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Toxicity of Smokeless Tobacco in Human Oral Epithelium with Emphasis on Carcinogen Metabolism and Regulation of Programmed Cell Death

by

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Till mina föräldrar

Abstract

The oral mucosa is globally a common site for cancer development. Primary risk factors include tobacco smoking and alcohol consumption whereas the contribution from usage of smokeless tobacco remains debated. The susceptibility of the human oral epithelium to carcinogens in tobacco likely depends on the presence of biotransformation enzymes, capable of metabolically activating or detoxifying these agents as well opposing influences from oxidative stress. Induction of programmed cell death (PCD), including function of tumor suppressor p53, may also modulate smokeless tobacco toxicity. On this basis, the purpose of this study was to investigate the expression of biotransformation enzymes as well as the roles of PCD and p53 in smokeless tobacco toxicity in oral epithelium.

Various qualitative and quantitative analyses of oral tissue specimens and normal, immortalized and malignant oral keratinocytes indicated presence of multiple biotransformation enzymes. Several cytochrome P450 (CYP450) transcripts were demonstrated including 1A1, 1A2, 2C, 2D6, 2E1, 3A4/7 and 3A5. Typical CYP450 substrates, including ethoxyresorufin, methoxyresorufin and chlorzoxazone, were detectably oxidized *in vitro* and metabolism of the tobacco-specific N-nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and aflatoxin B₁ (AFB₁) resulted in covalently bound adducts. Moreover, normal keratinocytes and SV40T antigen-immortalized keratinocytes (SVpgC2a) were shown to express enzymes catalyzing conjugation reactions and detoxification of reactive oxygen. Notably, SVpgC2a showed higher expression levels than normal keratinocytes of some enzymes, e.g. CYP1B1. By RT-PCR, the CYP450s were generally shown to be expressed at levels <50 molecules, the conjugation enzymes at levels between 50-1000 molecules and the enzymes involved in detoxification of reactive oxygen at levels >1000 molecules, using 10⁶ molecules of β-actin as reference. Microarray analysis confirmed expression of these enzymes at levels >300 molecules per 10⁶ molecules of β-actin. The results indicated the presence of several biotransformation enzymes in oral buccal mucosa *in vivo* and *in vitro*, suggesting the usefulness of oral keratinocyte cell lines for studies of both single agents as well as complex mixtures in human oral epithelium.

Studies of smokeless tobacco toxicity involved cultured oral keratinocyte cell lines and oral tissue specimens obtained from healthy controls, snuff users (SDL) and patients diagnosed for oral lichen planus (OLP). Assessments of net growth rates, apoptosis, necrosis and terminal differentiation *in vitro* showed that aqueous smokeless tobacco extract prepared from "Ettans snus" (STE) primarily caused necrotic death without substantial involvement of PCD. Carcinoma cells (SqCC/Y1) were more resistant to necrosis from STE as compared to normal cells. Extract prepared from "Kentucky

standard reference tobacco” caused similar toxicity as STE. The latter extract induced increases in p53 content that did not associate to increased apoptosis, whereas in contrast, the DNA damaging agent mitomycin C (MMC) increased both p53-content and apoptosis. STE and nicotine separately, significantly inhibited apoptosis induced by various regimens. Slight increases in bcl-2 transcripts in STE-exposed keratinocytes indicated the involvement of this gene. Analysis of Jurkat cells implied that reactive smokeless tobacco chemicals might also block apoptosis by inhibiting caspase activity. Oral tissue analysis agreed with the concept that smokeless tobacco may inhibit apoptosis, i.e. increased mitosis in SDL (relative to normal controls) was not associated with increased apoptosis, whereas OLP exhibited increases in both mitosis and apoptosis. Finally, expression of the p53 and Bcl-2 proteins was noted in SDL whereas OLP expressed p53 but not bcl-2.

In summary, the analysis of the expression of biotransformation enzymes and smokeless tobacco toxicity generally demonstrated similar results in tissue and cultured cell lines implying the usefulness of cell culture technology in the investigation of mechanisms underlying carcinogenesis and other oral disease processes. Thus, keratinocytes actively expressing multiple biotransformation enzymes were susceptible to smokeless tobacco toxicity. The toxicity mechanism of smokeless tobacco likely involves metabolism of carcinogenic agents, including N-nitrosamines, and inhibition of p53-mediated apoptosis. Thus, this study suggests several mechanisms whereby smokeless tobacco usage may contribute to adverse health effects including those associated with cancer development in the oral epithelium.

Contents

Abstract	4
Contents	6
List of original articles	8
Abbreviations	9
Introduction	11
Oral epithelium and oral cancer	11
<i>Structure of the oral mucosa</i>	11
<i>Oral cancer</i>	12
<i>Oral premalignancies</i>	13
Multistep model of carcinogenesis	14
Risk factors for oral cancer	15
<i>Tobacco smoking and alcohol</i>	15
<i>Smokeless tobacco</i>	16
<i>Tobacco specific nitrosamines and polycyclic aromatic hydrocarbons</i>	16
<i>Human papilloma virus</i>	17
<i>Other factors</i>	18
In vitro models for oral carcinogenesis	18
Enzymes involved in carcinogen metabolism	20
<i>Cytochromes P450</i>	21
<i>Conjugation enzymes</i>	22
<i>Protective enzymes against reactive oxygen</i>	24
Processes regulating tissue homeostasis	26
<i>Proliferation</i>	26
<i>Programmed cell death</i>	27
Apoptosis mechanisms	29
<i>p53</i>	30
<i>Death receptors</i>	31
<i>The Bcl-2 family</i>	32
<i>Caspases</i>	33

Aim of the present study	35
Results and discussion	36
Metabolic competence of oral tissue and cultured cells.....	36
<i>Expression of CYP450-forms in oral keratinocytes and tissue specimens</i>	36
<i>CYP450-dependent metabolism in cultured oral keratinocytes</i>	36
<i>Activation of pro-carcinogens in cultured oral keratinocytes</i>	37
<i>Transcript profiling by StaRT-PCR</i>	38
<i>Transcript profiling of detoxification enzymes in oral keratinocytes</i>	38
Smokeless tobacco toxicity in oral epithelium.....	40
<i>Smokeless tobacco toxicity in cultured oral keratinocytes</i>	40
<i>p53 associated apoptosis in cultured oral keratinocytes</i>	40
<i>Expression of apoptosis related genes in oral keratinocytes exposed to STE</i>	41
<i>Inhibition of apoptosis in cultured oral keratinocytes</i>	41
<i>Assessment of snuff induced oral lesions</i>	42
<i>Influences of STE on caspase activity</i>	43
<i>Smokeless tobacco toxicity in cultured malignant oral keratinocytes</i>	44
<i>Inhibition of receptor-mediated apoptosis in oral keratinocytes</i>	44
<i>Toxicity comparison of North American and Scandinavian STE</i>	45
Conclusions	46
Acknowledgements	48
References	50
Original articles	64

List of original articles

This thesis is based on the following articles which will be referred to by their Roman numerals:

