 97 mg of NNN (1,400 µmol/kg) and 19 mg of NNK (240 µmol/kg). Based on the analytical data for the best-selling U.S. snuff brand in 1980 (Hoffmann and Adams, 1981), consumption of 10 g of snuff per day exposes the oral cavity of a snuff dipper during a lifetime to about 5,700 mg of NNN (460 µmol/kg) and to about 360 mg of NNK (25 µmol/kg). This comparison supports the concept that the TSNA greatly contribute to the increased risk of snuff dippers for cancer of the oral cavity. The estimate of exposure of snuff dippers to the carcinogenic NNN and NNK did not consider the likely event that additional amounts of TSNA are formed endogenously during chewing (Tsuda and Kurasima, 1991).

DISCUSSION The bioassay data strongly support the epidemiological observation that ST is carcinogenic to humans. Twenty-eight carcinogens have been identified in chewing tobacco and snuff. The high concentrations of *N*-nitrosamines in ST, and especially the high levels of TSNA, are of great concern. The TSNA derive exclusively from the tobacco alkaloids, predominantly from the pharmacologically active nicotine, and are formed during tobacco processing. A bioassay has shown that a mixture of NNN and NNK induces oral tumors in rats. The orally applied amounts of NNN and NNK are comparable to the cumulative doses to which a snuff dipper is exposed during a lifetime.

Emphasis should be placed on educating the public to the hazards of tobacco chewing and snuff dipping. Because of the millions of ST users throughout the world, urgent support for the recommendations of the World Health Organization to regulate the harmful substances in ST is needed (WHO, 1988). As discussed in Brunnemann and Hoffmann (1992), significant reductions in the unacceptably high concentrations of carcinogens in tobacco, especially those of the nitrosamines, are feasible.

Table 5
Carcinogenicity of NNK in laboratory animals

| | Route of Application | Principal Target Organ | Dose, mmol/Animal | Reference |
|------------------------|---------------------------|-----------------------------|-------------------|--|
| Mouse (Sencar) | Topical (TI) ^a | Skin | 0.028 | Hecht and Hoffmann, 1989 |
| | Oral | Lung, nasal cavity, liver | 0.6 - 1.2 | Prokopczyk et al., 1992 |
| Mouse (A/J) | Intraperitoneal | Lung | 0.01 - 0.12 | Hecht and Hoffmann, 1989 Morse et al., 1991 |
| Rat (F-344) | Subcutaneous | Lung, nasal cavity, liver | 0.2 - 2.8 | Hecht and Hoffmann, 1989 |
| | | Lung, nasal cavity | 0.0025 - 3.6 | Belinsky et al., 1990 |
| | Oral (in drinking water) | Lung, liver, pancreas | 0.075 - 0.31 | Hecht and Hoffmann, 1989 |
| | Oral (by gavage) | Liver, lung | 1.3 | Lijinsky et al., 1990 |
| | Intravesical | Lung, liver | 1.5 | Lijinsky et al., 1990 |
| Syrian Golden Hamsters | Subcutaneous | Trachea, lung, nasal cavity | 0.005 - 0.9 | Hoffmann et al., 1991 |

^a TI = tumor-initiating assay with TPA as a promoter.

REFERENCES

- Albert, R.E., Sellakumar, A.R., Laskin, S., et al. Gaseous formaldehyde and hydrogen chloride induction of nasal cancer in the rat. *Journal of the National Cancer Institute* 68: 597-603, 1982.
- Anderson, L.M., Hecht, S.S., Dixon, D.E., et al. Evaluation of the transplacental tumorigenicity of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in mice. *Cancer Research* 49: 3770-3775, 1989.
- Belinsky, S.A., Foley, J.F., White, C.M., et al. Dose-response relationship between O⁶-methylguanine formation in Clara cells and induction of pulmonary neoplasia in the rat by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. *Cancer Research* 50: 3772-3780, 1990.
- Brunnemann, K.D., Hoffmann, D. Analytical studies on tobacco-specific N-nitrosamines in tobacco and tobacco smoke. *Critical Reviews of Toxicology* 2: 235-240, 1991.
- Campbell, J.M., Lindsey, A.J. Polycyclic hydrocarbons extracted from tobacco: The effect upon total quantities found in smoking. *British Journal of Cancer* 10: 649-652, 1956.
- Campbell, J.M., Lindsey, A.J. Polycyclic hydrocarbons in cigar smoke. *British Journal of Cancer* 11: 192-195, 1957.
- Chung, F.-L., Tanaka, T., Hecht, S.S. Induction of liver tumors in F344 rats by crotonaldehyde. *Cancer Research* 46: 1285-1289, 1986.
- Correa, A., Joshi, P.A., Castonguay, A., et al. The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone is an active transplacental carcinogen in Syrian golden hamsters. *Cancer Research* 50: 3435-3438, 1990.
- Gothoskar, S.V., Sant, S.M., Ranadive, K.J. Effect of tobacco and lime on oral mucosa of rats fed on vitamin B deficient diet. *Indian Journal of Cancer* 12: 424-429, 1975.

- Grafström, R.C., Curren, R.D., Yang, L.L. Acetaldehyde-induced inhibition of DNA repair and potentiation of N-nitroso compounds-induced mutagenesis in cultured human cells. *Progress in Clinical and Biological Research* 209A: 255-264, 1986.
- Hecht, S.S., Hoffmann, D. The relevance of tobacco-specific nitrosamines to human cancer. *Cancer Surveys* 8: 273-294, 1989.
- Hecht, S.S., Rivenson, A., Braley, J., et al. Induction of oral cavity tumors in F344 rats by tobacco-specific nitrosamines and snuff. *Cancer Research* 46: 4162-4166, 1986.
- Hirsch, J.-M., Johansson, S.L. Effect of long-term application of snuff on the oral mucosa: An experimental study in the rat. *Journal of Oral Pathology* 12: 187-198, 1983.
- Hirsch, J.-M., Johansson, S.L., Vahlne, A. Effect of snuff and herpes simplex virus-1 on rat oral mucosa: Possible associations with the development of squamous cell carcinoma. *Journal of Oral Pathology* 13: 52-62, 1984.
- Hirsch, J.-M., Thilander, H. Snuff-induced lesions of the oral mucosa: An experimental model in the rat. *Journal of Oral Pathology* 10: 342-353, 1981.
- Hoffmann, D., Adams, J.D. Carcinogenic tobacco-specific N-nitrosamines in snuff and in the saliva of snuff dippers. *Cancer Research* 41: 4305-4308, 1981.
- Hoffmann, D., Castonguay, A., Rivenson, A., et al. Comparative carcinogenicity and metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and N'-nitrosornicotine in Syrian golden hamsters. *Cancer Research* 41: 2386-2393, 1981.
- Hoffmann, D., Djordjevic, M.V., Brunnemann, K.D. On the control of toxic substances in smokeless tobacco. *Journal of Smoking-Related Disease* 3: 165-172, 1991.
- Hoffmann, D., Rivenson, A., Adams, J.D. Effects of route of administration and dose on the carcinogenicity of N-nitrosodiethanolamine in the Syrian golden hamster. *Cancer Research* 43: 2521-2524, 1983.
- Homburger, F. Mechanical irritation, polycyclic hydrocarbons, and snuff. Effects on facial skin, cheek pouch, and oral mucosa in Syrian hamsters. *Archives of Pathology* 91: 411-417, 1971.
- Homburger, F., Hsueh, S.-S., Russfield, A.B., et al. Absence of carcinogenic effects of chronic feeding of snuff in inbred Syrian hamsters. *Toxicology and Applied Pharmacology* 35: 515-521, 1976.
- International Agency for Research on Cancer. *IARC Monographs on the Evaluation of the Carcinogenic Risk to Humans: Benzo[a]pyrene* (volume 3). Lyon: IARC, 1973, pp. 91-136.
- International Agency for Research on Cancer. *IARC Monographs on the Evaluation of the Carcinogenic Risk to Humans: Urethane* (volume 7). Lyon: IARC, 1975, pp. 111-140.
- International Agency for Research on Cancer. *IARC Monographs on the Evaluation of the Carcinogenic Risk to Humans: Coumarin* (volume 10). Lyon: IARC, 1976, pp. 113-119.
- International Agency for Research on Cancer. *IARC Monographs on the Evaluation of the Carcinogenic Risk to Humans: Formaldehyde* (volume 29). Lyon: IARC, 1982, pp. 345-389.
- International Agency for Research on Cancer. *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Tobacco Habits Other Than Smoking; Betel-Quid and Areca-Nut Chewing; and Some Related Nitrosamines* (volume 37). Lyon: IARC, 1985.
- International Agency for Research on Cancer. *IARC Monographs on the Evaluation of the Carcinogenic Risks to Humans: Overall Evaluations of Carcinogenicity: An Updating of IARC Monographs* (volume 1 to 42, supplement 7). Lyon: IARC, 1987.
- International Agency for Research on Cancer. *IARC Monographs on the Evaluation of the Carcinogenic Risk to Humans: Man-Made Mineral Fibres and Radon* (volume 43). Lyon: IARC, 1988.
- Johansson, S.L., Hirsch, J.-M., Larsson, P.-A., et al. Snuff-induced carcinogenesis: Effect of snuff in rats initiated with 4-nitroquinoline-N-oxide. *Cancer Research* 49: 3063-3069, 1989.
- Kabat, G.C., Dieck, G.S., Wynder, E.L. Bladder cancer in nonsmokers. *Cancer* 57: 362-367, 1986.
- Kandarkar, S.V., Hasegkar, N.N., Sirsat, S.M. Optical and ultrastructural pathology of vitamin A pretreated hamster cheek pouch exposed to lime [Ca(OH)₂] and tobacco over total lifespan. *Neoplasia* 28: 729-737, 1981.
- Lijinsky, W., Farnsworth, D., Farrelly, J., et al. Tumors of the bladder and other organs induced by intravesical administration of N-nitroso compounds to rats. Annual Report, Basic Research in Progress NCI, Frederick Cancer Research and Development Center, September 1990, pp. 76-78.
- Morse, M.A., Eklind, K.I., Hecht, S.S., et al. Structure-activity relationship for inhibition of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone lung tumorigenesis by arylalkyl isothiocyanates in A/J mice. *Cancer Research* 51: 1846-1850, 1991.
- Park, N.H., Sapp, J.P., Herbosa, E.G. Oral cancer induced in hamsters with herpes simplex infection and simulated snuff dipping. *Oral Surgery, Oral Medicine, Oral Pathology* 62: 164-168, 1986.
- Peacock, E.D., Jr., Greenberg, B.G., Brawley, B.W. The effect of snuff and tobacco on the production of oral carcinoma: An experimental and epidemiological study. *Annals of Surgery* 151: 542-550, 1960.

- Peto, R., Gray, R., Brantom, P., et al. Nitrosamine carcinogenesis in 5120 rodents: Chronic administration of sixteen different concentrations of NDEA, NDMA, NPYR and NPIP in the water of 4,440 inbred rats, with parallel studies on NDEA alone of the effect of age of starting (3, 6 or 20 weeks) and of species (rats, mice or hamsters). *IARC Scientific Publications* 57: 627-665, 1984.
- Preussmann, R., Stewart, B.W. N-Nitroso carcinogens. In: *Chemical Carcinogens* (volume 132), E.C. Searle (Editor). Washington, DC: American Chemical Society, 1984, pp. 643-828. (American Chemical Society Monographs)
- Prokopczyk, B., Rivenson, A., Hoffmann, D. Comparative carcinogenicity of 3-(methylnitrosamino)propionitrile, and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone upon local application to mouse skin and rat oral mucosa. *Cancer Letters* 60: 153-157, 1991.
- Rivenson, A., Djordjevic, M.V., Amin, S., et al. Bioassay in A/J mice of some N-nitrosamines. *Cancer Letters* 47: 111-114, 1989.
- Roberts, D.L. Natural tobacco flavor. *Recent Advances in Tobacco Science* 14: 49-81, 1988.
- Robertson, J.B., Bray, J.T. Development of validation test for self-reported abstinence from smokeless tobacco products: Preliminary results. *Preventive Medicine* 17: 496-502, 1988.
- Tsuda, M., Kurashima, Y. Tobacco smoking, chewing and snuff dipping: Factors contributing to the endogenous formation of N-nitroso compounds. *Critical Reviews of Toxicology* 21: 243-253, 1991.
- U.S. Department of Health and Human Services. *The Health Consequences of Using Smokeless Tobacco: A Report of the Advisory Committee to the Surgeon General*. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, National Cancer Institute. DHHS Publication No. (NIH) 86-2874, 1986.
- U.S. Department of Health and Human Services. *Reducing the Health Consequences of Smoking: 25 Years of Progress. A Report of the Surgeon General*. U.S. Department of Health and Human Services, Public Health Service, Centers for Disease Control, Center for Chronic Disease Prevention and Health Promotion, Office on Smoking and Health. DHHS Publication No. (CDC) 89-8411, 1989.
- Weybrew, J.A., Stephens, R.L. Survey of carbonyl contents of tobacco. *Tobacco Science* 6: 53-57, 1962.
- World Health Organization. *Smokeless Tobacco Control. Report of a WHO Study Group*. Geneva: WHO, 1988. World Health Organization Technical Report 773.
- Wynder, E.L., Hoffmann, D. *Tobacco and Tobacco Smoke. Studies in Experimental Carcinogenesis*. New York: Academic Press, 1967.

Oncogenes in Head and Neck Cancer¹

Kenneth D. Somers, Suzanne E. Glickman, Suzanne L. Cartwright,
Bryan G. Stoll, Stella L. Maurizio, and Gary L. Schechter

ABSTRACT Squamous cell carcinomas of the head and neck (SCCHN) were analyzed for activated oncogenes by DNA transfection assay and Southern blot hybridization. Transforming activity was not detected in SCCHN DNAs (n=31) by two DNA transfection assays. A cluster of proto-oncogenes (*int-2*, *hst-1*, *bcl-1*) localized to chromosome 11 band q13 was amplified two- to eightfold in approximately 30 percent of SCCHN (n=45); *c-erbB-1* was amplified in 10 percent of the same tumors. Adjacent clinically and histologically normal tissue from the same patients had single proto-oncogene copy number. No amplification or rearrangement of *c-erbB-2/HER2*, *c-myc*, *N-myc*, *N-ras*, *H-ras-1*, or *K-ras-2* was detected in any of the SCCHN. The 11q13 amplicon in SCCHN consisted of *int-2*, *hst-1*, and *bcl-1*, but did not include *c-sea*, also on 11q13, or extend to the collagenase gene locus (11q21-22), *c-ets-1* (11q23), or *H-ras-1* (11p15.5). The data implicate amplification of *int-2/hst-1/bcl-1* as one of the genetic alterations underlying head and neck tumor formation.

INTRODUCTION Proto-oncogenes are normal cellular genes known to function in the control of cell growth and differentiation. When mutated or abnormally expressed, proto-oncogenes can be activated to oncogenic potential resulting in aberrant cell signaling and unrestrained cell proliferation (Bishop, 1991). Increasingly, activated oncogenes are being identified as one of the genetic alterations underlying human tumor pathogenesis. The most informative studies derive from the genetic analysis of colorectal cancer in which the stepwise accumulation of genetic damage in the form of activation of proto-oncogenes and inactivation of tumor suppressor genes leads to tumor development and progression (Fearon and Vogelstein, 1990).

There are few reports of activated oncogenes in head and neck tumors. Activated *ras* genes have been detected in < 10 percent of head and neck tumors (Howell et al., 1990; Sheng et al., 1990). We reported amplification of the *int-2* proto-oncogene in squamous cell carcinomas of the head and neck (SCCHN) (Somers et al., 1990) and recently demonstrated that *hst-1* is frequently co-amplified with *int-2* in SCCHN; *int-2* and *hst-1* are in the fibroblast growth factor family (Dickson et al., 1990), whose members function in angiogenesis and as mitogenic growth factors (reviewed in Burgess and Maciag, 1989). Although the expression and function of *int-2* and *hst-1* in SCCHN is uncertain, amplification of these genes in head and neck tumors implicates their role in tumor formation. In this study, we analyzed SCCHN for biologically active oncogenes by DNA transfection and extended our analysis of the *int-2* gene amplification unit in SCCHN.

¹ Supported by Smokeless Tobacco Research Council grant no. 0112 and the Medical College of Hampton Roads Foundation.

- MATERIALS AND METHODS** Surgical specimens were obtained from patients with histologically identified SCCHN. Adjacent normal tissue was obtained whenever possible. Tissue specimens were stored at -70 °C for subsequent DNA extraction. Human laryngeal SCC cell lines UMSCC10A, UMSCC10B, and UMSCC16 were provided by T.E. Carey, and Hep 2 was obtained from the American Type Culture Collection (ATCC). Cell lines were grown in Eagle's minimum essential medium supplemented with 10 percent fetal bovine serum. High molecular weight DNA was prepared as described by Somers and coworkers (1990).
- Tissue Specimens and DNA Extraction**
- Transfection Assays** DNA transfection was performed by the calcium phosphate precipitation technique (Graham and van der Eb, 1973) using NIH3T3 mouse fibroblasts as recipients. Foci of morphologically transformed cells were counted after 14 to 21 days. The nude mouse tumorigenicity assay was performed as described (Fasano et al., 1984) using pSV2_{neo} as a dominant selectable marker, thereby permitting G418 antibiotic selection of stable transfectants. Positive control DNA for transfection assays was extracted from H-*ras*-transformed NIH3T3 cells, 44-9. DNA extracted from human diploid fibroblasts or placenta served as the negative control. DNA extracted from primary transfectants and tumors was used in a second cycle transfection and examined for human DNA sequences by Southern blot analysis with the human *Alu* repetitive DNA probe BLUR 8.
- Southern Blot Analysis** DNA (10 µg) was digested with *EcoRI* or *BamHI*, separated by electrophoresis in 0.8 percent agarose gels, and blotted to nylon filters (Biotrans, ICN). Filters were baked for 2 h at 80 °C. Filters were prehybridized for 1 h at 42 °C in 50 percent formamide, 5X SSC (1X SSC is 0.15 M NaCl and 0.015 M sodium citrate), 5X Denhardt's solution, 0.05 M sodium phosphate (pH 6.5), 0.1 percent sodium dodecyl sulfate (SDS), and 250 µg/mL denatured salmon sperm DNA. Hybridization was performed with 1x10⁶ cpm of heat-denatured ³²P-labeled oncogene DNA probes per milliliter of prehybridization solution overnight at 42 °C. Filters were washed for 20 min at room temperature in 2X SSC and 0.1 percent SDS and at 50 °C for 30 min in 0.1X SSC and 0.1 percent SDS and then exposed at -70 °C to Kodak XAR-5 film with an intensifying screen. DNA probes used for hybridization included the following: *int-2* probe SS6 (Casey et al., 1986) provided by C. Dickson; *hst-1* probe pORF1 (Taira et al., 1987) provided by M. Terada; *bcl-1* probe b (Tsujiimoto et al., 1985) provided by Y. Tsujiimoto; *c-sea* probe p6.2 (Williams et al., 1988) provided by M. Hayman; and human collagenase (CLG) probe pClase 1 (Whitman et al., 1986) obtained from ATCC. Probes for *c-ets-1*, *H-ras-1*, *K-ras-2*, *N-ras*, *N-myc*, *c-myc*, *c-erbB-1*, and *c-erbB-2* were described previously (Somers et al., 1990). Cloned insert DNA (50 ng) was labeled with [³²P]dCTP by the random primer method to a specific activity of 5x10⁷ cpm/µg of DNA.
- RESULTS**
- Transforming Activity of SCCHN DNA** We used two general oncogene detection techniques, the NIH3T3 focus assay and the nude mouse tumorigenicity assay, to analyze proto-oncogene activation in 31 primary or metastatic SCCHN and four human cell lines derived from SCC of the larynx (Hep 2, UMSCC10A, UMSCC10B, and UMSCC16). Provisional evidence for

the transfer of oncogenes by DNA transfection could not be confirmed by analysis of transformant or nude mouse tumor DNAs for the presence of human *Alu* repetitive DNA sequences (data not shown). Control transfections of NIH3T3 cells with 44-9 DNA containing activated *H-ras* produced foci of morphologically transformed cells and induced tumors in nude mice with a latency of 1 to 2 wk.

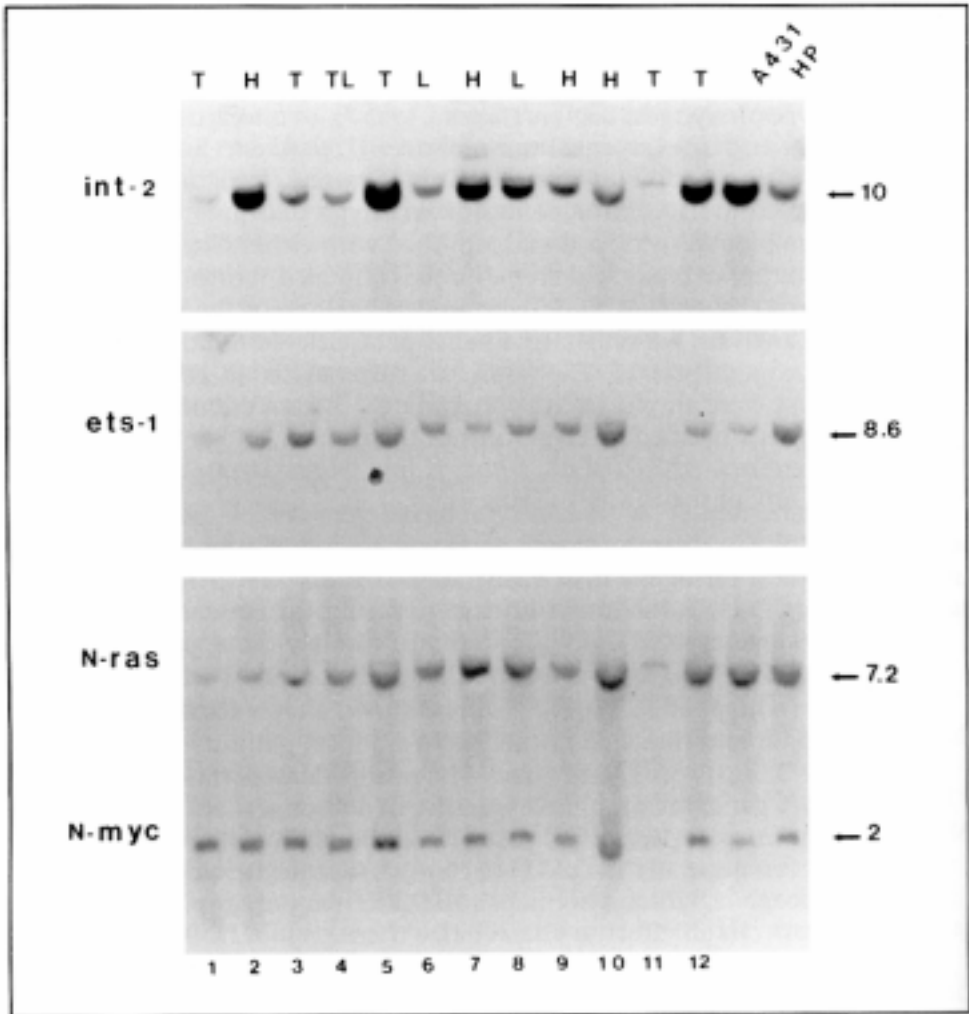
Southern Blot Analysis of Activated Oncogenes In an alternative approach, DNA extracted from SCCHN and tumor-derived cell lines was analyzed for amplified or rearranged proto-oncogenes by Southern blot hybridization. An example of such an analysis using probes corresponding to *int-2*, *c-ets-1*, *N-ras*, and *N-myc* is shown in Figure 1. *int-2* was amplified two- to fivefold in two hypopharyngeal tumors (lanes 2 and 7), two tongue tumors (lanes 5 and 12), and one laryngeal tumor (lane 8), relative to human placenta DNA used as a single copy *int-2* gene control (Figure 1, top). *int-2* was amplified threefold in A431 epidermoid carcinoma cells used as a positive control. The blot was stripped and reprobed with either *c-ets-1* (Figure 1, middle) or a mixture of *N-ras* and *N-myc* probes (Figure 1, bottom). No alterations of *c-ets-1*, *N-myc*, or *N-ras* were detected in any of the tumors. Using this type of analysis, we subjected a panel of SCCHN to Southern blot analysis using oncogene probes. The results are summarized in Table 1. *int-2*, *hst-1*, and *bcl-1* were amplified in approximately 30 percent of SCCHN, whereas *c-erbB-1* was amplified in 10 percent of the tumors. No amplification or rearrangement of *c-erbB-2/HER2*, *c-myc*, *N-myc*, *N-ras*, *H-ras-1*, or *K-ras-2* was detected in any of the SCCHN (Table 1).

Gene Amplification on 11q13 We demonstrated previously amplification of the *int-2* proto-oncogene in SCCHN (Somers et al., 1990). As shown in Table 1, *int-2*, *hst-1*, and *bcl-1* were the most frequently amplified proto-oncogenes in SCCHN. To define accurately the region of DNA amplification on 11q13, we analyzed two other gene loci, *bcl-1* and *c-sea*, which have also been mapped to 11q13. Figure 2 shows a representative *Bam*HI digest of tumor and matched normal DNA from six patients serially probed with *int-2*, *hst-1*, *bcl-1*, and *c-sea*. The results demonstrate that the *int-2*, *hst-1*, and *bcl-1* genes are amplified together in a laryngeal and pharyngeal tumor but not in adjacent normal tissue from the same patients. The degree of amplification generally ranged between two- and eightfold. The *c-sea* proto-oncogene, which also maps to 11q13, was not amplified in any of the tumors. In an effort to characterize the region of DNA amplification, filters were reprobed with the collagenase gene (CLG) located at segment 11q21-22 (Figure 2). No amplification of the CLG gene was detected in tumors where *int-2*, *hst-1*, and *bcl-1* were clearly amplified.

A map of chromosome 11 and the region of amplification is shown in Figure 3. The 11q13 amplicon contains four previously recognized oncogenes (*int-2*, *hst-1*, *bcl-1*, *c-sea*). Their location and map distances are shown. *hst-1* is located 35 kb from *int-2*, and *bcl-1* is approximately 1,000 kb proximal to the centromere from *int-2* (Nguyen et al., 1988). The precise location of *c-sea* relative to the other loci in 11q13 is unknown. We examined a group of *int-2* amplified head and neck tumors for amplification of *hst-1*, *bcl-1*, and *c-sea*. The results of this analysis are depicted in Figure 3.

Figure 1

Amplification of *int-2* in SCCHN. DNA (10 μ g) was digested with *Eco*RI and analyzed by Southern blot hybridization sequentially with the *int-2* probe (top), *c-ets-1* probe (middle), and mixed *N-ras* and *N-myc* probes (bottom). Lanes 1 to 12: DNA from 12 separate tumors derived from the tongue (T), hypopharynx (H), tonsil (TL), and larynx (L); A431: DNA from A431 cells; HP, human placenta DNA. Numbers on the right indicate DNA fragment sizes in kb detected by the probes.



Eleven of 12 tumors contained DNA coamplified for the *int-2* and *hst-1* gene; one tumor of the larynx exhibited *int-2*, but not *hst-1*, amplification. Eight of the *int-2* amplified tumors were evaluated for *bcl-1* and *c-sea* DNA amplification. All eight contained amplified *bcl-1* DNA, whereas none of eight had amplified *c-sea* DNA.

Table 1

Frequency of oncogene amplification in head and neck squamous cell carcinoma

| | Chromosome Location | Total | Amplification |
|-----------------|------------------------|-------|---------------|
| Oncogene | | | |
| <i>int-2</i> | 11 q13 | 45 | 14 (31%) |
| <i>hst-1</i> | 11 q13 | 39 | 11 (28%) |
| <i>bcl-1</i> | 11 q13 | 28 | 8 (29%) |
| <i>c-sea</i> | 11 q13 | 29 | 0 |
| <i>c-ets-1</i> | 11 q23 | 24 | 0 |
| H- <i>ras-1</i> | 11 p15.5 | 33 | 0 |
| K- <i>ras-2</i> | 12 p12.1 | 21 | 0 |
| N- <i>ras</i> | 1 p22 | 24 | 0 |
| N- <i>myc</i> | 2 p24 | 24 | 0 |
| <i>c-myc</i> | 8 q24 | 21 | 0 |
| <i>c-erbB-1</i> | 7 p12-13 | 21 | 2 (10%) |
| <i>c-erbB-2</i> | 17 q11-12 | 21 | 0 |

DISCUSSION In this study we examined a series of SCCHN for activated oncogenes. We were unable to detect transforming activity in 31 SCCHN DNAs by transfection assays. Detection of activated oncogenes by the biological transformation assay may underestimate the frequency of activated oncogenes (Barbacid, 1987). Few studies have examined head and neck cancer for activated *ras* genes. Tadokoro and associates (1989) detected activated H-*ras-1* by DNA transfection in two cell lines established from metastatic lymph nodes of patients with palatal or floor-of-mouth SCC. Activated K-*ras-2* was detected in 1 gingival SCC of 11 tumor DNAs tested by DNA transfection (Howell et al., 1990). Recently, the use of allele-specific oligonucleotide hybridization assays or RNase A mismatch cleavage analysis has significantly increased the detection of mutated *ras* genes in human malignancies (Bos, 1989). Using polymerase chain reaction and oligonucleotide hybridization to detect *ras* mutations at codons 12, 13, and 61, Sheng and associates (1990) detected a mutation at codon 12 of the H-*ras-1* gene in 2 of 54 head and neck tumors. No mutations were detected at positions 12, 13, and 61 of the K-*ras-2* gene or at positions 12 and 61 of the N-*ras* gene (Sheng et al., 1990). Although further studies are needed to confirm these findings, the combined data indicate that *ras* mutations are an uncommon genetic alteration in SCCHN.

Proto-oncogenes can be activated to oncogenic potential by mutational alterations or overexpression (Bishop, 1991). Overexpression of oncogenes at the level of gene amplification is a frequent observation in human tumors (Schwab and Amler, 1990). In certain tumors, oncogene amplification is associated with a more aggressive tumor phenotype and a poor clinical prognosis. Notable examples of oncogene amplification correlated with decreased survival include N-*myc* amplification in neuroblastomas (Brodeur et al., 1984; Seeger et al., 1985) and *c-erbB-2/HER2* amplification in breast and ovarian cancers (Slamon et al., 1989).

Figure 2

Coamplification of *int-2*, *hst-1*, and *bcl-1* in SCCHN. DNA (10 μ g) was digested with *Bam*HI and the filter probed sequentially with *int-2*, *hst-1*, *bcl-1*, *c-sea*, and CLG probes. Tumor (T) and adjacent normal tissue (N) from individual patients with tumors of the larynx (L), floor of mouth (FOM), and pharynx (PX). A431 and HP are the same as in Figure 1. Numbers on the right indicate DNA fragment sizes in kb detected by the probes.

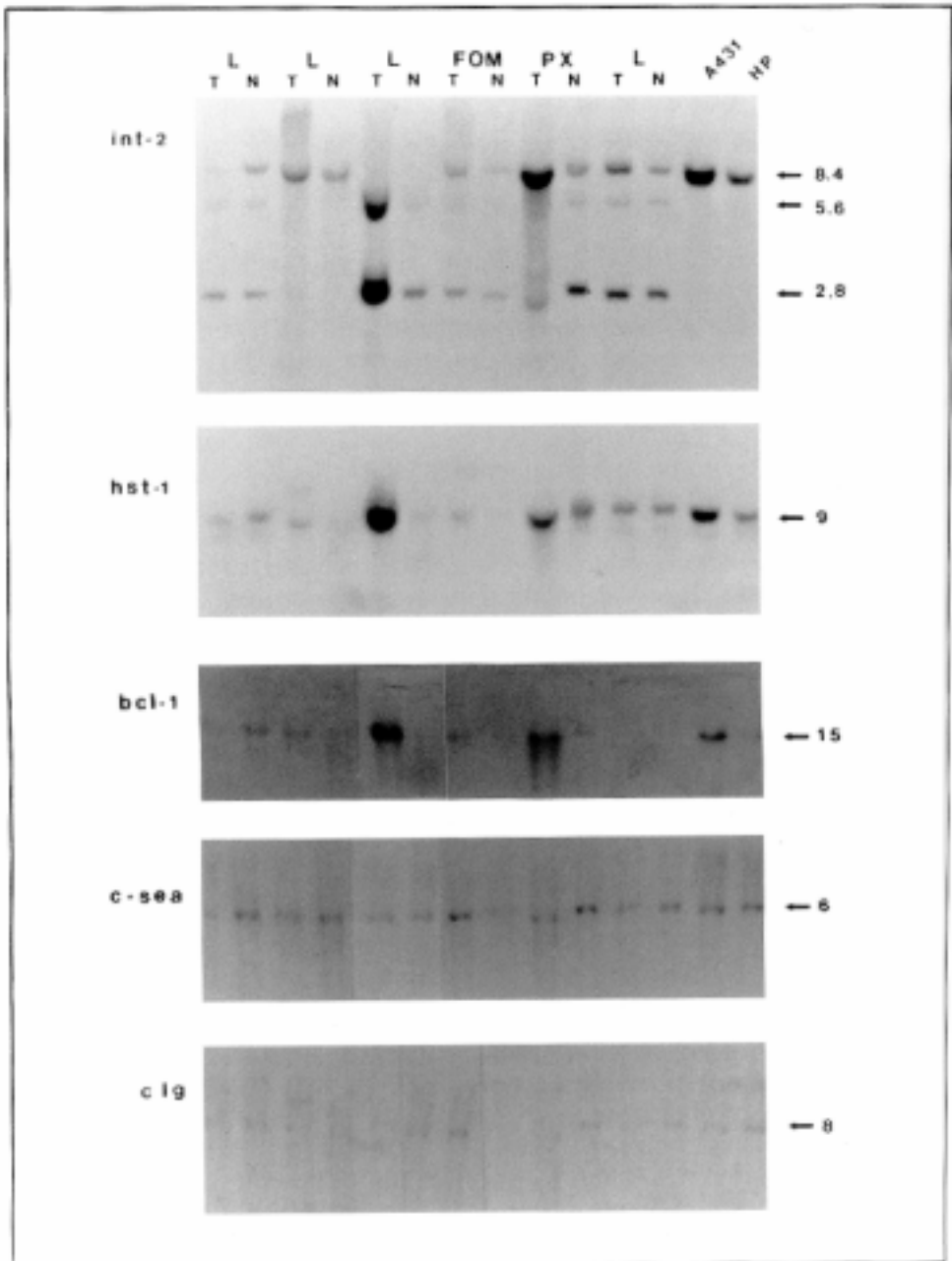
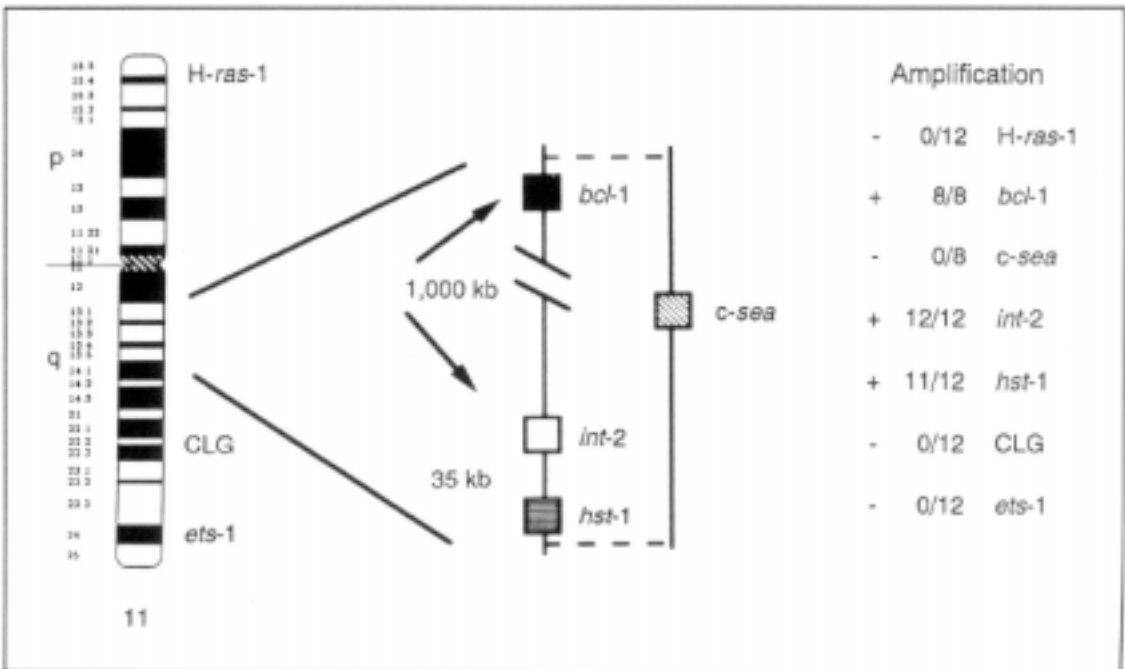


Figure 3

Map of chromosome 11 with the region of gene amplification in band q13 expanded. The table on the right indicates the presence (+) or absence (-) and frequency of amplification of specific gene loci on chromosome 11 in a panel of SCCHN.



There are few studies that report oncogene amplification and over-expression in head and neck cancer. Amplification of *N-myc* and *N-ras* was observed in approximately 40 percent of 23 cases of oral cavity SCC (Saranath et al., 1989). We were unable to confirm these findings. Increased transcription of *c-myc* was detected in 14 SCCHN (Field and Spandidos, 1987) and in 8 of 9 oral and laryngeal SCC (Riviere et al., 1990), but the number of cases is too small to make any meaningful clinical correlations. Results of the present study reveal the coamplification of *int-2*, *hst-1*, and *bcl-1* in about 30 percent of SCCHN. The three genes comprise a rather large amplicon on 11q13. We have broadly defined the limits of the 11q13 amplicon in SCCHN to include *int-2*, *hst-1*, and *bcl-1*, but not *c-sea*, which also maps to 11q13. The amplicon does not extend to the collagenase locus 11q21-22, the *c-ets-1* locus 11q23, or the pepsinogen cluster mapped to 11p11-q13 (Somers et al., 1990). Further work is needed to map more accurately the size of the 11q13 amplicon and to identify additional genes that reside in this region. It is also important to define the selective mechanisms that drive and maintain the 11q13 amplicon in SCCHN. Because *int-2* and *hst-1* are members of the FGF gene family, one model would predict that overexpression of *int-2* or *hst-1* might confer a growth advantage on the tumor cell. Studies of *int-2/hst-1* gene expression in SCCHN are needed to address this possibility. However, data from other studies have shown low or undetectable levels of *int-2* or *hst-1* transcripts in

human breast cancer, regardless of any amplification of the gene (Fantl et al., 1990). Alternatively, maintenance of the 11q13 amplicon would be driven by the expression of an unidentified gene on 11q13. Ultimately, it will be necessary to express *int-2* and *hst-1*cDNAs in squamous epithelial cells of the upper aerodigestive tract and examine these cells for properties that distinguish the malignant tumor phenotype.

Finally, it is important that amplification of *int-2*, *hst-1*, and *bcl-1* on 11q13 may be involved in different human tumors. One or more of the three proto-oncogenes within the amplicon have been reported to be amplified in breast carcinoma (Ali et al., 1989; Theillet et al., 1990), melanoma (Adelaide et al., 1988), esophageal carcinoma (Tsuda et al., 1989), stomach cancer (Yoshida et al., 1988), and SCCHN (Berenson et al., 1989; Merritt et al., 1990; Somers et al., 1990; Zhou et al., 1988). Thus, the elucidation of the role of the 11q13 amplicon in SCCHN might provide clues in understanding the development and progression of other human malignancies. Clearly, this marker in SCCHN should encourage future studies to determine if 11q13 gene amplification might be useful to predict tumor behavior and disease progression.

REFERENCES

- Adelaide, J., Mattei, M.-G., Marics, I., Raybaud, F., Planche, J., de Lapeyriere, O., Birnbaum, D. Chromosomal localization of the *hst* oncogene and its co-amplification with the *int-2* oncogene in human melanoma. *Oncogene* 2: 413-416, 1988.
- Ali, I.U., Merlo, G., Callahan, R., Lidereau, R. The amplification unit on chromosome 11q13 in aggressive primary human breast tumors entails the *bcl-1*, *int-2* and *hst* loci. *Oncogene* 4: 89-92, 1989.
- Barbacid, M. *ras* genes. *Annual Review of Biochemistry* 56: 79-82, 1987.
- Berenson, J.R., Yang, J., Mickel, R.A. Frequent amplification of the *bcl-1* locus in head and neck squamous cell carcinomas. *Oncogene* 4: 1111-1116, 1989.
- Bishop, J.M. Molecular themes in oncogenesis. *Cell* 64: 235-248, 1991.
- Bos, J.L. *ras* oncogenes in human cancer: A review. *Cancer Research* 49: 4682-4689, 1989.
- Brodeur, G.M., Seeger, R.C., Schwab, M., Varmus, H.E., Bishop, J.M. Amplification of N-*myc* in untreated human neuroblastomas correlates with advanced disease stage. *Science* 224: 1121-1124, 1984.
- Burgess, W.H., Maciag, T. The heparin binding (fibroblast) growth factor family proteins. *Annual Review of Biochemistry* 58: 575-606, 1989.
- Casey, G., Smith, R., McGillivray, D., Peters, G., Dickson, C. Characterization and chromosome assignment of the human homolog of *int-2*: A potential proto-oncogene. *Molecular and Cellular Biology* 6: 502-510, 1986.
- Dickson, C., Acland, P., Smith, R., Dixon, M., Deed, R., MacAllan, D., Walther, W., Fuller-Pace, F., Kiefer, P., Peters, G. Characterization of *int-2*: A member of the fibroblast growth factor family. *Journal of Cell Science* (Suppl.) 13: 87-96, 1990.
- Fantl, V., Richards, M.A., Smith, R., Lammie, G.A., Johnstone, G., Allen, D., Gregory, W., Peters, G., Dickson, C., Barnes, D.M. Gene amplification on chromosome band 11q13 and oestrogen receptor status in breast cancer. *European Journal of Cancer* 26: 423-429, 1990.
- Fasano, O., Birnbaum, D., Edlund, L., Fogh, J., Wigler, M. New human transforming genes detected by a tumorigenicity assay. *Molecular and Cellular Biology* 4: 1695-1705, 1984.
- Fearon, E.R., Vogelstein, B. A genetic model for colorectal tumorigenesis. *Cell* 61: 759-767, 1990.
- Field, J.K., Spandidos, D.A. Expression of oncogenes in human tumors with special reference to the head and neck region. *Journal of Oral Pathology* 16: 97-197, 1987.
- Graham, F.L., van der Eb, A.J. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 52: 456-467, 1973.
- Howell, R.E., Wong, F.S.H., Fenwick, R.G. A transforming Kirsten *ras* oncogene in an oral squamous carcinoma. *Journal of Oral Pathology and Medicine* 19: 301-305, 1990.
- Merritt, W.D., Weissler, M.C., Turk, B.F., Gilmer, T.M. Oncogene amplification in squamous cell carcinoma of the head and neck. *Archives of Otolaryngology—Head and Neck Surgery* 116: 1394-1398, 1990.