- I. **Vondracek M**, Xi Z, Larsson P, Baker V, Mace K, Pfeifer A, Tjälve H, Donato MT, Gomez-Lechon MJ, Grafström RC. Cytochrome P450 expression and related metabolism in human buccal mucosa. *Carcinogenesis*. **22**(3):481-8. (2001)
- II. Crawford EL, Peters GJ, Noordhuis P, Rots MG, **Vondracek M**, Grafström RC, Lieuallen K, Lennon G, Zahorchak RJ, Georgeson MJ, Wali A, Lechner JF, Fan PS, Kahaleh MB, Khuder SA, Warner KA, Weaver DA, Willey JC. Reproducible gene expression measurement among multiple laboratories obtained in a blinded study using standardized RT (StaRT)-PCR. *Mol Diagn*. **6**(4):217-25. (2001)
- III. **Vondracek M**, Weaver DA, Sarang Z, Hedberg JJ, Willey JC, Wängard L, Grafström RC. Transcript profiling of enzymes involved in detoxification of xenobiotics and reactive oxygen in human normal and simian virus 40 T antigen-immortalized oral keratinocytes. *Int J Cancer*. **99**(6):776-82. (2002)
- IV. **Vondracek M**, Hansson A., Zheng X, Sand L, Noren U, Hirsch JM, Lindholm J. and Grafström RC. Smokeless Tobacco Toxicity and Influences on Programmed Cell Death in Human Oral Mucosa *In Vivo* and *In Vitro*. Manuscript
- V. **Vondracek M**, Zheng X, Noren U, Elfving Å and Grafström RC. Influences of Smokeless Tobacco Extract on Growth and Apoptosis in Cultured Human Normal and Malignant Buccal Keratinocytes. Manuscript

Abbreviations

BaP	benzo(a)pyrene
BH	Bcl-2 homology domain
Cdk	cyclin dependent kinase
CT	competitive template
CYP450	cytochrome P450
DISC	death-inducing signaling complex
EGF	epidermal growth factor
GST	glutathione S-transferase
HPV	human papilloma virus
IGF-1	insulin-like growth factor-1
MMC	mitomycin C
NNK	N-nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
OLP	oral lichen planus
PAH	polycyclic aromatic hydrocarbons
PCD	programmed cell death
ROS	reactive oxygen species
SCC	squamous cell carcinoma
SDL	snuff dippers lesion
STE	smokeless tobacco extract
TGF- α	transforming growth factor- α
TGF- β	transforming growth factor- β
TSD	terminal squamous differentiation
TSNA	tobacco specific nitrosamine
XME	xenobiotic metabolizing enzyme

Introduction

Oral epithelium and oral cancer

Structure of the oral mucosa

Three basic types of mucosa exist in oral cavity: (i) the thick and keratinized mucosa in gingiva and hard palate, (ii) the thinner non-keratinizing mucosa in cheek (bucca), ventral tongue, lip, floor of the mouth and soft palate, and (iii) the specialized mucosa on the dorsal surface of the tongue (Burkhart and Maerker, 1981; Sloan et al., 1991). Regardless of the type of mucosa, the lining cells are of epithelial origin. The oral epithelium is separated from the underlying connective tissue (lamina propria) by the basal membrane (basal lamina), an extracellular structure composed of fibronectin, collagen and other proteins (Yurchenco and O'Rear, 1994). This extracellular structure is important for the behavior of the keratinocytes e.g. during the process of wound healing (Clark, 1990; Freedberg et al., 2001). The proliferative progenitor keratinocytes in epithelia are situated on the basal lamina and termed basal cells. These cells generate two populations of cells: the stem cells, which maintain the population of dividing cells, and the transiently amplifying cells which migrate from the basal cell layers to the superficial layers and are finally shed into the oral cavity (Hume and Potten, 1979; Watt, 2001). As these latter cells move through the epithelial layers they undergo terminal squamous differentiation (TSD), i.e. they become enlarged, flattened and produce various proteins that eventually may become crosslinked and give rise to the crosslinked envelope (Presland and Dale, 2000; Presland and Jurevic, 2002). The crosslinked envelope is a heat and detergent resistant structure that serves as protection for the underlying cells. The precursors of the crosslinked envelope include SPR1 and involucrin. Both proteins are normally expressed in the supra basal layers of the oral mucosa and their expression can therefore be used as a marker for commitment to TSD (Watt and Green, 1981; Itoiz et al., 1986; Steinert and Marekov, 1997; Lee et al., 2000). Characteristics and structural features of the oral mucosa are summarized in figure 1.

Keratins comprise the most abundant protein family in keratinocytes, and make up the largest subgroup of the intermediate filament multigene family. Keratins are typically expressed in pairs to form the intermediate filaments that provide a flexible and adaptable cellular scaffold (Coulombe and Omary, 2002). Differential expression of keratins are characteristic for the

various stages of development in epithelial tissues. Similar to SPR1 and involucrin, the expression of various keratins can therefore be used as markers for the various stages of cellular maturation and differentiation in keratinocytes (Clausen et al., 1983; Clausen et al., 1986).

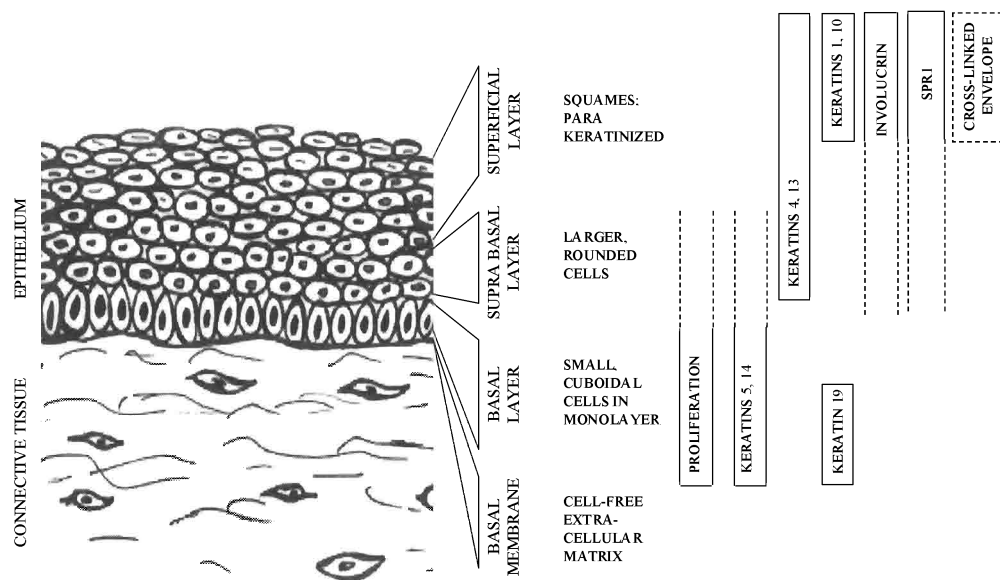


Figure 1. Characteristics and structural features of the oral mucosa.