- Nguyen, C., Roux, D., Mattei, M.-G., de Lapeyriere, O., Goldfarb, M., Birnbaum, D., Jordan, B.R. The FGF-related oncogenes *hst* and *int-2*, and the *bcl-1* locus are contained within one megabase in band q13 of chromosome 11, while the *fgf-5* oncogene maps to 4q21. *Oncogene* 3: 703-708, 1988.
- Riviere, A., Wilckens, C., Loning, T. Expression of *c-erbB2* and *c-myc* in squamous epithelial and squamous cell carcinomas of the head and neck and the lower female genital tract. *Journal of Oral Pathology and Medicine* 19: 408-413, 1990.
- Saranath, D., Panchal, R.G., Nair, R., Mehta, A.R., Sanghavi, V., Sumegi, J., Klein, G., Deo, M.G. Oncogene amplification in squamous cell carcinoma of the oral cavity. *Japanese Journal of Cancer Research* 80: 430-437, 1989.
- Schwab, M., Amler, L.C. Amplification of cellular oncogenes: A predictor of clinical outcome in human cancer. *Genes Chromosomes Cancer* 1: 181-193, 1990.
- Seeger, R.C., Brodeur, G.M., Sather, H., Dalton, A., Siegel, S.E., Wong, K.Y., Hammond, D. Association of multiple copies of *N-myc* oncogene with rapid progression of neuroblastomas. *New England Journal of Medicine* 313: 1111-1116, 1985.
- Sheng, Z.M., Barrois, M., Kljanienko, J., Micheau, C., Richard, J.M., Riou, G. Analysis of the *c-Ha-ras-1* gene for deletion, mutation, amplification and expression in lymph node metastases of human head and neck carcinomas. *British Journal of Cancer* 62: 398-404, 1990.
- Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., Ullrich, A., Press, M.F. Studies of the *HER2/neu* proto-oncogene in human breast and ovarian cancer. *Science* 244: 707-712, 1989.
- Somers, K.D., Cartwright, S.L., Schechter, G.L. Amplification of the *int-2* gene in human head and neck squamous cell carcinomas. *Oncogene* 5: 915-920, 1990.
- Tadokoro, K., Ueda, M., Ohshima, T., Fujita, K., Rikimaru, K., Takahashi, N., Enomoto, S., Tsuchida, N. Activation of oncogenes in human oral cancer cells: A novel codon 13 mutation of *c-H-ras-1* and concurrent amplification of *c-erbB-1* and *c-myc*. *Oncogene* 4: 499-505, 1989.
- Taira, M., Yoshida, T., Miyagawa, K., Sakamoto, H., Terada, M., Sugimura, T. DNA sequence of human transforming gene *hst* and identification of the coding sequence required for transforming activity. *Proceedings of the National Academy of Sciences* 84: 2980-2984, 1987.
- Theillet, C., Adnane, J., Szeptowski, P., Simon, M.-P., Jeanteur, P., Birnbaum, D., Gaudray, P. *BCL-1* participates in the 11q13 amplification found in breast cancer. *Oncogene* 5: 147-149, 1990.
- Tsuda, T., Tahara, E., Kajiyama, G., Sakamoto, H., Terada, M., Sugimura, T. High incidence of coamplification of *hst-1* and *int-2* genes in human esophageal carcinomas. *Cancer Research* 49: 5505-5508, 1989.
- Tsujimoto, Y., Jaffe, E., Cossman, J., Gorham, J., Nowell, P.C., Croce, C.M. Clustering of breakpoints on chromosome 11 in human B cell neoplasms with the t(11;14) chromosome translocation. *Nature* 315: 340-343, 1985.
- Whitham, S.E., Murphy, G., Angel, P., Rahmsdorf, H.-J., Smith, B.J., Lyons, A., Harris, T.J.R., Reynolds, J.J., Herrlich, P., Docherty, J.P. Comparison of human stromelysin and collagenase by cloning and sequence analysis. *Biochemistry Journal* 240: 913-916, 1986.
- Williams, B.P., Shipley, J.M., Spurr, N.K., Smith, D.R., Hayman, M.J., Goodfellow, P.N. A human sequence homologous to *v-sea* maps to chromosome 11, band q13. *Oncogene* 3: 345-348, 1988.
- Yoshida, M.C., Wada, M., Satoh, H., Yoshida, T., Sakamoto, H., Miyagawa, K., Yokota, J., Koda, T., Kakinuma, M., Sugimura, T., Terada, M. Human HST1 (HSTF1) gene maps to chromosome band 11q13 and coamplifies with the *INT2* gene in human cancer. *Proceedings of the National Academy of Sciences* 85: 4861-4864, 1988.
- Zhou, D.J., Casey, G., Cline, M.J. Amplification of human *int-2* in breast cancers and squamous cell carcinomas. *Oncogene* 2: 279-282, 1988.

Oncogenes in Tobacco-Induced Oral Cancer

Madhav G. Deo and Dhananjaya Saranath

ABSTRACT This paper reviews the status and implications of oncogenes in oral cancer caused by smokeless tobacco (chewing), which accounts for 40 percent of malignancies in India. A variety of abnormalities consisting of amplification, overexpression, point mutation, deletion, and rearrangement of oncogenes, particularly of *myc* and *ras* families, are seen in more than 90 percent of the cancers in India. A longitudinal study of oncogenes at various stages of oral carcinogenesis, particularly in leukoplakias and early cancers, will not only throw light on the molecular biology of malignant transformation but also provide clues to the early diagnosis of this cancer through molecular biology techniques.

INTRODUCTION Oral cancer, which is among the 10 most prevalent cancers in the world, is a common cancer in South and Southeast Asia, where the habit of tobacco chewing is widely prevalent. In India, tobacco chewing is a major cause of cancer mortality, accounting for about 40 percent of the total malignancies. Clinical, epidemiological, and laboratory studies indicate a causal relationship between prolonged tobacco chewing and oral cancer, smokeless tobacco being the *sine qua non* (Gupta et al., 1987; Jussawalla and Deshpande, 1971). A latent period of 5 to 15 yr is common, and almost every tobacco-related oral cancer is preceded by a phase of leukoplakia.

Several constituents of tobacco, such as tobacco-specific *N*-nitrosamines, polycyclic aromatic hydrocarbons, and polonium-210 (a radioactive alpha emitter), are capable of inducing preneoplastic and neoplastic changes in the oral cavity (IARC, 1985; Mattson and Winn, 1989). Although much information is available about their chemistry, the critical link between the tobacco-specific carcinogens and the cellular macromolecules leading to the malignant phenotype is an enigma. Tobacco-specific nitrosamines are known to form DNA adducts, resulting in mutations that could profoundly affect cellular genes (Hecht et al., 1988).

The importance of proto-oncogenes in cellular growth and differentiation is well documented (Bishop, 1987; Klein and Klein, 1985). One of the mechanisms involved in the process of carcinogenesis is the activation of proto-oncogenes (oncogenes), which has been observed in a number of human and experimental cancers (Klein, 1988). Illegitimate activation of proto-oncogenes may occur by various mechanisms, including gene amplification, chromosomal translocation, point mutation, and retroviral insertion, that in turn contribute to tumor development and progression in several systems (Klein, 1988).

This review elucidates the various molecular lesions that involve oncogenes in oral cancers. Such studies can be grouped into four categories: (1) examination of genomic DNA of the primary tumor for oncogene

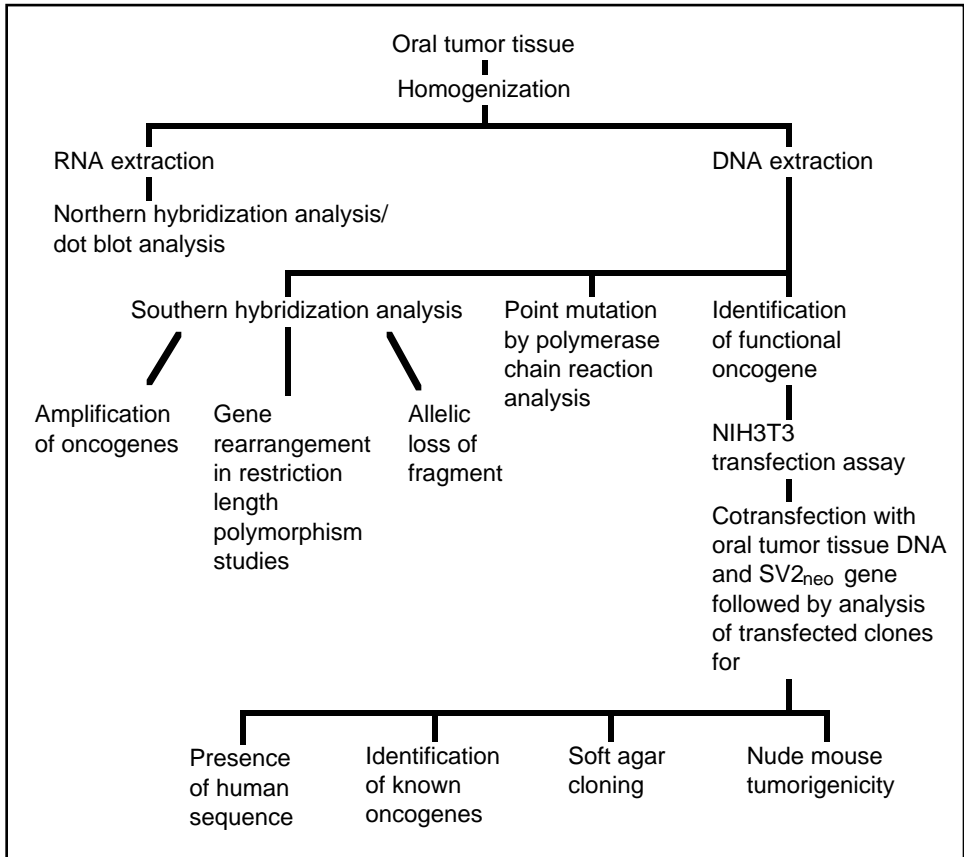
amplification; (2) aberrant oncogene expression as seen by an increase in mRNA transcript level or alteration in the transcript size or an increase in the protein product; (3) allelic loss, rearrangement, and point mutation; and (4) detection, isolation, and cloning of a functional oncogene in oral cancers in a transfection assay. The methodology used in our studies is outlined in Figure 1.

ONCOGENE AMPLIFICATION We investigated the status of the commonly amplified oncogenes of the *myc* and *ras* families in 102 primary oral tumor tissues (Saranath et al., 1989). All tumors were squamous cell carcinomas (SCCs) involving various regions: buccal mucosa (46 patients), lower alveolus (29 patients), tongue (23 patients), and floor of the mouth (4 patients). All patients had chewed tobacco regularly for 10 to 15 yr. Although histological grading varied from well to poorly differentiated, the majority of the tumors were large and in an advanced stage (TNM III/IV). Lymph node metastases were frequent, particularly in the T3 and T4 lesions. However, none of the patients showed distant metastasis. Peripheral blood cells (PBCs) from the patients were analyzed for oncogene involvement. PBCs from healthy volunteers and human placental DNA were included in the study as controls. Vimentin, a single copy gene, was used as an internal control gene.

Southern hybridization analysis of the *Eco*RI-digested DNA samples revealed a 3-fold to 10-fold amplification of *c-myc*, *N-myc*, *K-ras*, or *N-ras* in 49 of the 102 (48 percent) primary oral tumor tissues screened (Table 1). *H-ras* and *L-myc* oncogenes were not amplified in any of the samples. The controls—PBC DNA from the patients and healthy volunteers and normal human placental DNA—showed the presence of a single copy of the oncogenes.

Amplification of *myc* oncogenes was observed in 37 of the 102 patients. *N-myc* and *c-myc* were amplified in 26 percent (27 of 102) and 21 percent (21 of 102) of the patients, respectively (Table 1). An unusual finding was the coamplification of *c-myc* and *N-myc* in 11 of 37 (30 percent) of the patients. It has been hypothesized that the products of *myc* family oncogenes, *c-myc*, *N-myc*, and *L-myc*, down-regulate each other (Alt et al., 1986). As a consequence, it is predicted that tumor cells would have amplification or increased expression of only one of the *myc* oncogenes. Indeed, this appears to be the rule. However, oral cancer seems to be unique in this respect, with the two *myc* oncogenes coamplified in a number of patients. However, it is not possible from these studies to know if the coamplification represents the existence of two different clones that each contain a single amplified *myc*. Alternatively, the two *myc* oncogenes could be amplified in the same cell. Coexpression of *c-myc* and *N-myc* has been reported in the early undifferentiated pre- β cells in mice (Zimmerman et al., 1986), implying coexpression during a particular developmental stage or in a particular differentiation window of the cell. In our studies, amplification bore no correlation with clinicopathological features such as degree of differentiation, tumor size, TNM staging, nodal metastasis, or recurrence.

Figure 1

Flow chart of methods used to study oncogene aberrations in oral cancers

Opinion is divided on the status of *c-myc* amplification or its increased expression in oral cancer. Although Berenson and colleagues (1989) found no amplification, Yokota and coworkers (1986) reported a fivefold to eightfold amplification of the oncogene in SCCs of the head and neck. They also found that, compared with the primary tumor, the metastatic tumor tissue showed higher *myc* amplification. Field et al. (1986) observed an elevated *c-myc* mRNA expression correlating well with the stage of the disease. In another study, tumors with poor prognosis exhibited increased *c-myc* oncoprotein (Field et al., 1989). Oncogenes from the *myc* family have been involved in regulating cellular proliferation, and their amplification is associated with certain aggressive tumors (Alitalo et al., 1985).

Our data also demonstrate amplification of *K-ras* or *N-ras* in 33 percent (34 of 102) of the total samples analyzed. *K-ras* showed a threefold to eightfold amplification in 18 percent of the samples, whereas *N-ras* was amplified in 28 percent (Table 1). Amplification of *ras* oncogene did not correlate with any clinical parameters, including nodal metastasis. *H-ras* was not amplified. Other investigators also have observed no amplification of *H-ras* in oral cancer (Sheng et al., 1990; Sommers et al., 1990).

Table 1
Oncogene amplification in oral cancers^a

| Oncogenes | Range of Amplification | Cellular DNA Fragment (kb) | Samples Amplified n (%) |
|--------------|------------------------|---|-------------------------|
| <i>c-myc</i> | 3-6 | 12.7 | 21 (21) |
| <i>N-myc</i> | 3-10 | 3.8 | 27 (26) |
| <i>L-myc</i> | 1 | 10.0, 6.6 | 0 |
| <i>K-ras</i> | 3-8 | 2.7 | 18 (18) |
| <i>N-ras</i> | 3-9 | 7.2 | 28 (28) |
| <i>H-ras</i> | 1 | 23-30 | 0 |
| EGF-R | 3-8 | 7.8, 7.0, 6.1, 5.6, 3.7, 2.5, 2.1, 1.9, 1.6, and 1.3 | 19 (29) |

^a Total number of samples analyzed was 102 for *myc* and *ras* and 66 for EGF-R gene.

In our study, coamplification of multiple oncogenes was an important feature, being observed in 24 of the 49 samples showing amplification (Table 2). Two oncogenes were coamplified in 10 tumor tissues and 3 in an additional 10 samples; 5 patients showed concurrent amplification of the four oncogenes—*c-myc*, *N-myc*, *K-ras*, and *N-ras*. Multiple oncogene amplification indicates the complex, multistage process of oral carcinogenesis, and it implies alternate or simultaneous activation of the different oncogenes in oral carcinogenesis.

There are very few studies on the status of other oncogenes in oral cancer. In the studies of Sommers and colleagues (1990), amplification of *int-2* oncogene correlated well with frequency of recurrence. However, no correlation was observed with clinical staging or histological grading of the malignancy. Berenson and coworkers (1989) have reported a 2-fold to 10-fold amplification of the *bcl-1* oncogene in poorly differentiated tumors, but no correlation was seen with the TNM staging.

The epidermal growth factor-receptor (EGF-R) gene is considered to be the proto-oncogene of the *erb-B* gene of avian erythroblastosis virus, which is involved in transformation of chicken cells (Downward et al., 1984). Its ligand EGF is a potent mitogen for a variety of cells. EGF-R is commonly elevated in squamous cell carcinomas. In our study, in which all tumors were SCCs, the EGF-R gene was amplified in 29 percent (19 of 66) of the samples screened (Table 1). Preliminary results, from mRNA dot blot hybridization, indicate that amplification is associated with increased expression of the gene. Amplification and overexpression of the EGF-R gene, through interaction with the ligand, could be an additional step in oral carcinogenesis.

Table 22-2

Amplification of oncogenes *c-myc*, *N-myc*, *N-ras*, and *K-ras*

| | Number of Patients ^a | | | | |
|---|---------------------------------|---|--------------|--------------|--------------|
| | Total | <i>c-myc</i> | <i>N-myc</i> | <i>N-ras</i> | <i>K-ras</i> |
| Number of Patients | 102 | | | | |
| Patients Showing Oncogene Amplification | 49 ^b | 21 | 27 | 28 | 18 |
| One Oncogene | 24 | 9 | 5 | 7 | 3 |
| Two Oncogenes | 10 | | | | |
| <i>myc/myc</i> | 1 | | | | |
| <i>ras/ras</i> | 7 | (c-myc/K-ras 1; N-myc/K-ras 1; N-myc/K-ras 5) | | | |
| <i>ras/ras</i> | 2 | | | | |
| Three Oncogenes | 10 | | | | |
| <i>myc/myc/ras</i> | 5 | (N-myc/c-myc/K-ras 1; N-myc/c-myc/N-ras 4) | | | |
| <i>myc/ras/ras</i> | 5 | (N-myc/N-ras/K-ras 5) | | | |
| Four Oncogenes | 5 | | | | |

^a In many patients, more than one oncogene was amplified. The number derived by addition of the individual figures is therefore higher than the total number of positive samples.

EXPRESSION OF CELLULAR ONCOGENES

Expression of cellular oncogenes in oral cancers has been investigated through a variety of techniques, such as Northern hybridization/dot blot analysis for mRNA transcript in tumor tissue, in situ hybridization for detecting mRNA specific to oncogenes, and immunohistochemical demonstration of oncoproteins using monoclonal antibodies. Using dot blot hybridization, Spandidos and coworkers (1985) reported multiple transcriptional activation of *H-ras*, *K-ras*, and *c-myc* in oral cancers compared with normal tissue and premalignant pleomorphic salivary adenomas. The authors also observed a significant increase in *c-myc* oncogene expression in advanced stages III and IV compared with stages I and II. Recently, the authors also demonstrated correlation of increased *c-myc* expression with poor prognosis. No correlation was seen with the patient age, sex, TNM staging, site of tumor, histopathological grading, lymph node metastasis, or capsular rupture of the tumor (Field et al., 1989).

Using immunohistochemical techniques and oncoprotein-specific monoclonals, Azuma and colleagues (1987) studied *ras* p21 protein in oral cancers. Although 59 of 121 specimens reacted positively to Y13-259-*ras* p21-specific Mab, 44 oral leukoplakias and 58 normal mucosa showed a negative staining. Only 43 patients with oral cancer were followed up.

In these persons, *ras* p21 expression correlated well with poor prognosis and only weakly with regional lymph node metastasis. Furthermore, *ras* p21 expression was higher in the patients with a tobacco habit.

**RFLP ANALYSIS
OF L-*myc* AND
H-*ras* ONCOGENES**

L-*myc*

Researchers have examined restriction fragment length polymorphism (RFLP) in a number of human cancers to establish a specific association of a particular allele with an increased incidence of cancer, suggesting genetic predisposition to the cancer, or as an indicator of clinical behavior, in particular, metastatic potential or prognosis. In our studies, we used Southern hybridization analysis of 76 oral cancer primary tumor tissues and PBCs from the corresponding patients, and PBCs from 101 healthy volunteers, to classify the Indian population into three genotypes—L-L, S-L, S-S—according to the polymorphic patterns defined by the two L-*myc* alleles (Saranath et al., 1990). The pattern of the alleles in the PBC DNA of the patients was identical to the corresponding tumor DNA. The relative ratios of the three genotypes in the oral cancer patients were not significantly different from those seen in the healthy Indian population ($\chi^2=3.06$, $df=2$, $p > 0.25$), implying no predisposition to oral cancer by the presence of either allele. The L and S alleles were equally distributed in the population, with the frequency of each allele being 0.50, consistent with the Hardy-Weinberg law.

A striking correlation was observed between the RFLP pattern and the stage of differentiation, as well as the size of the tumors (Saranath et al., 1990). Thus, a preponderance of the S-fragment was observed in the moderate to poorly differentiated tumors ($\chi^2=4.97$, $df=1$, $p < 0.05$) and the larger sized (> 4 cm) tumors ($\chi^2=5.65$, $df=1$, $p < 0.025$). These observations suggest that the S allele product may be arresting the cells in a particular differentiation window, providing a further proliferative advantage to the cells, resulting in the larger sized tumors. There is also the possibility for involvement of another gene in proximity to the S-allele, or for a partially different S-fragment-coded L-*myc* protein, or a crucial role for the regulatory region of the S-fragment protein in the tumors. Lung and kidney tumors associated with S-fragment show aggressive behavior and higher tendency for metastasis (Takehi and Yoshida, 1989; Kawashima et al., 1987). However, this is not the case with malignancies of other organs (Ikeda et al., 1988).

H-*ras*

The H-*ras* locus includes a hypervariable region, designated as variable tandem repetition region (VTR), consisting of a series of 28 base pair repeats 3' to the gene (Capon et al., 1983). The VTR has been implicated in the regulation of the H-*ras* oncogene (Spandidos and Holmes, 1987). RFLP of the human H-*ras* oncogene has been ascribed to changes in the size of the VTR and can be defined by several restriction enzymes, including *Bam*HI, *Pvu*II, and *Taq*I (Capon et al., 1983; Pierotti et al., 1986).

In our studies, the status of H-*ras* locus was investigated in 62 patients with oral cancers (Saranath et al., 1991a). Southern blot analysis on *Bam*HI digestion of the tumor tissue DNA revealed 23 patients with H-*ras* heterozygosity. *Bam*HI digestion identified restriction fragments ranging from 6.6 kb to 8.6 kb. The allelic heterozygosity was better resolved by *Pvu*II and *Taq*I digestion. The former yielded an invariable fragment of 2.6 kb and variable

fragments ranging from 2.7 kb to 5.3 kb. *TaqI* digestion of the samples and hybridization with the VTR region probe (1 kb *MspI*-digested, VTR-specific *H-ras* probe) resolved four variable fragments of 2.4 kb, 3.0 kb, 3.6 kb, and 5.1 kb. In three samples, *TaqI* restriction analysis also demonstrated presence of a unique VTR rearrangement as indicated by 2.1 kb, 0.9 kb, and 0.6 kb fragments, substituting a 3.6 kb fragment, implying additional *TaqI* sites. Such a variant VTR fragment could be generated by either mutational events creating the *TaqI* sites or reiteration of the existing *TaqI* site present 18 bp from the 3' end of the VTR region during its amplification/duplication. This rearrangement, which suggests altered function of the VTR, could be one of the molecular lesions in tobacco-induced oral cancer.

Analysis of matched tumor tissue and PBC DNA from the same patient demonstrated tumor-associated loss of one of the allelic fragments in 7 of 23 patients (30 percent) with *H-ras* heterozygosity. Similar observations have been made by Howell and colleagues (1989) in oral SCCs. *H-ras* allelic loss has been reported in a number of human cancers (Ali et al., 1987); however, its implication in carcinogenesis is not yet clear. Tumor suppressor genes or anti-oncogenes have been implicated in the pathogenesis of some human tumors (Klein, 1988). Ali and coworkers (1987) identified a putative suppressor gene on chromosome 11, in the vicinity of the *H-ras* locus, localized between the β -globin and parathyroid hormone loci. The loss of *H-ras* allele observed in our patients also may encompass functional loss of the potent tumor suppressor gene on chromosome 11, further influencing the process of oral carcinogenesis in the patients. Alternatively, the loss could involve the normal *H-ras* gene, giving a selective functional advantage to the mutated *H-ras* allele involved in cell transformation and a consequent influence in oral carcinogenesis. In this respect, it is significant that 6 of 7 patients showing *H-ras* mutation at codon 12.2 also exhibited associated loss of the wild type gene. Such a loss may not be evident in RFLP studies or if the alleles are in a homozygous state.

H-ras POINT MUTATIONS Point mutations of *ras* oncogenes are observed in a variety of human cancers (Bos, 1989). We employed the polymerase chain reaction (PCR) technique for in vitro amplification of specific sequences followed by allele-specific oligonucleotide hybridization to examine *ras* activation by point mutations in 57 primary oral tumors (Saranath et al., 1991b). The mutational activation was studied in all three *ras* family members (*K-ras*, *N-ras*, and *H-ras*) at codons 12, 13, and 61, the codons affected in human cancers (Bos, 1989). Suitable primers were used to amplify sequences of 111 bp and 178 bp for the regions flanking codons 12, 13, and 61, respectively. The amplified sequences were initially screened with sets of mixed probes, each set covering one nucleotide position of a particular codon. Probes used to identify mutations in *H-ras* have been described elsewhere (Saranath et al., 1991b). On indication of a mutation in the sample DNA, a duplicate PCR of the genomic DNA was performed, and the two independently amplified DNAs were screened simultaneously with a set of single oligonucleotide probes specific for the nucleotide position. The presence of wild type codons was screened for in every set.

Mutations were detected in 20 to 57 (35 percent) of the samples and were restricted exclusively to the *H-ras* oncogene at codons 12, 13, and 61 (Table 3). None of the samples showed mutations in the *K-ras* or *N-ras* oncogenes. The mutations demonstrated an equal number (11 each) of nucleotide transitions and transversions. The mutations were seen primarily in codons 61.2 and 12.2. Nine samples showed A → G transition in codon 61.2 resulting in glutamine to arginine substitution. G → T transversion was observed in seven samples at codon 12.2 with glycine to valine substitution. Two patients concurrently carried mutations at codons 61.2 and 12.2. Six samples with a point mutation at codon 12.2 and two samples at codon 61.2 also demonstrated loss of the corresponding wild type codons, as judged by the absence of signals on allele-specific oligonucleotide hybridization. In addition, three novel mutations, not yet reported in human malignancies, were seen. These include the G → A (glycine to serine) substitution at codon 12.2, G → A (glycine to aspartate) at codon 13.2, and G → T (glutamine to histidine) (three cases) at codon 61.3.

In contrast to the very high frequency (35 percent) of *H-ras* mutations we observed, Sheng and coworkers (1990) detected mutations in only 3.7 percent (2 of 54) of their samples. Although mutations in *H-ras* occurred in the studies by Sheng and colleagues, the mutations were restricted to codon 12. Similarly, Johnson and coworkers (personal communication, 1991) and Rumsby and colleagues (1990) very rarely encountered mutations in oral SCCs from patients in the United Kingdom. Thus, it is clear that mutational frequencies in oral cancers in the West are infrequent, and perhaps only codon 12 is affected. The differences in oral cancers in India and the West may be attributed to differing tobacco habits. Whereas tobacco chewing appears to be the prime factor in the development of oral cancer in India, alcohol consumption contributes substantially in the West, where smoking also is a confounding factor in the pathogenesis of oral cancer. The mode of tobacco usage, strain or species of tobacco used, and curing process (which differs between East and West) also may contribute to these differences.

There appears to be, to a certain extent, organ specificity in activation of *ras* family oncogenes (Bos, 1989). Thus, *N-ras* point mutations are seen primarily in hematological malignancy, whereas in cancers of the lung, colon, and pancreas, *K-ras* frequently is affected. *H-ras* mutations are observed in bladder cancers (Bos, 1989) and experimentally induced skin tumors (Quintanilla et al., 1986). No explanation is readily available for this phenomenon, but it could be attributable to relative expression of different *ras* oncogenes in various organs (Leon et al., 1987). In lung cancer, another important tobacco-related cancer, mutation is observed primarily in codon 12 of *K-ras* (Slebos et al., 1990). However, in tobacco-related oral cancer, both codons 12 and 61 of *H-ras* are affected. In experimentally induced animal tumors, carcinogens show positional preference for *ras* mutations. Nitrosamines and DMBA preferentially affect codons 12 and 61, respectively (Quintanilla et al., 1986; Zarbl et al., 1985).

Table 3
H-ras mutations

| Codon Position of Nucleotide | Number of Patients | Number of Patients With Wild Type Signal Missing | Nucleotide Change | Amino Acid Change | Number of Patients With Amplification of Oncogenes ^a | | | |
|------------------------------------|-----------------------|--|----------------------|-------------------------|--|---------------|---------------|---------------|
| | | | | | L- <i>myc</i> | N- <i>myc</i> | K- <i>ras</i> | N- <i>ras</i> |
| 12.1 | 1 | 0 | GGC→AGC | Gly→Ser | 1 | N | N | N |
| 12.2 ^b | 7 | 6 | GGC→GTC | Gly→Val | N | N | N | 1 |
| 13.2 | 1 | 0 | GGC→GAC | Gly→Asp | N | N | N | N |
| 61.2 ^b | 9 | 2 | CAG→CGG | Gln→Arg | 3 | 2 | N | 2 |
| 61.2 | 1 | 0 | CAG→CTG | Gln→Leu | N | N | N | N |
| 61.3 | 3 | 0 | CAG→CAT | Gln→His | N | 2 | 2 | 2 |

^a N = nil.

^b Two patients showed mutations at both codon 12.2 and 61.2.

As mentioned earlier, tobacco contains several potent mutagens and carcinogens such as *N'*-nitrosornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Nitrosamines are known to produce metabolites capable of binding DNA, resulting in products such as *O*⁶-methylguanine, which can lead to miscoding during DNA replication (Topal, 1988). In India, tobacco is chewed generally as betel quid, which contains areca nut, slaked lime, catechu, and flavoring agents in addition to tobacco. Whereas mutation of codon 12 could be attributable to tobacco-specific nitrosamines, other chemical constituents may be responsible for mutation at codon 61. This aspect needs further elucidation.

IDENTIFICATION OF A FUNCTIONAL ONCOGENE

NIH3T3 mouse fibroblast transfection combined with the nude mice tumorigenicity assay is widely used to identify activated oncogenes (Shih and Weinberg, 1982). Using this system, Friedman and colleagues (1983) reported the presence of transforming genes in DNA from head and neck SCCs. However, the oncogene has yet to be characterized. Recently, Howell and coworkers (1990) identified the *K-ras* oncogene in head and neck SCCs from primary and secondary NIH-transformed cells. The oncogene was isolated from only 1 of the 11 tumors studied by the investigators.

The NIH3T3 transfection assay shows a bias toward detection of *ras* oncogenes. However, with the use of selectable markers such as SV2_{neo} gene, in cotransfection followed by tumorigenicity assays, several novel genes, besides the *ras* family oncogenes, also have been detected in human cancers (Fasano et al., 1984).

We recently carried out the NIH3T3 cotransfection assay, using oral tumor tissue DNA and SV2_{neo} selectable marker gene, followed by nude mouse tumorigenicity assay. Calf thymus DNA and *H-ras* pEJ6.6 clone were used as negative and positive controls, respectively. The *H-ras* oncogene-transfected cells induced tumors in the nude mice within 3 to 5 weeks.

These tumors and the cell lines established from them showed the presence of H-*ras* DNA in Southern hybridization analysis as well as a 1.2 kb H-*ras* RNA transcript in Northern hybridization analysis, validating the absence of technology errors in our studies.

Transfection assay was carried out with oral tumor DNA from three patients. The samples from the three patients induced transformation of NIH-3T3 cells, forming colonies in soft agar. The transfected cells induced tumors in nude mice within 5 to 10 wk. Further, DNA isolated from the nude mice tumors hybridized with human Alu Blur-2 probe in Southern analysis and cell lines established from the tumors retained the human Alu sequences. However, DNA isolated from oral tumor DNA transfected nude mice tumors, as well as their established tumor cell lines, revealed no hybridization with any of the *ras* or *myc* oncogenes. This was surprising because N-*ras* was amplified in two of the original primary tumors and one of them also showed H-*ras* point mutation at codon 12.2; the third tumor did not show any aberration of *ras* or *myc* oncogenes. As mentioned earlier, positive results were obtained in three of three oral tumor samples. Generally, a maximum of 30 percent transformation rate has been observed in NIH3T3 transfection/nude mouse tumorigenicity assays, using DNA from solid tumors (Krontiris and Cooper, 1981). Very high transfection and nude mice tumorigenicity frequency, in our studies, indicate the possibility of a highly potent, tobacco-induced activated oncogene, different from the *myc* or *ras* oncogenes. The situation may be similar to the presence of N-*myc* oncogene in neuroblastomas or L-*myc* in small cell lung carcinomas. We are currently in the process of cloning this gene from the nude mouse tumors to isolate, sequence, and identify the gene.

CONCLUSIONS AND FUTURE PROSPECTS The observation that more than 90 percent of the oral cancers caused by smokeless tobacco (chewing) show some sort of oncogene aberration (Table 4) indicates that tobacco-induced oncogene changes are closely linked to the pathogenesis of these cancers. The occurrence of multiple oncogene aberrations suggests that the process of malignant transformation results, probably, as a consequence of diverse molecule alterations. In our studies, the functional lesions in oncogenes could be grouped into (1) overproduction of oncogene effector molecules through amplification or overexpression of the genes, (2) production of abnormal oncogene products through point mutations, and (3) deletion of normal oncogene allele or putative tumor suppressor, as evidenced by allelic loss. These perturbations may act independently or in concert, resulting in deregulation of cellular growth.

A distinct difference emerges in the mode of activation of oncogenes in oral cancers in the Indian population and Western countries. Low levels of amplification of *myc* and *ras* oncogenes and low incidence of point mutations are observed in the West. Comparative studies of molecular biology of oral cancers vis-a-vis the varied tobacco habits and the associated confounding factors, such as alcohol in the West and different constituents of betel quid in India, may throw light on the pathogenic mechanisms responsible for these differences. Studies in suitable laboratory models using individual agents may be equally fruitful.

Table 4
Oncogenes in oral cancers

| Summary Analysis | Percentage | Comments |
|---|------------|--|
| Amplification <i>c-myc</i> , <i>N-myc</i> , <i>K-ras</i> , <i>N-ras</i> (n=102) | 48.0% | |
| Point Mutations <i>H-ras</i> (n=57) | 23.0 | Excluding 12% samples showing, in addition, amplification/allelic loss/ gene rearrangement |
| <i>H-ras</i> Allelic Loss or VTR Rearrangement (n=62; heterozygous=23) | 14.5 | Excluding 21.5% samples showing other oncogene aberrations |
| EGF-R Amplification (n=66) | 9.0 | Excluding 20% samples showing other oncogene aberrations |
| Samples Showing at Least One Oncogene Aberration | 94.5 | |

Transfection assays, in our studies, indicate involvement of a highly potent activated oncogene, outside the *ras* and *myc* families, in tobacco-induced oral carcinogenesis. The oncogene must be fully characterized. A high incidence of point mutations is observed in Indian patients at codons 12 and 61 of the *H-ras* oncogene. Some of the tobacco-specific carcinogens, such as nitrosamines, which form DNA adducts, are known to have positional preference for inducing point mutation in *ras* oncogenes. The precise role of point mutation, which is an early event in certain human malignancies, in the pathogenesis of oral cancer needs further elucidation.

Some tobacco chewers develop oral cancer at a relatively young age, a situation reminiscent of smoking and lung cancer. There is evidence that an autosomal gene, showing Mendelian codominant inheritance, is responsible for early-onset lung cancers (Bonney, 1990; Sellers et al., 1990). Interaction of the gene, which has yet to be characterized, with smoking accounts for 69 percent and 47 percent of lung cancers occurring at ages 50 and 60, respectively. Although this in no way negates the crucial role of smoking and should not be a deterrent to antitobacco efforts, similar studies should be conducted with tobacco chewers.

Our study comprised primarily large and advanced tumors. To get an idea of the absolute and relative importance of these perturbations, it is essential to investigate the status of oncogenes in a longitudinal study encompassing different stages of tobacco carcinogenesis, including leukoplakia. Such a study is also required to define the molecular counterpart of

precancerous lesions to identify leukoplakias that run a high risk of malignant transformation. In this respect, a retrospective study, using PCR techniques to examine paraffin blocks of leukoplakias and oral cancers in different stages of evolution, would be fruitful. Such studies are currently in progress in our laboratory.

REFERENCES

- Ali, I.U., Liderau, R., Theillet, C., Callahan, R. Reduction to homozygosity of genes on chromosome 11 in human breast neoplasia. *Science* 238: 185-188, 1987.
- Alitalo, K., Koskinen, P., Makela, T.P., Saksela, K., Sistonen, L., Winqvist, R. *myc* oncogene: Activation and amplification. *Biochimica et Biophysica Acta* 907: 1-32, 1985.
- Alt, F.W., DePinho, R., Zimmerman, K., Legouy, E., Hatton, K., Ferrier, P., Tesfaye, A., Yancopoulos, G., Nisen, P. The human *myc* gene family. *Cold Spring Harbor Symposia on Quantitative Biology* LI: 931-941, 1986.
- Azuma, M., Furumoto, N., Kawamata, H., Yoshida, H., Yanagawa, T., Yura, Y., Hayashi, Y., Takegawa, Y., Sato, M. The relation of *ras* oncogene product p21 expression to clinicopathological status criteria and clinical outcome in squamous cell head and neck cancer. *Cancer Journal* 1: 375-380, 1987.
- Berenson, J.R., Yang, J., Mickel, R.A. Frequent amplification of the *bcl-1* locus in head and neck squamous cell carcinomas. *Oncogene* 4: 1111-1116, 1989.
- Bishop, J.M. The molecular genetics of cancer. *Science* 235: 311, 1987.
- Bonney, G.E. Interactions of genes, environment and life-style in lung cancer development. *Journal of the National Cancer Institute* 82: 1236-1237, 1990.
- Bos, J.L. *ras* oncogenes in human cancer: A review. *Cancer Research* 49: 4682-4689, 1989.
- Capon, D., Chen, E., Levinson, A., Seeburg, P., Goeddel, D. Complete nucleotide sequences of the t24 human bladder carcinoma oncogene and its normal homologue. *Nature* 302: 33-37, 1983.
- Downward, J., Yarden, Y., Mayeot, S. Close similarity of epidermal growth factor receptor and *v-erbB* oncogene protein sequences. *Nature* 37: 521-528, 1984.
- Fasano, O., Birnbaum, D., Edlund, L., Fogh, J., Wigler, M. New human transforming genes detected by a tumorigenicity assay. *Molecular and Cellular Biology* 4: 1695-1705, 1984.
- Field, J.K., Lamothe, A., Spandidos, D.A. Clinical relevance of oncogene expression in head and neck tumors. *Anticancer Research* 6: 595-600, 1986.
- Field, J.K., Spandidos, D.A., Stell, P.M., Vaughan, E.D., Evan, J.I., Moore, J.P. Elevated expression of the *c-myc* oncoprotein correlates with poor prognosis in head and neck squamous cell carcinoma. *Oncogene* 4: 1463-1468, 1989.
- Friedman, W.H., Rosenblum, B., Lowenstein, P., Thornton, H., Katsantonis, G., Green, M. Oncogenes: Preliminary studies in head and neck cancer. *Laryngoscope* 93: 1441-1444, 1983.
- Gupta, P.C., Mehta, F.S., Pindborg, J.J., Aghi, M.B., Bhonsle, R.B., Murti, P.R. An educational intervention study for tobacco chewing and smoking habits among Indian villagers. In: *Smoking and Health*, A.M. Hisamichi and S. Tominaga (Editors). Amsterdam: Excerpta Medica, 1987, pp. 623-627.
- Hecht, S., Spratt, T.E., Trushin, N. Evidence for 4-(3-pyridyl)-4-oxobutylolation of DNA in F344 rats treated with the tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N'*-nitrososornicotine. *Carcinogenesis* 9: 161-165, 1988.
- Howell, R.E., Wong, F.S.H., Fenwick, R.G. Loss of Harvey *ras* heterozygosity in oral squamous carcinoma. *Journal of Oral Pathology and Medicine* 18: 79-83, 1989.
- Howell, R.E., Wong, F.S.H., Fenwick, R.G. A transforming Kirsten *ras* oncogene in an oral squamous carcinoma. *Journal of Oral Pathology and Medicine* 19: 301-305, 1990.
- Ikeda, I., Ishizaka, Y., Ochiai, M., Sakai, R., Itabashi, M., Onda, U., Sugimura, T., Nagao, M. No correlation between *l-myc* restriction fragment length polymorphism and malignancy of human colorectal cancers. *Japanese Journal of Cancer Research* 79: 674-676, 1988.
- International Agency for Research on Cancer. *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Tobacco Habits Other Than Smoking; Betel-Quid and Areca-Nut Chewing; and Some Related Nitrosamines* (volume 37). Lyon: IARC, 1985.
- Jussawalla, D.J., Deshpande, V.A. Evaluation of cancer risk in tobacco chewers and smokers: An epidemiologic assessment. *Cancer* 28: 469-476, 1971.