Oral cancer

Cancer in the oral cavity (including the pharynx) has been rated as the 6th most common cancer worldwide (Parkin et al., 1988; Moore et al., 2000). The incidence is relatively low in developed countries whereas it is one of the most common cancers on the Indian subcontinent and other parts of Asia. The incidence is higher in men than in women and increases with age. More than 80% of all oral cancers lesions are squamous cell carcinomas (SSC) arising in the mucosal membranes (Muir and Weiland, 1995; Ostman et al., 1995). Even in developed countries with advanced medical treatment, five-year survival rates of oral SSC are poor. This is mainly due to late diagnosis and subsequently to a large number of advanced-staged tumors. Moreover, a relatively high incidence of second primary tumors, a phenomenon termed field cancerization, further decreases the survival rates of oral cancer patients (Slaughter et al., 1954;

Silverman and Sugerma, 2000). In this perspective, prevention rather than treatment is a crucial priority.

Oral premalignancies

Leukoplakia is one of the most common oral lesions. It is defined as a whitish patch or plaque that can not be rubbed off and can not be characterized as any other lesion (Burkhardt and Maerker 1981). Like most epithelial lesions, leukoplakia can be classified as mild, moderate or severe according to the extent of dysplastic changes (Kramer et al., 1978; Kuffer and Lombardi, 2002). Therefore, the percentage of leukoplakias that progress into SSC can vary from 5% for leukoplakia with a mild degree of dysplasia up to 43% for leukoplakia with a severe degree of dysplasia (Liu and Klein-Szanto, 2000). Less common than leukoplakia are the reddish lesions termed erythroplakia. Erythroplakia can be classified according to similar criteria as for leukoplakia but is generally considered a high risk lesion (Pindborg et al., 1997).

Another oral lesion of possible premalignant nature is oral lichen planus (OLP). OLP affects one to two percent of the adult population and is characterized by epithelial basal cell destruction and subepithelial lymphocytic infiltration (Sugerma et al., 2000). Clinically, the whitish striated or reticular appearance, sometimes accompanied by dark red areas with epithelial erosions, differentiate the condition from leukoplakia (Burkhardt and Maerker, 1981). The basal cell destruction in OLP has been ascribed to increases in apoptosis rates (Sugerma et al., 2000). Moreover, high expression of the tumor suppressor p53 (discussed below) is generally detected in OLP specimens (Dekker et al., 1997; Bloor et al., 1999). On this basis, OLP specimens have been used as a reference lesion in the present study.

An oral lesion associated with tobacco usage is the snuff dippers lesion (SDL). This lesion is commonly found at the site of quid application in snuff consumers and is characterized by local thickening of the oral mucosa accompanied by hyperkeratosis (Pindborg et al., 1980; Hirsch et al., 1982; Frithiof et al., 1983; Andersson et al., 1991; Axell, 1993). SDL can be classified according to a four grade scale originally proposed by Axell (Axell et al., 1976). An increase in the clinical degree of this lesion seems to be associated with increasing consumption of snuff (Axell, 1993). The possible premalignant nature of SDL remains debated. An important reason for this being the partial or complete regression of the lesion when snuff consumption is interrupted (Larsson et al., 1991).

A number of additional oral lesions involving varying dysplastic degree can be distinguished in the oral mucosa. Examples include erythema multiforme, smokers palate and white sponge naevus (Burkhart and Maerker, 1981).

Multistep model of carcinogenesis

Development of cancer is generally considered to be a multistep process. Several lines of evidence support this hypothesis. Firstly, pathological analyses of multiple tissues reveal lesions that seem to represent intermediate steps in the process of cancer development, e.g. leukoplakia (van der Waal et al., 1997). Secondly, genomes of tumor cells contain multiple alterations indicating that several genetic events are required to generate a fully malignant phenotype (Kinzler and Vogelstein, 1996). Finally, studies in laboratory animals are able to reproduce and define the various phases of carcinogenesis (Yuspa and Poirier, 1988; Yuspa, 2000). Accordingly, a normal cell may undergo initiation followed by promotion, conversion and progression into a malignant phenotype. The initial step involves a genetic alteration that irreversibly results in an initiated cell. The subsequent promotion phase involves the clonal growth of the initiated cell into a cluster of preneoplastic cells, i.e. a preneoplastic lesion. The events of this stage are dependent on dose and are generally considered to be reversible upon removal of the promotive stimuli. The progression phase involves further genetic changes resulting in various phenotypic alterations such as desensitization to anti-growth signals, evasion of apoptosis and self-sufficiency for growth signals. This phase is characterized by genetic instability and generally results in the conversion of a homogenous preneoplastic lesion into a heterogeneous neoplastic tumor. Finally, occasional cells from the primary tumor acquire the capability to invade adjacent tissue and to enter the blood stream, thereby giving rise to metastasis (Figure 2). The above model, with special reference to the genetic changes occurring during development of cancer, is often referred to as the somatic mutation theory for cancer development. This model is widely accepted. However, in recent years several researchers have suggested that the somatic mutation theory needs modification or even replacement. The main argument has been that cell-cell communication and environmental adaptation are of considerable importance during the process of cancer development. In this regard, studies have shown that normal, non-tumor cells are sometimes able to suppress a tumor phenotype in adjacent neoplastic cells (Coleman et al., 1993; Sonnenschein and Soto, 2000).

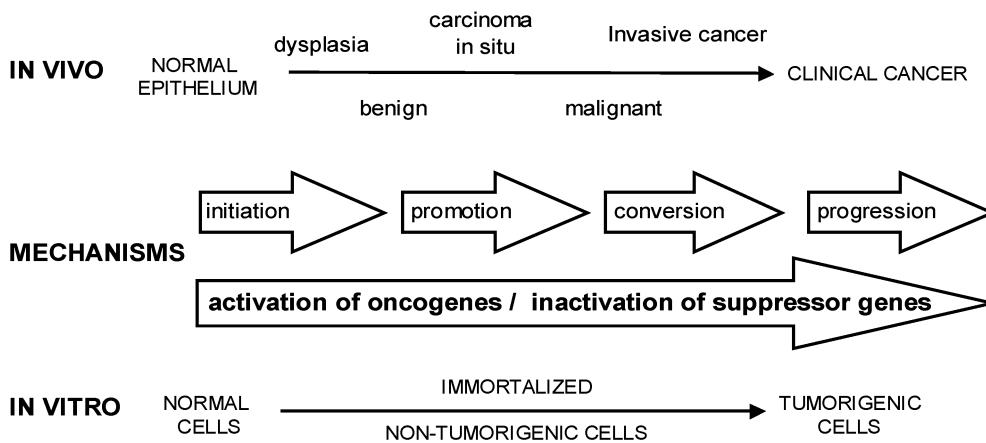


Figure 2. Multistep model of carcinogenesis.