- Takehi, Y., Yoshida, O. Restriction fragment length polymorphism of the L-*myc* gene and susceptibility to metastasis in renal cancer patients. *International Journal of Cancer* 43: 391-394, 1989.
- Kawashima, K., Shikama, H., Imoto, K., Izawa, M., Naruke, T., Okahayashi, K., Nishimura, S. Close correlation between restriction fragment length polymorphism of the L-*myc* gene and metastasis of human lung cancer to the lymph nodes and other organs. *Proceedings of the National Academy of Sciences* 85: 2353-2356, 1987.
- Klein, G. Oncogenes and tumor suppressor genes. Reviews in Oncology 1. *Acta Oncologica* 27: 427-437, 1988.
- Klein, G., Klein, E. Evolution of tumors and the impact of molecular oncology. *Nature* 315: 190-195, 1985.
- Krontiris, T.G., Cooper, G.M. Transforming activity of human tumor DNA. *Proceedings of the National Academy of Sciences* 78: 1181-1184, 1981.
- Mattson, M.E., Winn, D.M. Smokeless tobacco: Association with increased cancer risk. *National Cancer Institute Monographs* 8: 13-16, 1989.
- Pierotti, M., Radice, P., Biunno, I., Borrello, M., Cattadori, M., Porta, G. Detection of two *TaqI* polymorphisms in the VTR region of the human H-*ras*-1 oncogene. *Cytogenetics and Cell Genetics* 43: 174-180, 1986.
- Quintanilla, M., Brown, K., Ramsden, D., Balmain, A. Carcinogen-specific mutation and amplification of Ha-*ras* during mouse skin carcinogenesis. *Nature* 322: 78-82, 1986.
- Rumsby, G., Carter, R.L., Gusterson, B.A. Low incidence of *ras* oncogene activation in human squamous cell carcinomas. *British Journal of Cancer* 61: 365-368, 1990.
- Saranath, D., Panchal, R.G., Nair, R., Mehta, A.R., Sanghavi, V., Sumegi, J., Klein, G., Deo, M.G. Oncogene amplification in squamous cell carcinoma of the oral cavity. *Japanese Journal of Cancer Research* 80: 430-437, 1989.
- Saranath, D., Panchal, R.G., Nair, R., Mehta, A.R., Sanghavi, V., Deo, M.G. Restriction fragment length polymorphism of the L-*myc* gene in oral cancer patients. *British Journal of Cancer* 61: 530-533, 1990.
- Saranath, D., Bhoite, L.T., Mehta, A.R., Sanghavi, V., Deo, M.G. Loss of allelic heterozygosity at the Harvey *ras* locus in human oral carcinomas. *Journal of Cancer Research and Clinical Oncology* 117: 484-488, 1991a.
- Saranath, D., Chang, S.E., Bhoite, L.T., Panchal, R.G., Kerr, I.B., Mehta, A.R., Johnson, N.W., Deo, M.G. High frequency mutation in codons 12 and 61 of H-*ras* oncogene in chewing tobacco-related human oral carcinoma in India. *British Journal of Cancer* 63: 573-578, 1991b.
- Sellers, T.A., Bailey-Wilson, J.E., Elston, R.C., Wilson, A.F., Elston, G.Z., Ooi, W.L., Rothschild, H. Evidence for Mendelian inheritance in the pathogenesis of lung cancer. *Journal of the National Cancer Institute* 82: 1272-1279, 1990.
- Sheng, Z.M., Barrois, M., Klijanienko, J., Micheau, C., Richard, J.M., Riou, G. Analysis of the c-Ha-*ras*-1 gene for deletion, mutation, amplification and expression in lymph node metastasis of human head and neck carcinomas. *British Journal of Cancer* 62: 398-404, 1990.
- Shih, C., Weinberg, R.A. Isolation of a transforming sequence from a human bladder carcinoma cell line. *Cell* 29: 161-169, 1982.
- Slebos, R.J.C., Kibbelaar, R.E., Dalesio, O., Kooistra, A., Stam, J., Meijer, C.J.L.M., Wagenaar, S.S., Vanderschueren, R.G., Zandwijk, N., Mooi, W.J., Bos, J.L., Rodenhuis, S. H-*ras* oncogene activation as a prognostic marker in adenocarcinoma of the lung. *New England Journal of Medicine* 323: 561-565, 1990.
- Sommers, K.D., Cartwright, S.L., Schechter, G.L. Amplification of the *int-2* gene in human head and neck squamous cell carcinoma. *Oncogene* 5: 915-920, 1990.
- Spandidos, D.A., Holmes, L. Transcriptional enhancer activity in the variable tandem repeat DNA sequence downstream of the human Ha-*ras*-1 gene. *FEBS Letters* 218: 41-46, 1987.
- Spandidos, D.A., Lamothe, A., Field, J. Multiple transcriptional activation of cellular oncogenes in human head and neck solid tumours. *Anticancer Research* 5: 221-224, 1985.
- Topal, M.D. DNA repair, oncogenes and carcinogenesis. *Carcinogenesis* 9: 691-696, 1988.
- Yokota, J., Tsunetsugu-Yokota, Y., Baltitora, H., Leferre, C., Cline, M.J. Alterations of *myc*, *myb* and *ras*-ha protooncogene in cancers are frequent and show clinical correlation. *Science* 231: 261-265, 1986.
- Zarbl, H., Sukumar, S., Arthur, A.V., Martin-Zanca, D., Barbacid, M. Direct mutagenesis of Ha-*ras*-1 oncogenes by n-nitroso-n-methylurea during initiation of mammary carcinogenesis in rats. *Nature* 315: 382-385, 1985.
- Zimmerman, K.A., Yancopoulos, G.D., Collum, R.G., Russell, K.S., Kohl, N.E., Denis, K.A., Nau, M.M., Witte, O.N., Toran-Allerand, D., Gee, C.E., Minna, J.D., Alt, F.W. Differential expression of *myc* family genes during murine development. *Nature* 319: 780-783, 1986.

Metabolism and Macromolecular Binding of NNK and NNN, Important Carcinogens in Smokeless Tobacco¹

Stephen S. Hecht, Steven G. Carmella, and Sharon E. Murphy

ABSTRACT This paper describes the pathways of metabolic activation and macromolecular binding of two tobacco-specific nitrosamines, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N'*-nitrosornicotine (NNN). NNK and NNN are important because of their tumorigenicity in the oral cavity and their presence in significant quantities in snuff and the saliva of snuff dippers. Studies in cultured rat oral tissue have shown that both compounds are metabolically activated by α -hydroxylation. Methylation of DNA has been detected in rat oral tissue incubated with NNK. Human oral tissue also can metabolize these nitrosamines. Analysis of hemoglobin isolated from snuff dippers has demonstrated the presence of adducts resulting from the metabolic activation of NNK and NNN. Together, these results support the hypothesis that NNK and NNN are involved in the induction of oral cancer by snuff dipping.

INTRODUCTION Epidemiological studies have established that snuff dipping is a cause of oral cancer (IARC, 1985; Winn et al., 1981). Two tobacco-specific nitrosamines—4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and *N'*-nitrosornicotine (NNN)—are the most prevalent strong carcinogens in oral snuff and the saliva of snuff dippers (Hoffman and Adams, 1981; Hoffman et al., 1987). A mixture of NNK and NNN swabbed daily for life in the oral cavities of rats induced a significant incidence of oral tumors (Hecht et al., 1986). The total dose was similar to that encountered by snuff dippers (Hecht and Hoffman, 1989). NNK and NNN are the only tobacco constituents known to induce oral tumors in animals. These data support the hypothesis that NNK and NNN are causative factors in human oral cancer induced by snuff dipping (Hecht and Hoffman, 1989).

In this paper, we summarize our current understanding of the mechanisms by which NNK and NNN are metabolized to intermediates that bind to cellular macromolecules such as DNA and hemoglobin. We focus on data that relate to oral cavity carcinogenesis. The metabolism of carcinogens to DNA-reactive compounds is called metabolic activation. Its hallmark is the generation of electrophilic intermediates that can bind to nucleophilic centers in DNA, RNA, or protein. Binding to DNA with formation of covalently attached residues called “DNA adducts” is important in carcinogenesis. DNA adducts have miscoding properties and can interfere with the normal processes of replication. The presence of DNA adducts in proto-oncogenes can lead to their activation; the presence of DNA adducts in tumor suppressor genes can lead to their inactivation. Both processes are

¹ These studies were supported by National Cancer Institute grants no. CA-29580 and no. CA-44377.

involved in the transformation of normal cells to neoplastic cells and the eventual development of tumors. Although hemoglobin adducts, in contrast to DNA adducts, are not believed to play any part in tumor development, they can serve as surrogates for estimating levels of DNA adducts in human tissues.

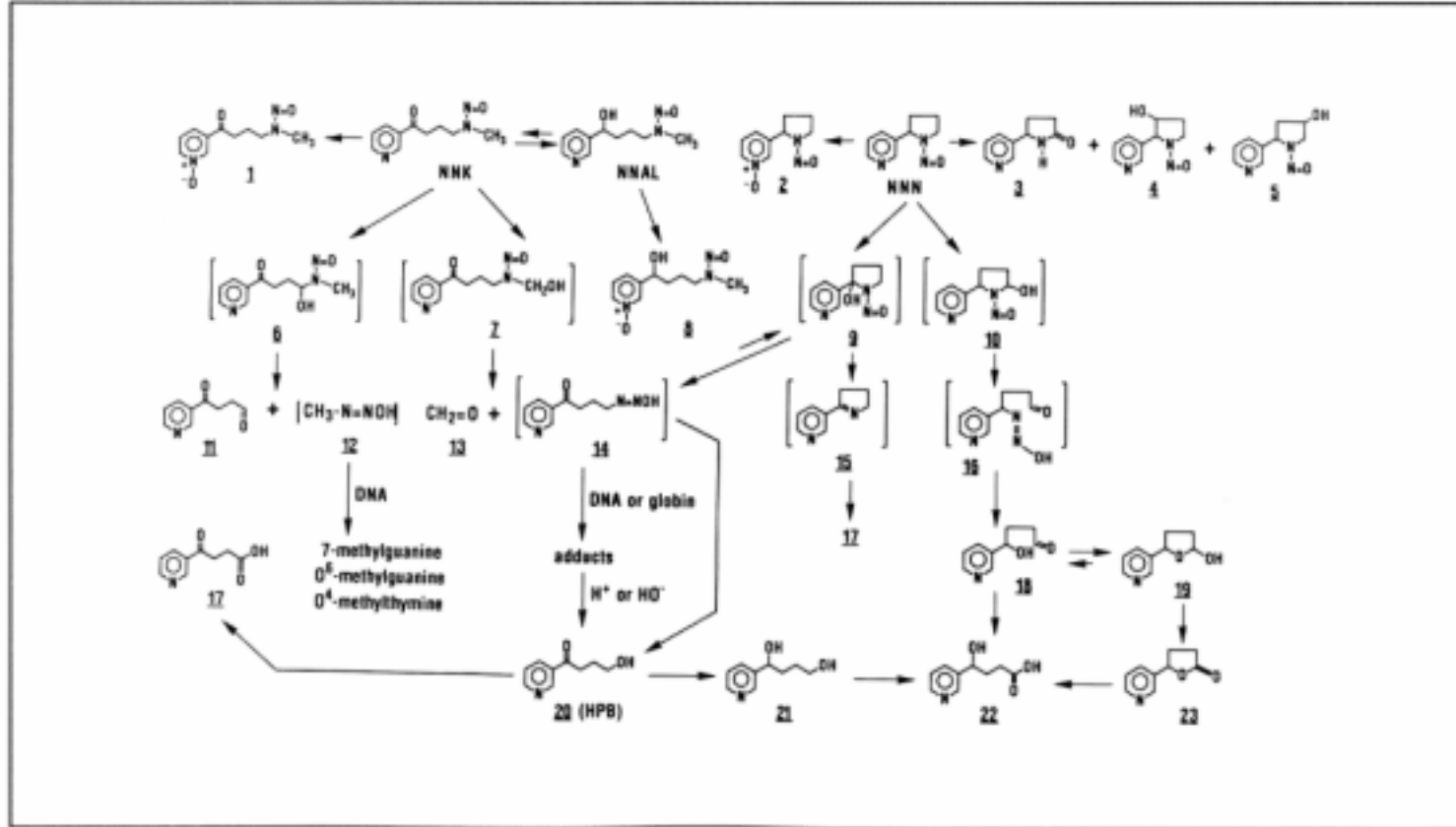
METABOLISM OF Figure 1 summarizes metabolic pathways of NNK and NNN.

NNK AND NNN Metabolites have been identified in the urine of rats, mice, and hamsters and in *in vitro* experiments with cultured tissues or subcellular fractions (Hecht and Hoffman, 1988; Hecht et al., 1983). There are three major metabolism pathways for NNK—pyridine N-oxidation to give NNK-1-N-oxide (marked 1 in Figure 1), carbonyl reduction to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), and α -hydroxylation to the unstable α -hydroxy-NNK derivatives 6 and 7. α -Hydroxylation is the major metabolic activation pathway of NNK because compounds 6 and 7 spontaneously decompose to the electrophiles methyl diazohydroxide (12) and 4-(3-pyridyl)-4-oxobutyl diazohydroxide (14) with simultaneous formation of the keto aldehyde (11) and formaldehyde (13). Methyl diazohydroxide reacts with DNA, producing a mixture of methylated DNA bases among which 7-methylguanine, *O*⁶-methylguanine, and *O*⁴-methylthymidine have been identified in the tissues of animals treated with NNK. The diazohydroxide (14) reacts with DNA, producing adducts of unknown structure that release 4-hydroxy-1-(3-pyridyl)-1-butanone (HPB, 20) upon acid hydrolysis. This process is referred to as pyridyloxobutylation. Both diazohydroxides also react with hemoglobin, producing globin adducts (Carmella and Hecht, 1987). The adduct formed from reaction of 14 with globin releases HPB upon mild base hydrolysis. The aldehyde 11 and HPB, which are initial products of α -hydroxylation of NNK, undergo further oxidation and reduction *in vivo* and in certain *in vitro* systems producing keto acid 17, diol 21, and hydroxy acid 22.

NNAL is formed from NNK in most tissues and also methylates and pyridyloxobutylates DNA (Hecht and Trushin, 1988). These results occur either by reversion to NNK or by α -hydroxylation. The latter pathways are not illustrated in Figure 1, but give rise to diol 21 and hydroxy acid 22. NNAL also undergoes pyridine N-oxidation producing NNAL-N-oxide 8. Another pathway of NNAL metabolism not shown in Figure 1 is formation of its *O*-glucuronide, which is a major metabolite in rodent urine (Morse et al., 1990).

Metabolism of NNN occurs by pyridine-N-oxidation to NNN-1-N-oxide 2, by denitrosation and oxidation yielding norcotinine 3, and by hydroxylation of the four positions of its pyrrolidine ring. The hydroxylation products 4 and 5 are minor metabolites and appear to be stable. The α -hydroxylation products 9 and 10 are major metabolites and are unstable. Compound 9 spontaneously decomposes to the same diazohydroxide 14 that is formed from methyl hydroxylation of NNK. Therefore, NNN also pyridyloxobutylates DNA and globin to give adducts that release HPB upon hydrolysis (Carmella and Hecht, 1987). Compound 10 generates the hydroxy aldehyde 18, which exists in the cyclic form as lactol 19. The initial

Figure 1
Metabolic pathways of NNK and NNN in rodents



Source: Hecht et al., 1991. Copyright 1991, CRC Press; used with permission.

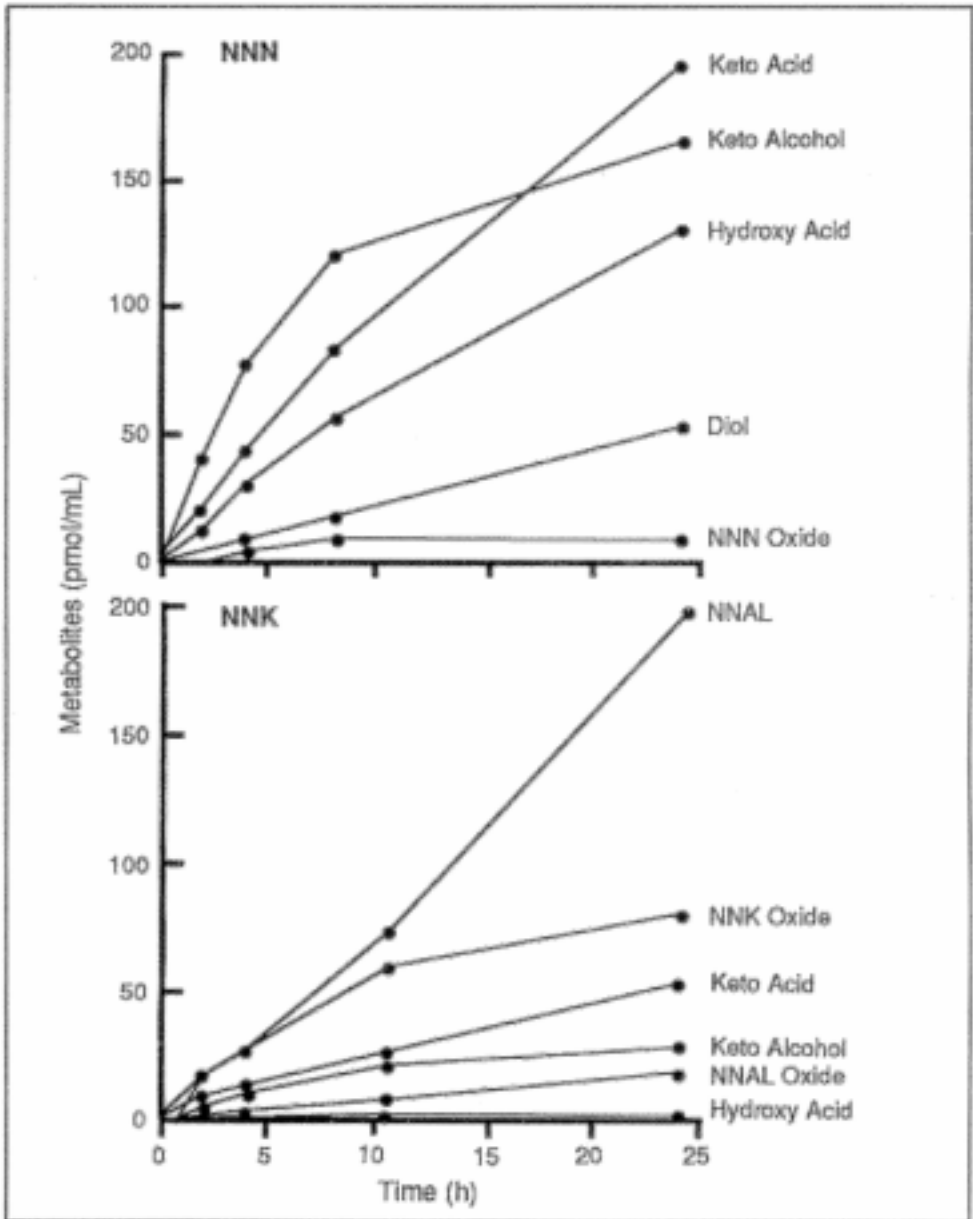
products of NNN α -hydroxylation, HPB and lactol 19, undergo further oxidation, producing keto acid 17 and hydroxy acid 22. These two acids are good indicators of the extent of α -hydroxylation of NNN by the two pathways illustrated in Figure 1.

Methylation and pyridyloxobutylation of DNA, via 12 and 14, are the two most likely pathways by which NNK would initiate tumorigenesis. The importance of the DNA methylation pathway in NNK tumorigenesis has been demonstrated in certain systems, whereas the role of the pyridyloxobutylation pathway is less clear (Bebinsky et al., 1989); Hecht et al., 1990. For NNN, which cannot methylate, DNA pyridyloxobutylation is likely to be important in tumorigenesis, but the mechanism is unclear at present.

In Rat Oral Tissue The metabolism of NNK and NNN can be conveniently studied by incubation of the tritium-labeled compounds with rat oral tissue (Murphy et al., 1990). Oral tissue is obtained from the insides of both cheeks, the roof of the mouth, and the inside of the lips. It is trimmed of muscle and connective tissue. Pieces of tissue are placed epithelial side up on a dry filter in a 60-mm culture dish and are then covered with 5 mL of Williams' medium with additives. They are incubated with [5-³H]NNK or [5-³H]NNN, which have tritium at the 5 position of the pyridine ring. Incubations are carried out for various periods up to 24 h; tissue viability is excellent under these conditions. At the end of the incubation, the medium is analyzed for metabolites by high-performance liquid chromatography (HPLC) with radioflow detection. Figure 2 illustrates the time course of metabolite formation from 1 μ M NNK or NNN in rat oral tissue. The major products of NNK metabolism were NNAL and NNK-1-N-oxide (1 in Figure 1). α -Hydroxylation produced keto acid (17) and HPB. Metabolism experiments with HPB demonstrated that the keto acid was formed from both keto aldehyde (11) and HPB, indicating that both α -hydroxylation pathways of NNK were operative. α -Hydroxylation of NNN was six times greater than α -hydroxylation of NNK. Keto acid (17) and HPB were the major α -hydroxylation products. In contrast to pyridine-N-oxidation of NNK, pyridine-N-oxidation of NNN was barely detectable. These results indicate that metabolic activation of NNN to intermediates capable of binding to DNA exceeded metabolic activation of NNK in cultured rat oral tissue.

DNA binding was examined in these cultured tissues, and 7-methylguanine was detected in oral tissue incubated with NNK labeled with tritium in the methyl group. This is consistent with the metabolic activation pathway that produces keto aldehyde (11) and keto acid (17). Although O⁶-methylguanine is known to be formed by the same pathway, it was not detectable under these conditions. It was also not possible to detect HPB in acid hydrolysates of DNA isolated from rat oral tissue incubated with [5-³H]NNK or [5-³H]NNN. Surprisingly, in rat esophagus, which metabolizes NNN to a similar extent by α -hydroxylation as oral tissue, HPB was detected in acid hydrolysates of isolated DNA (Murphy et al., 1990b). These results suggest that cellular constituents of oral tissue other than DNA may have a particular affinity for reaction with diazohydroxide (14).

Figure 2
Formation of NNK and NNN metabolites by cultured rat oral tissue as a function of time



Source: Murphy et al., 1990b. Copyright 1990, Cancer Research; used with permission.

The enzymology of NNK and NNN activation in oral tissue also requires further study. Cytochrome P₄₅₀ isozymes are believed to be responsible for the α -hydroxylation of these nitrosamines, but the forms present in oral tissue have not been characterized. Comparative studies of NNK and NNN

metabolism in rat oral tissue indicated that at least two different enzymes were involved in α -hydroxylation (Murphy et al., 1990b).

In Human Oral Tissue Limited information is available on the metabolism of NNK and NNN by human oral tissue. In one study, buccal mucosa obtained at immediate autopsy was cultured with labeled NNK or NNN (Cantonguay et al., 1983). NNK was extensively converted to NNAL, as in all human tissues examined to date. Hydroxy acid (22 in Figure 21-1) produced by α -hydroxylation of NNAL, was also detected. Similar results have been obtained in ongoing experiments with oral tissue obtained surgically. NNN was metabolized to NNN-1-N-oxide and to hydroxy acid (22). α -Hydroxylation of both NNK and NNN was far less extensive in human oral tissue than in rat oral tissue. However, it is not clear whether this is attributable to differences in the conditions under which the rat and human tissues were obtained and cultured.

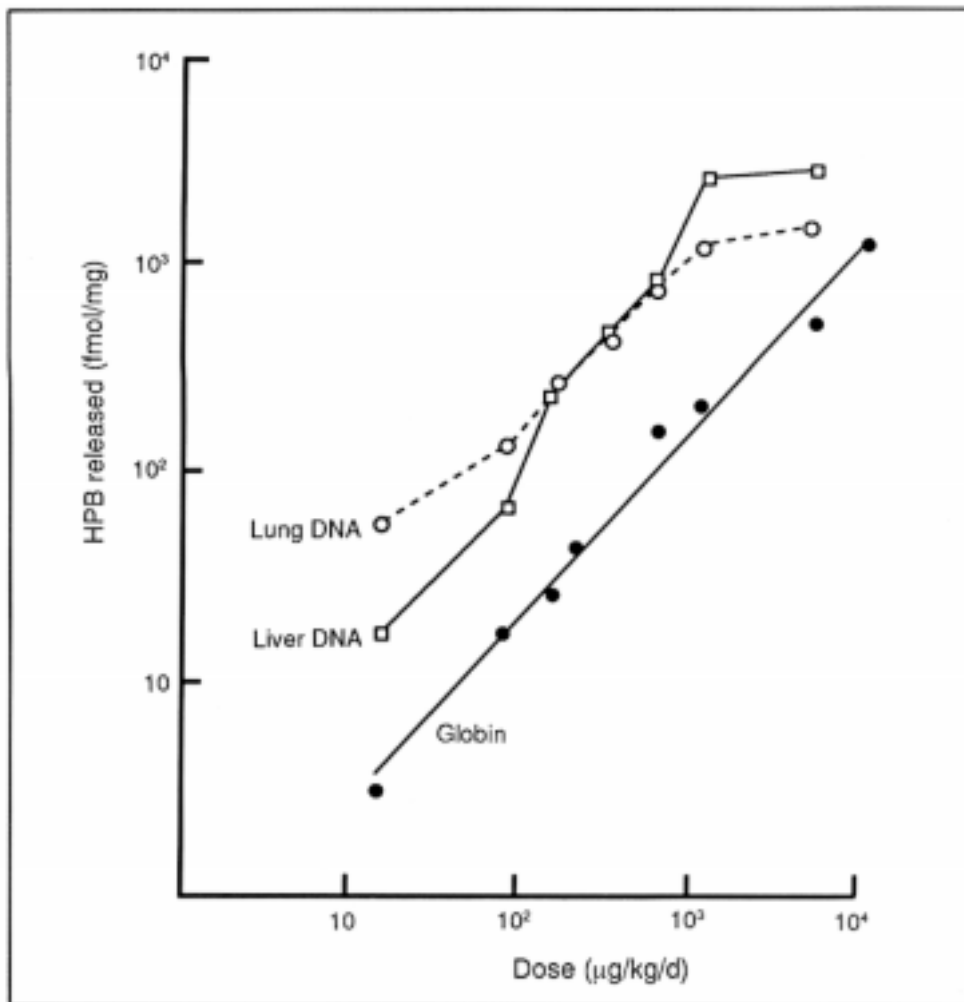
No information is available on oncogene activation by NNK or NNN in oral cavity tumors. This information could be useful in assessing their role in human oral cancer. Amplification of several oncogenes including *c-myc*, *N-myc*, *N-ras*, *K-ras*, and *int-2* have been observed in carcinomas of the oral cavity (Saranath et al., 1989; Somers et al., 1990).

DETECTION OF NNK/NNN-HEMOGLOBIN ADDUCTS As illustrated in Figure 1, the diazohydroxide 14, which is formed from both NNK and NNN, forms adducts in globin that release HPB upon mild-base hydrolysis. This was established by experiments in which labeled NNK or NNN was administered to rats (Carmella and Hecht, 1987). For NNK, approximately 0.1 percent of the dose was bound to hemoglobin, and about 20.0 percent of this could be released as HPB. The half-life of the adduct in rats is approximately 9 d. As shown in Figure 3, the formation of the HPB-releasing adduct was linear over doses ranging from 15 to 10,000 $\mu\text{g}/\text{kg}/\text{d}$, administered i.p. for 4 d (Murphy et al., 1990a). The release of HPB from lung and liver DNA by acid hydrolysis also increased over this dose range, but the increase was not linear. No data are available for comparison of oral tissue DNA adduct formation to hemoglobin adducts.

Measurement of hemoglobin adducts in humans could serve as a biomarker of the internal dose of a given electrophile (Ehrenberg and Osterman-Golkar, 1976). In this case, the electrophile of interest is the diazohydroxide 14. Its formation would be specifically related to uptake and metabolic activation of NNN and NNK, in contrast to methyl diazohydroxide 12, which could have many environmental sources. Advantages of hemoglobin adducts as surrogates for DNA adducts include the ready availability of hemoglobin in quantities sufficient for accurate analysis and the long lifetime of the erythrocyte in humans (120 d), which permits integration of dose (Ehrenberg and Osterman-Golkar, 1976). Because HPB is easily released from hemoglobin upon base hydrolysis and can be separated from the protein by extraction, it appeared to be a suitable compound for analysis.

Figure 3

Log log plot of HPB released from globin, after base hydrolysis, and from lung and liver DNA, after hydrolysis. Globin and DNA were obtained from rats treated with [5-³H]NNK (15 to 10,000 µg/kg/d, for 4 d).

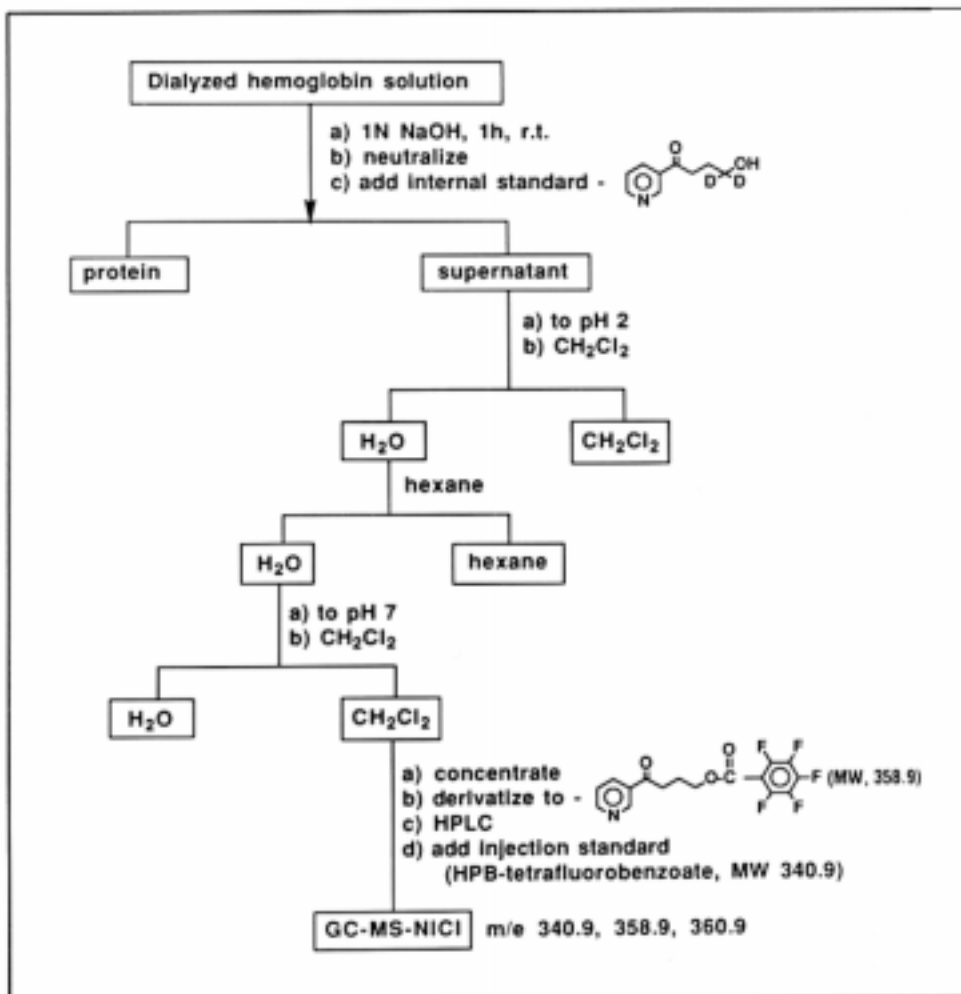


Source: Murphy et al., 1990b. Copyright 1990, Cancer Research; used with permission.

Figure 4 summarizes the scheme used for analysis of HPB released from human hemoglobin (Carmella et al., 1990). The key step is derivatization to its pentafluorobenzoate, which can be analyzed with high sensitivity (detection limit, approximately 1 fmol) by gas chromatography-negative ion chemical ionization mass spectrometry (GC-NICI-MS) with selective ion monitoring. With this method, HPB can readily be detected in samples of human hemoglobin that have been dialyzed and treated with mild base.

Figure 4

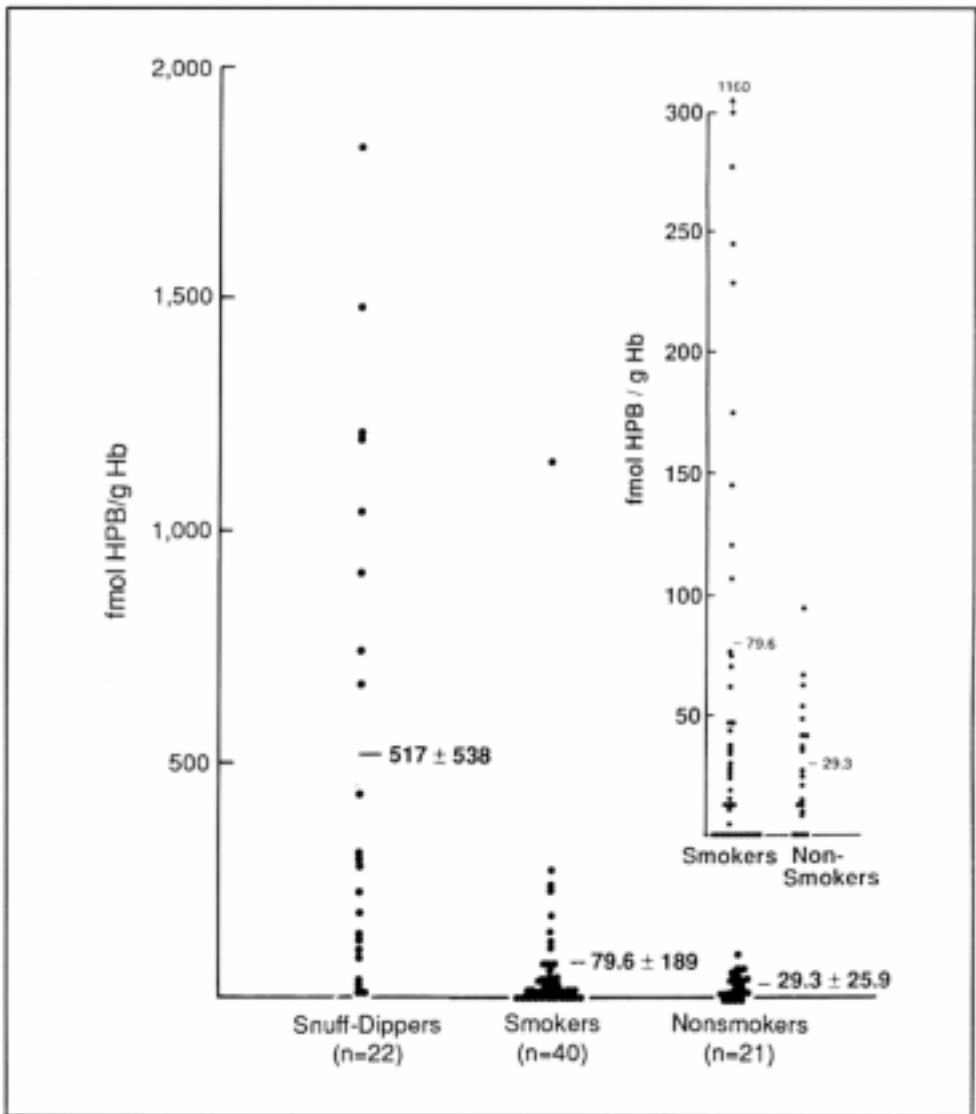
Scheme for analysis of HPB in hydrolysates of human hemoglobin



Source: Carmella et al., 1990. Copyright 1990, Cancer Research; used with permission.

Figure 5 illustrates results from analysis of hemoglobin of snuff dippers, smokers, and nonsmokers (Carmella et al., 1990). In this group, snuff dippers had the highest adduct levels; it is not known whether this will be a general phenomenon. The mean adduct level observed in snuff dippers was higher than expectations based on studies with rats. The effective daily dose of NNK and NNN producing HPB in these snuff dippers could be estimated as approximately 0.07 $\mu\text{g}/\text{kg}/\text{d}$. Injection of rats i.p. with 1 $\mu\text{g}/\text{kg}$ NNK for 5 wk resulted in 517 ± 3.2 fmol/g HPB. The higher levels of HPB-releasing adducts in snuff dippers than in rats could result from the endogenous formation of NNN and NNK. NNN may be formed by nitrosation of nor-nicotine, which is present in tobacco or produced in metabolism of nicotine. NNK could be formed by nitrosation of nicotine. The wide variation

Figure 5
Levels of HPB released from hemoglobin of snuff dippers, smokers, and nonsmokers



Source: Carmella et al., 1990. Copyright 1990, Cancer Research; used with permission.

in levels of hemoglobin adducts among the snuff dippers suggests that there are differences in individual capacities for metabolic activation of NNK and NNN and, possibly, related differences in risk.

The presence of HPB-releasing adducts in snuff dippers' hemoglobin supports our hypothesis that NNK and NNN are causative agents for oral cancer in snuff dippers. The data demonstrate that snuff dippers can metabolically activate NNK and NNN to diazohydroxide (14 in Figure 1).

It will be important to investigate the presence of HPB-releasing adducts in oral DNA from snuff dippers and to determine whether their levels correlate with levels of hemoglobin adducts. A method for the analysis of HPB released by acid hydrolysis of human DNA has recently been developed (Foiles et al., 1991).

**GAPS IN
ASSESSING
THE ROLE OF
NNK AND NNN**

Although the metabolic activation pathways of NNK and NNN have been characterized in a number of tissues, there are relatively limited data for the oral cavity. It would be important to determine which P₄₅₀ isozymes are involved in NNK and NNN metabolism in the rat oral cavity as well as in human oral tissues. This would allow one to more accurately assess human risk by determining an individual's level of expression of these particular isozymes in oral tissues. The DNA adducts that result from the metabolic activation of NNK and NNN in oral tissue have not been thoroughly examined. More sensitive methods are needed to detect these adducts. It would be useful to understand the spectrum of adducts formed from these nitrosamines in oral tissue; the presence of particular adducts could perhaps be related to oncogene activation, tumor suppressor gene inactivation, and risk for oral cancer.

The characteristics of the model in which a mixture of NNK and NNN induces oral tumors upon lifetime swabbing in the rat oral cavity should be more clearly defined. Is NNK or NNN responsible for the tumorigenic effect, or are both compounds necessary? Does irritation caused by continuous swabbing contribute to the tumorigenic effect? Could the protocol be varied in some way to decrease the latent period and improve tumor yield in order to provide a more practical model? It is not clear how the route of administration affects tumor incidence in this model. Lung tumors were observed, consistent with the organospecificity of NNK, but no tumors of the esophagus and nasal cavity were observed. They might have been expected, given the known target tissues of NNK and NNN.

The potent pulmonary tumorigenicity of NNK suggests that snuff dippers may be at risk for lung cancer. This should be investigated.

The role of cofactors in tumorigenesis by NNK and NNN, as components of a complex mixture like snuff, is not clear. Snuff extract inhibited the tumorigenicity of NNK and NNN (Hecht et al., 1986). This could be attributable to the inhibitory effect of nicotine on their metabolic activation or to other compounds in the extract. Snuff is tumorigenic when instilled in the rat lip canal; the precise role of NNK and NNN in this model is not clear (Hecht et al., 1986). It seems likely that the irritant properties of snuff would enhance NNK and NNN tumorigenesis by increasing cell replication. This should be tested. Viruses may also play a role as cofactors in NNK and NNN tumorigenesis (Park et al., 1986).

Hemoglobin adducts appear to provide a method for assessing the metabolic activation of NNK and NNN in humans. What contributes to a high adduct level and what are its consequences? Does endogenous nitrosation increase the dose of NNK and NNN beyond what is found in snuff? Or are high adduct levels strictly a function of an individual's ability

to metabolically activate the compounds? What are the separate roles of NNK and NNN in contributing to adduct levels? These questions must be answered before the hemoglobin adduct data can be fully interpreted.

SUMMARY The evidence that NNK and NNN play a role in human oral cancer induced by snuff is strong. Both compounds are present in significant amounts in snuff and in the saliva of snuff dippers. They are metabolically activated in snuff dippers to intermediates that bind to hemoglobin. They cause oral tumors in rats and are metabolically activated by rat and human oral tissue. Although there are many questions about the mechanisms by which snuff causes oral tumors in rats and humans, there is no doubt that the presence of NNK and NNN in snuff is an unacceptable risk to people who choose to use these products.