Risk factors for oral cancer

Considerable geographical differences in oral cancer incidence indicate that social and cultural lifestyle factors as well as dietary habits may play an important role in the etiology of oral cancer (Zain, 2001). World wide, the use of various tobacco products and alcohol consumption are considered major risk factors for oral cancer development (Hirayama, 1966; Mehta et al., 1969; IARC, 1986; Bundgaard et al., 1995; Johnson et al., 1996; Moreno-Lopez et al., 2000). In various parts of Asia, where tobacco and betel quid chewing is common, oral cancer incidence is considerably higher than in western countries (Parkin et al., 1988; Moore et al., 2000). Other risk factors for oral cancer development include human papilloma virus, candida infections, and possibly also poor oral hygiene (Reichart, 2001).

Tobacco smoking and alcohol

Tobacco smoking alone is a major risk factor for oral cancer development (IARC, 1986; Rassekh, 2001; Winn, 2001). Several studies support this association and some also indicate a dose-response relationship (IARC, 1986). The relative risk of developing oral cancer seems to have increased over time since Cancer Prevention Study I (1959-1965) in the US estimated the risk to be 6.33 and 1.96 for men and women respectively, while Cancer Prevention Study II (1982-1986) estimated the risk to be 27.5 and 5.59, respectively (Gupta et al., 1996).

Notably, tobacco smoking and alcohol consumption often coexist and many studies consider both factors together. Some of these studies have reported an additive effect of joint exposure (Graham et al., 1977; Llewelyn and Mitchell, 1994), whereas others report synergistic effects (Schottenfeld, 1979; Blot et al., 1988). The effect of alcohol alone has received less attention and presents a more complicated issue since only few non-smokers are high alcohol consumers. However, most studies seem to indicate that alcohol consumption alone has an increasing effect on oral cancer risk (IARC, 1988).

Smokeless tobacco

The role of smokeless tobacco use in the etiology of oral cancer remains a debated issue. In western countries, smokeless tobacco mainly comes in two forms: chewing tobacco and snuff. Within these groups, several different types exist. Chewing tobacco is mainly available as loose leaf, plugs (pressed into bricks) or twists (dried, ropelike strands). Snuff is made from finely cut tobacco leaves and comes in a moist or dry form. Many additional types of smokeless tobacco exist in Asia and the Middle east. Several studies in the west have indicated that habitual use of smokeless tobacco can increase the risk of oral cancer (Peacock and Greenberg, 1960; Winn et al., 1981; IARC, 1985). Moreover, in Southeast Asia where chewing of quids containing tobacco is common, the association to high incidences of oral cancer is well documented (Samuel et al., 1969; Jussawalla and Deshpande, 1971; Gupta et al., 1982; IARC, 1985; Sankaranarayanan et al., 1989). In contrast, recent studies in Sweden did not correlate smokeless tobacco use to an increased risk for oral cancer (Lewin et al., 1998; Schildt et al., 1998). Such findings indicate that differences in composition between smokeless tobacco products in various parts of the world significantly influence their carcinogenicity. In this regard, Swedish products were recently shown to contain several-fold lower concentrations of tobacco specific nitrosamines (discussed below) as compared to their North American counterparts (Brunnemann et al., 2002).

Tobacco specific nitrosamines and polycyclic aromatic hydrocarbons

While tobacco seems to be the most important etiological factor for oral cancer development, the major cancer causing agents in tobacco are the polycyclic aromatic hydrocarbons (PAHs) and tobacco specific nitrosamines (TSNAs) (Hecht, 1999). PAHs are mainly formed from combustion of tobacco in smoked cigarettes but can also be formed during processing of various smokeless tobacco products (IARC, 1985). One of the most potent PAHs is benzo(a)pyrene (BaP). BaP has long been considered as one of the major carcinogens in

tobacco and has been directly linked to human lung cancer via adduct formation analysis of the p53 gene in human cells (Denissenko et al., 1996). Although BaP is mainly considered to be a lung carcinogen, its presence in tobacco smoke as well as in smokeless tobacco may also contribute to carcinogenic effects in the oral cavity. TSNAs are the most abundant and likely the main carcinogens in smokeless tobacco products. They are formed by N-nitrosation of nicotine and other alkaloids during smoking and also during storage and processing of tobacco (IARC, 1985; IARC, 1986). TSNAs are highly carcinogenic in animal studies and many different types of TSNAs are present in saliva from tobacco users (Wenke et al., 1984; Nair et al., 1985; Prokopczyk et al., 1987; Idris et al., 1992; Hecht, 1998). One of the most potent TSNA is N-nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). NNK is considered to be a model compound for lung cancer studies and is also present at relatively high levels in various smokeless tobacco products (IARC, 1985; Osterdahl, 1991; Brunnemann et al., 2002). NNK induces oral tumors in rats when administered together with N-nitrosoornicotine (NNN), supporting its role as a potent carcinogen for oral cancer development (Hecht et al., 1986). Moreover, levels of NNK hemoglobin adducts in snuff users are clearly increased over controls (Carmella et al., 1990). Both PAHs and TSNAs are procarcinogens that require metabolic activation to exert their carcinogenic effect. In this regard, the competency of human oral keratinocytes to activate BaP and NNK to reactive intermediates was previously demonstrated in cultured cells (Autrup et al., 1985; Liu et al., 1993).

Human papilloma virus

High risk human papilloma viruses (HPV) 16 and 18 are well known for their oncogenic potential in genital cancers (IARC, 1995). HPV infections are also found in a substantial proportion of both benign and malignant lesions of the oral cavity (Balaram et al., 1995; Bouda et al., 2000). Moreover, patients diagnosed with HPV associated genital cancers exhibit an increased risk for subsequent development of oral cancer (IARC, 1995). Experimentally, HPV-immortalized human oral keratinocytes were previously shown to convert to a malignant phenotype upon exposure to chemical carcinogens whereas non-HPV-immortalized oral keratinocytes did not (Shin et al., 1994). Although such epidemiological and experimental evidence support the role for HPV in the etiology of oral cancer, many uncertainties and limitations hamper the interpretation of available data. Therefore, the role of HPV in the etiology of oral cancer remains questioned.

Other factors

Other factors that may influence the susceptibility to oral cancer development include diet, oral hygiene, contaminants and hereditary factors. A diet rich in antioxidants is known to have protective effects (Enwonwu and Meeks, 1995). In contrast, heavy consumption of nitrite and nitrosamine-containing food may be coupled to increased risk (De Stefani et al., 1994; Rogers et al., 1995). Poor oral hygiene may add to the risk, possibly through increased acetaldehyde production from ethanol in saliva (Homann et al., 2000). Air pollution from fossil fuels has been shown to increase the risk (Dietz et al., 1995). Contaminants in foods and fluids may also increase the risk, e.g. storage of food and tobacco crops has been shown involve fungal contamination and the presence of Aflatoxin B₁ (Varma et al., 1991; Warke et al., 1999). Clearly, hereditary factors such as mutations in oncogenes and tumor-suppressor genes and/or differential expression of genes involved in carcinogen metabolism will influence the risk of developing oral cancer. These factors are discussed below.