REFERENCES

- Belinsky, S.A., Devereux, T.R., Maronpot, R.R., et al. Relationship between the formation of pro-mutagenic adducts and the activation of the *K-ras* proto-oncogene in lung tumors from A/J mice treated with nitrosamines. *Cancer Research* 49: 5305-5311, 1989.
- Carmella, S.G., Hecht, S.S. Formation of hemoglobin adducts upon treatment of F344 rats with the tobacco-specific nitrosamines 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and *N'*-nitrosornicotine. *Cancer Research* 47: 2626-2630, 1987.
- Carmella, S.G., Kagan, S.S., Kagan, M., et al. Mass spectrometric analysis of tobacco-specific nitrosamine hemoglobin adducts in snuff dippers, smokers, and nonsmokers. *Cancer Research* 50: 5438-5445, 1990.
- Castonguay, A., Stoner, G.D., Schut, H.A.J., et al. Metabolism of tobacco-specific *N'*-nitrosamines by cultured human tissues. *Proceedings of the National Academy of Sciences* 80: 6694-6697, 1983.
- Ehrenberg, L., Osterman-Golkar, S. Alkylation of macromolecules for detecting mutagenic agents. *Teratogenesis, Carcinogenesis, and Mutagenesis* 1: 105-127, 1976.
- Foiles, P.G., Akerkar, S.A., Carmella, S.G., et al. Mass spectrometric analysis of tobacco-specific nitrosamine DNA adducts in smokers and nonsmokers. *Chemical Research in Toxicology*, 4: 364, 1991.
- Hecht, S.S., Castonguay, A., Rivenson, A., et al. Tobacco specific nitrosamines: Carcinogenicity, metabolism, and possible role in human cancer. *Journal of Environmental Science and Health. Part C. (CI)* 1: 1-54, 1983a.
- Hecht, S.S., Haley, N.J., and Hoffmann, D. Monitoring exposure to tobacco products by measurement of nicotine metabolites and derived carcinogens. In: *Molecular Dosimetry and Human Cancer: Analytical, Epidemiological, and Social Considerations*, J.D. Groopman and P.L. Skipper (Editors). Boca Raton, FL: CRC Press, 1991, pp. 325-361.
- Hecht, S.S., Hoffmann, D. Tobacco-specific nitrosamines: An important group of carcinogens in tobacco and tobacco smoke. *Carcinogenesis* 9: 875-884, 1988.
- Hecht, S.S., Hoffmann, D. The relevance of tobacco-specific nitrosamines to human cancer. *Cancer Surveys* 8: 273-294, 1989.
- Hecht, S.S., Jordan, K.G., Choi, C.-I., et al. Effects of deuterium substitution on the tumorigenicity of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol in A/J mice. *Carcinogenesis* 11: 1017-1020, 1990.
- Hecht, S.S., Rivenson, A., Braley, J., et al. Induction of oral cavity tumors in F344 rats by tobacco-specific nitrosamines and snuff. *Cancer Research* 46: 4162-4166, 1986.
- Hecht, S.S., Trushin, N. DNA and hemoglobin alkylation by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) in F344 rats. *Carcinogenesis* 9: 1665-1668, 1988.
- Hoffmann, D., Adams, J.D. Carcinogenic tobacco-specific N-nitrosamines in snuff and saliva of snuff dippers. *Cancer Research* 41: 4305-4308, 1981.
- Hoffmann, D., Adams, J.D., Lisk, D., Fiseune, I., Brunnemann, K. Toxic and carcinogenic agents in dry and moist snuff. *Journal of the National Cancer Institute* 79: 1281-1286, 1987.
- International Agency for Research on Cancer. *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Tobacco Habits Other Than Smoking; Betel-Quid and Areca-Nut Chewing; and Some Related Nitrosamines* (volume 37). Lyon: IARC, 1985, pp. 113-116.
- Morse, M.A., Eklind, K.I., Toussaint, M., et al. Characterization of a glucuronide metabolite of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and its dose dependent excretion in the urine of mice and rats. *Carcinogenesis* 11: 1819-1823, 1990.

- Murphy, S.E., Heiblum, R., Trushin, N. Comparative metabolism of *N'*-nitrosornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone by cultured rat oral tissue and esophagus. *Cancer Research* 50: 4685-4691, 1990b.
- Murphy, S.E., Palomino, A., Hecht, S.S., et al. Dose-response study of DNA and hemoglobin adduct formation by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in F344 rats. *Cancer Research* 50: 5446-5452, 1990a.
- Park, N.H., Sapp, J.P., Herbosa, E.G. Oral cancer induced in hamsters with *herpes simplex* infection and simulated snuff-dipping. *Oral Surgery, Oral Medicine, Oral Pathology* 62: 164-168, 1986.
- Saranath, D., Panchal, R.G., Nair, R., et al. Oncogene amplification in squamous cell carcinoma of the oral cavity. *Japanese Journal of Cancer Research* 80: 430-437, 1989.
- Somers, K.D., Cartwright, S.L., Schechter, G.L. Amplification of the *int-2* gene in human head and neck squamous cell carcinomas. *Oncogene* 5: 915-920, 1990.
- Winn, D.M., Blot, W.J., Shy, C.M., Pickle, L.W., Toledo, A., Frameni, J.F., Jr. Snuff dipping and oral cancer among women in southern United States. *New England Journal of Medicine* 304: 745-749, 1981.

Role of Nicotine as a Cofactor In Smokeless Tobacco Carcinogenesis¹

Christopher A. Squier and Georgia K. Johnson

ABSTRACT Nicotine is a constituent of all tobacco but is present at higher concentrations in smokeless tobacco than in other forms. The ST user is likely to have nicotine come into contact more frequently with the oral lining than is the smoker. Studies with animals suggest that the presence of nicotine enhances the effect of known carcinogens in the development of oral carcinoma. In vitro studies show that 2 percent and 6 percent nicotine applied topically to oral mucosa causes epithelial damage and an increase in permeability. We suggest that the method by which nicotine contributes to tumor development involves initial tissue damage, followed by a reparative response, accompanied by increased permeability to carcinogens. Continued exposure will lead to malignant transformation of the tissue.

INTRODUCTION Nicotine is the component of tobacco that causes the psychoactive effects that lead to addiction as well as to a variety of physiological changes (Benowitz, 1986 and 1988). The concentration of nicotine in tobacco ranges from 1 to 2 percent of the dry weight of the processed leaf in cigarette tobacco (Gritz et al., 1981), between 1.45 and 8.00 percent in smokeless tobacco (snuff and plug tobacco) in the United States (Gritz et al., 1981), and between 0.5 and 3.0 percent in snuff worldwide (Hoffmann and Adams, 1981). However, unlike smoking tobacco where only 15 to 20 percent of nicotine is transferred into mainstream smoke (Schmeltz et al., 1979), which is present intermittently in the oral cavity, all of the nicotine in ST is present in the mouth for extended periods of time. The possibility that the nicotine in smokeless tobacco may be a precursor of the tobacco-associated nitrosamines, some of which are known carcinogens (Hoffmann and Adams, 1981), has been thoroughly explored (Hoffmann et al., 1985 and 1986). However, little attention has been given to the effect that high levels of topical nicotine might have on the oral mucosa.

IN VIVO STUDIES WITH NICOTINE In a series of studies using skin, Bock (1980) was able to show that carcinogenesis by benzo[*a*]pyrene was enhanced by nicotine, although nicotine did not initiate or promote tumors; nicotine acted as a cofactor but was not a carcinogen by itself. We were able to demonstrate a similar effect in oral mucosa (Chen and Squier, 1990) by painting hamster cheek pouches with either 7,12-dimethylbenz[*a*]anthracene (DMBA) alone or in combination with 6 percent nicotine for 12 wk. Animals treated with DMBA and nicotine showed significantly ($p < 0.001$) more tumors and a significantly ($p < 0.05$) greater than expected proportion of large tumors (> 3 mm diameter) in the cheek pouch than hamsters treated with DMBA alone. Animals treated with nicotine alone did not develop tumors. In a similar experiment, using 6 percent nicotine and either *N*'-nitrosonor-nicotine (NNN) or 4-(methylnitrosamino)-1-(3 pyridyl)-1-butanone (NNK),

¹ Supported in part by National Institute of Dental Research grants no. R01-DE07930 and R29-DE/AI10153-01.

we have observed greater epithelial hyperkeratosis and dysplasia in the oral mucosa and the presence of papillomas in gastric mucosa as compared with treatment with nicotine alone (Chen and Squier, unpublished results).

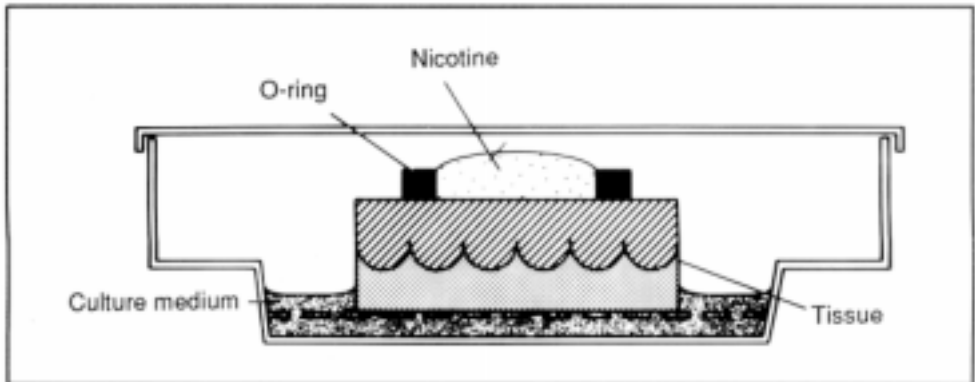
Studies in which snuff was applied topically to the rat periodontium have indicated decreased gingival blood flow (Schroeder and Milo, 1989) and bone loss (Hill et al., 1990). However, other workers have reported increased gingival blood flow in dogs after topical application of nicotine (Johnson et al., 1989). These differences may represent a paradoxical vasoactive effect of nicotine at various dose levels, but the studies do show that nicotine can exert a local tissue effect.

IN VITRO STUDIES WITH NICOTINE To examine the local acute effects of nicotine applied topically to oral mucosa, we have developed an in vitro model system (Figure 1). Nicotine is prepared in phosphate-buffered saliva (PBS) at concentrations of 2 or 6 percent and placed in a circular rubber gasket attached to the surface of specimens of oral mucosa with cyanoacrylate. The tissue is maintained in culture medium and incubated at 37 °C for 1 or 2 h; controls are incubated with PBS alone at the same pH (7.5) and osmolarity (450 mosm) as the nicotine solution. The tissue consists of pig oral mucosa taken at death from the gingiva, floor of the mouth, and the buccal mucosa, which have a structure very similar to that of the corresponding human tissue (Lesch et al., 1989). Specimens either are processed for morphological examination by light and electron microscopy, or the functional capacity of the tissue is assessed by measure of the permeability. We accomplish this by clamping specimens between the halves of a glass perfusion chamber and determining the amount of tritiated water that passes across per unit time. From these data, a permeability constant (K_p) can be calculated (Siegal et al., 1971).

Morphologically, in all mucosal regions there was more damage after treatment with 6 percent than with 2 percent nicotine and greater damage after 1 h incubation with 6 percent nicotine than after 2 h with 2 percent nicotine. Changes in controls were minimal. The epithelium of gingiva (Figure 2) and floor-of-mouth mucosa (Figure 3) showed greater destruction than buccal mucosa (Figure 4). Changes were usually more evident in the deeper cellular layers than at the surface, and this type of damage was particularly striking for gingiva. The changes represented acantholysis and separation of epithelial strata, cell shrinkage, and nuclear pyknosis. It is notable that the in vitro appearances frequently resemble those illustrated for biopsies from oral mucosa of chronic snuff users (Andersson, 1991).

Those tissues treated with 2 percent nicotine that showed only slight alterations at the light microscope level (Figures 3 and 5) revealed considerable ultrastructural changes when examined with the transmission electron microscope. Changes included peripheral condensation of chromatin in cell nuclei and the presence of frequent intracellular vacuoles (Figure 5, left) that appear to represent damaged organelles such as mitochondria (Figure 5, right) as well as discontinuities in the basal lamina. Such changes seem to represent the initial cellular responses to nicotine and are of particular importance because 2 percent nicotine is found in many types of snuff worldwide.

Figure 1
The model used to expose oral mucosa to topical nicotine



Source: Squier and Lesch, 1988; used with permission.

The pattern of damage may reflect the chemical action of the nicotine molecule, as controls did not suggest any effect caused by pH or osmolarity. Nicotine may disrupt phospholipid-containing membranes, such as those of the deeper cell layers, whereas the superficial layers contain greater amounts of glycolipid or pure lipid rather than phospholipid (Squier et al., 1991).

Measurement of permeability to tritiated water of tissues treated with 6 percent nicotine for 2 h (Table 1) revealed significant ($p > 0.05$) increases in K_p values in all regions compared with controls incubated in PBS or normal, untreated mucosa (values from Lesch and coworkers, 1989). Because the putative carcinogens in ST (the tobacco-associated nitrosamines) are present in an aqueous environment and can be extracted by saliva (Hecht et al., 1974), our results indicate that nicotine-induced damage could increase the access of such carcinogens to the deeper, proliferative compartment of the oral epithelium.

**CLINICAL
 IMPLICATIONS
 OF STUDIES
 WITH NICOTINE**

One of the characteristic features of chronic ST use is the presence of a hyperkeratotic oral mucosal lesion at the site of regular tobacco placement. Characterized clinically as a white lesion, leukoplakia, or snuff-dipper's lesion, these lesions show an increased likelihood of malignant transformation such that 4 percent of lesions may become carcinoma (Schafer et al., 1983). The pathogenesis of these lesions may reflect the acute epithelial damage brought about by the presence of nicotine in tobacco that is applied to an area of oral mucosa. The initial damage would evoke a hyperplastic reparative response and would be associated with an increased permeability to any carcinogens. Chronic irritation, as a result of continued placement of tobacco at the same site, would lead to hyperkeratosis that appears as a white patch. Hyperplasia and hyperkeratosis are protective responses of skin and oral mucosa to mechanical or chemical irritation. However, hyperkeratotic regions of skin (Grice, 1980) and oral mucosa (Squier et al., 1985) tend to show increased permeability so that tobacco-associated carcinogens will continue to enter the lesional tissue. Thus, the tissue represents a site of elevated cell proliferation that is exposed to higher levels of carcinogens than the adjacent normal mucosa. This will increase the likelihood of development of carcinoma.

Figure 2

Sections of porcine gingiva after exposure for 2 h with PBS (left), 2 percent nicotine (center), and 6 percent nicotine (right). Note the gradation of damage from left to right, all of which occurs in the deeper cell layers (magnification x200).

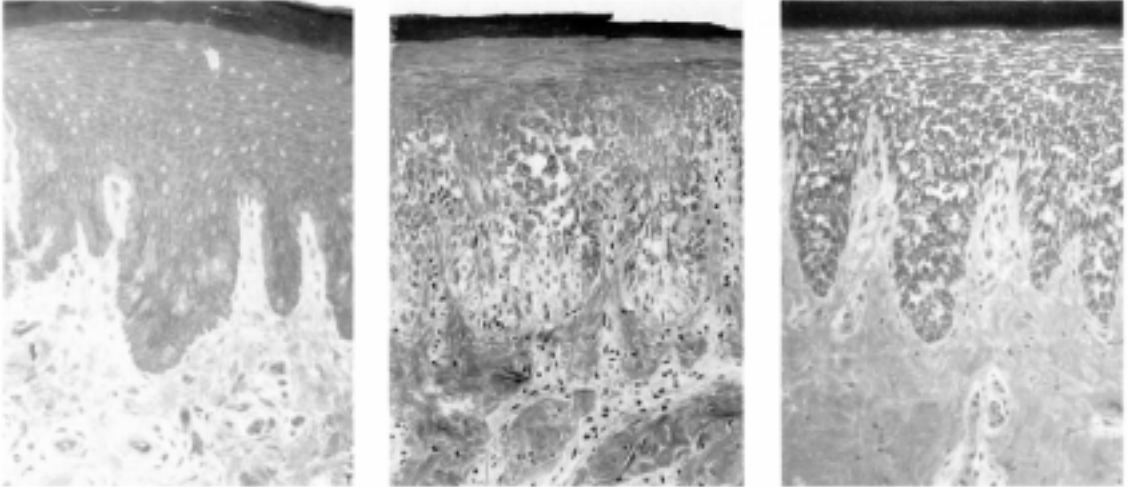
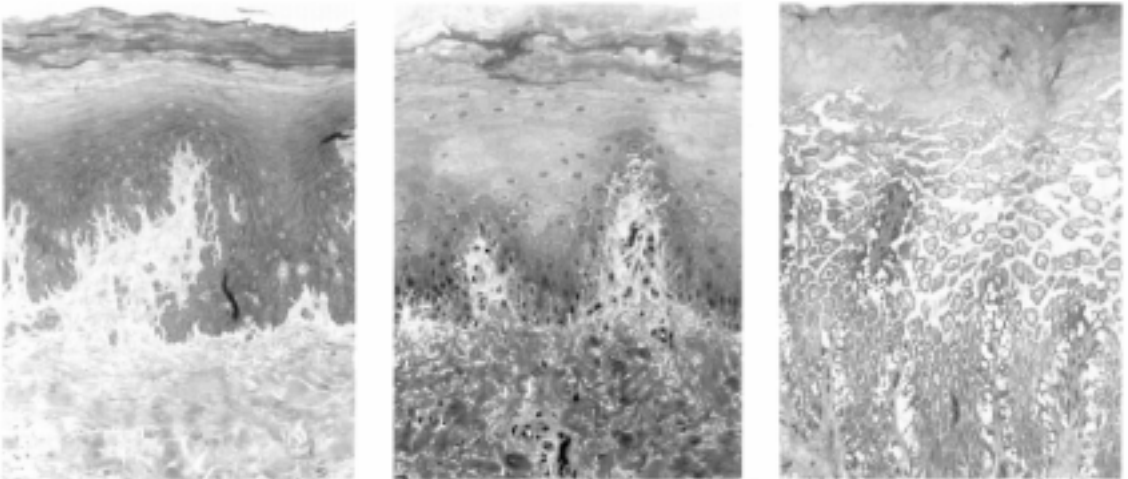


Figure 3

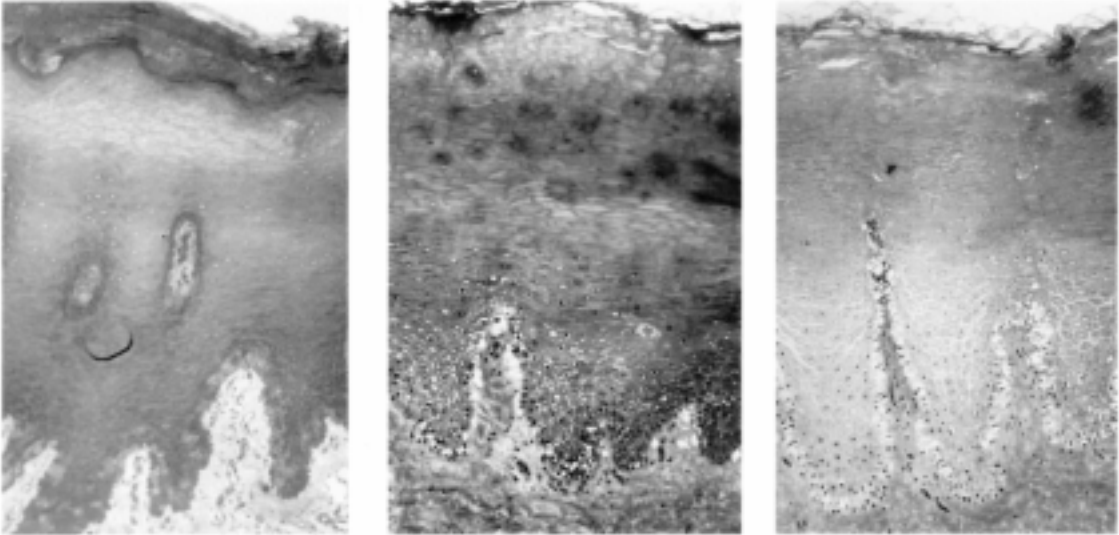
Sections of porcine floor-of-mouth mucosa after exposure for 2 h with PBS (left), 2 percent nicotine (center), and 6 percent nicotine (right). There is greater intercellular disruption after treatment with 6 percent (right) than with 2 percent nicotine (center) (magnification x200).



Although this sequence of events is conceptual, it is consistent with clinical evidence. When smokeless tobacco is first placed on the oral mucosa, there is erythema and, histologically, evidence of acute inflammation (G.K. Johnson, personal communication). If a habitual tobacco user places tobacco at a new site in the mouth, the original lesion usually resolves clinically and histologically (Andersson, 1991), but another develops at the

Figure 4

Sections of porcine buccal mucosa after exposure for 2 h with PBS (left), 2 percent nicotine (center), and 6 percent nicotine (right). There is marked intercellular disruption after treatment with 6 percent nicotine, but the deepest cells show nuclear shrinkage and separation with 2 percent nicotine (magnification x125).



new site of placement. Such events clearly implicate tobacco as the causative agent in pathological changes in the oral mucosa and lead to the hypothesis that nicotine may be the component that initiates the morphological changes described. The events proposed above would also be consistent with suggestions that ST carcinogenesis involves factors other than a carcinogen (Park et al., 1988) and that the development of oral carcinoma requires extended exposure to tobacco (Winn et al., 1981).

If nicotine is involved in the production of the snuff-induced lesion, then the local effect of nicotine gum, often prescribed during smoking cessation, might be of importance. In a study that evaluated gingival response to nicotine chewing gum (2 mg or 4 mg per portion), Silver and coworkers (1989) reported transient gingival blanching in all subjects. When the gum was repeatedly held in the same location, vesicle formation occurred that resolved when the gum was placed in different positions. The absence of severe, prolonged changes in nicotine gum chewers (Christen et al., 1985; Silver et al., 1989), in contrast to the tissue changes described in our experiments, can probably be explained by the levels of nicotine exposure. Although bioavailability of nicotine may vary, nicotine concentration in chewing gum (0.2 percent by weight) is almost an order less than that found in typical ST products (0.5 to 3.0 percent nicotine) (Hoffmann and Adams, 1981). Furthermore, the gum is typically used for only 5 to 6 h daily. This represents a much lower nicotine exposure than that of individuals with an established ST habit who may continually use the product, with its higher concentrations of nicotine, for more than 12 h/d (Andersson, 1991).

Figure 5

Transmission electron micrographs of floor-of-mouth mucosa exposed for 2 h to 2 percent nicotine. Epithelial cells show condensation of nuclear chromatin (top, magnification x17,000) and numerous vacuoles that appear to represent damaged cell organelles, including mitochondria (bottom, x28,500).

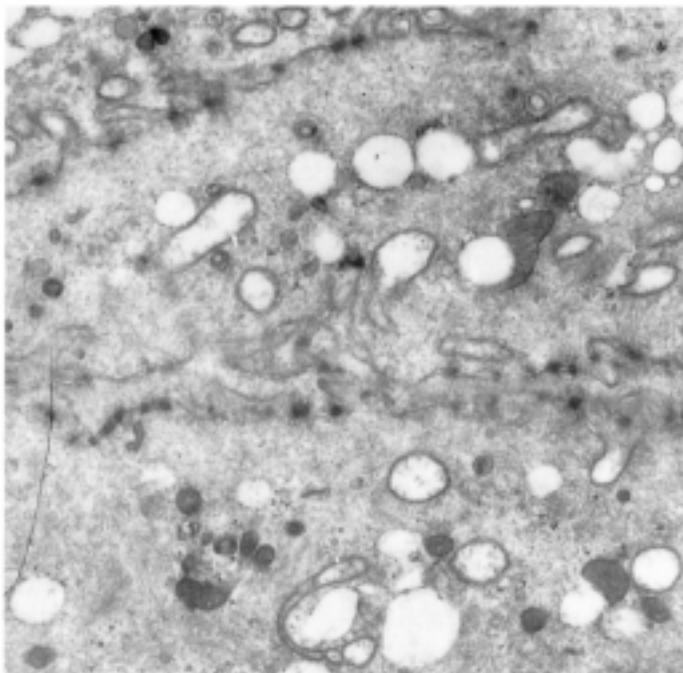
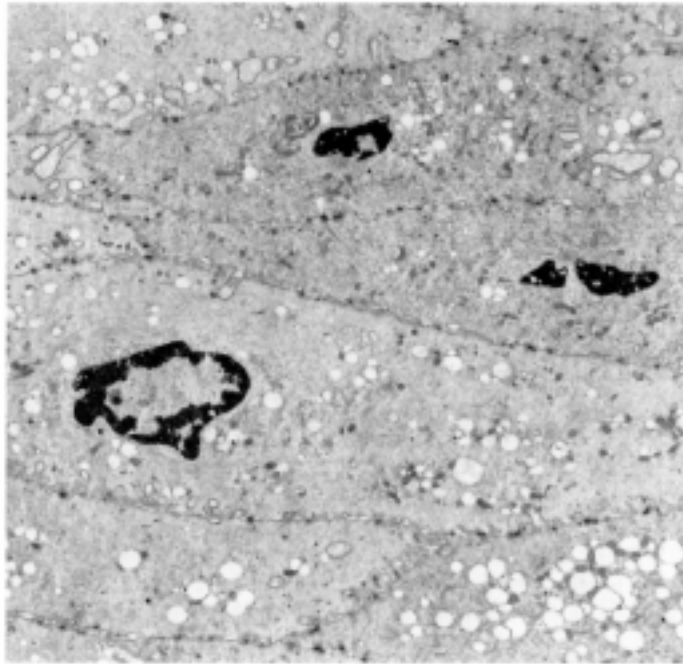


Table 1
Effect of nicotine on mucosal permeability to tritiated water^a

| | Untreated Control | PBS Control | Nicotine Treated ^b |
|----------------|-------------------|-------------|-------------------------------|
| Tissue Region | | | |
| Gingiva | 364 ± 18 | 351 ± 54 | 667 ± 77 |
| Buccal mucosa | 634 ± 19 | 692 ± 49 | 781 ± 46 |
| Floor of mouth | 808 ± 23 | 814 ± 46 | 971 ± 94 |

^a K_p SEM $\times 10^{-7}$ cm/min.

^b Values significantly greater than controls ($p > 0.05$).

FUTURE DIRECTIONS A logical consequence of the implication of nicotine in ST carcinogenesis would be to reduce its concentration to levels that do not cause tissue damage. As this threshold is unknown, it is not possible to predict whether such a strategy would still provide adequate nicotine levels to offer the psychoactive effect that is the major reason for ST use while limiting the pathological effects on the oral mucosa.

ACKNOWLEDGMENTS We thank Mary Kremer and Chuck Lesch for technical assistance.

REFERENCES

- Andersson, G. Snuff-induced changes associated with the use of loose and portion-bag-packed Swedish moist snuff: A clinical, histological, and followup study. *Swedish Dental Journal Suppl.* 75: 1-88, 1991.
- Benowitz, N.L. Clinical pharmacology of nicotine. *American Review of Medicine* 37: 21-32, 1986.
- Benowitz, N.W. Nicotine and smokeless tobacco. *CA—A Cancer Journal for Clinicians* 38: 224-256, 1988.
- Bock, F.G. Cocarcinogenic properties of nicotine. *Banbury Report* 3: 129-139, 1980.
- Chen, Y.-P., Squier, C.A. Effect of nicotine on 7,12-dimethylbenz(a)anthracene carcinogenesis in hamster cheek pouch. *Journal of the National Cancer Institute* 82: 861-864, 1990.
- Christen, A.G., Beiswanger, B.B., Mallatt, M.E., et al. Effects of nicotine-containing chewing gum on oral soft and hard tissues: A clinical study. *Oral Surgery, Oral Medicine, Oral Pathology* 59: 37-42, 1985.
- Grice, K.A. Transepidermal water loss in pathological skin. In: *The Pathophysiology of Skin*, A. Jarrett (Editor). London: Academic Press, 1980, p. 2147.
- Gritz, E.R., Baer-Weiss, V., Benowitz, N.L., Yunakis, H.V., Jarvik, M.E. Plasma nicotine and cotinine concentrations in habitual smokeless tobacco users. *Clinical Pharmacology Therapy* 30: 201-209, 1981.
- Hecht, S.S., Orna, R.M., Hoffmann, D. Chemical studies of tobacco smoke XXXIII. *N*'-nitrosornicotine in tobacco: Analysis of possible contributing factors and biologic implications. *Journal of the National Cancer Institute* 54: 1237-1244, 1974.
- Hill, L.N., Milo, A.G., Schroeder, K.L. Bone loss relative to gingival blood flow in snuff-treated versus control rats (abstract 707). *Journal of Dental Research* 69: 197, 1990.
- Hoffmann, D., Adams, J.D. Carcinogenic tobacco-specific N-nitrosamines in snuff and in the saliva of snuff dippers. *Cancer Research* 41: 4305-4308, 1981.
- Hoffmann, D., Hecht, S.S., Melikian, A.A., Haley, N.J., Brunnemann, K.D., Adams, J.D., Wynder, E.L. Tumorigenic agents in tobacco products and their uptake by chewers, smokers and nonsmokers. In: *Biochemical and Molecular Epidemiology of Cancer*. New York: Alan R. Liss, 1986, pp. 191-204.
- Hoffmann, D., Lavoic, E.J., Hecht, S.S. Nicotine: A precursor for carcinogens. *Cancer Letters* 26: 67-75, 1985.
- Johnson, G.K., Todd, G.L., Fung, E.Y.K. Effects of nicotine on gingival blood flow (abstract 834). *Journal of Dental Research* 68: 285, 1989.

- Lesch, C.A., Squier, C.A., Cruckley, A., Williams, D.M., Speight, P. The permeability of human oral mucosa and skin to water. *Journal of Dental Research* 68: 1345-1349, 1989.
- Park, N.H., Akoto-Amanfu, E., Paik, D.I. Smokeless tobacco carcinogenesis: The role of viral and other factors. *CA—A Cancer Journal for Clinicians* 38: 224-233, 1988.
- Schafer, W.G., Hine, M.K., Levy, B.M. *A Textbook of Oral Pathology* (4th edition). Philadelphia: W.B. Saunders, 1983, p. 104.
- Schmeltz, I., Wenger, A., Hoffmann, D., Tso, T.C. On the fate of nicotine during pyrolysis and in a burning cigarette. *Agricultural and Food Chemistry* 27: 602-608, 1979.
- Schroeder, K.L., Milo, A.G. Topically applied smokeless tobacco effects on rat gingival blood flow (abstract 879). *Journal of Dental Research* 68: 291, 1989.
- Siegal, I.A., Hall, S.H., Stambaugh, R. Permeability of the oral mucosa. In: *Current Concepts of the Histology of Oral Mucosa*, C.A. Squier and J. Meyer (Editors). Springfield, IL: Charles C. Thomas, 1971, pp. 274-186.
- Silver, K.J., Sachs, D.P.L., Hottel, T.L. Gingival response to nicotine polacrilex. *Journal of the American Dental Association* 118: 53-56, 1989.
- Squier, C.A., Hall, B.K. The permeability of hyperplastic oral epithelium. *Journal of Oral Pathology* 15: 357-362, 1985.
- Squier, C.A., Wertz, P.W., Cox, P. Thin-layer chromatographic analysis of lipids in different layers of porcine epidermis and oral epithelium. *Archives of Oral Biology* 36: 647-653, 1991.
- Winn, D.M., Blot, W.J., Shy, C.M., Pickle, L.W., Toledo, A., Fraumeni, J.F., Jr. Snuff dipping and oral cancer among women in the southern United States. *New England Journal of Medicine* 304: 745-749, 1981.

Perinatal Carcinogenesis by Constituents Of Smokeless Tobacco: Animal Models And Potential Human Risk

Lucy M. Anderson and Jerry M. Rice

ABSTRACT Carcinogenic risk for human perinates from parental use of smokeless tobacco would be implied if (1) increased risk in organs distant from the oral site of exposure were demonstrated in adults; (2) increased cancer risk from parental cigarette smoking were found; and (3) carcinogens in ST were active perinatal carcinogens in animal models. Epidemiological evidence for the first two points is limited but increasing. In regard to the third point, in animal models, the most potent carcinogen present in significant quantity in ST, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), has been demonstrated to be a relatively potent carcinogen in fetal hamsters, especially in the respiratory tract, and in newborn mice (lung and liver). NNK was significantly but weakly positive in lungs and livers of fetal mice. Interpreted with other studies on perinatal effects of nitrosamines and other carcinogens, the results with NNK are consistent with metabolic activation in perinatal tissues being a limiting factor in risk for the tobacco-specific nitrosamines and other smokeless tobacco carcinogens, such as benzo[*a*]pyrene and *N*-nitrosodimethylamine. Investigation of this biochemistry with rodent tissue and human or primate perinatal tissues or both is therefore of high priority in evaluation of human risk, along with analysis of amniotic fluid and breast milk of ST users. The possibility of preconception effects, especially in males, predisposing to cancer in the offspring may be studied with animal models. In addition, epidemiological studies of childhood and adult cancers as related to the amount and kind of ST used by both parents are indicated.

INTRODUCTION Increasing use of smokeless tobacco by women of child-bearing age (Orlandi and Boyd, 1989) presents the possibility of adverse effects, including cancer, for their children. Such a possibility exists if effective doses of the toxic constituents of these topically applied products reach the fetus transplacentally via blood circulation or the suckling infant through milk. This possibility is confirmed by epidemiology linking ST with increased cancer at sites other than the head and neck. Available evidence suggests an association with cancers of the bladder, pancreas, and kidney (Bjalke and Schuman, 1982; Goodman et al., 1986; Heuch et al., 1983; Kabat et al., 1986). Both transplacental (Alaoui-Jamali et al., 1989; Anderson et al., 1989a; Castonguay et al., 1984; Correa et al., 1990; Rossignol et al., 1989) and transmammary (Diaz Gomez et al., 1986; LaVoie et al., 1987) passage of tobacco product carcinogens occurs in rodents. In humans, an increase in 4-aminobiphenyl hemoglobin adducts in fetuses of smoking mothers was recently demonstrated (Coghlin et al., 1991). It is therefore necessary to consider risk of tumorigenesis in the fetuses and neonates of ST users.

SENSITIVITY AND SUSCEPTIBILITY FACTORS A wide variety of chemicals cause tumors when delivered transplacentally to rodent fetuses or administered directly to newborns (Rice, 1981; Toth, 1968). The tumors that result are mostly adult rather than embryonal type and present in tissues characteristic of the chemical and the species. Fetal sensitivity varies from minimal at toxic adult doses to much greater than that observed in the adult (see Anderson et al., 1985a). The most effective transplacental carcinogens are

the rapidly penetrating, direct-acting alkylating agents, especially *N*-ethylnitrosourea (ENU), to which the nervous systems of rat fetuses are approximately fiftyfold more sensitive than those of the adult animal. In mice, transplacental ENU not only initiated more lung and liver tumors in fetuses than in adults at the same dose, but the prenatally initiated neoplasms became larger and more malignant (Branstetter et al., 1988 and 1989). ENU was also found to be a transplacental carcinogen in two non-human primates, patas and rhesus monkeys, causing, in addition to neoplasms of the vasculature, tumors that were not seen in the mothers or after treatment of other adult monkeys (Rice et al., 1989), including hepatocellular carcinoma, leukemia, lung adenoma, nephroblastoma, and an assortment of tumors of the brain.

These results indicate that animal fetuses, both rodent and primate, have high innate sensitivity to tumor initiation by genotoxic carcinogens that are in an activated state and an effective dose. The reasons for this special sensitivity probably include high rates of cell division, which permits fixation of lesions before repair can occur, and multiple divisions after initiation to give rise to a large clone of initiated cells. Other factors, such as limited ability to repair DNA damage, high proportion of undifferentiated cells, and immaturity of immune surveillance, hormonal, and growth control systems, have also been suggested to play a role, although convincing evidence is lacking.

More variable results are obtained for transplacental carcinogens that are activated or detoxified or both by enzymes in the mother, the placenta, or the fetus. Nitrosamines, in contrast to nitrosoureas, require metabolic activation and are generally ineffective transplacental carcinogens; they seem to elicit tumors in direct proportion to the capacity of fetal tissues to activate them metabolically. Thus, *N*-nitrosodiethylamine (NDEA) causes respiratory tumors in mice and hamsters with steadily increasing efficiency as the end of gestation nears, correlated with the acquisition of an unusually high ability to metabolize NDEA by fetal hamster lungs at term (Lofberg and Tjalve, 1984).

Larger metabolism-dependent carcinogens such as polycyclic aromatic hydrocarbons (PAHs) present an even more complex picture, because their effectiveness may be limited by poor placental penetration (Neubert and Tapken, 1988) or detoxification in the placenta (Pelkonen, 1985; Remmer, 1987). Nevertheless, certain PAHs, including 3-methylcholanthrene (MC) and 7,12-dimethylbenz[*a*]anthracene (DMBA), are more active transplacentally than in adult rodents (Anderson et al., 1985b; Rice et al., 1978). In mice, fetal risk of lung and liver carcinogenesis by MC is influenced strongly by both maternal and fetal ability to respond to induction of a cytochrome P₄₅₀ (1A1) that metabolizes MC; maternal induction protected against and fetal induction potentiated MC's effect (Anderson et al., 1985b and 1989c). Biochemical analysis of this model suggests that extensive induction in maternal liver reduces the effective dose of unchanged carcinogen reaching the fetuses, but in the fetus, the more limited induction in liver does not outweigh the increased level of activation to ultimate carcinogen in the lung (Chauhan et al., 1991; Miller et al., 1989, 1990a, and 1990b).

The newborn infant is a particularly vulnerable target for carcinogens, often being more sensitive to carcinogens than adults (Toth, 1968) or than late fetuses after an equivalent dose to the mother. The reasons for this include high rates of cell division, as for fetuses, and long half-life of chemicals, because the newborn's detoxifying enzymes are generally inadequate. For the carcinogens in ST, newborn sensitivity would be of greatest concern if these were secreted in milk in significant quantities. This would seem to be a possibility, because tumorigenesis after transmammary delivery has been demonstrated for a chemically diverse assortment of carcinogens (Maekawa and Odashima, 1975; Mohr and Althoff, 1971; Nomura, 1973; Nomura et al., 1974; Vesselinovitch et al., 1979).

Activated oncogenes have been found in transplacentally induced tumors and show tissue specificity, but the type and incidence of activated oncogenes were similar to those in tumors induced in the same tissues in adults by these agents (Loktionov et al., 1990; Perantoni et al., 1987; Sukumar and Barbacid, 1990; Yamasaki et al., 1987). Special fetal susceptibility does not appear to relate to unique features of oncogene activation.

In some cases the appearance of prenatally initiated tumors may be dependent on or hastened by postnatal exposure to tumor promoters. This has been demonstrated for a variety of tumors in epithelial tissue and several promoters (Anderson et al., 1985a; Diwan et al., 1989; Napalkov et al., 1987; Suganuma et al., 1987). Hormones and tissue regeneration also provided a promotional stimulus (Ogawa et al., 1982; Rice, 1981). On the other hand, postnatal exposures to such diverse agents as hormones, barbiturates, growth factors, or nicotine sometimes suppressed tumor development (Alexandrov et al., 1989; Anderson et al., 1985a; Beniashvilli and Zedgenidze, 1989; Berger et al., 1987; Naito et al., 1985).

In sum, the perinate can present a high intrinsic sensitivity to tumorigenesis, which may be counterbalanced by maternal or placental detoxification, poor transplacental or transmammary delivery, limitations in metabolic activation, other poorly understood species- and organ-specific constraints, lack of necessary postnatal stimulation, or postnatal inhibitory influences. No doubt other factors influencing the process in both directions remain to be discovered. All of these must be considered in evaluation of potential human perinatal risk from ST.

TOBACCO-SPECIFIC NITROSAMINES IN ANIMAL MODELS Cigarette smoke condensate administered to pregnant Syrian hamsters caused a significant increase in tumors, including neoplasms of the adrenal gland, pancreas, liver, respiratory system, ovary, and connective tissue (Nicolov and Chernozemsky, 1979), primarily in the female offspring. In short-term studies with mice, exposure of mothers during pregnancy to mainstream or sidestream cigarette smoke resulted in increased sister chromatid exchanges in fetal livers (Karube et al., 1989), and extract of ST administered before and during gestation resulted in reduced fetal weight, decreased ossification, and increased resorptions (Paulson et al., 1991).

Other efforts have focused on the chemicals present in tobacco products. The carcinogens of greatest concern in ST are the tobacco-specific

nitrosamines, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), *N'*-nitrosonornicotine (NNN), *N'*-nitrosoanabasine (NAB), and *N'*-nitrosoanatabine (NAT); all occur in ST in the parts-per-million range, being especially high in snuff (Hecht and Hoffmann, 1988). Thus far, perinatal carcinogenicity studies have been carried out only with NNK, the most potent in adult rodents. NNK administered to pregnant Syrian golden hamsters was detected in placenta, along with a twentyfold greater quantity of its metabolite, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). Both were present, at about one-half the placental level in the fetal lung, and were found in amniotic fluid (Rossignol et al., 1989). Explants of exposed fetal lung and trachea showed a high incidence of chromosomal aberrations.

These findings predicted the positive outcome of the bioassay in hamsters (Correa et al., 1990); transplacental NNK caused a significant incidence of respiratory tract tumors at doses of 50 to 300 mg/kg, with the greatest effect shown in 61 percent of females after a single dose of 200 mg/kg on the last day of gestation. There was also a significant number of pheochromocytomas of the adrenal glands and a few hepatocellular carcinomas and pancreatic ductular adenomas. Thus NNK joins other nitrosamines as an effective transplacental carcinogen of the respiratory system in hamsters, again correlated with development of capacity for metabolic activation. The fetal hamster lung and tracheal tissues were capable of metabolizing NNK *in vitro* to a markedly increased extent over the last 4 d of gestation; the amount of most metabolites doubled between days 14 and 15 (Rossignol et al., 1989). In a comparison of methylated bases in DNA of lung explants from fetuses and adults, fetal DNA contained slightly more *O*⁶- and *N*⁷-methylguanines. These findings are consistent with another report, that fetal hamster lung metabolized NDEA at a threefold greater rate than adult lung (Lofberg and Tjalve, 1984).

Metabolism of [¹⁴C]NNK was similarly examined in fetal C57BL/6 mice (Castonguay et al., 1984). The compound and its metabolites were detected in all fetal tissues and amniotic fluid. Macromolecule-bound label was found in fetal nose and liver and to a lesser extent in lung and kidney. Fetal liver, lung, and nasal tissue, incubated *in vitro* with NNK, formed the keto acid product of α C-hydroxylation (carcinogen activation), but only at levels 3 to 4 percent those of the corresponding maternal tissue. Nasal tissue was most active.