In vitro models for oral carcinogenesis

The use of cultured human cells provides a valuable tool for studying both normal cellular processes and cancer development *in vitro*. Experimental models for head and neck carcinogenesis have traditionally involved animal studies, e.g. application of carcinogens onto the rat mucosa and the hamster cheek pouch (Gimenez-Conti, 1993; Grasso and Mann, 1998). However, such animal systems differ significantly from their human counterpart both morphologically and metabolically and therefore make extrapolations to humans less reliable. Moreover, even when studying human cell cultures, significant differences between cultures from various tissues are apparent (Harris et al., 1982; Brysk et al., 1995; Elmore et al., 2001). Together, these findings emphasize the importance of using human cell cultures originating from relevant tissues in the study of normal biology and development of cancer.

Culture conditions for human keratinocytes have commonly involved the use of serum in the media. However, during the 1980s techniques for growing human keratinocytes under serum-free conditions were developed (Tsao et al., 1982). Serum-free culturing offers several advantages over serum containing culture techniques, e.g. less experimental variability, possibility of identifying factors that regulate proliferation and differentiation, and utilization of selective growth conditions for different cell types. Moreover, serum is a potent inducer of

differentiation in keratinocytes, possibly involving multiple stimuli, e.g. Ca^{2+} , transforming growth factor- β (TGF- β) and retinoids (Masui et al., 1986; Ke et al., 1990; Sundqvist et al., 1991; Blumenberg and Tomic-Canic, 1997). Therefore, serum-free culture conditions allow for the maintenance of a proliferative cellular state and delayed senescence.

Monolayer cultures of normal human oral keratinocytes can be obtained by dissociation of tissue specimens with trypsin and subsequent serum-free culture of the fragmented tissue and individual cells (Grafstrom, 2002). The epithelial cells obtained from this procedure exhibit several characteristics of normal basal keratinocytes *in vivo*, (i) they have a high proliferative capacity, (ii) they are relatively small in size and they express keratins typical of basal cells *in vivo*, and (iii) they show low expression of various differentiation markers (Grafstrom et al., 1997). Importantly, these cells also retain the ability to undergo squamous differentiation *in vitro* as demonstrated by decreased proliferation, increased cell area and expression of involucrin upon treatment with Ca^{2+} or serum (Sundqvist et al., 1991).

The serum-free culture methods applied to normal oral keratinocytes can also be applied to transformed and malignant oral keratinocytes (Sacks, 1996; Grafstrom, 2002). In this regard, the immortalized SVpgC2a cell line was previously established by transfection of normal buccal keratinocytes with a plasmid containing the SV40 large T antigen gene (Kulkarni et al., 1995). This cell line has maintained a high proliferative rate during more than two years in culture. It is partly resistant to serum-induced involucrin expression and growth inhibition by TGF- β . However, it does not induce tumors when inoculated into athymic nude mice and thus, does not represent a fully malignant phenotype (Kulkarni et al., 1995; Kulkarni et al., 1996). Moreover, its expression pattern of keratins is similar to a severe epithelial dysplasia *in vivo* (Hansson et al., 2001). A more common method for developing oral cell lines has been to establish cell cultures from tumors. One such line, SqCC/Y1, was derived in 1981 from a SSC of the cheek (Pitman et al., 1983). This cell line was later adapted to serum-free culture conditions (Sundqvist et al., 1991). The SqCC/Y1 cell line is immortal, it shows invasive properties in athymic nude mice and has a keratin expression pattern similar to SSC *in vivo* (Hansson et al., 2001). On this basis, available normal, transformed and malignant oral cell lines may constitute a step-wise model for oral cancer development *in vitro* (see Figure 2).

In addition to the above described monoculture systems for oral keratinocytes, normal, transformed and malignant oral keratinocytes can also be grown as an organized tissue-like

structure using organotypic culturing techniques (Grafstrom et al., 1997; Hansson et al., 2001). Organotypic culturing allows for studying aspects like epithelial-mesenchymal interactions and epithelial growth and differentiation in an *in vitro* tissue-like state. Therefore, this technique may provide an *in vitro* cell system for normal biology and carcinogenesis studies that is more similar to the *in vivo* situation as compared to regular monocultures.

Besides modeling the various stages of cancer development, human cell cultures are also valuable tools in pharmaco-toxicological studies. In this regard, the majority of cell lines with human origin have traditionally been established from tumors. A main deficiency of such cell lines is their malignant phenotype, which apparently is far from normal. Moreover, commonly used tumor cell lines rarely originate from relevant target tissues. An alternative and likely a more accurate approach is the use of immortalized cell lines that do originate from relevant target tissues. However, immortalized cell lines usually exhibit less metabolic activity as compared to their normal counterparts (Gomez-Lechon et al., 1997). In this regard, few studies have considered the metabolic competence of the oral mucosa and cell lines originating from the oral mucosa.

Enzymes involved in carcinogen metabolism

The human body is continuously exposed to a vast number of foreign compounds collectively known as xenobiotics. Although the liver is the major organ for detoxification, the oral cavity represents an important site in the human body where epithelial cells are continuously exposed to xenobiotics. Many xenobiotics are lipophilic and are therefore easily absorbed through various epithelial barriers such as the skin, gastrointestinal tract and the upper aerodigestive tract. Their lipophilicity also presents a major obstacle for their subsequent elimination. Therefore, such compounds have to be converted to more hydrophilic products in order to be efficiently excreted via urine and faeces. Several enzymes, often referred to as xenobiotic metabolizing enzymes (XME), catalyse these reactions. The various steps involved in the conversion of lipophilic compounds to more hydrophilic ones include initial activation by exposure or introduction of a functional group (-OH, NH₂, SH or COOH), followed by subsequent conjugation to various hydrophilic molecules such as glutathione, glucuronic acid and sulphate. The initial activation step is generally carried out by cytochrome P450 enzymes and gives rise to reactive intermediates, more suitable for conjugation. However, these reactive

intermediates also present a threat to the organism since they can react with cellular macromolecules such as proteins and DNA. In this regard, the balance between activation and conjugation reactions in various tissues (often referred to as phase I and phase II metabolism) may critically determine the burden of reactive intermediates and thus the sensitivity to various toxic agents and carcinogens (Parkinson, 1996).

Cytochromes P450

The cytochromes P450 (CYP450s) are a superfamily of heme containing enzymes whose carbon monoxide complexes show an absorption maximum at 450nm. They are the major enzymes responsible for phase I metabolism of a vast number of xenobiotics but also play a major role in endogenous metabolism. CYP450 expression is mainly hepatic since the liver represents the main organ for detoxification. However, many extrahepatic tissues also show significant expression (Park et al., 1995; Guengerich et al., 1998). The reaction catalyzed by CYP450s can often be summarized as the introduction of a hydroxyl group into the substrate or the unmasking of a hydroxyl or amino group within the substrate. The catalytic cycle involves (i) binding of the substrate, (ii) electron transfer from NADPH to CYP450 via the flavoprotein NADPH-P450 reductase, (iii) binding of oxygen, (iv) a second electron transfer via the reductase or cytochrome b₅, and (v) splitting of the oxygen molecule with one atom being transferred to the substrate and the other being reduced to water. The CYP450s and their electron donating proteins are located mainly in the endoplasmic reticulum although significant levels have also been found in other cellular compartments, e.g. golgi and mitochondria. Currently, more than 50 different CYP450s have been identified in humans and almost 2000 in the whole biological kingdom (<http://dnelson.utmem.edu/cytochromep450.html>). Due to the large number of CYP450s, their nomenclature has been organized into a standardized system based on sequence identities (Nelson et al., 1996). The enzymes are named "CYP" followed by an arabic number representing the family (>40% identity in protein sequence), a letter for the subfamily (>55% identity) and finally another arabic number for the actual enzyme. On this basis, the human CYP450s belonging to family 1, 2 and 3 are mainly involved in xenobiotic metabolism whereas other CYP450 families are involved in metabolism of endogenous substrates.

While the main function for CYP450s is to add reactive groups to xenobiotics, thus facilitating subsequent conjugation and excretion, many pro-carcinogens also require CYP450 dependent metabolism to become truly carcinogenic agents. Oral carcinogens such as TSNAs

have been shown to be metabolized by several CYP450s to generate reactive and dangerous metabolites. Therefore, individual expression of CYP450s and other XMEs are of great importance when determining susceptibility to various carcinogens. Moreover, differential tissue-expression of XMEs may critically regulate organ specific susceptibility to various carcinogens.

Several observations in both laboratory animals and human material have implied that oral epithelium exhibits a capacity for CYP450-dependent metabolic activation reactions. Oral cancers associated to both smoked and smokeless tobacco may clearly be related to CYP450 dependent activation of procarcinogens present in tobacco (IARC, 1985; IARC, 1986). Furthermore, exposure of laboratory animals to TSNAs have been shown to induce oral squamous cell carcinoma (Hecht et al., 1986). *In vitro*-studies have shown that BaP and NNK are metabolized to reactive intermediates in cultured oral keratinocytes (Autrup et al., 1985; Liu et al., 1993) and that CYP450 transcripts are present in normal and HPV-immortalized oral keratinocytes (Farin et al., 1995). Furthermore, polymorphic variants of CYP450s involved in PAH and alcohol metabolism, i.e. CYP1A1, 1B1 and 2E1 have been linked to an increased risk for head and neck cancer development (Hung et al., 1997; Park et al., 1997; Ko et al., 2001; Sreelekha et al., 2001). These results thus suggest that CYP450 enzymes play a critical role in the development of oral cancers.

Conjugation enzymes

Phase II metabolizing enzymes are enzymes that conjugate glutathione, sulfate, glucuronic acid and other molecules to functional groups of xenobiotics. These enzymes are highly concentrated in the liver but are also present in extrahepatic tissues. With the important exception of methyl and acetyl conjugations, these reactions result in a large increase in hydrophilicity of the xenobiotic compounds and therefore facilitate their subsequent excretion. Several enzymes, including sulfotransferases, glucuronosyltransferases, acetyltransferases and glutathionetransferases, catalyze these reactions (Parkinson, 1996). Some of the enzymes involved in phase II conjugation are briefly described below.

Sulfotransferases

Sulfotransferases conjugate sulphate to mainly but not exclusively phenols and aliphatic alcohols. The enzymes use 3'-phosphoadenosine-5'-phosphosulfate (PAPS) as a cofactor. The reaction involves a nucleophilic attack of oxygen or nitrogen (from the functional group of the

xenobiotic) on the sulphur atom in PAPS and subsequent cleavage of the phosphosulfate bond in PAPS. Three major forms of the enzyme have been isolated from human liver. Two are phenol sulfotransferases distinguished by their thermal stability whereas the third is an alcohol preferring sulfotransferase.

Glucuronosyltransferases

These enzymes transfer glucuronic acid to a vast number of substrates including endogenous compounds such as steroids and bilirubin. Glucuronidation reactions require uridine diphosphate-glucuronic acid (UDP-GA) as a cofactor. The enzyme catalyses conjugation of the glucuronic acid portion from UDP-GA to a nucleophilic oxygen, nitrogen or sulphur atom in the xenobiotic or endogenous substrate. In humans, multiple forms of this enzyme arise from alternative splicing of the UGT1 gene transcript or from multiple UGT2 genes (Parkinson, 1996).

N-acetyltransferases

Unlike sulfation and glucuronidation reactions, acetylations (and methylations) generally do not generate a more hydrophilic product. However, N-acetyltransferase catalyzed N-acetylation of certain xenobiotics does facilitate their subsequent excretion. The reaction requires the cofactor acetylcoenzyme A (acetyl-CoA) and occurs in two steps. The first step involves transfer of the acetyl group to a cysteine residue in the active site of the enzyme and subsequent release of CoA. The second step involves transfer of the acetyl moiety from the enzyme to the amino group of the substrate. Humans express two major forms of this enzyme, i.e. NAT1 and NAT2.

Glutathione S-transferases

Glutathione S-transferases (GSTs) catalyze the transfer of the tripeptide glutathione (γ -glu-cys-gly) to a vast number of different electrophilic compounds. The importance of GSTs and glutathione in detoxification reactions is apparent from their high abundance in various tissues, especially in the liver where up to 10% of the total protein can be GSTs (Parkinson, 1996). Many GST substrates are products of CYP450-dependent phase I metabolism. However, several other reactive compounds, such as endogenously produced reactive intermediates during normal metabolism and respiration also serve as GST substrates. Typical GST substrates generally share three common features: (i) they are hydrophobic, (ii) they contain an electrophilic atom, and (iii) they are able to react non-enzymatically with glutathione at a measurable rate (Parkinson, 1996). Because of the high abundance of glutathione (and GST) in

many tissues, non-enzymatic reactions of glutathione with electrophilic compounds are also important in the cellular defense against toxic compounds.

GST enzymes are mostly homodimers, composed of identical subunits although heterodimers also exist. The nomenclature system is designed such that each gene giving rise to one specific protein has its own identity (Mannervik et al., 1992). The various GST subunits are grouped into six recognized classes or families including, for the soluble GSTs, alfa, kappa, mu, pi, omega, sigma, theta and zeta (Hayes and Strange, 2000). In addition to the soluble GSTs, distinct microsomal GSTs also exist. These enzymes belong to the MAPEG family of enzymes (membrane associated proteins in eicosanoid and glutathione metabolism) which include prostaglandin E synthase, leukotriene C₄ synthase, FLAP and the trimeric microsomal GSTs (MGST1,2,3) (Jakobsson et al., 2000).