These biochemical findings suggested that NNK would be a positive but weak transplacental carcinogen in the mouse, and bioassay confirmed this prediction (Anderson et al., 1989b). Pregnant females of three mouse strains of differing characteristics were given three NNK doses of 100 mg/kg. Transplacentally exposed offspring of strain A mice, highly susceptible to lung tumor initiation, showed a tenfold increase in incidence of lung tumor bearers among females and a twofold change in males, with a small effect on multiplicity. Although of statistical significance, the degree of carcinogenicity was much less than that in the mothers, who exhibited a tumor multiplicity of > 20. It is of interest that a greater effect was seen in the

female offspring, as was noted above for both cigarette smoke condensate and NNK in hamsters.

Progeny of C3H/He x C57BL/6 mice (C3B6F₁) and of outbred Swiss [Cr:NIH(s)] mice, exposed transplacentally to NNK, were held for 18 mo and included groups treated postnatally with barbital or polychlorinated biphenyls (PCBs) as tumor promoters. Prenatal NNK treatment of male C3B6F₁ mice caused a significant increase in incidence of hepatocellular tumors (40 vs. 17 percent in controls), with no further effect of barbital and PCBs. Male offspring of Swiss mice had a low incidence of liver tumors (5 percent) after transplacental NNK; this increased to 19 percent in those receiving PCBs postnatally, a significant tumor-promotive effect. Although the mothers again developed lung tumors in high multiplicity, there was no increase in these in the C3B6F₁ or Swiss mice offspring.

The results from the three strains together show convincingly that NNK is a relatively weak transplacental carcinogen in the mouse, affecting primarily fetal organs of high sensitivity. The postnatal effects of the PCBs in Swiss mice are of special interest, indicating that, in certain genetic situations, tumors initiated prenatally by NNK could be promoted to expression postnatally by exposure to other environmental agents.

NNK was also tested in neonatal Swiss mice, with 50 mg/kg doses given five times between days 1 and 14. NNK was much more effective with neonatal treatment than after transplacental exposure, causing liver tumors in 57 percent of males and 14 percent of females (none in controls) and lung tumors in 57 percent of males and 37 percent of females (21 to 22 percent in controls) (Anderson et al., 1991a). Here, the males were more susceptible to lung tumorigenesis than were females. Although neonates appear to be more sensitive than fetuses, the actual dose received may be greater, because maternal and placental metabolism and the physical placental barrier probably reduce the amount of NNK reaching the fetus.

Overall the studies of perinatal carcinogenesis by NNK in hamsters and mice show that this chemical is a weak (transplacentally in mice) to moderate (in neonatal mice and transplacentally in hamsters) perinatal tumorigen, that its efficacy correlates with ability of target tissue to activate it metabolically, and that the tumors caused were those characteristic of the species and strain.

OTHER ST CONSTITUENTS

Other carcinogens are present in ST at lower levels than tobacco-specific nitrosamines (Hecht and Hoffmann, 1988) but could contribute nonetheless to a perinatal carcinogenic effect. Of those found at levels > 1 ppb, *N*-nitrosodimethylamine (NDMA) and benzo[*a*]pyrene (B[*a*]P) have been tested for perinatal carcinogenicity. In rats, hamsters, and mice, NDMA was a weak transplacental carcinogen, giving a significant but low yield of tumors (Anderson et al., 1985a and 1989a). NDMA is quite fetotoxic, so only relatively low doses (5 to 30 mg/kg) could be tolerated for testing. The limited prenatal effectiveness of NDMA as either a teratogen or carcinogen may relate to lack of capacity for metabolic activation, because a spontaneously reacting derivative, acetoxymethyl-methylnitrosamine, was active as a teratogen (Platzek et al., 1983) and as a transplacental carcinogen in rats (J.M. Rice, unpublished observations).

In contrast, NDMA is highly carcinogenic in neonatal mice (Frei, 1970; Toth et al., 1964), resulting in many lung and liver tumors. Coccia and coworkers (1988) found that NDMA demethylase activity in neonatal Swiss mouse liver, compared to adult liver, was two-thirds less, the rate of cell division twenty-fivefold greater, the amount of *O*⁶-methylguanine adduct in DNA 2 and 4 h after a carcinogenic NDMA dose fourfold greater, and the level of *O*⁶-methylguanine transferase repair enzyme fivefold less. Thus the factors suggested as predisposing to tumor initiation in the neonate were all confirmed.

B[a]P is a moderate transplacental carcinogen in mice, even though transplacental passage of B[a]P may be limited (Neubert and Tapken, 1988). Experiments in which B[a]P or its metabolites were injected directly into mouse fetuses demonstrated that B[a]P itself caused lung tumors, but the diol-epoxide metabolite, the ultimate carcinogenic form, was more effective, suggesting that ability to activate B[a]P is limiting in the fetus (Rossi et al., 1983). B[a]P, like the nitrosamines, is a potent carcinogen in neonatal mice (Truhaut et al., 1966). As a highly soluble lipid compound, B[a]P would be expected to partition readily into breast milk and has been quantified there (LaVoie et al., 1987).

In contrast to B[a]P, DMBA is a potent transplacental carcinogen in the rat (Rice et al., 1978). The possibility thus exists that other naturally occurring PAHs, including some that occur in tobacco products, may be significantly potent transplacentally. Most such substances are not readily available and have not been tested.

POTENTIAL RISK AND FUTURE RESEARCH A conclusion from the assays of perinatal carcinogenesis by NNK in animal models is that this chemical and similar ones in ST may contribute to the risk of human perinatal carcinogenesis, depending on factors about which we have little or no information. Some of these information gaps are as follows:

- Presence, ontogeny, and variability of cytochromes P₄₅₀ activating NNK, NDMA, and PAHs in human fetuses and placentas, especially cytochrome P₄₅₀ 1A1/2, 2A3, 2D6, and 3A7
 - Activation of PAH diols by cytochrome P₄₅₀ 3A7
 - DNA and hemoglobin adducts as markers of activation
 - Capacity of primate placenta to detoxify NNK, and influence of other factors such as smoking and coexposure to other xenobiotics; induction and competitive inhibition;
- Occurrence of aromatic amine carcinogens in ST;
- Toxicokinetics of NNK during pregnancy and lactation and modulating effects of other chemicals in nonhuman primate studies;
- Concentration of NNK and metabolites, other ST carcinogens in amniotic fluid and breast milk of users;
- Preconception effects of NNK on cancer in animal models; and

- Relations of childhood or adult cancers to use of ST by parents as revealed by epidemiological studies.

These gaps might be bridged by application of current technologies, as discussed below.

Biochemistry: By extrapolation of conclusions reached with rodent models, human perinatal risk as a result of carcinogens present in ST is probably determined in part by the levels of activating cytochrome **Carcinogen** **Metabolism and** **DNA Adducts** P_{450} enzymes in the target tissues. Of cytochromes P_{450} in human fetal liver, 40 to 85 percent is the fetus-specific P_{450} 3A7, related but not identical to the predominant isoform of this family in adult liver, 3A4 (Cresteil et al., 1985; Kitada et al., 1985a; Komori et al., 1989a and 1989b; Wrighton and Vandenbranden, 1989). Which P_{450} 's constitute the remaining 15 to 60 percent is an important unresolved question. P_{450} 2D6 is detectable in human fetal liver by both immunoblot and Northern blot, although at low levels compared with adults (Treluyer et al., 1991). Low levels of P_{450} 1A1 gene expression were detected by use of the polymerase chain reaction in the majority of a small sample of fetal adrenals, livers, and lung, but not kidneys (Omiecinski et al., 1990). P_{450} 1A2 mRNA was reported to be absent (Cresteil, reported in Anderson et al., 1991b); however, a protein cross-reacting with an antibody to 1A2 was partially purified (Kitada et al., 1990), and a 1A isoform, suggested to be 1A2, was recognized by a monoclonal antibody to rat P_{450} 1A1 in human fetal hepatocytes (Murray et al., 1992). P_{450} 2E1 was not expressed in fetal (Komori et al., 1989b) or newborn (Morel et al., 1990) livers.

To relate these observations to carcinogens in ST, activation of NNK to a mutagen in cell expression systems is supported by human cytochromes P_{450} 1A2, 2A3, 2D6, and 2E1 (Crespi et al., 1991). As noted above, in human perinatal liver 2E1 is probably absent, 2D6 is present at low levels, and a P_{450} related to 1A2 may occur. Perinatal expression of 2A3 should be investigated, because it acts not only on NNK but also on NDMA (Crespi et al., 1990). P_{450} 3A7, the predominant human fetal form, also exhibits considerable NDMA demethylase activity (Kitada et al., 1985a).

With regard to PAHs such as B[a]P, B[a]P hydroxylase activity, generally considered a detoxification step, correlated with levels of 3A7 and was inhibitable by anti-3A7 antibody in human fetal livers (Kitada et al., 1985b and 1987); phenols but not diols were formed (Blanck et al., 1983). It is not certain which human P_{450} provides the initial activation of B[a]P to a diol; both 2C8,9 (Cresteil et al., 1985) and 1A1 (Shimada et al., 1989b) have been suggested. P_{450} 1A1 may be effectively induced in human placentas by smoking (Pasanen et al., 1990; Pasanen and Pelkonen, 1990), and placental microsomes induced by smoking do produce B[a]P-7,8-diol (Blanck et al., 1983). Whether ST brings about this induction is not known and should be determined.

P_{450} 3A4 carries out the further activation of B[a]P-7,8-diol to the ultimate diol-epoxide carcinogen (McManus et al., 1990; Shimada et al., 1989a). Whether the fetal form, 3A7, also has this capacity should be determined. If

so, PAH-diols formed by induced placenta might cross to the fetus where they could be further activated by the P₄₅₀ 3A7 present in some abundance in the fetal tissues.

In sum, for carcinogens in ST, there seems reason for concern about tumor initiation in human fetuses by NDMA, as a result of its metabolism by P₄₅₀ 3A7, and by PAHs, because P₄₅₀ 1A1 in fetal tissues and especially in induced placenta could provide the initial activation, followed by possible ultimate activation by the substantial levels of 3A7 in the fetus. Potential effects of the more abundant NNK catalyzed by the low level of P₄₅₀ 2D6 present is more problematic; it is particularly important to discover whether there is a 1A2-like protein present with activity toward NNK.

DNA and hemoglobin adducts have been a popular exposure biomarker. Thus far, of the tobacco-related carcinogens, only 4-aminobiphenyl adducts on hemoglobin have been detected in fetuses in association with maternal cigarette smoking (Coghlin et al., 1991). The occurrence of aromatic amines in ST has not been reported and should be investigated. O⁶-methylguanine adducts of DNA from NNK or NDMA correlate with tumorigenesis in fetal hamster and newborn mouse and are consistent with the known oncogene mutations in the resultant tumors. Sensitive methods for detection of this adduct in human tissues are becoming available (Shields et al., 1990) and might profitably be applied to human fetuses and newborns, as related to tobacco use and other parameters. O⁶-methyldeoxyguanosine has been detected in human placental DNA (Foiles et al., 1988). The pyridyl-oxobutyl adduct could be an indicator of exposure specifically to NNK. Repair and persistence of adducts after perinatal exposure will be difficult to evaluate with human material, but this question might be addressed with nonhuman primates.

Distribution and Toxicokinetics As noted above, NNK and a metabolite were present in the amniotic fluid of hamsters and mice. Fetuses both breathe and ingest amniotic fluid. Human amniotic fluid has the capacity to activate carcinogens (Daubeze et al., 1986) and contains Ames-test mutagens, with a significantly higher level for heavy smokers (Rivrud et al., 1986). It might be worthwhile to analyze human amniotic fluid for tobacco-specific nitrosamines and their metabolites.

The dose of NNK incurred by the fetus or nursing infant will be influenced significantly by metabolic clearance in maternal liver and possibly in placenta, and both enzyme induction and competitive inhibition are possible. Are there NNK-metabolizing enzymes in placenta and, if so, are they induced by cigarette smoking, snuff dipping, or both? This important question is addressable now, by use of placentas from normal pregnancies to search for relevant P₄₅₀ isoforms (discussed above) and for measurement of total NNK substrate conversion. This *in vitro* system could also be used to study competitive inhibition of metabolism by chemicals such as hormones, ethanol, pharmaceutical drugs, and industrial and household solvents.

The question of induction and inhibition in maternal liver, with an influence on clearance, will be more difficult for researchers to address using human tissue. A reasonable start may be made with nonhuman primates.

Epidemiology No epidemiological studies of cancer associated with parental use of ST have been reported. Suggestions may be sought from the epidemiology of cigarette smoking, which entails the same set of chemical carcinogens, and from the animal model work described above. Reviews of the epidemiological evidence have been given by Preston-Martin (1989) and John et al. (1991). Relative risks of 1.1 to 3.0 were frequently associated with maternal or paternal cigarette smoking or both, with positive effects of statistical significance in three studies: childhood leukemia associated with numbers of cigarettes smoked daily by the mothers (Stjernfeldt et al., 1986); in a prospective study, a relationship between all cancers and maternal smoking (Golding et al., 1990); and increased adult lung cancers among children of smoking mothers (Correa et al., 1983). Increased risk of hematopoietic cancers in adulthood from maternal or paternal smoking (Sandler et al., 1985) had a particularly strong association if both parents smoked. In studies implicating fathers, there was a positive association between mothers living with a smoker and brain tumors (Preston-Martin et al., 1982) and between paternal smoking and rhabdomyosarcomas (Grufferman et al., 1982). An effect of paternal smoking could imply (1) enhanced placental penetration of the smoke constituents in nonsmoking mothers with uninduced detoxification enzymes; (2) transmammary exposure of the infant; (3) direct respiratory exposure of the infant; or (4) a germ cell effect on the father.

There are corresponding possibilities for ST, especially snuff, that epidemiologists should pursue, and both childhood and adult cancers should be included. Populations in India and southeast Asia, where use of ST by women is common, could be especially fruitful to study. In addition to use of ST during pregnancy, use during lactation should receive attention, in light of the high sensitivity of rodent neonates. Paternal use should also be examined because preconception paternal exposures are becoming increasingly implicated in various childhood cancers (Bunin et al., 1992), with germ cell effects being the most likely mechanism. This possibility is supported by the finding of increased tumors in the offspring of male rodents exposed to carcinogens before conception (Mohr et al., 1989; Turusov et al., 1989). Considering the high concentration of mutagens present in some smokeless tobacco, epidemiological investigation of the children of male as well as female users seems warranted.

REFERENCES

- Alaoui-Jamali, M.A., Rossignol, G., Schuller, H.M. Transplacental genotoxicity of a tobacco-specific N-nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in Syrian golden hamster. *Mutation Research* 223: 65-72, 1989.
- Alexandrov, V.A., Popovich, I.G., Anisimov, V.N., Napalkov, N.P. Influence of hormonal disturbances on transplacental and multigeneration carcinogenesis in rats. *IARC Scientific Publication* 96: 35-49, 1989.

- Anderson, L.M., Donovan, P.J., Rice, J.M. Risk assessment for transplacental carcinogenesis. In: *New Approaches in Toxicity Testing and Their Applications in Human Risk Assessment*, A.P. Li (Editor). New York: Raven Press, 1985a, pp. 179-202.
- Anderson, L.M., Hagiwara, A., Kovatch, R.M., Rehm, S., Rice, J.M. Transplacental initiation of liver, lung, neurogenic, and connective tissue tumors by N-nitroso compounds in mice. *Fundamental and Applied Toxicology* 12: 604-620, 1989a.
- Anderson, L.M., Hecht, S.S., Dixon, D.E., Dove, L.F., Kovatch, R.M., Amin, S., Hoffmann, D., Rice, J.M. Evaluation of the transplacental tumorigenicity of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in mice. *Cancer Research* 49: 3770-3775, 1989b.
- Anderson, L.M., Hecht, S.S., Kovatch, R.M., Amin, S., Hoffmann, D., Rice, J.M. Tumorigenicity of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in infant mice. *Cancer Letters* 58: 177-181, 1991a.
- Anderson, L.M., Jones, A.B., Rice, J.M. Perinatal carcinogenesis: Current directions (meeting report). *British Journal of Cancer* 63: 1025-1028, 1991b.
- Anderson, L.M., Jones, A.B., Riggs, C.W., Kovatch, R.M. Modification of transplacental tumorigenesis by 3-methylcholanthrene in mice by genotype at the *Ah* locus and pretreatment with β -naphthoflavone. *Cancer Research* 49: 1676-1681, 1989c.
- Anderson, L.M., Jones, A.B., Riggs, C.W., Ohshima, M. Fetal mouse susceptibility to transplacental lung and liver carcinogenesis by 3-methylcholanthrene: Positive correlation with responsiveness to inducers of aromatic hydrocarbon metabolism. *Carcinogenesis* 6: 1389-1393, 1985b.
- Berger, M.R., Petru, E., Habs, M., Schmahl, D. Influence of perinatal nicotine administration on transplacental carcinogenesis in Sprague Dawley rats by N-methylnitrosourea. *British Journal of Cancer* 55: 37-40, 1987.
- Beniashvili, D.S., Zedginidze, T.G. Significance of modifying factors in the development of transplacental renal tumors in rabbits. *IARC Scientific Publication* 96: 51-55, 1989.
- Bjalke, E., Schuman, L.M. Chewing of tobacco and use of snuff. Relationship to cancer of the pancreas and other sites in two prospective studies. In: *Proceedings of the 13th International Cancer Congress*. Seattle: 13th International Congress, 1982, p. 207.
- Blanck, A., Rane, A., Toftgard, R., Gustafsson, J. Biotransformation of benzo[a]pyrene and 7-ethoxyresorufin and heme-staining proteins in microsomes from human fetal liver and placenta. *Biochemical Pharmacology* 32: 1547-1552, 1983.
- Branstetter, D.G., Stoner, G.D., Budd, C., Conran, P.B., Goldblatt, P.J. Effect of gestational development on lung tumor size and morphology in the mouse. *Cancer Research* 48: 379-386, 1988.
- Branstetter, D.G., Stoner, G.D., Budd, C., Conran, P.B., Goldblatt, P.J. Relationship between in utero development of the mouse liver and tumor development following transplacental exposure to ethylnitrosourea. *Cancer Research* 49: 3620-3626, 1989.
- Bunin, G., Noller, K., Rose, P., Smith, E. Carcinogenesis. In: *Occupational and Environmental Reproductive Hazards: A Guide for Clinicians*, M. Paul (Editor). Baltimore: Williams and Wilkins, 1992.
- Castonguay, A., Tjalve, H., Trushin, N., Hecht, S.S. Perinatal metabolism of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in C57BL mice. *Journal of the National Cancer Institute* 72: 1117-1126, 1984.
- Chauhan, D.P., Miller, M.S., Owens, I.S., Anderson, L.M. Gene expression, ontogeny and transplacental induction of hepatic UDP-glucuronosyl transferase activity in mice. *Developmental Pharmacology and Therapeutics* 16: 139-149, 1991.
- Coccia, P., Salmona, M., Diomedea, L., Citti, L., Marioni, L., Romano, M. Liver DNA alkylation after a single carcinogenic dose of dimethylnitrosamine to newborn and adult CFW Swiss mice. *Chemico-Biological Interactions* 68: 259-271, 1988.
- Coghlin, J., Gann, P.H., Hammond, S.K., Skipper, P.L., Taghizadeh, K., Paul, M., Tannenbaum, S.R. 4-Aminobiphenyl hemoglobin adducts in fetuses exposed to the tobacco smoke carcinogen in utero. *Journal of the National Cancer Institute* 83: 274-280, 1991.
- Correa, E., Joshi, P.A., Castonguay, A., Schuller, H.M. The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone is an active transplacental carcinogen in Syrian golden hamsters. *Cancer Research* 50: 3435-3438, 1990.
- Correa, P., Pickle, L.W., Fontham, E., Lin, Y., Haenszel, W. Passive smoking and lung cancer. *Lancet* 2: 595-597, 1983.
- Crespi, C.L., Penman, B.W., Gelboin, H.V., Gonzalez, F.J. A tobacco smoke-derived nitrosamine, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, is activated by multiple human cytochrome P₄₅₀s including the polymorphic human cytochrome P₄₅₀ 2D6. *Carcinogenesis* 12: 1197-1201, 1991.
- Crespi, C.L., Penman, B.W., Leakey, J.A.E., Arlotto, M.P., Stark, A., Parkinson, A., Turner, T., Steimel, D., Rudo, K., Davies, R.L., Langenbach, R. Human cytochrome P₄₅₀ II A3: cDNA sequence, role of the enzyme in the metabolic activation of promutagens, comparison to nitrosamine activation by human cytochrome P₄₅₀ IIE1. *Carcinogenesis* 11: 1293-1300, 1990.

- Cresteil, T., Beaune, P., Kremers, P., Celier, C., Guengerich, F.P., Leroux, J. Immunoquantification of epoxide hydrolase and cytochrome P-450 isozymes in fetal and adult human liver microsomes. *European Journal of Biochemistry* 151: 345-350, 1985.
- Daubeze, M., Cassand, P., Migaud, M.L., Garrigue, P., Leng, J.J., Narbonne, J.F. Metabolic activation of benzo[a]pyrene by human amniotic fluid. *Comptes Rendus de l'Academie des Sciences* 302: 625-628, 1986.
- Diaz Gomez, M.I., Tamayo, D., Castro, J.A. Administration of *N*-nitrosodimethylamine, *N*-nitrosopyrrolidine, or *N*-nitrosornicotine to nursing rats: Their interactions with liver and kidney nucleic acids from sucklings. *Journal of the National Cancer Institute* 76: 1133-1136, 1986.
- Diwan, B.A., Ohshima, M., Rice, J.M. Effects of postnatal administration of tumour-promoting barbiturates on the development of tumours initiated by prenatal exposure of fetal rats and mice to *N*-alkylnitrosoureas. *IARC Scientific Publication* 96: 75-80, 1989.
- Foiles, P.G., Miglietta, L.M., Akerkar, S.A., Everson, R.B., Hecht, S.S. Detection of O⁶-methyldeoxyguanosine in human placental DNA. *Cancer Research* 48: 4184-4188, 1988.
- Frei, J.V. Toxicity, tissue changes, and tumor induction in inbred Swiss mice by methyl-nitrosoamine and -amide compounds. *Cancer Research* 30: 11-17, 1970.
- Golding, J., Paterson, M., Kinlen, L.J. Factors associated with childhood cancer in a national cohort study. *British Journal of Cancer* 62: 304-308, 1990.
- Goodman, M.T., Morgenstern, H., Wynder, E.L. A case-control study of factors affecting the development of renal cell cancer. *American Journal of Epidemiology* 124: 926-941, 1986.
- Grufferman, S., Wang, H.H., DeLong, E.R., Kimm, S.Y.S., Delzell, E.S., Falletta, J.M. Environmental factors in the etiology of rhabdomyosarcoma in childhood. *Journal of the National Cancer Institute* 68: 107-113, 1982.
- Hecht, S.S., Hoffmann, D. Tobacco-specific *N*-nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. *Carcinogenesis* 9: 875-884, 1988.
- Heuch, I., Kvale, G., Jacobsen, B.K., Bjelke, E. Use of alcohol, tobacco and coffee, and risk of pancreatic cancer. *British Journal of Cancer* 48: 637-643, 1983.
- John, E.M., Savitz, D.A., Sandler, D.P. Prenatal exposure to parents' smoking and childhood cancer. *American Journal of Epidemiology* 133: 123-132, 1991.
- Kabat, G.C., Dieck, G.S., Wynder, E.L. Bladder cancer in nonsmokers. *Cancer* 57: 362-367, 1986.
- Karube, T., Odagiri, Y., Takemoto, K., Watanabe, S. Analyses of transplacentally induced sister chromatid exchanges and micronuclei in mouse fetal liver cells following maternal exposure to cigarette smoke. *Cancer Research* 49: 3550-3552, 1989.
- Kitada, M., Kamataki, T., Itahashi, K., Rikihisa, T., Kanukubo, Y. Significance of cytochrome P-450 (P-450HFLA) of human fetal livers in the steroid and drug oxidations. *Biochemical Pharmacology* 36: 453-456, 1987.
- Kitada, M., Kamataki, T., Itahashi, K., Rikihisa, T., Kato, R., Kanakubo, Y. Purification and properties of cytochrome P-450 from homogenates of human fetal livers. *Archives of Biochemistry and Biophysics* 241: 275-280, 1985a.
- Kitada, M., Kamataki, T., Itahashi, K., Rikihisa, T., Kato, R., Kanakubo, Y. Immunochemical examinations of cytochrome P-450 in various tissues of human fetuses using antibodies to human fetal cytochrome P-450, P-450 HFLA. *Biochemical and Biophysical Research Communications* 131: 1154-1159, 1985b.
- Kitada, M., Taneda, M., Ohta, K., Nagashima, K., Itahashi, K., Kamataki, T. Metabolic activation of aflatoxin B₁ and 2-amino-3-methylimidazo[4,5-f]quinoline by human adult and fetal livers. *Cancer Research* 50: 2641-2645, 1990.
- Komori, M., Nisho, K., Ohi, H., Kitada, M., Kamataki, T. Molecular cloning and sequence analysis of cDNA containing the entire coding region for human fetal liver cytochrome P-450. *Journal of Biochemistry* 105: 161-163, 1989a.
- Komori, M., Nishio, K., Fujitani, T., Ohi, H., Kitada, M., Mima, S., Itahashi, K., Kamataki, T. Isolation of a new human fetal liver cytochrome P450 cDNA clone: Evidence for expression of a limited number of forms of cytochrome P450 in human fetal livers. *Archives of Biochemistry and Biophysics* 272: 219-225, 1989b.
- LaVoie, E.J., Stern, S.L., Choi, C., Reinhardt, J., Adams, J.D. Transfer of the tobacco-specific carcinogens *N*'-nitrosornicotine and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and benzo[a]pyrene in the milk of lactating rats. *Carcinogenesis* 8: 433-437, 1987.
- Lofberg, B., Tjalve, H. The disposition and metabolism of *N*-nitrosodiethylamine in adult, infant, and foetal tissues of the Syrian golden hamster. *Acta Pharmacologica et Toxicologica* 54: 104-114, 1984.
- Loktionov, A., Hollstein, M., Martel, M., Galendo, D., Cabral, R.P., Tomatis, L., Yamasaki, Y. Tissue-specific activating mutations of Ha- and Ki-ras oncogenes in skin, lung, and liver tumors induced in mice following transplacental exposure to DMBA. *Molecular Carcinogenesis* 3: 134-140, 1990.

- Maekawa, A., Odashima, S. Induction of tumors of the nervous system in ACI/N rat with 1-butyl-1-nitrosourea administered transplacentally, neonatally, or via maternal milk. *Gann* 66: 175-183, 1975.
- McManus, M.E., Burgess, W.M., Veronese, M.E., Huggett, A., Quattrochi, L.C., Tukey, R.H. Metabolism of 2-acetylaminofluorene and benzo[*a*]pyrene and activation of food-derived heterocyclic amine mutagens by human cytochromes P-450. *Cancer Research* 50: 3367-3376, 1990.
- Miller, M.S., Jones, A.B., Anderson, L.M. The formation of 3-methylcholanthrene-initiated lung tumors correlates with induction of cytochrome P₄₅₀ IA1 by the carcinogen in fetal but not adult mice. *Toxicology and Applied Pharmacology* 104: 235-245, 1990a.
- Miller, M.S., Jones, A.B., Chauhan, D.P., Anderson, L.M. Role of the maternal environment in determining susceptibility to transplacentally induced chemical carcinogenesis in mouse fetuses. *Carcinogenesis* 11: 1979-1984, 1990b.
- Miller, M.S., Jones, A.B., Chauhan, D.P., Park, S.S., Anderson, L.M. Differential induction of fetal mouse liver and lung cytochromes P-450 by β -naphthoflavone and 3-methylcholanthrene. *Carcinogenesis* 10: 875-883, 1989.
- Mohr, U., Althoff, J. Carcinogenic activity of aliphatic nitrosamines via the mother's milk in the offspring of Syrian golden hamsters. *Proceedings of the Society for Experimental Biology and Medicine* 136: 1007-1009, 1971.
- Mohr, U., Emura, M., Aufderheide, M., Riebe, M., Ernst, H. Possible role of genetic predisposition in multigeneration carcinogenesis. *IARC Scientific Publication* 89: 93-103, 1989.
- Morel, F., Beaune, P.H., Ratanasavanh, D., Flinois, J., Yang, C.S., Guengerich, F.P., Guillouzo, A. Expression of cytochrome P-450 enzymes in cultured human hepatocytes. *European Journal of Biochemistry* 191: 437-444, 1990.
- Murray, G.I., Foster, C.O., Barnes, T.S., Weaver, R.J., Snyder, C.P., Ewen, S.W.B., Melvin, W.T., Burke, M.D. Cytochrome P₄₅₀ IA expression in adult and human fetal liver. *Carcinogenesis* 13: 165-169, 1992.
- Naito, M., Aoyama, H., Ito, A. Inhibitory effect of phenobarbital on the development of gliomas in WF rats treated neonatally with *N*-ethyl-*N*-nitrosourea. *Journal of the National Cancer Institute* 74: 725-728, 1985.
- Napalkov, N.P., Likhachev, A., Anisimov, V., Loktionov, A., Zabezinski, M., Ovsyannikov, A., Wahrendorf, J., Becher, H., Tomatis, L. Promotion of skin tumours by TPA in the progeny of mice exposed pre-natally to DMBA. *Carcinogenesis* 8: 381-385, 1987.
- Neubert, D., Tapken, S. Transfer of benzo[*a*]pyrene into mouse embryos and fetuses. *Archives of Toxicology* 62: 236-239, 1988.
- Nicolov, I.G., Chernozemsky, I.N. Tumors and hyperplastic lesions in Syrian hamsters following transplacental and neonatal treatment with cigarette smoke condensate. *Journal of Cancer Research and Clinical Oncology* 94: 249-256, 1979.
- Nomura, T. Carcinogenesis by urethan via mother's milk and its enhancement of transplacental carcinogenesis in mice. *Cancer Research* 33: 1677-1683, 1973.
- Nomura, T., Okamoto, E., Tateishi, N., Kimura, S., Isa, Y., Manabe, H., Sakamoto, Y. Tumor induction in the progeny of mice receiving 4-nitroquinoline 1-oxide and *N*-methyl-*N*-nitrosourethan during pregnancy or lactation. *Cancer Research* 34: 3373-3378, 1974.
- Ogawa, K., Yokokawa K., Tomoyori, T., Onoe, T. Induction of α -glutamyltranspeptidase-positive altered hepatocyte lesions by combination of transplacental-initiation and postnatal-selection. *International Journal of Cancer* 29: 333-336, 1982.
- Omicinski, C.H., Redlich, C.A., Costa, P. Induction and developmental expression of cytochrome P₄₅₀ IA1 messenger RNA in rat and human tissues: Detection by the polymerase chain reaction. *Cancer Research* 50: 4315-4321, 1990.
- Orlandi, M.A., Boyd, G. Smokeless tobacco use among adolescents: A theoretical overview. *National Cancer Institute Monographs* 8: 5-12, 1989.
- Pasanen, M., Haaparanta, T., Sundin, M., Sivonen, P., Vakakangas, K., Raunio, H., Hines, R., Gustafsson, J., Pelkonen, L. Immunohistochemical and molecular biological studies on human placental cigarette smoke-inducible cytochrome P₄₅₀-dependent monooxygenase activities. *Toxicology* 62: 175-187, 1990.
- Pasanen, M., Pelkonen, O. Human placental xenobiotic and steroid biotransformations catalyzed by cytochrome P450, epoxide hydrolase, and glutathione S-transferase activities and their relationships to maternal cigarette smoking. *Drug Metabolism Reviews* 21: 427-461, 1990.
- Paulson, R.M., Shanfel, J., Prause, L., Iranpour, S., Paulson, J.O. Pre-conceptual and post-conceptual tobacco effects on the CD-1 mouse fetus. *Journal of Craniofacial Genetics and Developmental Biology* 11: 48-58, 1991.
- Pelkonen, O. Fetoplacental biochemistry—xenobiotic metabolism and pharmacokinetics. In: *Occupational Hazards and Reproduction*, K. Hemminki, M. Sorsa, and V. Vainio (Editors). Washington: Hemisphere Publishing, 1985, pp. 113-126.

- Perantoni, A.O., Rice, J.M., Reed, C.D., Watatani, M., Wenk, M.F. Activated *neu* oncogene sequences in primary tumors of the peripheral nervous system induced in rats by transplacental exposure to ethylnitrosourea. *Proceedings of the National Academy of Sciences* 84: 6317-6321, 1987.
- Platzek, T., Bochert, G., Rahm, U. Embryotoxicity induced by alkylating agents. Teratogenicity of acetoxymethyl-methylnitrosamine: Dose-response relationship, application route dependency and phase specificity. *Archives of Toxicology* 52: 45-69, 1983.
- Preston-Martin, S. Epidemiological studies of perinatal carcinogenesis. *IARC Scientific Publication* 89: 289-313, 1989.
- Preston-Martin, S., Yu, M.C., Benton, B., Henderson, B.E. N-Nitroso compounds and childhood brain tumors: A case-control study. *Cancer Research* 42: 5240-5245, 1982.
- Remmer, H. Passively inhaled tobacco smoke: A challenge to toxicology and preventive medicine. *Archives of Toxicology* 61: 89-104, 1987.
- Rice, J.M. Effects of prenatal exposure to chemical carcinogens and methods for their detection. In: *Developmental Toxicology*, C.A. Kimmel and J. Buelke-Sam (Editors). New York: Raven Press, 1981, pp. 191-212.
- Rice, J.M., Joshi, S.R., Shenefelt, R.E., Wenk, M.L. Transplacental carcinogenic activity of 7,12-dimethylbenz[a]anthracene. In: *Carcinogenesis, Polynuclear Aromatic Hydrocarbons* (volume 3), P.W. Jones and R.I. Freudenthal (Editors). New York: Raven Press, 1978, pp. 413-422.
- Rice, J.M., Rehm, S., Donovan, P.J., Perantoni, A.O. Comparative transplacental carcinogenesis by directly acting and metabolism dependent alkylating agents in rodents and nonhuman primates. *IARC Scientific Publication* 96: 17-34, 1989.
- Rivrud, G.N., Berg, K., Anderson, D., Blowers, S., Bjro, K. Mutagenic effect of amniotic fluid from smoking women at term. *Mutation Research* 171: 71-77, 1986.
- Rossi, L., Barbieri, O., Sanguineti, M., Staccione, A., Santi, L.F., Santi, L. Carcinogenic activity of benzo[a]pyrene and some of its synthetic derivatives by direct injection into the mouse fetus. *Carcinogenesis* 4: 153-156, 1983.
- Rosignol, G., Alaoui-Jamali, M.A., Castonguay, A., Schuller, H.M. Metabolism and DNA damage induced by 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in fetal tissues of the Syrian golden hamster. *Cancer Research* 49: 5671-5676, 1989.
- Sandler, D.P., Everson, R.B., Wilcox, A.J., Browder, J.P. Cancer risk in adulthood from early life exposure to parents' smoking. *American Journal of Public Health* 75: 487-492, 1985.
- Shields, P.G., Povey, A.C., Wilson, V.L., Weston, A., Harris, C.C. Combined high-performance liquid chromatography/³²P-postlabeling assay of N⁷-methyldeoxyguanosine. *Cancer Research* 50: 6580-6584, 1990.
- Shimada, T., Iwasaki, M., Martin, M.V., Guengerich, F.G. Human liver microsomal cytochrome P-450 enzymes involved in the bioactivation of procarcinogens detected by *umu* gene response in *Salmonella typhimurium* TA 1535/pSK1002. *Cancer Research* 49: 3218-3228, 1989a.
- Shimada, T., Martin, M.V., Pruess-Schwartz, D., Marnett, L.J., Guengerich, F.P. Role of individual human cytochrome P-450 enzymes in the bioactivation of benzo[a]pyrene, 7,8-dihydroxy-7,8-dihydrobenzo[a]pyrene, and other dihydrodiol derivatives of polycyclic aromatic hydrocarbons. *Cancer Research* 49: 6304-6312, 1989b.
- Stjernfeldt, M., Berglund, K., Lindsten, J., Ludvigsson, J. Maternal smoking during pregnancy and risk of childhood cancer. *Lancet* 1: 1350-1352, 1986.
- Suganuma, M., Fujiki, H., Morino, K., Takayama, S., Sugimura, T. Tumor promoting activity of teleocidin in skin and forestomach of mice initiated transplacentally with 7,12-dimethylbenz[a]anthracene. *Journal of Cancer Research and Clinical Oncology* 113: 123-125, 1987.
- Sukumar, S., Barbacid, M. Specific patterns of oncogene activation in transplacentally induced tumors. *Proceedings of the National Academy of Sciences* 87: 718-722, 1990.
- Toth, B. Critical review of experiments in chemical carcinogenesis using newborn animals. *Cancer Research* 28: 727-738, 1968.
- Toth, B., Magee, P.N., Shubik, P. Carcinogenesis study with dimethylnitrosamine administered orally to adult and subcutaneously to newborn BALB/c mice. *Cancer Research* 24: 1712-1721, 1964.
- Treluyer, J.M., Jacqx-Aigrain, E., Alvarez, F., Cresteil, T. Expression of CYP2D6 in developing human liver. *European Journal of Biochemistry* 202: 583-588, 1991.
- Truhaut, R., Lesca, P., Dechambre, R.P., Gerard-Marchant, P. Sur les modalités de manifestation du pouvoir cancerogène du benzo-3-4-pyrene chez la souris nouveau-née ou traitée immédiatement après le sevrage. *Pathologie et Biologie (Paris)* 14: 955-959, 1966.
- Turusov, V.S., Cardis, E. Review of experiments of multigeneration carcinogenicity: Discussion of design, experimental models and analyses. *IARC Scientific Publication* 89: 105-120, 1989.
- Vesselinovitch, S.D., Rao, K.V.N., Mihailovich, N. Transplacental and lactational carcinogenesis by safrole. *Cancer Research* 39: 4378-4380, 1979.

Wrighton, S.A., Vandenbranden, M. Isolation and characterization of human fetal liver cytochrome P₄₅₀ HLp2: A third member of the P₄₅₀ III gene family. *Archives of Biochemistry and Biophysics* 268: 144-151, 1989.

Yamasaki, H., Hollstein, M., Martel, N., Galenda, D., Cabral, R.P., Tomatis, L. Transplacental induction of a specific mutation in fetal Ha-*ras* and its critical role in postnatal carcinogenesis. *International Journal of Cancer* 40: 818-822, 1987.

Interaction Between Smokeless Tobacco-Related Carcinogens and Human Papillomaviruses in the Pathogenesis of Oral Dysplasia and Cancer¹

Joel M. Palefsky, John S. Greenspan, Troy E. Daniels, Jennifer Berline, Deborah G. Grady, and Virginia L. Ernster

ABSTRACT Several lines of evidence point to a role for smokeless tobacco-related carcinogens in the pathogenesis of oral cancer. Previous studies of oral cancer suggest that other factors may play a role as well, including use of alcohol and infection with human papillomavirus (HPV). To examine the role of HPV in the pathogenesis of ST-related oral cancer, we sought HPV DNA in three different sets of formalin-fixed biopsy tissues, using the polymerase chain reaction (PCR) technique: (1) nondysplastic oral lesions associated with short-term ST use, (2) oral cancers and dysplasias associated with long-term use of ST, and (3) oral cancers and dysplasias not related to the use of ST. In these studies HPV was not detected in early, nondysplastic lesions associated with short-term use. However, HPV was detectable in approximately 30 percent of the oral cancers—whether or not they were associated with ST use. These results suggested that HPV infection may be one of several factors contributing to the pathogenesis of ST-related oral cancer, and that HPV infection does not occur at the earliest stages of ST lesion development.

INTRODUCTION It has been estimated that 30 percent of all cancers in the United States are associated with the use of tobacco in its various forms (Doll and Peto, 1981; Wynder and Gori, 1977). Approximately 53 million Americans are believed to smoke, and approximately 12 million to use smokeless tobacco (Hecht and Hoffmann, 1988). Smoking is strongly associated with malignancy of the respiratory tract, upper digestive tract, bladder, kidney, and pancreas (Doll and Peto, 1981; Wynder and Gori, 1977). There is also a large body of evidence linking smoking to oral cancer (Doll and Peto, 1981; Wynder and Gori, 1977), and death rates from oral cancer are four times higher among smokers than nonsmokers (Doll and Peto, 1976; Hammond, 1966; Rogot and Murray, 1980). Oral cancer is also strongly associated with the use of smokeless tobacco, especially snuff (Consensus Conference, 1986; IARC, 1985; US DHHS, 1986; Vogler et al., 1962; Winn, 1988); the incidence of cancer of the cheek and gums has been shown to be as much as 50 times higher among long-term ST users than among age-matched controls (Winn, 1986).

The mechanisms by which smoking and ST contribute to the development of oral cancer or its precursors are not yet known. One potential mechanism is the direct effect of carcinogens in cigarette smoke or ST on the oral epithelium. Tobacco products are known to contain a number of substances that may give rise to carcinogens (Hecht and Hoffmann, 1988).

¹ This study was supported by National Institute of Dental Research grant no. PO1 DE08547.

The most important of these are nicotine, a tertiary amine, and nornicotine, anabasine, and anatabine, which are secondary amines. These compounds may react with nitrosating agents to form nitrosamines (NNN, NNK, NNA, NAB, and NAT), which have been shown to be carcinogenic in mice, rats, and hamsters (Hecht and Hoffmann, 1988; Hoffmann and Hecht, 1985). Other potential carcinogens found in smoked tobacco products include formaldehyde, acetaldehyde, croton aldehyde, and benzopyrene. The mechanism by which nitrosamines and other potential carcinogens act to promote malignancy is not yet known. One possibility is modification of cellular DNA by nitrosamines. There are several mechanisms by which this could occur; for example, nitrosamines may be α -hydroxylated by specific cytochrome P₄₅₀ isozymes to yield methyldiazohydroxide, which is capable of methylating DNA to produce O⁶-methylguanine and other point mutations.

A second mechanism may involve induction of chromosomal DNA breakage. It has been shown that tobacco-related products may induce single-stranded DNA breaks because of the reduction of oxygen to superoxide radicals (Borish et al., 1987), and the resulting repair process may increase the likelihood of a mutation (Richter et al., 1988). These mutations may then contribute to carcinogenesis by altering the levels or functions of genes involved in the control of cell growth.