Although several enzymes involved in phase II metabolism may play an important role in the cellular defense against carcinogens and toxic chemicals, studies on individual enzymes and their role in oral cancer development have mainly involved the abundant GSTs. In this regard, several observations have indicated that GSTM1 and GSTT1 may play an important role in the protection against development of cancer in the upper aerodigestive tract as individuals carrying null mutants for these enzymes have a slightly increased risk for cancer development (Jahnke et al., 1996; Cheng et al., 1999; Nair et al., 1999; Hamel et al., 2000; Park et al., 2000). Moreover, polymorphic variants of the GSTP1 gene also seem to correlate with an increased cancer risk (Matthias et al., 1998; Katoh et al., 1999; Park et al., 1999), and GSTP1 overexpression has been reported in oral carcinomas as compared to control tissue (Sarkar et al., 1997; Bentz et al., 2000). Finally, total GST activities have been shown to progressively increase with increasing degree of oral dysplasia and carcinomas as compared to normal tissue (Chen and Lin, 1997). On this basis, it is evident that phase II metabolizing enzymes may play an important role in the development of oral cancer.

Protective enzymes against reactive oxygen

Although reactive oxygen species (ROS) are not classical carcinogens, they are highly reactive molecules and can therefore be very harmful to cells. In this regard, enzymes involved in the protection against ROS also play an important role in protection against cancer. ROS are widely generated in biological systems as a consequence of aerobic respiration and lipid peroxidation. Moreover, several xenobiotics can induce formation of large amounts of ROS

through a process known as redox cycling (Gregus and Klaasen, 1996). A typical example is that of doxorubicin which can accept electrons from reductases such as NADPH-oxidoreductase to give rise to radicals. These radicals can subsequently transfer the extra electron to molecular oxygen, thus generating ROS (Kappus, 1986). Furthermore, toxicity of tobacco products variably involve generation of ROS (IARC, 1985; IARC, 1986). While small amounts of ROS actually can act as a proliferation inducer, the sustained presence of large amounts of ROS, a phenomenon known as oxidative stress, induces oxidative damage to critical biological macromolecules and can therefore result in severe cellular injury (Cerutti, 1985; Segal and Greenberg, 1996; Bae et al., 1997; Mills et al., 1998).

To avoid oxidative stress, several enzyme systems involved in detoxification of ROS have evolved. These enzymes primarily include the superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidases (GPX). SOD converts the highly reactive superoxide radical to the less reactive molecule hydrogen peroxide. Two major forms of SOD include the cytosolic, dimeric Cu/ZnSOD, also termed SOD1 and the mitochondrial, tetrameric MnSOD, also termed SOD2. However, since hydrogen peroxide can react with molecular oxygen to form the even more potent hydroxyl radical, sequential detoxification of hydrogen peroxide is necessary. This step involves either the peroxisomal enzyme CAT, or the cytosolic enzyme GPX. CAT is a tetrameric protein that catalyses the conversion of hydrogen peroxide into water and molecular oxygen. On the other hand, GPX can catalyze the conversion of several various organic hydroperoxides, including hydrogen peroxide, into alcohols or water. Moreover, in contrast to CAT, GPX has an absolute requirement for glutathione as a cofactor. Various forms of GPX include both intracellular and excreted forms of this enzyme (Cotgreave et al., 1988; Gregus and Klaasen, 1996).

In addition to inducing direct toxicity in cells, oxidative stress is believed to be a major source driving the promotion phase of carcinogenesis (Cerutti, 1985; Halliwell et al., 1992; Wiseman and Halliwell, 1996; Mates and Sanchez-Jimenez, 2000). In this regard, evidence for the involvement of ROS in development of head and neck cancers mainly comes from studies showing altered activities of ROS detoxification enzymes in tumors as compared to normal tissue and demonstrations that antioxidants can antagonize the initiation and promotion phases of carcinogenesis (Durak et al., 1993; Enwonwu and Meeks, 1995; Winn, 1995; Saroja et al., 1999). A possible mechanism whereby oxidative stress may promote tumor progression is the induction of genomic instability, leading to activation of oncogenes and inactivation of tumor

suppressor genes. Other mechanisms include damage to proteins such as DNA repair enzymes and influences on intracellular signaling (Sundaresan et al., 1995; Goldman et al., 1998; Mates and Sanchez-Jimenez, 2000).

Processes regulating tissue homeostasis

In a stable and mature organ, tissue homeostasis is maintained by a balance between cellular proliferation and death. As discussed earlier, continuously renewing epithelial tissues like the oral mucosa naturally contain highly proliferative cells in the basal compartments and non-proliferative, differentiating and thus dying cells in the suprabasal layers. Death in normal epithelia occurs by two programmed mechanisms, TSD and apoptosis. Additionally, if cells are seriously injured, necrotic death may occur. The processes of proliferation and loss of cells through various types of programmed cell death (PCD), including TSD and apoptosis, are normally in a dynamic steady state, thus maintaining epithelial stability and function. However, imbalances in these processes may lead to development of severe pathological conditions including cancer.

Proliferation

As cells divide, they go through a series of controlled events collectively known as the cell cycle. These processes mainly include DNA replication (S-phase) and mitosis (M-phase). Regulation of the cell cycle is of critical importance during development and normal tissue homeostasis and is controlled mainly by a number of heterodimeric protein kinases, each containing both a regulatory and a catalytic subunit. The regulatory proteins, the cyclins, are synthesized and degraded in a highly regulated manner during the various stages of the cell cycle and are each associated with a specific cyclin dependent kinase (cdk). These cyclin/cdk complexes in turn activate transcription of proteins needed for progression through the various stages of the cell cycle and also induce degradation of various cell cycle inhibitors, thus irreversibly driving the cell cycle in one direction. An important step in the regulation of the cell cycle is the restriction point when cells pass through the G₁ (gap 1-phase) to S transition. This involves activation of the transcription factor E2F through phosphorylation of the Rb protein which normally binds to and inactivates E2F. Another important checkpoint involves the p53-mediated G₁ and G₂ arrest (discussed below). Other crucial regulators of the cell cycle

are the cyclin-kinase inhibitors, including the cdk inhibitors, e.g. p21, that bind to and inactivate various cdks, and the INK4 family that bind to and inactivate cdk4/6 (Lodish, 2000).

Keratinocyte proliferation is dependent on several factors, including the balance between available growth factors and growth inhibitors, the state of keratinocyte differentiation and cellular adhesion to various matrixes. Several growth factors, including epidermal growth factor (EGF), its homologue transforming growth factor α (TGF- α) and insulin-like growth factor-1 (IGF-1), have been shown to mediate keratinocyte proliferation (Rheinwald and Green, 1977; Coffey et al., 1987; Liu et al., 1993). Some of these growth factors are produced by the keratinocytes themselves and therefore act in an autocrine manner, e.g. various members of the EGF family (Pittelkow et al., 1993; Piepkorn et al., 1994). Others are mainly produced by fibroblasts and diffuse into the epithelium, thus acting in a paracrine manner, e.g. IGF-1 (Krane et al., 1992; Tavakkol et al., 1992). These factors all exert their proliferative effect by binding to specific cellular receptors. The mechanisms whereby various growth factors stimulate cell growth are not yet fully understood, although they are likely to result in upregulation of various cyclins and inhibition of cdk inhibitors, thereby ultimately leading to cell cycle progression. In contrast, other factors may act by inhibiting proliferation. One extensively studied inhibitor of keratinocyte proliferation is TGF- β . Binding of this peptide to its receptors has been shown to induce increased levels of cyclin-kinase inhibitors and thus inhibition of the cell cycle (Reynisdottir et al., 1995). Moreover, overexpression of TGF- β has been shown to induce epithelial atrophy in mice (Sellheyer et al., 1993). Importantly, in addition to acting as an inhibitor of keratinocyte proliferation, TGF- β can also induce TSD and apoptosis in oral keratinocytes (Min et al., 1999).