In addition to the use of tobacco products, viral infection is increasingly implicated in the pathogenesis of oral cancer. Oral cancer bears significant resemblance to other mucosal epithelial cancers, such as cancer of the cervix and anus, both of which have been associated with infection with human papillomavirus (HPV) (Beaudenon et al., 1986; Boshart et al., 1984; Crum et al., 1985; Lorincz et al., 1986 and 1987; Reid et al., 1987). The association between HPV infection and mucosal cancer has been characterized best for anogenital cancer. Of the more than 60 known HPV types, infection with HPV types 16 and 18 is most closely associated with anogenital cancer and high-grade intraepithelial neoplasia (grades 2-3); types 31, 33, and 35 are associated with an intermediate risk of cancer; and types 6, 11, 42, 43, and 44 are associated with low-grade intraepithelial neoplasia (grade 1) and condyloma, both of which carry a low risk of progression to invasive disease.

The relationship between HPV infection and oral cancer was first described in 1983, when cytopathic changes of HPV infection (koilocytosis) similar to those seen in cervical HPV infection were noted in oral lesions (Syrjanen et al., 1983). Subsequent to that report, several investigators have looked for HPV DNA in oral lesions (Adler-Storthz et al., 1986; Dekmezian et al., 1987; Kashima et al., 1990; Loning et al., 1985 and 1987; Lookingbill et al., 1987; Maitland et al., 1987; Milde and Loning, 1986; Ostrow et al., 1987; Scully et al., 1985; Syrjanen et al., 1988; de Villiers et al., 1985 and 1986), using a wide variety of HPV DNA hybridization techniques with differing sensitivities and specificities. In one study, involving dot blot hybridization, HPV DNA was detected in 8 of 22 oral cancers (36 percent): type 16/18 in 3 cancers, type 6/11 in 1, and other types in 4 other cancers (Loning et al., 1987). Using *in situ* hybridization, one group reported detection of HPV

DNA in 6 of 51 (12 percent) oral cancer specimens, and 6 of 21 (29 percent) oral dysplasia specimens (Syrjanen et al., 1988). Using Southern blot hybridization, another group detected HPV sequences in almost 50 percent of invasive cancers, but also detected HPV in a similar proportion of normal tissues (Maitland et al., 1987). Using a similar technique, Kashima et al. reported that 7 of 74 (9 percent) were positive for HPV DNA. In this series of tests, a wide range of types were identified, including HPV-16, -6, -57, -3, and -13 (Kashima et al., 1990). Similar to the report by Maitland et al. (1987), HPV DNA was detectable in 3 of 33 (9 percent) clinically normal tissues obtained from the mucosal surface contralateral to the lesion. Taken together, these studies suggest that HPV DNA is detectable in fewer than half of the specimens of oral cancer, and that a wide range of HPV types may be present. Moreover, detection of HPV DNA in clinically normal oral tissues in proportions similar to those of the cancerous lesions in some studies raises the question of the nature of the contribution of HPV to the pathogenesis of oral lesions.

The techniques used in the studies described above, that is, Southern blot hybridization, dot blot hybridization, and in situ hybridization, all have limited sensitivity. Thus, the inability to detect HPV DNA in a higher proportion of oral cancers and precancerous lesions may reflect a level of HPV infection below the limit of the sensitivity of these tests or infection with HPV types other than would be normally detected with currently available probes. To address the question of the prevalence of HPV in oral dysplasias and cancers, using DNA detection techniques that are both highly sensitive and capable of detecting a large number of HPV types, we used the PCR technique with HPV L1 consensus primers.

Studies of the role of HPV in ST-associated lesions provide a unique opportunity to determine the stage at which HPV infection may begin to play a role in the pathogenesis of oral cancer for two reasons: (1) the use of ST products is clearly associated with low-grade lesions such as hyperkeratosis and hyperorthokeratosis, which may represent precursors to dysplasia, given sufficient exposure; and (2) as described previously, use of ST products is also clearly a risk factor for development of oral cancer. Using in situ hybridization, Greer et al. (1990) studied 50 leukoplakias associated with the use of ST, and found 2 (4 percent) containing HPV-6 and 3 (6 percent) containing HPV-2. Therefore, to address the question of the stage of precancerous disease at which HPV may first be detected through more sensitive techniques, we used PCR also to study ST-associated lesions ranging from histologically normal to invasive cancer.

METHODS One hundred eight oral biopsies ranging from histologically normal to invasive cancer were obtained, fixed in formalin, and embedded in paraffin. To perform PCR, tissue sections 7 μm in thickness were cut from each paraffin block, and the paraffin was removed by suspending the section in 500 μL of xylene in its original Eppendorf tube. The tissue was dried, resuspended in 100 μL of water containing proteinase K at a concentration of 100 $\mu\text{g}/\text{mL}$, and digested overnight at 37 $^{\circ}\text{C}$. Consensus primers for the detection of the L1 region were employed as described elsewhere (Ting et al., 1990). Positive

controls consisted of the amplification of human beta-hemoglobin DNA from each tissue. Negative controls were used to minimize the possibility of false-positives attributable to contamination from one specimen to another in the laboratory, and consisted of amplification of each mixture with all components of the reaction except target DNA. Fifty cycles of amplification were performed with 800.0 μM dNTPs (United States Biochemical Corp., Cleveland, Ohio), 1.0 μM of each primer, 2.5 mM MgCl_2 , 0.5% Tween-20, 0.5% NP-40, and 50 units/mL AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Connecticut). Five microliters of 100 μL amplification product were applied to a nylon membrane and the presence of HPV DNA was sought with ^{32}P -labeled consensus probes. Positive samples identified positive with the consensus probes were then studied with probes specific for HPV-6, -11, -16, -18, -31, and -33.

RESULTS To begin to understand the stages of oral disease at which HPV infection may occur, we examined 52 biopsies obtained from professional baseball players who were short-term users of ST. The tissues represented 26 biopsies with histologically diagnosed lesions ranging from hyperparakeratosis to basal cell hyperplasia (Daniels et al., this volume; Grady et al., 1991) and 26 histologically normal biopsies adjacent to the lesion, which served as controls. None of the tissues demonstrated dysplastic changes. In these studies HPV-33 could be detected in only one hyperparakeratotic lesion, and HPV type 6/11 in the normal tissue adjacent to another hyperparakeratotic lesion, which was itself HPV-negative.

To study the prevalence of HPV in high-grade lesions associated with long-term use of ST, 10 high-grade dysplasia/oral cancer lesions and 4 histologically normal control tissues were obtained from the laboratory of Dr. George Kaugars of the University of Virginia. In contrast to the low-grade lesions obtained from the baseball players, 3 of 10 high-grade dysplasia/oral cancer lesions (30 percent) associated with long-term use of ST were found to be HPV-16 DNA-positive; none of the normal control tissues were positive for HPV DNA.

Thirty-two oral cancer and dysplasia tissues not associated with use of ST were studied, along with 10 negative controls. The results indicated that 8 of 25 of the oral cancer tissues (32 percent) were HPV DNA-positive and 3 of 7 (43 percent) of high-grade oral dysplasia were HPV DNA-positive (Palefsky et al., unpublished data). The HPV types detected were found to be heterogeneous; fewer than half of the HPV types detected were represented by the common anogenital HPV types. In contrast to the oral dysplasia and cancer tissues, none of seven fibromas and none of three normal tissues were positive for HPV.

DISCUSSION Studies of the prevalence of HPV in ST-associated lesions in short-term and long-term users are not strictly comparable, since the latter represented an older population from a specific geographic area of the United States. Nevertheless, results of these studies suggest that the low-grade non-dysplastic lesions associated with short-term ST use (< 5 yr) are not associated with HPV, whereas high-grade ST-associated lesions contain HPV DNA in a proportion similar to that seen in oral dysplasia and cancers that are not

associated with ST use. These results suggest that HPV does not play a significant role in the pathogenesis of ST-associated disease at its earliest stages, but instead begins to be detectable in association with dysplasia. While dysplastic and cancerous tissues not associated with use of ST were also found to be associated with HPV, a large number of cases of oral cancer and dysplasia were HPV-negative on PCR examination, consistent with studies using less sensitive techniques. Oral cancer therefore appears to be a more heterogeneous disease than cervical cancer with respect to etiology; while HPV may play a significant role in the pathogenesis of oral cancer, it is clear that it need not necessarily be present, and that other factors are likely to be important as well.

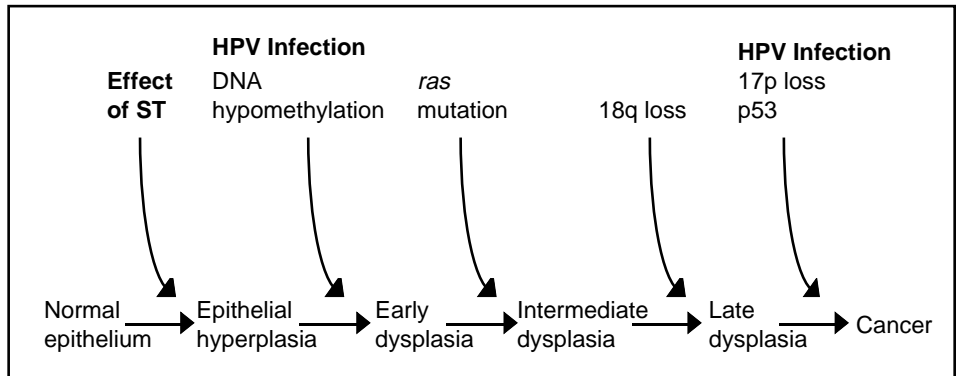
Among other factors that may play a role in the pathogenesis of oral cancer are genetic mutations. Reports have been published that document the sequence of cellular changes at the molecular level that occur as a benign colonic adenoma progresses to invasive colorectal cancer (Baker et al., 1990; Fearon et al., 1990). In these studies, a series of genetic mutations accumulated as the lesion progressed, and the accumulation of further abnormalities was necessary for progression to the next stage of disease. These included mutations in the *ras* oncogene, translocations of the short arm of chromosome 5, and breakage of the long arm of chromosome 11. A model such as this may also be applicable to the progressive changes that occur in the oral mucosa as a lesion develops from benign to increasing grades of dysplasia and, ultimately, to invasive cancer.

On the basis of the data described above, we propose a model for the development of ST-associated cancer (Figure 1). In this model, ST-related products are responsible for the development of low-grade nondysplastic lesions, including hyperparakeratosis, hyperorthokeratosis, and basal cell hyperplasia, that may progress to dysplasia with continued exposure. It is not yet known if these low-grade lesions are associated with chromosomal abnormalities or oncogenic mutations. With continued exposure to these products, as well as other cofactors such as alcohol, further genetic damage may accumulate, resulting in dysplasia. ST products may induce these chromosomal changes via DNA adduct formation or chromosomal breakage. The result of these changes may include activation of oncogenes such as *ras* or inactivation of a member of the anti-oncogene family, such as the RB or p53 proteins. We also propose that infection with HPV may represent one of the cofactors that play a role, in conjunction with ST products, in the pathogenesis of ST-related dysplasia and cancer. At this time, it is not known if dysplasia occurs as a result of HPV infection or if HPV infects a preestablished dysplastic lesion and potentiates its progression. However, recent studies of HPV-16-transfected cells with the floating raft model of epithelial differentiation have been conducted, in which transfection of HPV-16 DNA has been shown to result in epithelial changes consistent with dysplasia (McCance et al., 1988). This supports the hypothesis that dysplasia results from HPV infection.

Once established, HPV infection may contribute to the development and/or progression of dysplasia in a number of ways. Among these are the binding and inactivation of p53 gene product by HPV E6 protein, as well as

Figure 1

Proposed schema for the development of ST-related oral cancer. In this schema, several different etiologic factors may play a significant role. ST appears to play an important role and may continue to do so at any subsequent stage. HPV does not appear to play an important role at the earliest stages but may facilitate development and progression of dysplasia in some ST-related lesions. Like ST, HPV may also play a role in development of invasive cancer. A variety of other factors such as *ras* mutations or loss of chromosomal arms may also play a role at different stages of disease. The alterations depicted in this figure were adapted from studies of the development of colon cancer (Fearon and Vogelstein, 1990); similar studies of ST-related cancer have not yet been performed.



the inactivation of the retinoblastoma gene product by the HPV E7 protein (Munger et al., 1989; Werness et al., 1990). In addition, other mechanisms by which HPV contributes to the development of dysplasia almost certainly exist and remain to be characterized.

In summary, ST-related oral cancer may result from cumulative genetic damage induced by ST, with or without other cofactors. Among possible significant cofactors is HPV infection, which does not appear to play a role in pathogenesis of the earliest ST-related lesions, but may instead play a role at later stages, beginning with dysplasia.

This model suggests that the development of ST-related cancers may represent the end product of cooperation between a number of different factors, including HPV infection. Prevention of cancer development must therefore focus on interference with this process at a number of levels. Cessation of exposure to ST-related products remains the most important intervention, but interference with potential cofactors, including abstinence from alcohol, may play an important role as well. Currently, no therapies are available to interfere with HPV gene products, and the only method of preventing the consequences of HPV infection is the removal of HPV-infected lesional tissue. Nevertheless, if and when therapies directed against HPV do become available, they may constitute useful adjunctive measures for those cases in which HPV infection can be demonstrated.

Much remains to be learned about the pathogenesis of ST-related cancer at the molecular level. The evidence for an important role for HPV in the pathogenesis of oral cancer in general, and ST-related oral cancer in particular, remains incomplete; studies of larger numbers of dysplastic and cancerous oral tissues obtained from populations with well-matched controls are needed. Such studies should include analysis of histologically normal control tissues from matched control subjects, as well as of normal tissue from the individuals with oral lesions. Standardized methods of HPV detection are needed, as well as standardization of histopathologic criteria for grading the lesions. Adequate in vitro models for studying the interaction between ST products, HPV, and alcohol are needed; the recently developed floating raft system may be very useful for this purpose. Further research is needed to define which of the ST-related products are most toxic to epithelial cells; whether and how HPV gene products interact with ST products to induce dysplastic changes; characterization of chromosomal changes induced by ST; characterization of the role of oncogenic activators, or inactivation of anti-oncogenes in these cells; characterization of the HPV types present in ST-related cancer; and characterization of the risk factors for acquisition of HPV infection. Knowledge of cofactors that play a role along with ST products will permit a more effective approach to the prevention of ST-related dysplasia and cancer. Knowledge of the molecular mechanisms of the pathogenesis of these diseases will also be of great value in preventing progression or inducing regression of ST-related dysplasia, and in preventing progression to cancer among those in whom dysplasia has already occurred.

REFERENCES

- Adler-Storhiz, K., Newland, J.R., Tessin, B.A., et al. Identification of human papillomavirus types in oral verruca vulgaris. *Journal of Oral Pathology and Medicine* 15: 230-233, 1986.
- Baker, S.J., Preisinger, A.C., Jessup, J.M., et al. p53 gene mutations occur in combination with 17p allelic deletions as late events in colorectal tumorigenesis. *Cancer Research* 50: 7717-7722, 1990.
- Beaudenon, S., Kremsdorf, D., Croissant, O., et al. A novel type of human papillomavirus associated with genital neoplasias. *Nature (London)* 321: 246-249, 1986.
- Borish, E.T., Pryor, W.A., Venugopal, S., et al. DNA synthesis is blocked by cigarette tar-induced DNA single-strand breaks. *Carcinogenesis* 8: 1517-1520, 1987.
- Boshart, M., Gissmann, L., Ikenberg, H., et al. A new type of papillomavirus: Its presence in genital cancer biopsies and in cell lines derived from cervical cancer. *EMBO Journal* 3: 1151-1157, 1984.
- Consensus Conference. Health implications of smokeless tobacco use. *Journal of the American Medical Association* 255: 1045-1048, 1986.
- Crum, C., Mitao, M.R., Levine, R., et al. Cervical papillomaviruses segregate within morphologically distinct precancerous lesions. *Journal of Virology* 54: 675-681, 1985.
- Dekmezian, R.H., Batsakis, J.G., Goepfert, H. In situ hybridization of papillomavirus DNA in head and neck squamous cell carcinomas. *Archives of Otolaryngology and Head and Neck Surgery* 113: 819-821, 1987.
- Doll, R., Peto, R. Mortality in relation to smoking: 20 years' observations on male British doctors. *British Medical Journal* 2: 1525-1536, 1976.
- Doll, R., Peto, R. The causes of cancer: Quantitative estimate of avoidable risks of cancer in the United States today. *Journal of the National Cancer Institute* 66: 1191-1308, 1981.
- Fearon, E.R., Vogelstein, B. A genetic model for colorectal tumorigenesis. *Cell* 61: 759-767, 1990.
- Grady, D., Greene, J., Ernster, V.L., et al. Short-term changes a surprise with smokeless tobacco oral lesions. *Journal of the American Dental Association* 122: 62-64, 1991.
- Greer, R.O., Eversole, L.R., Crosby, L.K. Detection of human papillomavirus-genomic DNA in oral epithelial dysplasias, oral smokeless tobacco-associated leukoplakias and epithelial malignancies. *Journal of Oral and Maxillofacial Surgery* 48: 1201-1205, 1990.
- Hammond, E.C. Smoking in relation to the death rates of one million men and women. *National Cancer Institute Monograph* 19: 127-204, 1966.

- Hecht, S.S., Hoffmann, D. Tobacco-specific nitrosamines, an important group of carcinogens in tobacco and tobacco smoke. *Carcinogenesis* 9: 875-884, 1988.
- Hoffmann, D., Hecht, S.S. Nicotine-derived N-nitrosamines and tobacco-related cancer: Current status and future questions. *Cancer Research* 45: 935-944, 1985.
- International Agency for Research on Cancer. *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans: Tobacco Habits Other than Smoking: Betel-Quid and Areca-Nut Chewing; and Some Related Nitrosamines* (volume 37). Lyon: IARC, 1985, pp. 113-116.
- Kashima, H.K., Kutcher, M., Kessis, T., et al. Human papillomavirus in squamous cell carcinoma, leukoplakia, lichen planus, and clinically normal epithelium of the oral cavity. *Annals of Otolaryngology and Rhinology* 99: 55-61, 1990.
- Loning, T., Ikenberg, H., Becker, J., et al. Analysis of oral papillomas, leukoplakia and invasive carcinomas for human papillomavirus type related DNA. *Journal of Investigative Dermatology* 84: 417-420, 1985.
- Lookingbill, D.P., Kreider, J.W., Howitt, M.K., et al. Human papillomavirus type 16 in Bowenoid papulosis, intraoral papillomas, and squamous cell carcinoma of the tongue. *Archives of Dermatology* 123: 363-368, 1987.
- Lorincz, A.T., Lancaster, W., Temple, G. Cloning and characterization of a new human papillomavirus from a woman with dysplasia of the uterine cervix. *Journal of Virology* 58: 225-229, 1986.
- Lorincz, A.T., Quinn, A., Lancaster, W., et al. A new type of papillomavirus associated with cancer of the uterine cervix. *Virology* 159: 187-190, 1987.
- Maitland, N.J., Cox, M.F., Lynas, C., et al. Detection of human papillomavirus DNA in biopsies of human oral tissue. *British Journal of Cancer* 56: 245-250, 1987.
- McCance, D.J., Kopan, R., Fuchs, E., et al. Human papillomavirus type 16 alters human epithelial cell differentiation in vitro. *Proceedings of the National Academy of Sciences* 85: 7169-7173, 1988.
- Milde, K., Loning, T. The detection of papillomavirus DNA in oral papillomas and carcinomas: Application of in situ hybridization with biotinylated HPV 16 probes. *Journal of Oral Pathology* 15: 292-296, 1986.
- Munger, K., Werness, B.A., Dyson, N., et al. Complex formation of human papillomavirus E7 proteins with the retinoblastoma tumor suppressor gene product. *EMBO Journal* 8: 4099-4105, 1989.
- Ostrow, R.S., Manias, D.A., Fong, W.J., et al. A survey of human cancers for human papillomavirus DNA by filter hybridization. *Cancer* 59: 429-434, 1987.
- Reid, R., Greenberg, M., Jenson, A.B., et al. Sexually transmitted papillomaviral infections. I. The anatomic distribution and pathologic grade of neoplastic lesions associated with different viral types. *American Journal of Obstetrics and Gynecology* 156: 212-222, 1987.
- Richter, C., Park, J.-W., Ames, B.N. Normal oxidative damage to mitochondrial and nuclear DNA is extensive. *Proceedings of the National Academy of Sciences* 85: 6465-6467, 1988.
- Rogot, E., Murray, J.L. Smoking and the causes of death among U.S. veterans: 16 years of observation. *Public Health Reports* 95: 213-222, 1980.
- Scully, C., Prime, S., Maitland, N. Papillomaviruses: Their role in oral disease. *Oral Surgery, Oral Medicine, Oral Pathology* 60: 166-174, 1985.
- Syrjanen, K., Syrjanen, S.M., Lamberg, M., et al. Morphological and immunohistochemical evidence suggesting human papillomavirus (HPV) involvement in oral squamous cell carcinogenesis. *International Journal of Oral Surgery* 12: 418-424, 1983.
- Syrjanen, S.M., Syrjanen, K.J., Happonen, R.-P. Human papillomavirus (HPV) DNA sequences in oral precancerous lesions and squamous cell carcinoma demonstrated by in situ hybridization. *Journal of Oral Pathology* 17: 273-278, 1988.
- Ting, Y., Manos, M.M. In: *PCR Protocols and Applications*. New York: Academic Press, 1990, pp. 356-367.
- U.S. Department of Health and Human Services. *The Health Consequences of Using Smokeless Tobacco: A Report of the Advisory Committee to the Surgeon General*. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, National Cancer Institute. DHSS Publication No. (NIH) 86-2874, 1986, pp. 33-58.
- de Villiers, E.-M., Neumann, C., Le, J.-Y., et al. Infection of the oral mucosa with defined types of human papillomavirus. *Medical Microbiology and Immunology* 174: 287, 1986.
- de Villiers, E.-M., Wiedauer, H., Otto, H., et al. Papillomavirus in human tongue carcinomas. *International Journal of Cancer* 36: 575, 1985.
- Vogler, W.R., Lloyd, J.W., Milmore, B.K. A retrospective study of etiologic factors in cancer of the mouth, pharynx and larynx. *Cancer* 15: 246-258, 1962.
- Werness, B.A., Levine, A.J., Howley, P.M. Association of human papillomavirus types 16 and 18 E6 proteins with p53. *Science* 248: 76-79, 1990.
- Winn, D.M. Smokeless tobacco and oral/pharynx cancer: The role of cofactors. *Banbury Report* 23: 361-371, 1986.
- Winn, D.M. Smokeless tobacco and cancer: The epidemiologic evidence. *CA—A Cancer Journal for Clinicians* 38: 236-243, 1988.
- Wynder, E.L., Gori, G.B. Contribution of the environment to cancer incidence: An epidemiologic exercise. *Journal of the National Cancer Institute* 58: 825-832, 1977.

Identification of Human Papillomavirus DNA in Smokeless Tobacco Keratoses And Premalignant and Malignant Oral Lesions, by PCR Amplification With Consensus Sequence Primers¹

Robert O. Greer, Jr., Kenneth R. Shroyer, and Louise Crosby

ABSTRACT Human papillomaviruses (HPVs), trophic for cutaneous and mucosal epithelium, have been reported in association with benign, dysplastic, and malignant proliferations of the oral mucous membrane. More than 60 HPV types have been recognized on the basis of significant sequence divergence following molecular cloning as recombinant DNAs. To evaluate the role of HPV in oral neoplasia, completion of large-scale studies is needed to determine the diversity of papillomaviruses involved and studies implemented to identify patterns of transcription of transforming sequences in high-risk HPV types with a trophism for the oral cavity. The purpose of the current investigation was to test for the presence of HPVs in biopsies of oral epithelial dysplasia, verrucous hyperplasia, smokeless tobacco keratoses, and squamous cell carcinoma by examining lesional tissue by polymerase chain reaction (PCR) amplification techniques using L1 consensus sequence primers. HPV sequences were detected in all four categories of lesions and in no control samples. PCR amplification allows rapid and specific detection of HPV in oral biopsy specimens and may enhance our ability to evaluate large numbers of clinical samples and demonstrate a broad variety of HPV sequences and novel HPV types in oral cancer and precancer.

INTRODUCTION Papillomaviruses are tenacious, persistent viruses capable of residing latent within host cells for extended periods. HPV is an epitheliotrophic infectious agent with a genome consisting of approximately 7,900 base pairs (bp) of double-stranded circular DNA encapsulated within an icosahedral protein shell. HPV has been increasingly associated with mucosal cancers, particularly carcinoma of the cervix (Howley, 1987; Vousden, 1989; zur Hansen and Schneider, 1987). The cloned DNAs of a significant number of human and animal papillomavirus DNAs have been sequenced completely, and they are notable for their overall similarity in genetic organization, in spite of substantial sequence variation and diversity in host and tissue specificity, histological presentation, and malignant potential (Koutsky et al., 1989; zur Hansen and Schneider, 1987). Although human papillomaviruses are characterized by a low degree of pathogenicity, their synergistic effect with other carcinogens, such as tobacco and alcohol, enhances their carcinogenic potential.

¹ This investigation was supported in part by funding from Smokeless Tobacco Research Council grant no. 0278, National Cancer Institute grant no. CA-21098-14, and a grant from the Sands House Foundation.

In previous studies using *in situ* hybridization methods and dot blot hybridization, we have demonstrated human papillomavirus in ST keratoses, oral epithelial dysplasia, verrucous hyperplasia, verrucous carcinoma, and squamous cell carcinoma (Greer et al., 1987, 1990a, and 1990b).

Recently we used a PCR DNA amplification system for the identification of HPV type 16 E6 DNA sequence in formalin-fixed, paraffin-embedded biopsy material from 61 oral precancers and cancers, including squamous cell carcinoma, epithelial dysplasia, ST keratoses, verrucous hyperplasia, and verrucous carcinoma (Shroyer and Greer, 1991). This PCR amplification procedure allowed rapid and specific detection of low-abundance viruses and single-copy genes in biopsy specimens. The PCR amplification technique was judged simple and highly sensitive for analyzing specific HPV DNA sequences from archival material. The test can be correlated easily with the histological appearance seen in adjacent sections.

Several investigators (Bauer et al., 1991; Manos et al., 1990; Resnick et al., 1990) have developed a PCR DNA amplification system using two distinct consensus oligonucleotide primer sets for the improved detection and typing of a broad spectrum of human genital papillomavirus sequences including those of novel viruses. Their systems incorporate one consensus primer set designed to amplify the L1 domain from a wide variety of HPV types and a second consensus primer set designed to amplify a domain within the E6 open reading frame sequence of HPV. Resnick and associates (1990) have demonstrated that when consensus and type-specific oligonucleotide probes are used in hybridization analysis of amplified products, as few as 10 copies of HPV can be detected. The investigators have been able also to amplify many other known genital HPV types including 6, 11, 16, 18, 31, 33, 35, 39, 40, 42, 45, and 51 to 59, as well as a large number of other HPVs that have not yet been assigned a type.

In the current investigation, we analyzed paraffin-embedded tissue sections from squamous cell cancers, oral epithelial dysplasias, verrucous hyperplasias, and oral ST keratoses for the presence of HPV DNA by a PCR-based system, using consensus sequence primers that amplify an approximately 450-bp region of the L1 open reading frame. We also evaluated 18 PCR product bands in the four categories of lesions, using dot blot hybridization to confirm the presence of HPV-16 DNA.

MATERIALS AND METHODS Sections from 44 paraffin-embedded tissue blocks representing 8 squamous cell carcinomas, 11 oral epithelial dysplasias, 9 verrucous hyperplasias, and 16 ST keratoses were examined from the pathology archives of Western States Regional Oral Pathology Laboratory and the University of Colorado Oral Pathology Laboratory. All tissue samples were obtained during 1990. Sections 5 to 10 μm in thickness were cut from blocks and placed in 0.6-mL capped centrifuge tubes and prepared for PCR amplification as described by Wright and Manos (1990).

PCR Aliquots (10 μL) of the prepared samples were subjected to 40 cycles of amplification, with HPV L1 consensus sequence primers MY11 and MY09 with β -globin primers PC04 and GH20 (Perkin-Elmer Cetus, Norwalk, Connecticut), as previously described (Resnick et al., 1990). Recombinant

plasmid-containing HPV-16 DNA (Oncor, Gaithersburg, Maryland) and sections from cervical squamous cell carcinoma were used as positive controls. Great care was taken to avoid the possibility of sample contamination during preparation. Amplification was evaluated by agarose gel electrophoresis (10 μ L from each reaction), ethidium bromide staining, and visualization under ultraviolet light. Samples that failed to yield the 268-bp β -globin amplification product were excluded from the study.

HPV negative controls included tissue samples that had been previously characterized as negative for HPV DNA. In addition, multiple aqueous controls were included with each experiment to monitor for the possibility of reagent contamination.

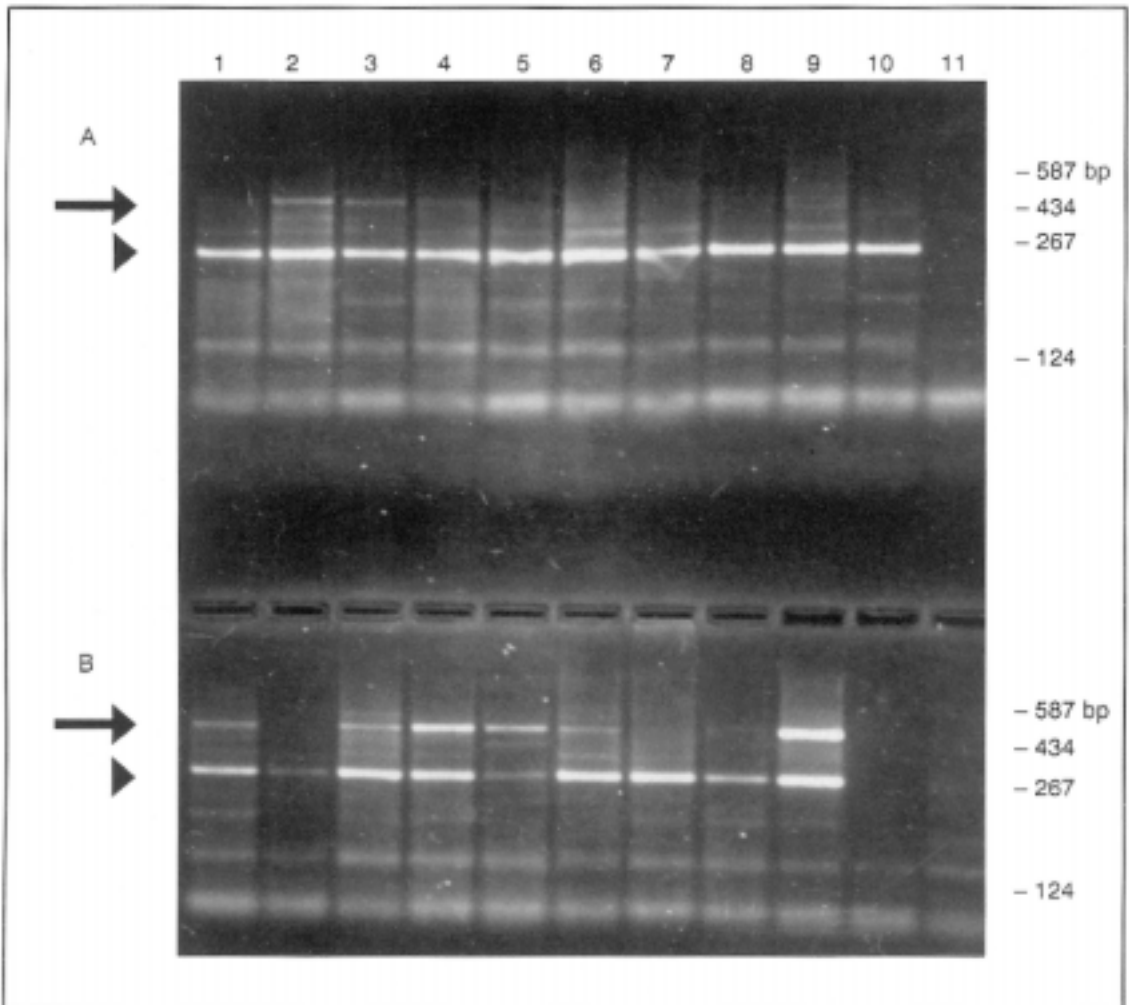
Dot blot hybridization for HPV-16 was carried out on 18 cases with BioRad's Bio-Dot minifold apparatus for six replicate membranes (Sambrook et al., 1989). DNA oligomer probes were end-labeled with γ -[32 P]ATP with hybridization carried out for 3 h at 56 °C in hybridization solution with each of the radio-labeled probes. Subsequent autoradiographic exposure was at -70 °C for 12 to 48 h.

RESULTS Biopsy specimens and positive controls were amplified with HPV L1 consensus primers (Figure 1). HPV amplification products were observed in smokeless tobacco keratoses, squamous cell carcinomas, oral epithelial dysplasia, and verrucous hyperplasias. From the total of 30 amplifiable specimens, 8 showed evidence of infection with HPV. The data reported in Table 1 represent the specific lesions analyzed and the determination of whether or not HPV was present. Subsequently, 18 cases representing each of the four categories of lesions were probed for HPV-16 using residual PCR product via dot blot hybridization. Of 18 cases, 8 were positive for HPV-16: 3 ST keratoses, 2 squamous cell carcinomas, 2 epithelial dysplasias, and 1 verrucous hyperplasia. Two cases positive by generic probe analysis were negative for HPV-16 and are currently being analyzed by dot blot for HPV-2, -4, -6, -11, -18, -31, -33, and -35. The eight HPV-16-positive cases are shown in Figure 2 in lanes A1, 2, and 3, B5, and C1 through 4.

DISCUSSION A distinct advantage of PCR lies in its ability to make large numbers of copies of DNA sequences from targets that are present in minute quantities in the original sample. As a result, the improved L1 consensus sequence amplification method is significantly more sensitive in detecting amplified types of HPV in oral tissue samples than in situ hybridization. Although the PCR method applied here allows a demonstration of only generic HPV, it is anticipated that we will subsequently be able to determine specific HPV types through the use of type-specific DNA probes in hybridization analysis of the PCR products. The increased sensitivity of the PCR method seems to be critical in detecting HPV in small tissue fragments from the oral cavity and in suboptimal tissue samples where low-level infections may be present. One disadvantage of the PCR technique is the potential for obtaining false positive results because of sample-to-sample contamination, or more importantly, because of the carryover of DNA from previous DNA amplifications (Bauer et al., 1991). To minimize this risk, we used only single-designated-use pipettes and separated pre- and post-PCR samples and reagents throughout all stages of the investigation.

Figure 1

PCR amplification with HPV L1 consensus sequence primers, representative cases. HPV L1 DNA was amplified over 40 cycles. β -Globin primers were included as an amplification control. Amplified products were analyzed by 4 percent (wt/vol) agarose gel electrophoresis, stained with ethidium bromide, and photographed under UV light. Molecular weight markers (Hae III cut pBR322) are shown in the right margin. HPV L1 positive cases (A2-4, B3-6) show a band at about 450 bp (arrow). β -Globin amplification products show a band at about 268 bp (arrowhead). HPV-16 plasmid DNA positive control (B9) and reagent controls (A11, B10, 11) are also shown.



Although the procedure may improve the likelihood of detecting a wide variety of HPV sequences, including novel types, it still does not answer the basic question of whether HPV assumes the function of a tumor promoter, as suggested by Amtmann (1987), or a causal agent, as suggested by Chow and associates (1987) and McCance (1988). To accomplish that, investigators must correlate expression of transforming sequences and lesional

Table 1
Identification of HPV in oral biopsy specimens through PCR analysis

| Pathological Diagnosis | HPV ^a |
|---------------------------|------------------|
| Squamous Cell Carcinoma | 2/8 |
| Oral Epithelial Dysplasia | 1/11 |
| Verrucous Hyperplasia | 2/9 |
| ST Keratosis | 3/16 |

^a Positive cases over total number of amplifiable cases tested.

presentation with patient historical data, including specific etiologic influences such as alcohol consumption, smoking habits, wart history, racial origin, age, and sex. While PCR is a highly sensitive technique for the demonstration of HPV DNA, it provides no information on the functional state of the viral genome. Investigation of the possible role of HPV in the neoplastic transformation of the oral mucosa will require an understanding of the pattern of gene expression in HPV DNA-positive cases. Such an analysis in the past has been encumbered by considerable problems with inadequate degrees of sensitivity in the detection of mRNA.

Another question to be answered is whether there is clinical significance in detecting HPV in the oral precancerous and cancerous lesion. Nuovo and associates (1990), in a study of HPV DNA in penile lesions histologically negative for condyloma, suggest that lesions with low copy numbers of the virus may in fact be either exceedingly early or regressing. They question whether these lesions, which are associated with small copy numbers of the virus, are truly infectious. The same questions apply to the oral cavity.

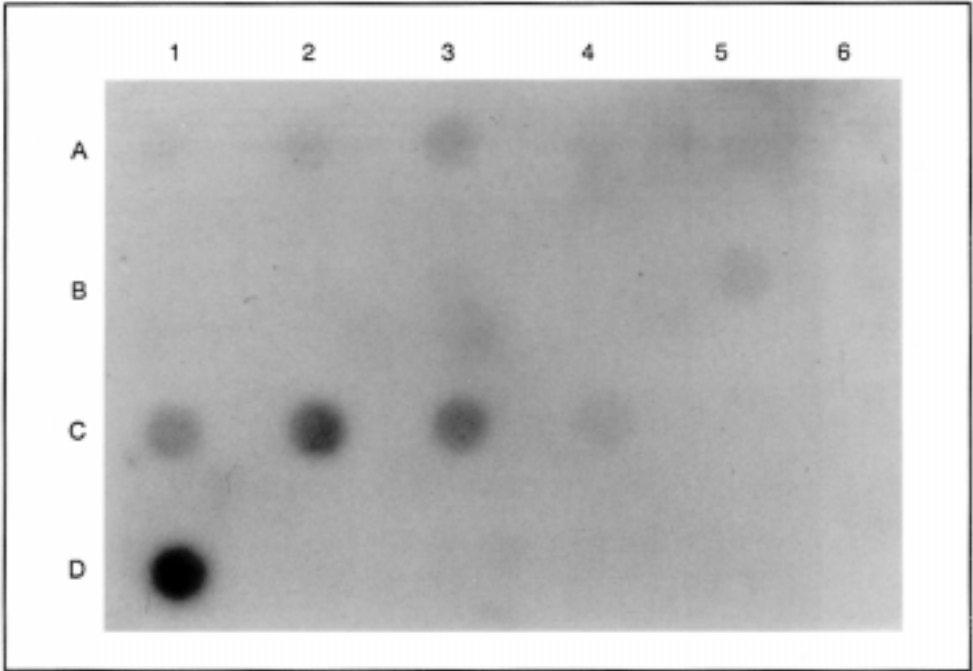
There is little doubt that HPV plays a significant role in the development of mucosal cancer. Accumulated evidence over the last several years links specific HPV types to the various manifestations of anogenital infection (Bartholoma et al., 1991). The seven most prevalent anogenital HPV types are HPV-6, -11, -16, -18, -31, -33, and -35. It is reasonable to suggest that some of these same viruses play a significant role in the development of oral cancer.

Because it is unlikely that all latent affected sites are eradicated with treatment, such as incisional biopsy, complete surgical excision, or stripping, it is possible that once someone is infected with HPV, the virus is harbored for life. It is also likely that HPV infections of the oral mucous membranes are not completely curable by surgical excision.

Bauer and associates (1991) have suggested that there may be an age-specific prevalence for cervical HPV infection. The same postulate may be made for HPV-infected oral mucous membranes. Although other genetic, environmental, and exogenous variables may be implicated in the progression to oral dysplasia or neoplasia, infection with specific HPVs may very well still be predictive of disease in certain subsets of patients. To determine

Figure 2

Dot blot hybridization for HPV-16 with residual PCR product. Eight positive cases are seen in lanes A1, 2, and 3, B5, and C1 through 4. A1, B5, and C4 are weakly positive. A2, 3, and C1, 2, and 3 are strongly positive. HPV-16 plasmid DNA positive control is D1.



whether HPV is responsible for preneoplastic or neoplastic mucosal changes, long-term prospective studies are needed to aid understanding of the cause of HPV oral infections in asymptomatic patients, patients who have immune disorders, and patients who are extensive users of exogenous agents such as tobacco and alcohol.

Clearly, more work is needed to obtain a complete catalog of oral HPV types. From an epidemiological standpoint, investigators need to determine the prevalence of infections with multiple HPV types in the oral cavity. Also, Bauer and associates (1991), in their evaluation of genital HPV infections, have shown that infections with multiple HPV types were underestimated both when a commercially available dot blot kit system was used and when they used the PCR method. These investigators are currently developing a system that utilizes restriction endonuclease digestion of amplification products to detect multiple infections.

There is little question that PCR will play a significant role in long-term epidemiological studies of HPV. PCR offers the advantage of type detection and high sensitivity in assessing the incidence and prevalence of HPV infection. Further analysis via PCR is needed to determine the demographic and behavioral risk factors for HPV infection in subsets of high-risk patients,

such as tobacco and alcohol abusers, patients with immune deficits, and perhaps even patients with long-term chronic oral infections, including periodontal disease. Such prospective studies should allow us to more adequately understand the natural history of HPV infection, its role in mucous membrane disease, and potential clinical applications of HPV detection by PCR methods.

Experimental data strongly suggest that, besides the presence of HPV DNA in cervical epithelium, other events are required for full malignant conversion of cells infected by so-called oncogenic HPVs. Bosch and co-workers (1990) have suggested that a change in the state of the viral DNA may be one factor in progression toward malignancy. Lehn and associates (1988) have suggested that in premalignant lesions of the cervix, viral DNA is present as an episome, with a tendency toward integration in high-grade cervical interepithelial neoplasias. Several studies have provided evidence that HPV-16 and HPV-18 E6 and E7 genes can profoundly influence proliferation and differentiation capacities of rodent and human cells and tissue culture. It is completely unknown, however, which cellular regulatory mechanisms are responsible for either downregulation of HPV-18 E6 and E7 genes or growth inhibition of the nontumorigenic hybrid cells *in vivo*, and whether these mechanisms are partially or completely different or identical (Bosch et al., 1990). Future studies are needed to analyze whether there is a causal relationship between the downregulation of the HPV-18 E6 and E7 gene expression and *in vivo* growth inhibition of nontumorigenic cells. Identification of cellular genes that interfere with the expression of HPV transforming genes will be required. Bosch and others suggest that this will contribute to a better understanding of the cellular growth-regulatory networks affected in HPV-associated carcinogenesis (Bosch et al., 1990).

REFERENCES

- Amtmann, E., Randeria, J., Wayss, K. Interactions of papillomaviruses with carcinogens and tumor promoters. In: *Cancer Cells 5, Papillomaviruses*, B.M. Steinberg, J.L. Brandsma, and L.B. Taichman (Editors). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1987, pp. 259-265.
- Bartholoma, N.Y., Adelson, M.D., Forbes, B.A. Evaluation of two commercially available nucleic acid hybridization assays for detection and typing of human papillomavirus in clinical specimens. *American Journal of Clinical Pathology* 95: 21-29, 1991.
- Bauer, H.M., Ting, Y., Greer, C., et al. Genital human papillomavirus infection in female university students as determined by a PCR-based method. *Journal of the American Medical Association* 265: 472-477, 1991.
- Bosch, F.X., Schwarz, E., Boukamp, P., et al. Suppression *in vivo* of human papillomavirus type 18 E6-E7 gene expression in nontumorigenic hela fibroblast hybrid cells. *Journal of Virology* 64: 4743-4754, 1990.
- Bouck, N.P., Good, D.L., Polverini, P.J., et al. Suppressor control of an inhibitor of angiogenesis. In: *Recessive Oncogenes and Tumor Suppression*, W. Cavenee, N. Hastie, and E. Stanbridge (Editors). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989, pp. 179-185.
- Chow, L.T., Hirochika, H., Nasser, M., et al. Human papillomavirus gene expression. In: *Cancer Cells 5, Papillomaviruses*, B.M. Steinberg, J.L. Brandsma, and L.B. Taichman (Editors). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1987, pp. 55-72.
- Elovic, A., Galli, S.J., Weller, P.F., et al. Production of transforming growth factor-alpha by hamster eosinophils. *American Journal of Pathology* 137: 1425-1434, 1990.
- Greer, R.O., Douglas, J.M., Breese, L., et al. Evaluation of oral and laryngeal specimens for human papillomavirus (HPV) DNA by dot blot hybridization. *Journal of Oral Pathology and Medicine* 19: 35-38, 1990.

- Greer, R.O., Eversole, L.R., Crosby, L.K. Detection of papillomavirus-genomic DNA in oral epithelial dysplasias, oral smokeless tobacco-associated leukoplakias, and epithelial malignancies. *Journal of Oral Maxillofacial Surgery* 48: 1201-1205, 1990.
- Greer, R.O., Eversole, L.R., Poulson, T.C., et al. Identification of human papillomavirus DNA in smokeless tobacco-associated keratoses from juveniles, adults and older adults using immunocytochemical and in situ DNA hybridization techniques. *Gerodontics* 3: 201-208, 1987.
- Howley, P.M. The role of papillomaviruses in human cancer. In: *Important Advances in Oncology*, V.T. DeVita, Jr., S. Hellman, and J.A. Rosenberg (Editors). Philadelphia: Lippincott, 1987, pp. 55-73.
- Koutsky, L.A., Galloway, D.A., Holmes, K.K. Epidemiology of genital human papillomavirus infection. *Epidemiology Review* 10: 122-163, 1989.
- Lehn, H., Villa, F., Marziona, M., et al. Physical state and biologic activity of human papillomavirus genomes in precancerous lesions of the female genital tract. *Journal of General Virology* 69: 187-196, 1988.
- Manos, M.M., Ting, Y., Wright, D.K., et al. Use of polymerase chain reaction amplification for the detection of genital human papillomaviruses. In: *Cancer Cells 7, Molecular Diagnostics of Human Cancer*, M. Furth and M. Greaves (Editors). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1990, pp. 209-214.
- McCance, D.J. Human papilloma virus (HPV) infections in the aetiology of cervical cancer. *Cancer Survey* 7: 499-506, 1988.
- Nuovo, G., Hochman, H., Yehuda, D., et al. Detection of human papillomavirus DNA in penile lesions histologically negative for condyloma. *American Journal of Surgical Pathology* 14: 829-836, 1990.
- Resnick, R.M., Cornelissen, M.T., Wright, D.K., et al. Detection and typing of human papillomavirus in archival cervical cancer specimens by DNA amplification with consensus primers. *Journal of the National Cancer Institute* 82: 1477-1484, 1990.
- Sambrook, J., Fritsch, E.F., Maniatis, T. (Editors) *Molecular Cloning: A Laboratory Manual* (2nd edition). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1989, pp. 1051-1957.
- Shroyer, K.R., Greer, R.O. Detection of human papillomavirus DNA by in situ DNA hybridization and polymerase chain reaction in premalignant and malignant oral lesions. *Oral Surgery, Oral Medicine, Oral Pathology* 71: 708-713, 1991.
- Ting, Y., Manos, M.M. Detection and typing of genital human papillomaviruses. In: *PCR Protocols: A Guide to Methods and Applications*, M. Innis, D. Gelfand, J. Sninsky, and T. White (Editors). San Diego: Academic Press, 1990, pp. 356-367.
- Vousden, K.H. Human papillomaviruses and cervical carcinoma. *Cancer Cells*. 1: 43-50, 1989.
- Wright, D.K., Manos, M.M. Sample preparation for paraffin-embedded tissues. In: *PCR Protocols: A Guide to Methods and Applications*. M. Innis, D. Gelfand, J. Sninsky, and T. White (Editors). San Diego: Academic Press, 1990, pp. 153-158.
- zur Hansen, H., Schneider, A. The role of papillomaviruses in human anogenital cancer. In: *Papoviridae: The Papillomaviruses*, volume 2, N.P. Salzman and P.M. Howley (Editors). New York: Plenum Press, 1987, pp. 245-264.

Lipids as Factors in the Cell Response to Tobacco Components¹

George S. Schuster, Scott Lubas, Thomas R. Dirksen, and John F. Erbland

ABSTRACT Lipids are recognized as major components of or act in concert with components of the intracellular signaling systems that regulate cell growth and behavior. Extracellular modulators, by altering signaling-associated lipids, can affect cell responses. The current study examines the effects of *N*'-nitrososornicotine (NNN), a tobacco-associated nitrosamine, and a tumor promoter, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), on cell lipids and on protein kinase C (PKC) activity in oral epithelial cells. Cells were exposed to NNN or TPA for 30 min and homogenized, and then their PKC and non-PKC histone phosphotransferase activity was assessed. NNN significantly increased PKC activity in the cells, especially in the particulate fraction, whereas TPA produced a slight decrease in activity compared with dimethyl sulfoxide (DMSO) solvent-treated cells. Both modulators produced increased non-PKC phosphotransferase activity compared with control cells. Lipid synthesis by cells during and after exposure to the modulators was determined through use of [¹⁴C]acetate. Cells treated concurrently with modulator and [¹⁴C]acetate showed varied responses, depending on the lipid class and dose of modulator. Generally both modulators stimulated labeling of phospholipids, whereas NNN increased and TPA decreased diglyceride labeling. Fatty acid labeling was decreased by both modulators, whereas triglyceride labeling was enhanced by TPA. Posttreatment labeling showed that modulator-induced changes were transient. The results indicate that NNN can alter some components of the signaling pathway, notably PKC, and may affect de novo synthesis of diglycerides, which are putative endogenous promoters.

INTRODUCTION Lipids are recognized as important participants in the responses of cells to a variety of modulators, that is, substances that alter cell behavior. Lipids function as components of cell membranes to which the modulators bind, specifically or nonspecifically; they are components of specific receptors for tumor promoters, and they act as part of or in concert with components of the intracellular signaling systems that are activated after binding of agents to specific receptors. These signaling pathways regulate cell growth and behavior. For example, studies have demonstrated that tumor promoters such as 12-*O*-tetradecanoylphorbol-13-acetate specifically bind to receptors. In the case of TPA, these receptors are composed of Ca²⁺, phospholipid, and protein kinase C (PKC) (Konig et al., 1985). Receptor binding then results in activation of the intracellular signaling pathways.

Intracellular signaling involves the closely interconnecting activities of the inositol phosphate-Ca²⁺ and diacylglycerol (DAG)-PKC pathways, which have been implicated in the control of cell proliferation. In these pathways there is activation of certain proteins through phosphorylation of tyrosine and hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP₂) with formation of 1,2-diacylglycerol and inositol-1,4,5-triphosphate (IP₃). The diglyceride activates PKC, and IP₃ promotes increased cytosolic free calcium. These latter two activities initiate a cascade of reactions that bring about

¹ Funded by Smokeless Tobacco Research Council grant no. 0056 and National Institutes of Health grant nos. DE-07143 and RR-05795.

various cellular responses (Berridge, 1987). Thus any stimuli or cell reactions that affect components of these pathways can in turn alter cell behavior. In addition there are a variety of associated reactions, such as prostaglandin production, that may contribute to the signaling-induced responses (Parker, 1987).

Nitrosamines from smokeless tobacco are carcinogens that may act independently or in synergy with other agents to induce esophageal, nasal, and oral tumors (Hecht and Hoffmann, 1988). Indeed, synergy or promotion should be considered critical factors in the activities of some of these nitrosamines, especially some of the weaker ones (Hecht and Hoffmann, 1988).

Previous studies in our laboratories have shown that modulators of cell behavior such as retinoic acid (RA), TPA, and the nitrosamine *N'*-nitrosornicotine may affect cell lipids and other components of the cell signaling system, either directly or indirectly. Ringler and associates (1984) demonstrated that RA decreased formation of cholesterol, sphingomyelin, phosphatidylinositol, and phosphatidylserine and increased formation of triglycerides of normal and transformed hamster fibroblast cell types. In hamster oral epithelial cells, RA also affected lipid synthesis from acetate, decreasing synthesis of cholesterol and fatty acids but increasing that of triglycerides and phosphatidylcholine. In these same cells, NNN enhanced acetate incorporation into phosphatidylcholine and decreased its incorporation into cholesterol (Schuster et al., 1988). TPA also has been shown to alter cell lipids in a variety of cell types. For example, TPA altered incorporation and release of arachidonic acid, especially that associated with the phospholipids of U937 cells. The pattern of change was to a great extent dependent on the state of cell differentiation (Wiederhold, 1988).

Additional studies have demonstrated that pretreatment of cells with one modulator can alter the subsequent cell surface binding of another modulator. For example, RA decreased binding of phorbol esters to oral epithelial cells, whereas NNN enhanced binding significantly (Table 1). These changes occurred at times that corresponded to the altered lipid synthesis induced by RA (Schuster et al., 1986 and 1988). The opposite has been shown to occur as well, that is, pretreatment of oral epithelial cells with TPA enhanced binding of NNN to the cells by 89 percent. These studies also demonstrated that, although TPA bound to receptors on the cell, NNN binding did not appear to involve receptors.

The results of these various studies suggested a relation between cell lipid metabolism and the extent and nature of cell behavior in the presence of these modulators. They also indicated that nitrosamines and promoters can interact to alter the cell responses to each other and that these may be related to their effects on cell lipids. Thus, various cell responses and perhaps tobacco-related carcinogenesis may be dependent on one agent altering the response to others. The current studies describe the effects of NNN and TPA on some components of the signaling system that may be related to transformation-associated responses.

Table 1
Specific binding of PDB to oral epithelial cells after pretreatment with RA or NNN (percent control)^a

| | RA | NNN |
|-----------------------|-----------------|------------------|
| Time of Pretreatment, | | |
| 4 | 68 ^b | - |
| 24 | 94 | 100 |
| 48 | 100 | - |
| 72 | - | 218 ^b |
| 168 | - | 170 ^b |

Source: Schuster et al., 1988.

^a Specific binding of [³H]phorbol dibutyrate (PDB) to hamster buccal pouch cells (HCP) was assayed after pretreatment of the cells for various periods with 10 μM NNN, 10 μM retinoic acid (RA), or 0.1 percent dimethyl sulfoxide solvent (control). Percentage of control was calculated based on pmol [³H]PDB specifically bound/10⁶ viable cells. Results were based on three samples per time point in three to five experiments.

^b Different from control (based on pmols/10⁶ cells), *p* < 0.05.

METHODS The cells used were a line of cloned normal hamster buccal pouch (HCP) cells, the characteristics of which are described elsewhere (Schuster et al., 1990). The cells were maintained in Dulbecco's modification of minimal essential medium (DMEM) supplemented with 5 percent fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. The same lot of FBS (GIBCO) was used for all studies.

Protein Kinase C Assay HCP cell suspensions (2x10⁶ cells) were exposed to 10 μM NNN or 0.1 μM TPA in DMSO such that the final concentration of the solvent was 0.1 percent. Control cultures contained the same amount of DMSO. Cells were incubated 30 min at 37 °C in an atmosphere of 95 percent air-5 percent CO₂ and then harvested by centrifugation. PKC was assayed by the methods described by Chida and associates (1988) and Ways and associates (1987). Cells were suspended in buffer consisting of 20 mM Tris-HCL (pH 7.4), 2 mM EDTA, 0.5 mM EGTA, 0.25 M sucrose, and 50 mg/mL phenylsulfonyl-methyl chloride (PMSF). They were disrupted by 50 strokes in a glass homogenizer, then centrifuged at 100,000 *g* for 1 h at 4 °C. The supernatant was separated, and the pellet resuspended in the same buffer as described above except that it lacked sucrose but contained 0.1 percent Triton X-100.

The final assay mixture consisted of 20 mM Tris-HCL (pH 7.4), 5 mM MgSO₄, 200 μg/mL histone (type V-S, Sigma), 10 μM ATP (including 1 μCi γ-[³²P]ATP), and 200 μL of enzyme preparation. Activators consisted of 64 μM phosphatidylserine, diglyceride (1.3 μM 1,2-sn-diolein), and 1 mM CaCl₂. The samples were incubated for 3 min at 30 °C. The reaction was stopped by the addition of 1 mL of ice-cold 25 percent trichloroacetic acid (TCA). Samples were filtered through membrane filters (Millipore type HA) and then washed three times with the TCA. They were then counted in a

liquid scintillation counter. Protein content of aliquots was measured by the Bio-Rad assay. We determined specific activity for PKC by subtracting ^{32}P incorporation into histone in the absence of activators from that in the presence of activators. The basal histone phosphotransferase activity (non-PKC) is the ^{32}P incorporated into histone in the absence of activators. Activity was calculated on the amount of protein in duplicate aliquots of cell supernatant or pellet.

Lipid Assays HCP cells were plated in 100-mm dishes at 5×10^6 cells/dish. After overnight attachment, the medium was removed by aspiration, and the plates refed with medium containing TPA (0.1 μM , 0.2 μM , or 0.5 μM), NNN (10 μM , 20 μM , or 50 μM), or DMSO (0.1 percent). The medium also contained 10 $\mu\text{Ci/mL}$ [^{14}C]acetate. At the end of 30 min, the media were removed, and the cells were harvested. An identical set of plates was pretreated for 30 min with 0.1 μM TPA or 10 μM NNN or 0.1 percent DMSO alone, but no isotope. After 30 min, the plates were refed with medium containing 1 $\mu\text{Ci/mL}$ of isotope but no modulator. These were incubated for an additional 4 h and then harvested.

At the end of the respective labeling periods, the cells were washed three times with saline, dislodged with Teflon scrapers, and harvested by centrifugation. Cell pellets were washed twice with saline, and the lipids extracted by the method of Bligh and Dyer (1959). Lipids were separated on SG-81 paper by use of the solvent system described by Marinetti (1965) for neutral lipids and the solvents of Rouser and coworkers (1970) for polar lipids. Radioactive lipids were located by autoradiography, and the spots cut from the paper and counted in a liquid scintillation spectrometer. Aliquots of the cells were assayed for protein as described above, and the number of cells counted in a hemocytometer. Results were compared by a *t* test based on three to five experiments.

PKC RESULTS PKC activity was assayed after 30 min of exposure to the modulator, when maximum NNN uptake was previously shown (Schuster et al., 1990). The total PKC activity in cells exposed to NNN was about 1.5 times that of control cells, 826 ± 67 (SD) cpm/ μg protein for NNN-exposed cells vs. 555 ± 84 (SD) cpm/ μg protein for control (DMSO-treated) cells ($n=12$). This difference was significant ($p < 0.05$). PKC activity was present in both the cytosol and particulate fractions, and NNN produced an apparent change in the distribution of this activity (Table 2). The majority of activity was present in the particulate fraction, and PKC activity in this fraction was enhanced in response to NNN; TPA had a minimal effect on distribution at this point.

Figure 1 shows the specific activity of PKC in the cell fractions after 30 min of modulator exposure. PKC activity was significantly reduced in the cytosol after treatment with NNN compared with DMSO exposure, whereas it was significantly increased in the particulate fraction. The increase in overall as well as percent of activity in the particulate fraction suggests that the major response to NNN occurs here, not only as *de novo* synthesis but also as a shift from one site to the other. After 30 min of incubation, TPA produced a slight overall decrease in total activity that was not statistically significant.

Table 2
Enzyme activity in cell fractions after modulator exposure^a

| | Cell Fraction | |
|-----------|--|-------------------------|
| | Cytosol | Solubilized particulate |
| | <i>PKC activity^b</i> | |
| Modulator | | |
| DMSO | 24.8% | 75.2% |
| TPA | 16.8 | 83.2 |
| NNN | 2.3 | 97.7 |
| | <i>Non-PKC histone phosphotransferase activity^b</i> | |
| DMSO | 51.6 | 48.4 |
| TPA | 41.9 | 58.1 |
| NNN | 48.4 | 51.6 |

^a Percentage of protein kinase C (PKC) activity and non-PKC histone phosphotransferase activity was assayed in cytosolic and solubilized particulate fractions of hamster buccal pouch cells (HCP) following exposure of the cells to 10 μ M N'-nitrosornicotine (NNN), 0.1 μ M 12-O-tetradecanoylphorbol-13-acetate (TPA), or 0.1 percent dimethyl sulfoxide solvent for 30 min.

^b Mean of six experiments.

Non-PKC Phosphotransferase

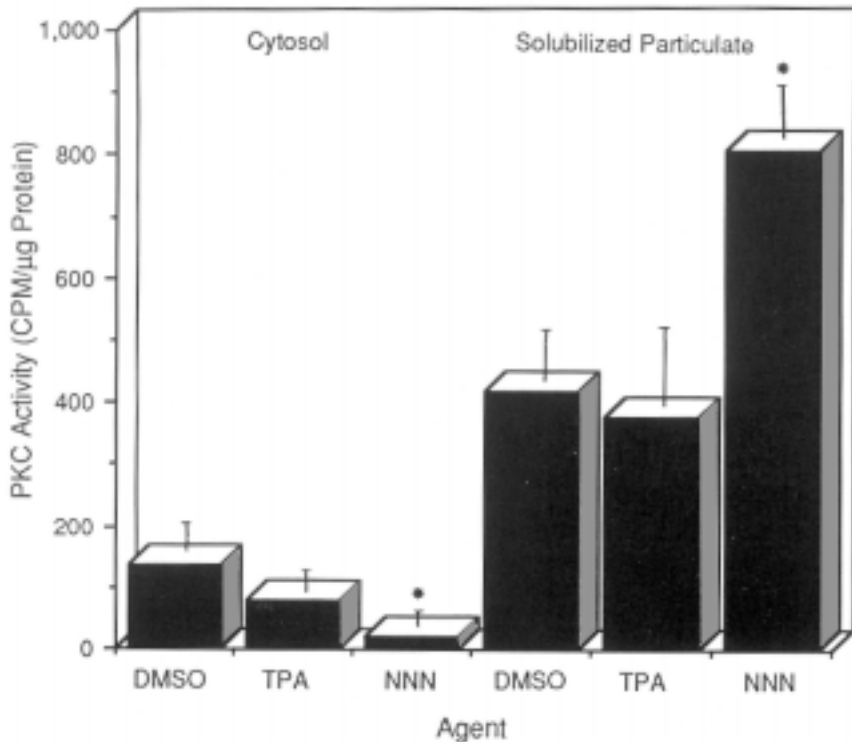
Both NNN and TPA did increase the levels of total non-PKC phosphotransferase activity somewhat. This was most notable in cells treated with NNN, where total activity was increased to 1.5 times that of DMSO-exposed cells (1,941 \pm 98 [SD] cpm/ μ g protein vs. 1,282 \pm 95 [SD] cpm/ μ g protein) (n=12). The increase was significant (p < 0.05). This increase in activity occurred in both cytosol and particulate fractions (Figure 2). The TPA-produced increase in enzymic activity was less; 1,425 \pm 139 (SD) cpm/ μ g protein for TPA-treated cells vs. 1,282 \pm 95 (SD) cpm/ μ g protein for DMSO-treated cells (n=12), but this change was evident in only the particulate fraction (Figure 2). Non-PKC phosphotransferase activity generally was about evenly distributed between the cytosol and particulate fractions. NNN did not appear to affect this distribution, whereas TPA caused a modest shift of activity from cytosol to particulate fraction (Table 2).

Diglyceride Formation

In addition to PKC, diglycerides are major components of cell signaling pathways and may serve as endogenous promoters. Diglycerides may be formed from phospholipids via the activities of phospholipases or synthesized de novo by cells. The current studies were directed at the latter source. Formation of diglycerides and other lipid classes in direct response to the modulators NNN and TPA was examined through labeling of the cells in the presence of the modulator or through pretreating of the cells, removing the modulator, and then labeling the cells. The latter study was done to permit assessment of the longevity of any responses. NNN increased cell lipid labeling by nearly 15 percent compared with solvent-treated cells, whereas TPA decreased labeling about 20 percent. This was not the result of differences in cell number, because direct cell counts and protein

Figure 1

The effects of 10 μM NNN, 0.1 μM TPA, or 0.1 percent DMSO on the specific activity of protein kinase C were measured in cytosolic and solubilized particulate fractions of hamster buccal pouch cells after 30 min exposure to the modulators. Values are the mean of six experiments ($\pm\text{SD}$).



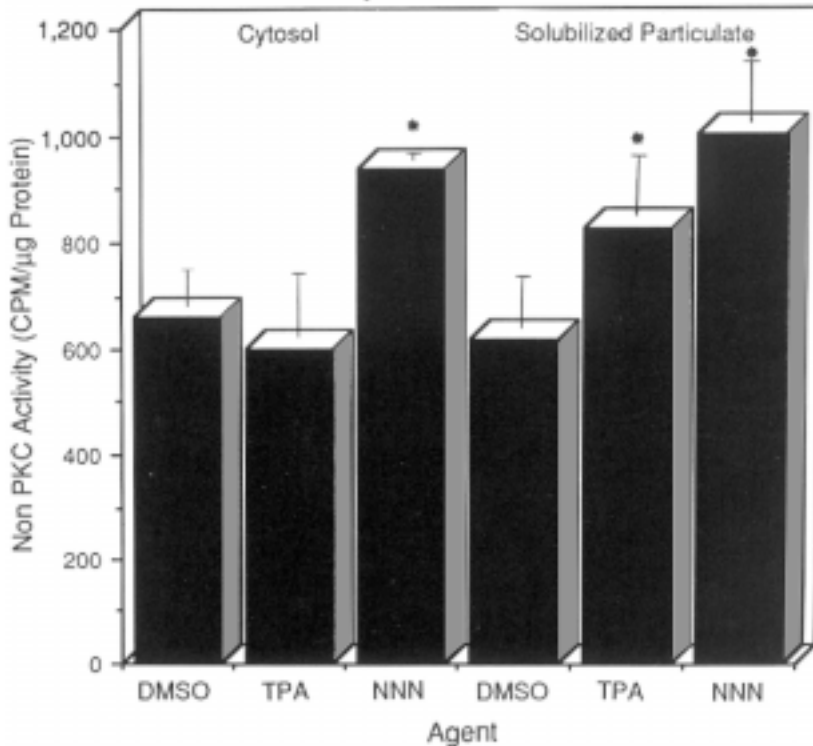
* $p < 0.05$ compared with DMSO-treated cells.

determination of aliquots from the samples revealed no significant differences in these parameters among the treatments.

Concurrent treatment and labeling of the cells with modulators and isotope for 30 min produced various responses at modulator concentrations equal to or above those previously shown (Schuster et al., 1986) to produce changes in cell lipids, the response depending on the dose of modulator and lipid class. Total cell lipid labeling and labeling of various classes by acetate differed somewhat between experiments; thus, when responses in various classes are taken as an aggregate of all experiments, some differences are not statistically significant. However, the labeling of lipid classes at a given concentration of modulator, compared with solvent-treated control cultures, was generally very consistent between experiments (Figures 3 and 4). Thus, phospholipid labeling was uniformly increased by both modulators. Diglyceride labeling was decreased by TPA (Figure 4) and stimulated at all except the highest dose of NNN (Figure 3). Cholesterol labeling was

Figure 2

The effects of 10 μM NNN, 0.1 μM TPA, or 0.1 percent DMSO on the specific activity of non-PKC histone phosphotransferase were determined in cytosolic and solubilized particulate fractions of HCP cells after 30 min exposure to the modulators. Values are the mean of six experiments ($\pm\text{SD}$).



* $p < 0.05$ compared with DMSO-treated cells.

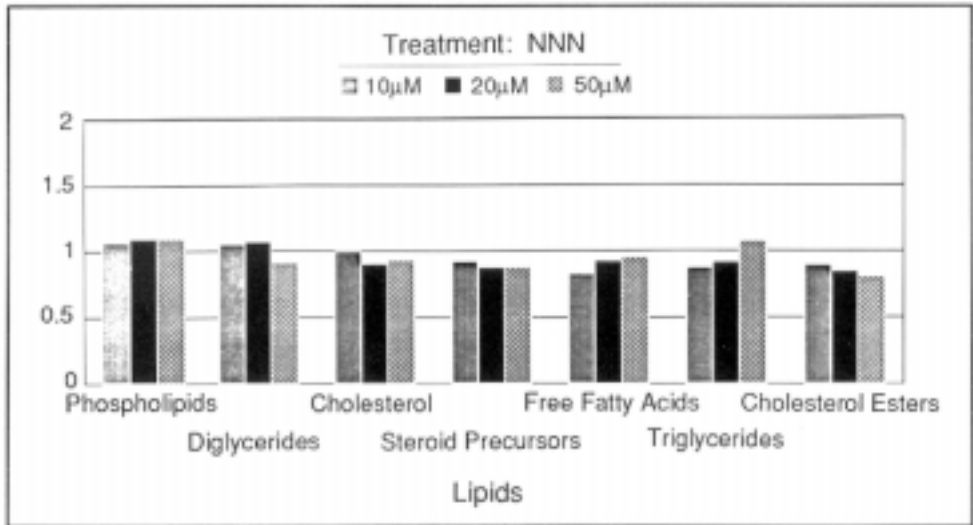
moderately but consistently decreased by both modulators, whereas triglyceride labeling was greatly increased and fatty acid labeling decreased by TPA. NNN decreased labeling of cholesterol esters.

Posttreatment labeling of cell lipids indicated that most of the alterations disappeared within 4 h after modulator removal, although labeling of phospholipids in TPA-treated cells remained slightly but significantly elevated as did that of sphingomyelin and glycolipids. At 4 h after NNN exposure, there was significantly elevated labeling only in steroid precursors.

DISCUSSION Some tobacco-derived substances may affect cell behavior only marginally in normal time or concentration exposures, and cofactors may therefore be critical for responses such as altered division or transformation to occur. Many of these cell responses are lipid associated, such as signaling pathways, or involve lipid-containing components, such as the cell membranes (Schuster et al., 1986, 1988, and 1990). Therefore, if lipid metabolism is altered it can affect the cell behavior.

Figure 3

Relative effects of various concentrations of NNN on [¹⁴C]acetate incorporation into lipids of HCP cells during 30 min exposure to the modulator plus 10 μ Ci/mL [¹⁴C]acetate. Treated cells are compared with cells exposed to 0.1 percent DMSO, which were given a value of 1.00.

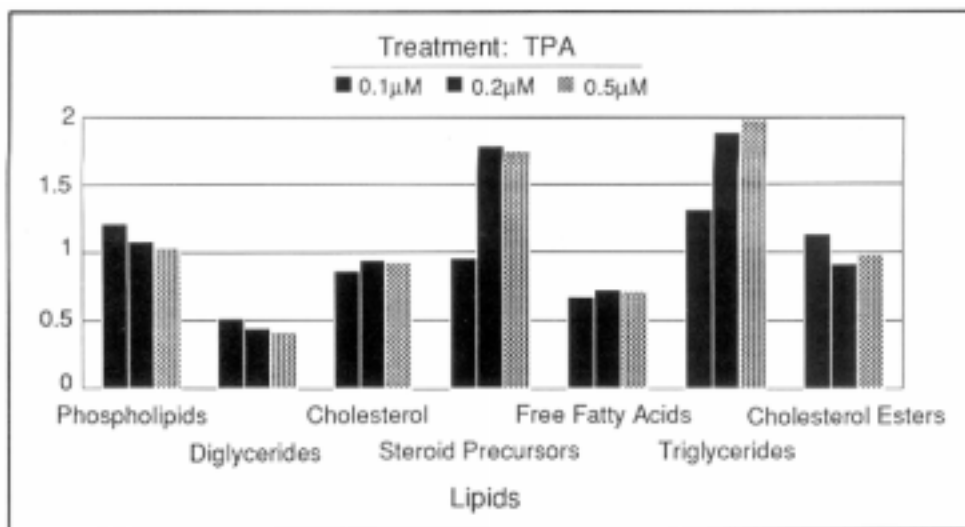


Although signaling pathway components such as PKC are usually activated by binding of agents to specific receptors, NNN binds nonspecifically (Schuster et al., 1990). However, it does significantly affect specific activity of both PKC and non-PKC histone phosphotransferase activity. PKC activity is increased in the particulate fraction, whereas activity in the cytosol decreases (Table 2 and Figure 1), suggesting that there may be a shift in activity to the particulate fraction as well as an increase in activity in this fraction. These results are similar to the shifts seen in MCF-7 cells in response to TPA and the diglyceride 1,2-dioctanoyl-sn-glycerol demonstrated by Issandou and associates (1988). The TPA-associated decrease in PKC activity in HCP cells was not significant, but a similar response in U937 cells, as shown by Ways and coworkers (1991), was more extensive, suggesting differences between cell types. The optimal time and dose of exposure to the NNN and TPA were based on our previous studies with HCP cells as well as studies by others (Issandou et al., 1988; Schuster et al., 1986; Ways et al., 1991). The present results demonstrate that changes in PKC activity can occur in oral epithelial cells in response to NNN, meaning that this nitrosamine and perhaps other modulators that do not bind to receptors are able to activate this component of the intracellular signaling system.

The significance of the NNN-induced increase in non-PKC phosphotransferase activity is not clear at this time, but Ways and coworkers (1987) suggest that such activity may mediate other effects produced by cell modulators.

Figure 4

Relative effects of various concentrations of TPA on [14 C]acetate incorporation into cellular lipids during 30 min exposure to the modulator plus 10 μ Ci/mL [14 C]acetate. Treated cells are compared with cells exposed to 0.1 percent DMSO, which were given a value of 1.00.



Cell signaling pathways also involve lipids as direct mediators in the responses to stimuli (Berridge, 1987). Diglycerides may serve as endogenous promoters (Rozenfurt, 1984). Although phospholipase activity likely is the major source for diglycerides involved in cell signaling, neosynthesized diglycerides are important in activation and downregulation of PKC and altered mitogenic signaling (Chiarugi, 1989a and 1989b; Peter-Riesch et al., 1988). Those studies showed increased diglyceride synthesis from glucose. In contrast, significantly increased synthesis from acetate was not evident in the HCP cells used in our studies, and indeed, TPA caused a significant decrease in diglycerides from this source. This may be a result of specific metabolic patterns of the HCP cell type; that is, the pathways used for neosynthesis of diglycerides from acetate are not affected by NNN and are downregulated by TPA. HCP cells apparently do not synthesize significant quantities of lipids from radiolabeled glucose, even in media totally lacking nonlabeled glucose (unpublished observations). Therefore, other pathways for lipid production in these cells will have to be examined for their response to the various modulators, because others also have shown cell-specific differences (Chida et al., 1988; Harris et al., 1982; Hecht and Hoffmann, 1988.) The changes in diglyceride formation appear to be transient because prior studies with longer periods of TPA exposure and labeling (Schuster et al., 1990) as well as the current posttreatment labeling showed no differences from control cells.

Altered labeling of lipid classes other than the diglycerides during the short term may relate to cells adjusting to the binding or embedment of the modulators in the cell membrane. Frezard and coworkers (1989) found that,

when TPA binds to cells, it becomes embedded in the cytoplasmic membranes. NNN does not bind to receptors but likely will at least transiently affect membrane organization. Therefore, the changes seen in phospholipids and fatty acids may reflect the cells' adjustment to the presence of the modulators, a result consistent with previous studies showing TPA-associated increases in 18:2, 18:3, 20:3, and 20:4 fatty acids in these cells (Schuster et al., 1990).

Although incorporation of acetate into cell lipids varied between experiments, the patterns of response to modulators are consistent. The variation may be the result of the nature of the HCP cells in culture. The cells were plated so as to be loose monolayers when used. However, even when thoroughly dispersed for plating, these epithelial cells would tend to stick together and settle to the surface of the dish in patches, resulting in areas where the cells may be locally dense. In such a situation the cells in the center of the clusters may be less active in their growth and synthetic activities, including their responses to the modulators. In general, metabolic or growth rate-associated responses are likely to be affected by culture density, resulting in interexperiment variability. Cell regulatory characteristics that have been shown to be density dependent include cyclic nucleotide levels, availability of various receptors, and lipid metabolic pathways (D'Armiento et al., 1973; Holley et al., 1978; Jetten et al., 1989; Ponc et al., 1987).

The previous and current studies taken together suggest that NNN and TPA can directly or indirectly affect the cell signaling pathways, including some of the lipid components. They also suggest that the combined effects of modulators may be a significant factor in the response of oral epithelial cells to initiators and promoters. Finally, the results suggest that these cells may differ from other cell types in some ways, necessitating the use of oral epithelial cells to fully define responses to tobacco exposure.

REFERENCES

- Berridge, M.J. Inositol lipids and cell proliferation. *Biochimica et Biophysica Acta* 907: 33-45, 1987.
- Bligh, E.G., Dyer, W.J. A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology* 37: 911-917, 1959.
- Chiarugi, V., Bruni, P., Pasquali, F., et al. Synthesis of diacylglycerol de novo is responsible for permanent activation and down-regulation of protein kinase C in transformed cells. *Biochemical and Biophysical Research Communications* 164: 816-823, 1989b.
- Chiarugi, V.P., Magnelli, L., Pasquali, F., et al. Signal transduction in EF-J-*ras*-transformed cells: de novo synthesis of diacylglycerol and subversion of agonist-stimulated inositol lipid metabolism. *FEBS Letters* 252: 129-134, 1989.
- Chida, K., Kato, N., Yamada, S., et al. Protein kinase C activities and bindings of a phorbol ester in 41 cell lines. *Biochemical and Biophysical Research Communications* 157: 1-8, 1988.
- D'Armiento, M., Johnson, G.S., Paston, I. Cyclic AMP and growth of fibroblasts: Effect of environmental pH. *Nature New Biology* 242: 78-80, 1973.
- Frezard, F., Garnier-Suillerot, A., Ballard, J., et al. Membrane-phorbol ester interaction monitored by circular dichroism. *Biochimica et Biophysica Acta* 979: 316-320, 1989.
- Harris, C.C., Trump, B.F., Grafstrom, R., et al. Differences in metabolism of chemical carcinogens in cultured human epithelial cells. *Journal of Cellular Biochemistry* 18: 285-294, 1982.
- Hecht, S.S., Hoffmann, D. Tobacco-specific nitrosamines: An important group of carcinogens in tobacco and tobacco smoke. *Carcinogenesis* 9: 875-884, 1988.
- Holley, R.W., Armour, R., Baldwin, J.H. Density-dependent regulation of growth of BSC-1 cells in cell culture: Growth inhibitors formed by the cells. *Proceedings of the National Academy of Sciences* 75: 1864-1866, 1978.

- Issandou, M., Bayard, F., Darbon, J.M. Inhibition of MCF-7 cell growth by 12-*O*-tetradecanoylphorbol-13-acetate and 1,2-dioctanoyl-sn-glycerol: Distinct effects on protein kinase C activity. *Cancer Research* 48: 6943-6950, 1988.
- Jetten, A.M., George, M.A., Nervi, C., et al. Increased cholesterol sulfate and cholesterol sulfotransferase activity in relation to the multi-step process of differentiation in human epidermal keratinocytes. *Journal of Investigative Dermatology* 92: 203-209, 1989.
- Konig, B., DiNitto, P.A., Blumberg, P.M. Phospholipid and Ca²⁺ dependency of phorbol ester receptors. *Journal of Cellular Biochemistry* 27: 255-265, 1985.
- Marinetti, G.V. Chromatography of lipids on commercial silica gel loaded filter paper. *Journal of Lipid Research* 6: 315-317, 1965.
- Parker, J., Daniel, L.W., Waite, M. Evidence of protein kinase C involvement in phorbol diester-stimulated arachidonic acid release and prostaglandin synthesis. *Journal of Biological Chemistry* 286: 5385-5393, 1987.
- Peter-Riesch, B., Fathi, M., Schlegel, W., et al. Glucose and carbachol generate 1,2-diacyl-glycerol by different mechanisms in pancreatic islets. *Journal of Clinical Investigations* 81: 1154-1161, 1988.
- Ponec, M., Kempenaar, J., Boonstra, J. Regulation of lipid synthesis in relation to keratinocyte differentiation capacity. *Biochimica et Biophysica Acta* 921: 512-521, 1987.
- Ringler, M.B., Erbland, J.F., Singh, B.B., et al. Effects of retinoic acid on ¹⁴C-acetate incorporation into lipids of normal and transformed hamster fibroblasts. *Experimental Cell Research* 154: 171-180, 1984.
- Rouser, G., Fleischer, S., Yamamoto, A. Two-dimensional thin layer chromatographic separation of polar lipids and determination of phospholipids by phosphorus analysis of spots. *Lipids* 5: 494-496, 1970.
- Rozengurt, E., Rodriguez-Pena, A., Coombs, M., et al. Diacylglycerol stimulates DNA synthesis and cell division in mouse 3T3 cells: Role of Ca²⁺-sensitive phospholipid-dependent protein kinase. *Proceedings of the National Academy of Sciences* 81: 5748-5752, 1984.
- Schuster, G.S., Erbland, J.F., Wyrick, S.D., et al. Oral epithelial cell lipid synthesis in the presence of retinoic acid or nitrosonornicotine. *Journal of Oral Pathology* 15: 430-433, 1986.
- Schuster, G.S., Erbland, J.F., Wyrick, S.D., et al. Phorbol ester binding to oral epithelial cells in the presence of retinoic acid on *N'*-nitrosonornicotine. *Cytobios* 54: 53-60, 1988.
- Schuster, G.S., Lubas, S., Erbland, J.F., et al. Binding and uptake of *N'*-nitrosonornicotine by oral epithelial cells. *Journal of Oral Pathology and Medicine* 19: 114-118, 1990.
- Ways, K.D., Dodd, R.C., Earp, H.S. Dissimilar effects of phorbol ester and diacylglycerol derivatives on protein kinase activity in the monoblastoid U937 cell. *Cancer Research* 47: 3344-3350, 1987.
- Ways, K., Riddle, R., Ways, M., et al. Effect of phorbol esters on cytosolic protein kinase C: Content and activity in the human monoblastoid U937 cell. *Journal of Biological Chemistry* 266: 1258-1264, 1991.
- Wiederhold, M.D., Anderson, K.M., Harris, J.E. Labeling of lipids and phospholipids with ³H-arachidonic acid and the biosynthesis of eicosanoids in U937 cells differentiated by phorbol ester. *Biochimica et Biophysica Acta* 959: 296-304, 1988.

Role of Viruses in Oral Carcinogenesis¹

No-Hee Park, Byung-Moo Min, Sheng-Lin Li,
Henry M. Cherrick, and Jay Doniger

ABSTRACT The role of herpes simplex virus and human papillomavirus in oral carcinogenesis was studied. Herpes simplex virus showed no carcinogenicity *in vivo*; repeated viral inoculation of hamster buccal pouch mucosa failed to produce tumors or histopathologic evidence of malignancy in pouches. However, herpes simplex virus demonstrated *in vivo* cocarcinogenicity; viral inoculation significantly enhanced the oncogenic capacity of benzo[*a*]pyrene, a tobacco-chemical carcinogen, in the oral cavity of hamsters. Human papillomavirus types 16 and 18 demonstrated oncogenicity by transforming normal human oral keratinocytes. While normal cells exhibited a limited lifespan, cells transformed by these viruses showed immortality and altered morphology in comparison with their normal counterparts. The transformed HOK-16A and B and HGK-18 cells contained intact type 16 or 18 human papillomaviral DNA integrated into cellular chromosomes, respectively. Further, these cells expressed several viral-specific poly(A⁺)RNAs including viral E6/E7 polyadenylated RNAs. Notably, these cells overexpressed cellular *myc* proto-oncogene compared to their normal counterparts; however, the immortalized cell lines were not able to produce tumors in nude mice, indicating that the cells are only partially transformed.

INTRODUCTION Clearly cigarette, cigar, and pipe smoking are causally associated with oral cancer (US DHHS, 1982) as is snuff dipping (US DHHS, 1986). The constituents of smoked tobacco tar and smokeless tobacco responsible for oral cancer are the tobacco-specific *N'*-nitrosamines (TSNA) and benzo[*a*]pyrene (B[*a*]P). TSNA are formed from nicotine and minor tobacco alkaloids during aging, curing, and fermentation of tobacco (Hecht et al., 1977). Among TSNA, high levels of *N'*-nitrosornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) are found in tobacco (Hoffmann et al., 1984). Some studies, however, indicate a lack of linkage between malignant changes and ST use by humans (Offenbacher and Weathers, 1985). Moreover, several laboratory studies report failure to develop oral malignancy with repeated intraoral placement of smokeless tobacco in animals (Park et al., 1985; Shklar et al., 1985). Possible involvement of other factors such as alcohol, caffeine, and viruses has therefore been postulated to be associated with development of tobacco-related oral malignancies in humans. Among these factors, the role of viruses, especially herpes simplex virus (HSV) and human papillomavirus (HPV), in oral carcinogenesis has been studied in our laboratory.

Oral cancers appear to be associated with an increased immune response to HSV-1 (Shillitoe et al., 1982). The expression of HSV-1 genes has been detected in oral cancer tissues (Eglin et al., 1983). HSV infection is extremely prevalent, with up to 90 percent of individuals having antibodies to HSV by age 10 (Overall, 1979). More than one-third of the world's population suffers from recurrent intraoral or orofacial herpetic infections (National Institutes of Health, 1973). Individuals with latent HSV infection in the sensory or autonomic ganglia actively shed infectious virions onto

¹ Supported in part by Smokeless Tobacco Research Council grants no. 0052 and no. 0231.

oral mucosae, yet are without clinical symptoms, providing an opportunity for HSV to interact with water-soluble components of smokeless tobacco in the oral cavity. Tobacco and HSV are synergistic in developing precancerous lesions in mice (Park et al., 1985). Furthermore, repeated HSV infection in combination with simulated snuff dipping leads to oral cancer in animals (Hirsch et al., 1983; Park et al., 1985). Also, HSV-1 infection significantly increases the carcinogenic activity of 7,12-dimethylbenz[*a*]anthracene (DMBA) in hamster buccal pouch mucosa by, in part, accelerating DMBA-induced activation of *c-erb-B-1* proto-oncogene in the pouch epithelium (Oh et al., 1989).

Human papillomavirus (HPV) is also linked to certain human malignancies. This association is based on the finding that up to 90 percent of cancer tissues from genital lesions contain viral DNA (Durst et al., 1983). Of the more than 60 genotypes of HPV, types 16 (HPV-16) and 18 (HPV-18), as well as recently isolated types 31 (HPV-31) and 33 (HPV-33), are most frequently associated with cervical cancer (Schwarz et al., 1985). In a high percentage of cervical carcinomas and in cell lines derived from these cancers, HPV-16 and -18 DNAs are integrated into cellular chromosomes, whereas the viral DNAs are generally retained as extrachromosomal episomes in premalignant dysplastic lesions (McCance, 1986). As in cervical cancers, HPV is also positively correlated with human oral malignancies, with up to 60 percent of cancer tissues from oral biopsies containing viral DNA (Dekemejian et al., 1987). Since the epithelia of oral and female genital mucosae are histologically similar, and both are continuously challenged by many environmental factors, close association of HPV with the development of oral malignancies is not surprising. Although human oral keratinocytes are undeniably major target cells for HPV infection and HPV-induced tumorigenesis, the *in vitro* transforming activity of HPV in human oral keratinocytes has never been studied because of the unavailability of a suitable culture system.

In the present study, we demonstrate the *in vivo* cocarcinogenic effect of HSV in the oral cavity of hamsters, and the carcinogenicity of HPV for one of its target cells, the oral keratinocytes.

METHODS

Viruses, Plasmids, And Primary Culture of Oral Keratinocytes

HSV-1 (F-strain; American Type Culture Collection, Rockville, Maryland) was propagated in Vero cell monolayers with viral titers being adjusted to 10^8 plaque-forming units (PFU) per milliliter. pMHPV-16d (a head-to-tail dimer of HPV-16 DNA inserted into the *Bam*HI cloning site of the plasmid pdMMT_{neo}) and pSHPV-18m (recombinant DNA containing single copy of HPV-18 DNA inserted into *Eco*RI cloning site of plasmid PSV2_{neo}) were constructed as described elsewhere (Woodworth et al., 1989; Park et al., 1991). The primary normal human oral keratinocytes (NHOK) and normal human gingival keratinocytes (NHGK) were established from the excised tissues of hard palate and retromolar areas of a healthy male volunteer as described elsewhere (Park et al., 1991).

Animals, HSV-1 Inoculation, and Application of Carcinogens To investigate the *in vivo* carcinogenicity of NNN, NNK, and B[a]P, alone or in combination with HSV-1 inoculation, we inoculated the right buccal pouch mucosa with HSV-1 or culture medium (mock inoculation), as described elsewhere (Park et al., 1988), and applied either mineral oil or chemical carcinogens topically as follows: group 1 (control), mock inoculation plus topical application of mineral oil; group 2, HSV-1 inoculation plus topical application of mineral oil; group 3, mock inoculation plus topical application of 1 percent NNK; group 4, HSV-1 inoculation plus topical application of 1 percent NNK; group 5, mock inoculation plus topical application of 1 percent NNN; group 6, HSV-1 inoculation plus topical application of 1 percent NNN; group 7, mock inoculation plus topical application of 1 percent B[a]P; group 8, HSV-1 inoculation plus topical application of 1 percent B[a]P. Approximately 100 μ L of mineral oil, NNK, NNN, or B[a]P solution were applied as described in Table 1. One hundred sixty hamsters were divided into eight equal groups, and right pouch mucosae were inoculated with HSV-1 (1×10^8 PFU per pouch) or culture medium (mock inoculation). Twenty-four hours after the inoculation, mineral oil, NNK, NNN, or B[a]P was topically applied to the inoculated pouch mucosa, three times per week for 15 (for B[a]P) or 20 (for mineral oil, NNK, and NNN) consecutive weeks. Left pouches were used as internal controls. Since our preliminary study showed that mild premalignant microscopic changes were developed by 15 wk and 20 wk of treatment of topical B[a]P and TSNA (NNN and NNK), respectively, in hamster buccal pouches, the chemical carcinogens were applied for 15 or 20 wk to demonstrate the cocarcinogenicity of HSV-1. The pouches were examined once a week for the appearance of tumors, and the animals were sacrificed at 30 wk after the initiation of topical application of mineral oil or chemical carcinogens. The above data were obtained just before the animals were sacrificed. Of animals receiving both HSV-1 inoculation and topical B[a]P, two hamsters died of HSV-1 encephalitis at 2 wk after inoculation. No changes were observed from the left pouch mucosae used as internal controls. At the end of the experimental period, the animals were sacrificed and the buccal pouches were fixed for light microscopic examination. Although NNN and NNK are hydrophilic compounds, they were dissolved in mineral oil to enhance the penetration of the compounds into tissue.

Transformation Of Primary NHOK And NHGK With HPV-16 and HPV-18 Primary NHOK and NHGK were transfected with pMHPV-16d (or pdMMT_{neo}) and pSHPV-18m (or pSV2_{neo}) using Lipofectin reagent (BRL Life Technologies, Gaithersburg, Maryland). Two G418-resistant cell colonies transfected with pMHPV-16d were isolated, subcultured, and named HOK-16A and HOK-16B lines. One cell colony transfected with pSHPV-18m was isolated and named HGK-18 (Park et al., 1991).

Analysis of Cellular DNA and RNA From HPV-Transformed Oral Keratinocytes High molecular weight cellular DNA from NHOK, NHGK, HOK-16A, HOK-16B, and HGK-18 lines was extracted. To determine the presence of viral DNA and, if present, its copy number per cell, cellular DNA were restricted and electrophoresed in 1 percent agarose, and Southern blot hybridization carried out under stringent condition using ³²P-labeled 7.9-kbp total HPV-16 DNA or total HPV-18

Table 1

Effect of tobacco-related chemical carcinogens and HSV-1, alone or in combination, on the development of oral cancer in hamster buccal pouches

| | Effect on Right Buccal Pouches | | | | | |
|---|--------------------------------|-------------------------------|---------------------------|------------------------------------|---|--|
| | Number of Pouches per Group | Number of Pouches With Tumors | Number of Discrete Tumors | Average Number of Tumors per Pouch | Average Tumor Size (mm diameter) ^a | Average Cumulative Tumor Diameter per Pouch(mm) ^a |
| Experimental Group ^b | | | | | | |
| 1. Control (mock inoculation + TA of mineral oil) | 20 | 0 | 0 | 0 | NA | NA |
| 2. HSV-1 inoculation + TA of mineral oil | 20 | 0 | 0 | 0 | NA | NA |
| 3. Mock inoculation + TA of 1% NNK | 20 | 0 | 0 | 0 | NA | NA |
| 4. HSV-1 inoculation + TA of 1% NNK | 20 | 0 | 0 | 0 | NA | NA |
| 5. Mock inoculation + TA of 1% NNN | 20 | 0 | 0 | 0 | NA | NA |
| 6. HSV-1 inoculation + TA of 1% NNN | 20 | 0 | 0 | 0 | NA | NA |
| 7. Mock inoculation + TA of 1% B[a]P | 20 | 4 | 6 | 1.5 | 0.5 | 0.75 ± 0.40 |
| 8. HSV-1 inoculation + TA of 1% B[a]P | 18 | 10 ^c | 30 ^c | 3.0 ^c | 0.8 ^d | 2.4 ± 0.5 ^d |

^a NA: not applicable.

^b TA: topical application.

^c Significantly different ($p < 0.05$) from group 7 (Fisher's exact test, double tailed).

^d Significantly different ($p < 0.05$) from group 7 (Student's *t* test, double pair).

DNA probes. After hybridization, the filter was washed and exposed to X-ray film. The physical state of viral DNA in the HOK-16A, HOK-16B, and HGK-18 lines was determined by Southern blot hybridization analysis as indicated in the figure legends. Northern blot hybridization was performed to determine expression of HPV DNA, *c-myc* proto-oncogene, and β -actin gene in the transformed cells (Park et al., 1991).

In Vivo Tumorigenicity of HPV-Transformed Oral Keratinocytes NHOK, NHGK, HOK-16A, HOK-16B, and HGK-18 monolayer cultures were trypsinized, resuspended in PBS, and subcutaneously injected into 25 athymic nude mice (*nu/nu*; 1×10^7 cells/0.1 mL per animal; five animals per cell type) 1 d after mice had been X-irradiated (300R). All mice were injected on the right flank and monitored twice weekly for the appearance of tumors over a period of more than 3 mo.

RESULTS

Cocarcinogenicity Of HSV-1 in B[a]P-Induced Oral Cancer

As we reported previously (Park et al., 1988), repeated HSV-1 inoculation induces neither tumors nor histopathologic changes in hamster buccal pouch mucosa. The present data also show that tumors or malignant histopathologic changes do not develop in the buccal pouch mucosa of animals receiving mineral oil, NNN, or NNK, alone or in combination with repeated HSV-1 inoculation. The body weights of animals were not altered by HSV-1 inoculation and/or topical application of chemical carcinogens. However, 20 percent of hamsters receiving topical B[a]P along with mock inoculation developed tumors in the right pouches, while 56 percent of animals treated with topical B[a]P and HSV-1 inoculation developed tumors in the right pouch mucosa (Table 1). HSV-1 inoculation also significantly hastened the appearance of B[a]P-induced tumor formation. The tumors appeared 16 wk after the initiation of topical B[a]P in animals receiving B[a]P plus mock inoculation, but the tumors occurred 11 to 12 wk after initiation of B[a]P treatment in animals treated with both topical B[a]P and HSV-1 inoculation. The average size of tumors in animals receiving both HSV-1 and B[a]P was significantly greater than in those receiving B[a]P treatment with mock inoculation (Table 1).

Microscopic findings also show that HSV-1 inoculation induced more malignant histopathologic changes in pouch mucosa receiving B[a]P. A significantly higher number of pouches showed epithelial atypia and cancer invasion in the group receiving both HSV-1 inoculation and topical B[a]P, than in the group receiving mock inoculation and topical B[a]P (Figure 27-1 and Table 2). These data indicate that NNN and NNK are not carcinogenic in hamster buccal pouches and that HSV-1 does not alter the noncarcinogenicity of these compounds in the pouch epithelium. B[a]P, alone, demonstrated weak carcinogenicity in buccal pouches, an effect that was significantly increased by HSV-1. These results also confirm that HSV-1 alone is not carcinogenic, but can selectively enhance the oncogenicity of certain chemical carcinogens in the oral cavity of hamsters.

Table 2

Effect of tobacco-related chemical carcinogens and HSV-1, alone or in combination, on the histopathologic changes of hamster buccal pouch mucosa

| Experimental Group ^b | Histopathologic Changes of Right Pouch Mucosa ^a | | | | | |
|---|--|---------------------------------|------------|---|--------------------|----------------------------------|
| | Hyper-keratosis | Granular Cell Layer Hyperplasia | Acanthosis | Inflammatory Infiltrate in Lamina Propria | Epithelial Atypia | Invasive Squamous Cell Carcinoma |
| 1. Control (mock inoculation + TA of mineral oil) | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 |
| 2. HSV-1 inoculation + TA of mineral oil | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 | 0/20 |
| 3. Mock inoculation + TA of 1% NNK | 10/20 | 5/20 | 5/20 | 0/20 | 0/20 | 0/20 |
| 4. HSV-1 inoculation + TA of 1% NNK | 9/20 | 6/20 | 6/20 | 0/20 | 0/20 | 0/20 |
| 5. Mock inoculation + TA of 1% NNN | 11/20 | 5/20 | 7/20 | 0/20 | 0/20 | 0/20 |
| 6. HSV-1 inoculation + TA of 1% NNN | 10/20 | 7/20 | 6/20 | 0/20 | 0/20 | 0/20 |
| 7. Mock inoculation + TA of 1% B[a]P | 11/20 | 9/20 | 9/20 | 7/20 | 4/20 | 1/20 |
| 8. HSV-1 inoculation + TA of 1% B[a]P | 12/18 | 10/18 | 12/18 | 15/18 ^c | 10/18 ^c | 4/18 ^c |

^a The excised pouch tissues were fixed in 10% neutral formalin, sectioned in paraffin, and stained with hematoxylin and eosin for light microscopic findings. Numerator is the number of pouches with the described histopathologic changes; denominator is the number of pouches examined.

^b TA: topical application.

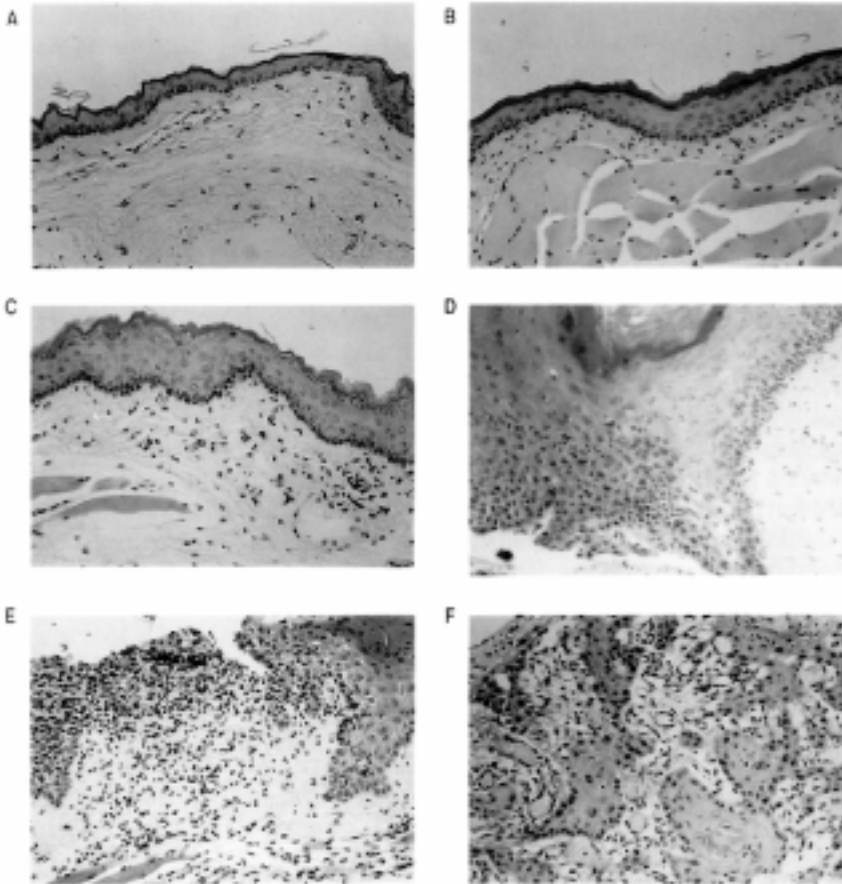
^c Significantly different ($p < 0.05$) from group 7 (Fisher's exact test, double tailed).

Proliferation Pattern and Morphology of Oral Keratinocytes

NHOK, NHGK, and G418-resistant cell colonies transfected with the vector plasmids (pdMMT_{neo} or pSV2_{neo}) were similar in their morphology and could not be subcultured beyond the fifth to sixth passage. The G418-resistant cell colonies transfected with recombinant pMHPV-16d or pSHPV-18m plasmids, however, appear to be immortal, these cells have now been maintained through 40 passages over

Figure 1

Microphotographs representing the induction of hyperkeratosis, hyperplasia, acanthosis, carcinoma in situ, and invasive cancer from the hamster buccal pouches. A., control; B., hyperplasia and hyperkeratosis; C. and D., acanthosis and hyperkeratosis; E., carcinoma in situ; F., invasive cancer (original magnification x100).



8 mo. The cells display keratinocyte morphology and are characterized by a lack of stratification. These cells continue to proliferate and to retain an undifferentiated morphology. The transformed cell lines proliferate faster than NHOK or NHGK, have a cobblestone-like morphology, and establish a higher density at confluence in comparison with the normal counterpart.

Viral DNA in HPV-Transformed Oral Keratinocytes DNA from NHOK and NHGK did not hybridize to HPV-16 DNA and HPV-18 DNA, respectively, indicating that NHOK and NHGK did not contain HPV-16 and HPV-18 DNA, respectively. DNA from HOK-16A, HOK-16B, and HGK-18 cell lines hybridized to the viral probes, suggesting the presence of viral DNA in the immortalized cell lines. Densitometric analysis showed that HOK-16A and HOK-16B cell lines

contain approximately 40 and 25 copies of HPV-16 DNA per cell, respectively, while the HGK-18 cell line harbors about 10 copies of HPV-18 DNA per cell (Figures 2 and 3).

After digestion of HOK-16A and HOK-16B cellular DNA with *EcoRV*, an enzyme that does not cut the pMHPV-16d plasmid, Southern blot analysis showed a single HPV-16-specific band, larger than 30 kbp, suggesting that HPV DNA exists as an integrated form, not an episomal form, in the HOK-16A and -16B cell lines. After *BamHI* digestion, which releases the HPV-16 DNA sequences from vector DNA, Southern analysis showed that HOK-16A and -16B cell lines contained 7.9-kbp HPV-16 DNA genome, indicating an integration of intact HPV-16 DNA into host chromosomes in these cell lines. In addition to the expected 7.9-kbp complete HPV-16 DNA genome, the cell lines contained rearranged HPV-16 DNA sequences; the hybridization of HOK-16A and -16B DNA digested with *BamHI* showed multiple HPV-16-specific bands that were bigger or smaller than 7.9-kbp (Figure 27-2). Similar to HOK-16A and -16B lines, HGK-18 DNA also contained intact and integrated HPV-18 DNA; after *EcoRI* digestion, which releases HPV-18 DNA from vector, Southern analysis showed 7.9-kbp band hybridized to ³²P-HPV-18 DNA. After digestion with *Sall*, an enzyme that does not cut the pSHPV-18m plasmid, a single HPV-18-specific band larger than 30 kbp was seen, suggesting that HPV-18 DNA exists as an integrated form in the HGK-18 cell line (Figure 3).

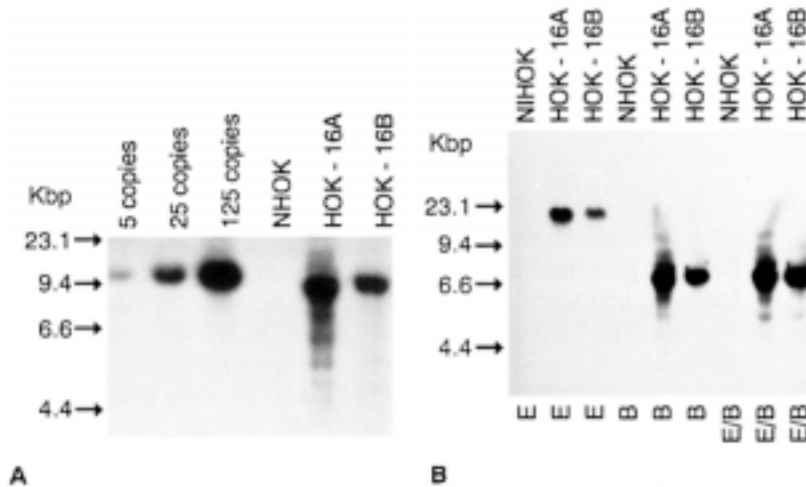
To further assess the integration of rearranged viral sequences into cellular genomic DNA, high molecular weight cellular DNA from the transformed, immortalized cell lines was double digested with two enzymes: *BamHI* and *EcoRV* (for HOK-16A and HOK-16B DNA) or *EcoRI* and *Sall* (for HGK-18 DNA). This treatment can generate smaller, viral-specific, rearranged DNA fragment(s) if *EcoRV* or *Sall* digestion sites exist in cellular DNA covalently linked to viral DNA. The double restriction profiles of HOK-16A and HGK-18 DNA were similar to *BamHI* and *EcoRI* restriction profiles of the DNA, respectively, indicating no rearranged fragments with junctions between cellular and viral DNA (Figures 2 and 3). This does not rule out integration, because *EcoRV* or *Sall* restriction sites do not necessarily exist in the rearranged HPV-16-specific *BamHI* fragments or HPV-18-specific *EcoRI* fragments in HOK-16A DNA and HGK-18 DNA, respectively. However, the double digestion of HOK-16B DNA generated smaller rearranged HPV-16-specific DNA fragments than were observed after the single *BamHI* digestion (Figure 2 and 3), providing conclusive evidence for integration into this line.

Northern blot hybridization using ³²P-HPV-16 DNA probe revealed that multiple HPV-16 poly(A⁺)RNAs were highly expressed from the HOK-16A and HOK-16B cell lines, whereas HPV-16 poly(A⁺)RNAs were not expressed from NHOK (Figure 4). The intense 1.6-1.8-kbp band is characteristic of the major HPV-16 E6/E7 message observed in HPV-16 immortalized human keratinocyte and cervical epithelial cell lines (Figure 3). The HGK-18 cell line also abundantly expressed HPV-18 E6/E7 polyadenylated RNAs (Figure 5).

Figure 2

Southern blot hybridization analysis of cellular DNA of NHOK, HOK-16A, and HOK-16B.

- A: Determination of the presence and copy numbers of HPV-16 DNA per cell in HOK-16A and HOK-16B cell lines. HPV-16 DNA (corresponding to 5, 25, and 125 copies of viral DNA per cell which were mixed with carrier DNA [*Bam*HI-digested 10 μ g of NHOK DNA]) and *Bam*HI-digested cellular DNA (10 μ g) extracted from NHOK, HOK-16A, and HOK-16B were electrophoresed in 1% agarose gel. The fragmented DNA was then transferred to a nitrocellulose filter and hybridized to 32 P-labeled 7.9-kbp total HPV-16 DNA. The filter was washed and exposed to Kodak SB-5 X-ray film.
- B: Determination of physical state of HPV-16 DNA in HOK-16A and HOK-16B cell lines. 10 μ g of high molecular weight cellular DNA were digested with *Bam*HI (B) and/or *Eco*RV (E) restriction enzymes. *Bam*HI enzyme separates vector from HPV-16 sequences, while *Eco*RV does not digest pMHPV-16d. The fragmented DNA was then transferred to nitrocellulose filter and hybridized to 32 P-labeled 7.9-kbp HPV-16 DNA. The filter was washed and exposed to X-ray film.



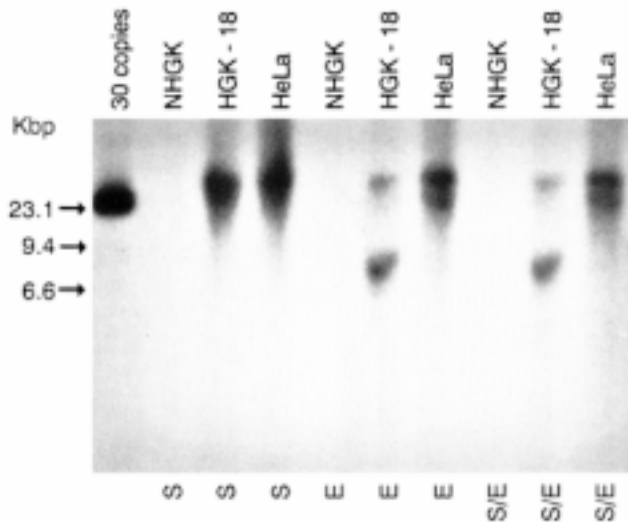
Source: Park et al., 1991; used with permission.

**Expression of
c-myc Proto-
oncogene and
 β -Actin Gene**

Figures 4 and 5 show cellular *myc* proto-oncogene and β -actin gene poly(A⁺)RNAs transcribed from NHOK, NHGK, and the immortalized cell lines. There are three polyadenylated, hybridized *c-myc* RNAs whose sizes are 5.2-kb, 2.4-kb, and 1.1-kb. Expression of the *myc* gene from the HOK-16A, HOK-16B, and HGK-18 cell lines was notably higher than that of the normal counterpart. The expression patterns of β -actin gene from NHOK, NHGK, HOK-16A, HOK-16B, and HGK-18 cell lines were somewhat similar and active. All cells expressed 2.0-kb mRNA in a similar manner, indicating that the cells were metabolically active (Figures 4 and 5).

Figure 3

Determination of the presence and physical state of HPV-18 DNA in HGK-18 and HeLa cell lines. HPV-18 DNA (corresponding to 30 copies of viral DNA per cell which were mixed with carrier DNA [*EcoRI*-digested 10 µg of NHGK DNA]) and *SaI* (S) and/or *EcoRI* (E)-digested NHGK, HGK-18, HeLa cell DNAs were electrophoresed in 1% agarose gel. *EcoRI* enzyme separates vector from HPV-18 sequences, while *SaI* does not digest pSHPV-18m. The fragmented DNA was transferred to a nitrocellulose filter and hybridized to ³²P-labeled 7.9-kbp total HPV-18 DNA. The filter was washed and exposed to X-ray film.



Tumorigenicity of HPV-Transformed Cells

The immortalized HOK-16A, HOK-16B, and HGK-18 cell lines were tested for tumorigenicity in nude mice. Mice injected with either the immortalized cells or normal cells did not develop tumors after being monitored for more than 3 mo.

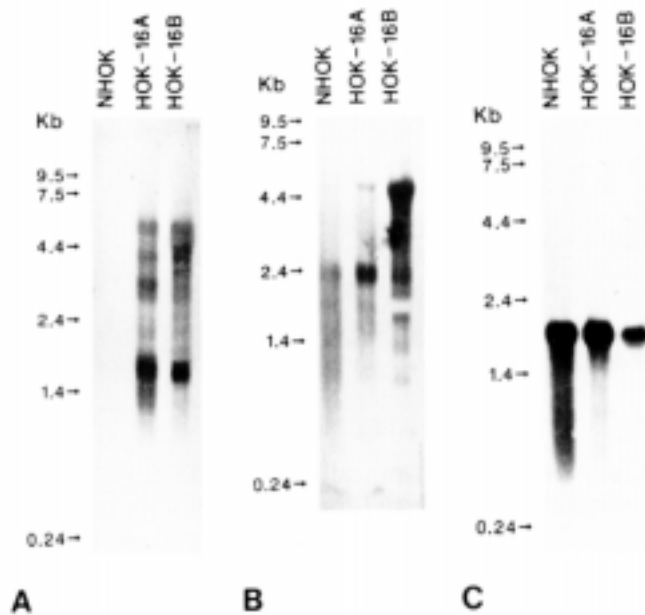
DISCUSSION Present data show that HSV-1 enhances the carcinogenicity of B[a]P, a tobacco-chemical carcinogen. The exact mechanism of HSV-1 cocarcinogenicity remains speculative, but HSV-1 has been proposed to stimulate the oncogenicity of chemical carcinogens by impairing the immunologic response of the host, by interfering with cellular chemical detoxification, by altering target cell permeability, or by causing proliferation of latent tumor cells (Casto and DiPaolo, 1973). Since HSV infection has been associated with chromosomal aberrations, mutations, and selective DNA amplification, HSV might alter cellular DNA, making interaction with B[a]P more favorable. However, rigorous laboratory investigations must be carried out to elucidate further the mechanism of HSV cocarcinogenicity.

Our results provide evidence that HPV-16 and HPV-18 participate in the carcinogenesis in one of their *in vivo* target cells, oral keratinocytes. NHOK and NHGK exhibited a limited *in vitro* lifespan, terminally differentiating after five to six passages. Liposomal transfection of these primary oral keratinocytes with pdMMT_{neo} or pSV2_{neo} plasmid did not extend their

Figure 4

Northern blot hybridization analysis of poly(A⁺)RNAs of NHOK, HOK-16A, and HOK-16B cell lines

- A: Determination of the expression of HPV-16 from HOK-16A and HOK-16B cell lines. Poly(A⁺)RNAs extracted from NHOK, HOK-16A, and HOK-16B cells were electrophoresed in 1.2% agarose gel containing 2.2 M formaldehyde, transferred to a nylon filter, and hybridized to ³²P-labeled 7.9-kbp total HPV-16 DNA. The filter was washed and exposed to X-ray film.
- B: The hybridized ³²P-HPV-16 DNA was stripped from the nylon filter and rehybridized with ³²P-labeled *v-myc* oncogene probe. The filter was washed and exposed to X-ray film.
- C: Hybridized ³²P-*v-myc* DNA was stripped from the filter and rehybridized to ³²P-labeled human β -actin gene. The filter was washed and exposed to X-ray film.



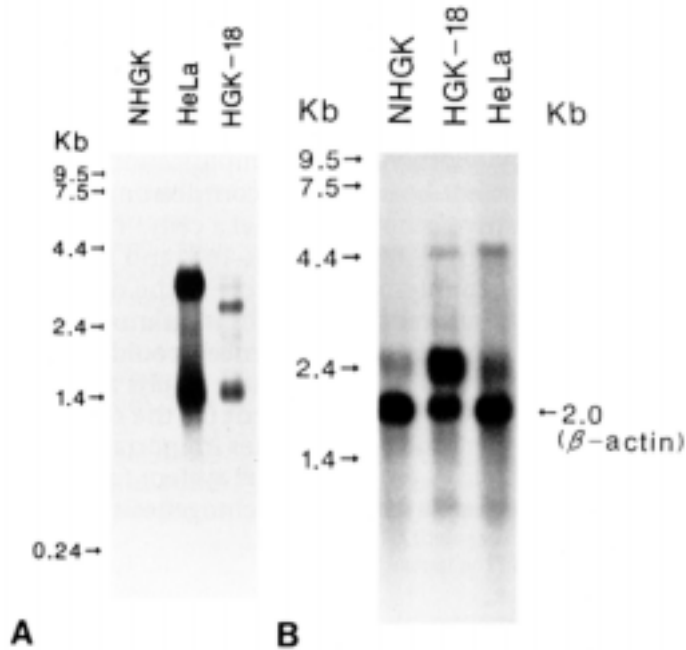
Source: Park et al., 1991; used with permission.

in vitro lifespan, but transfection with pMHPV-16d and pSHPV-18m, cloned HPV-16 and HPV-18 DNA, respectively, recombined into vector plasmid-conferred immortality. These data implicate the presence of HPV-16 or -18 DNA in the cells as a requirement for their immortality. This result supports findings of other reports showing generation of immortalized human keratinocytes by cloned HPV-16 or HPV-18 DNA (Kaur and McDougall, 1988; Woodworth et al., 1989). The transformation mechanism of keratinocytes by HPV-16 DNA transfection is not clear, but expression of HPV-16 E6/E7 gene products has been proposed as being responsible for the transformation (Kaur and McDougall, 1988). Our Northern blot analysis demonstrates that the viral E6 and E7 open reading frames are expressed in

Figure 5

Northern blot hybridization analysis of poly(A⁺)RNAs of NHGK, HGK-18, and HeLa cell lines

- A: Determination of the expression of HPV-18 from HGK-18 and HeLa cell lines. Poly(A⁺)RNAs extracted from NHGK, HGK-18, and HeLa cells were electrophoresed in 1.2% agarose gel containing 2.2 M formaldehyde, transferred to a nylon filter, and hybridized to ³²P-labeled *EcoRI-BamHI* 2.4-kbp fragment of HPV-18 DNA. This 2.4-kbp HPV-18 DNA fragment contains intact HPV-18 E6 and E7 genes which are responsible for cell transformation. The filter was washed and exposed to X-ray film.
- B: The hybridized ³²P-HPV-18 DNA was stripped from the nylon filter and rehybridized with ³²P-labeled *v-myc* oncogene and ³²P-labeled human β -actin gene probes. The filter was washed and exposed to X-ray film.



the HOK-16A, HOK-16B, and HGK-18 cell lines, indicating that the transformation mechanism of oral keratinocytes by HPV-16 and HPV-18 could be similar to that of other human cells.

The physical state of the viral DNA in the transformed cell lines in this study is also similar to that reported elsewhere (Kaur and McDougall, 1988; Woodworth et al., 1989). Southern blot hybridization analysis showed that both HPV-16 and HPV-18 DNA are retained in integrated form in the HOK-16B cell line and suggests the same for line HOK-16A and HGK-18 cell lines. Integrated DNA might be absolutely necessary for the morphological transformation and immortalization of the cells, because HPV-16 DNA found in benign lesions is always in the episomal form, whereas it is usually integrated into host chromosome in carcinomas, even though episomal viral DNA can persist concurrently.

Northern blot analysis of the immortalized cell lines shows that the viral DNA, including the early region encoding the E6/E7 genes, is actively transcribed. The E6 and E7 genes of HPV-16 and HPV-18 have been reported to be retained in many cancer cell lines (Schwarz et al., 1985) and may be responsible, in part, for the immortality of the HOK-16A, HOK-16B, and HGK-18 cell lines.

Since HPV-16 and -18 DNAs are integrated in cervical carcinomas and these tumors appear to retain the HPV enhancer region, it is possible that HPV insertion also results in an activation of certain cellular proto-oncogenes (Durst et al., 1987). In HeLa cell chromosomes, HPV-18 DNA has been found to integrate into four specific chromosomes: chromosomes 8, 9, 5, and 22. Of these, chromosomes 8, 9, and 22 correspond to the location of cellular *myc*, *abl*, and *sis* proto-oncogenes, respectively. Lazo and colleagues (1989) reported that the location of the HPV DNA integration site is near the *myc* proto-oncogene, but the *myc* gene is not rearranged or amplified. It may, however, be expressed through a viral regulatory sequence in its proximity. Both viral gene amplification and constitutive *c-myc* gene expression might be important contributing factors to the immortalization and proliferative properties of HeLa cells. Like HeLa cells, the *c-myc* gene is overexpressed in HOK-16A, HOK-16B, and HGK-18 cell lines compared to their normal counterpart. The role of the overexpressed *c-myc* gene products in the immortalized cell lines is unknown, but it, along with the expression of HPV-16 or HPV-18 sequences, could play a crucial role for the immortalization of the cells. These results also suggest that the overexpression of *c-myc* gene is not sufficient for the cells to be tumorigenic. The human oral keratinocyte cell lines immortalized by HPV-16 and HPV-18 provide, therefore, a useful model system for elucidating critical molecular changes associated with oral carcinogenesis.

REFERENCES

- Casto, B.C., DiPaolo, J.A. Virus, chemicals, and cancer. *Progress in Medical Virology* 16: 1-47, 1973.
- Dekmezian, R.H., Batsakis, J.G., Goepfert, H. In situ hybridization of papillomavirus DNA in head and neck squamous cell carcinomas. *Archives of Otolaryngology—Head and Neck Surgery* 113: 819-821, 1987.
- Durst, M., Croce, C.M., Gissmann, L., Schwarz, E., Huebner, K. Papillomavirus sequences integrate near cellular oncogenes in some cervical carcinomas. *Proceedings of the National Academy of Sciences* 84: 1070-1074, 1987.
- Durst, M., Gissmann, L., Ikenburg, H., zur Hausen, H. A papillomavirus DNA from a cervical carcinoma and its prevalence in cancer biopsy samples from different geographic regions. *Proceedings of the National Academy of Sciences* 80: 3812-3815, 1983.
- Eglin, R.P., Scully, C., Lehner, T., Ward-Booth, P., McGregor, I.A. Detection of RNA complementary to herpes simplex virus in human oral squamous cell carcinoma. *Lancet* 2: 766-768, 1983.
- Hecht, S.S., Chen, C.B., Dong, M., Ornaf, R.M., Hoffmann, D., Ts'o, T.C. Chemical studies on tobacco smoke: VI. Studies on nonvolatile nitrosamines in tobacco. *Beitrag Tabakforsch* 9: 1-6, 1977.
- Hirsch, J.M., Johansson, S.L., Vahlne, A. Effect of snuff and herpes simplex virus 1 on rat oral mucosa: Possible associations with the development of squamous cell carcinoma. *Journal of Oral Pathology* 12: 187-198, 1983.
- Hoffmann, D., Brunnenmann, K.D., Adams, J.D., Hecht, S.S. Formation and analysis of N-nitrosamines in tobacco products and their endogenous formation in consumers. In: *N-Nitroso Compounds: Occurrence, Biological Effects and Relevance to Human Cancer*, I.K. O'Neill, R.C. von Borsteel, C.T. Miller, J. Long, and H. Bartsch (Editors). New York: Oxford University Press, 1984, pp. 743-762.

- Kaur, P., McDougall, J.K. Characterization of primary human keratinocytes transformed by human papillomavirus type 18. *Journal of Virology* 62: 1917-1924, 1988.
- Lazo, P.A., DiPaolo, J.A., Popescu, N.C. Amplification of the integrated viral transforming genes of human papillomavirus 18 and its 5'-flanking cellular sequence located near the *myc* proto-oncogene in HeLa cells. *Cancer Research* 49: 4305-4310, 1989.
- McCance, D. Human papillomaviruses and cancer. *Biochimica et Biophysica Acta* 823: 195-205, 1986.
- National Institutes of Health. Workshop on the treatment and prevention of herpes simplex virus infection. *Journal of Infectious Diseases* 127: 117-119, 1973.
- Offenbacher, S., Weathers, D.R. Effects of smokeless tobacco on the periodontal, mucosal and caries status of adolescent males. *Journal of Oral Pathology* 14: 162-181, 1985.
- Oh, J.S., Paik, D.-I., Christensen, R., Akoto-Amanfu, E., Kim, K., Park, N.-H. Herpes simplex virus enhances the 7,12-dimethylbenz[*a*]anthracene (DMBA)-induced carcinogenesis and amplification and overexpression of *c-erb-B-1* proto-oncogene in hamster buccal pouch epithelium. *Oral Surgery, Oral Medicine, Oral Pathology* 68: 428-435, 1989.
- Overall, J.C., Jr. Dermatologic Diseases. In: *Antiviral Agents and Viral Diseases of Man*, G.J. Galasso, T.C. Merigan, and R.A. Buchanan (Editors). New York: Raven Press, 1979, pp. 305-384.
- Park, N.-H., Akoto-Amanfu, E., Paik, D.I. Smokeless tobacco carcinogenesis: The role of viral and other factors. *CA-A Cancer Journal for Clinicians* 38: 248-256, 1988.
- Park, N.-H., Min, B.-M., Li, S.-L., Cherrick, H.M., Doniger, J. immortalization of human oral keratinocytes with type 16 human papillomavirus. *Carcinogenesis* 12: 1627-1631, 1991.
- Park N.-H., Niukian, K., Shklar, G. Combined effect of herpes simplex virus and tobacco on the histopathologic changes in lips of mice. *Oral Surgery, Oral Medicine, Oral Pathology* 59: 154-158, 1985.
- Schwarz, E., Freese, U.K., Gissmann, L., Mayer, W., Roggenburk, B., Stremlau, A., zur Hausen, H. Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature (London)* 314: 111-114, 1985.
- Shillitoe, E.J., Greenspan, D., Greenspan, J.S., Hanson, L.S., Silverman, S., Jr. Neutralizing antibody to herpes simplex virus type 1 in patients with oral cancer. *Cancer* 49: 2315-2320, 1982.
- Shklar, G., Niukian, K., Hassan, M., Herbosa, E.G. Effects of smokeless tobacco and snuff on oral mucosa of experimental animals. *Journal of Oral and Maxillofacial Surgery* 43: 80-86, 1985.
- U.S. Department of Health and Human Services. *Health Consequences of Smoking: Cancer. A Report of the Surgeon General*. U.S. Department of Health and Human Services, Public Health Service, Office on Smoking and Health. DHHS Publication No. (PHS) 82-50179, 1982.
- U.S. Department of Health and Human Services. *Health Implications of Smokeless Tobacco Use: National Institutes of Health Consensus Development Conference Statement*. 6, No. 1. U.S. Department of Health and Human Services, Public Health Service, National Institutes of Health, 1986.
- Woodworth, C.D., Doniger, J., DiPaolo, J.A. immortalization of human foreskin keratinocytes by various human papillomavirus DNAs corresponds to their association with cervical carcinoma. *Journal of Virology* 63: 159-164, 1989.