Programmed cell death

As discussed above, most of the keratinocytes in continuously growing epithelia like the oral mucosa naturally undergo terminal differentiation. This occurs as the cells migrate to the outer epithelial layers and involves cellular enlargement and expression of several proteins such as involucrin and certain types of keratins (Watt and Green, 1982; Sloan et al., 1991; Bloor et al., 1998). Moreover, TSD is strongly associated with a decreased proliferation capacity. While the process of cellular differentiation in various epithelial tissues gives rise to the specific features of the epithelium, the ultimate outcome of TSD is cellular death. On this basis, TSD is a major programmed cell death mechanism whereby tissue homeostasis is maintained in a constantly growing epithelium (Haake and Polakowska, 1993; Evan and Vousden, 2001; Zhu and

Skoultchi, 2001). Several endogenous as well as exogenous agents have been shown to induce TSD in cultured oral keratinocytes including serum, Ca^{2+} and TGF- β (Sundqvist et al., 1991; Sundqvist et al., 1991).

The other main type of programmed cell death is apoptosis. Apoptosis has been shown to play a major role in various biological processes such as embryogenesis, normal tissue homeostasis and several pathological conditions including cancer. Apoptotic death differs from necrotic death in several ways. Firstly, apoptosis is a highly regulated suicide process that requires energy and is characterized by distinct morphological and biochemical changes. Secondly, apoptotic death eventually results in fragmentation of the dying cells into membrane-enclosed vesicles that are efficiently phagocytosed by macrophages and surrounding cells. On this basis, apoptosis is a cellular suicide program in which cells die “quietly” without injuring the surrounding tissue. Necrosis, on the other hand, constitutes a passive form of cell death occurring when cells are severely injured and thus can not maintain membrane integrity. As a consequence, influx of water into necrotic cells eventually causes them to swell and burst. Necrotic cells will therefore spill out various toxic cellular components (including proteases, nucleases and reactive metabolites) into the extracellular space and thereby cause injury to the surrounding tissue. Moreover, necrotic death generally attracts lymphocytes and thus causes inflammation.

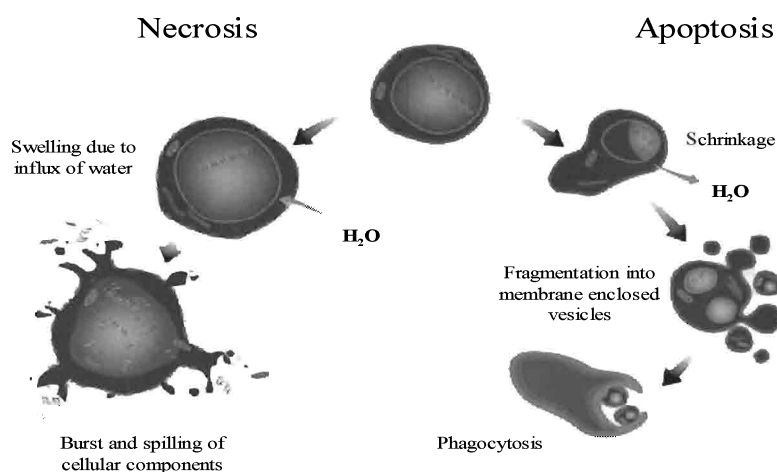


Figure 3. Apoptosis versus necrosis.

Interestingly, apoptosis and TSD share several common features, including DNA fragmentation and crosslinking of structural proteins by transglutaminases (Polakowska et al., 1994). Moreover, both apoptosis and TSD are likely to be regulated by the Bcl-2 family of proteins (Maruoka et al., 1997; Harada et al., 1998). In this regard, TSD has been suggested to be a specialized form of apoptosis (Haake and Polakowska, 1993; Maruoka et al., 1997). However, the precise relationship between apoptosis and differentiation is not clear and thus remains a matter of controversy.

Apoptosis was first described in 1972 by Kerr, Wyllie and Currie. They reported that a special type of cell death with distinct morphological features occurred in several normal biological processes as well as in pathological conditions and termed this death process apoptosis (Kerr et al., 1972). Later, Horvitz and colleagues provided evidence for the genetic regulation of programmed cell death as they mapped the fate of every cell in the organism *C. elegans* and showed that apoptosis was determined by several genes, including the pro-apoptotic *ced-3* and *ced-4* and the anti-apoptotic *ced-9* genes (Ellis and Horvitz, 1986). The counterparts to these three genes have later been shown to be major determinants of apoptosis in mammalian cells as well. In recent years, the area of apoptosis research has grown exponentially and there are now more than 200 new publications every week referring to “apoptosis” (Vaux, 2002). The next section summarizes some of the major mechanisms whereby the apoptotic process is now known to function.

Apoptosis mechanisms

Cellular suicide through apoptosis is a highly regulated, energy dependent process by which unnecessary or damaged cells can be eliminated. Several different types of stimuli have been shown to induce cell death by apoptosis. Firstly, DNA damaging agents, including ionizing radiation and alkylating chemicals are traditional inducers of apoptosis and often involve activation of the tumor-suppressor p53. Secondly, stimulation of “death-receptors”, such as the CD95 (Apo-1/Fas) receptor, often leads to activation of intracellular signal cascades leading to apoptosis. Furthermore, several biochemical agents that directly or indirectly affect the apoptotic cellular machinery are potent inducers of apoptosis, e.g. kinase inhibitors like staurosporine. Finally, physiological agents like heat and mechanical damage can also induce apoptosis. Importantly, when cells are exposed to excessive doses of these stimuli, death by

necrosis is common. Initiation of the apoptotic process clearly differs among the different types of stimuli. However, the various initiation signals seem to converge in a common execution pathway, involving proteolytic cleavage of various cellular substrates and mitochondrial cytochrome c release.

The main regulators of the apoptotic machinery may be arbitrarily divided into groups, including p53 and p53 regulated proteins, death receptors, the Bcl-2 family of proteins and the caspase family and other proteases. Although these groups are not distinct and p53 regulated proteins actually include members of the death receptors and Bcl-2 family of proteins, this grouping is used here to facilitate the description of apoptotic mechanisms.

p53

A common cellular response to various stress stimuli such as exposure to DNA damaging agents is upregulation of the tumor suppressor protein p53. This protein can direct several stress responses, including cell cycle arrest, DNA repair and cell death by apoptosis. Moreover, p53 mutations are the most common chromosomal aberrations in human cancers (Levine, 1997; Wiman, 1997). On this basis, the p53 protein has sometimes been referred to as “the guardian of the genome” (Lane, 1992).

The p53 protein functions as a tetrameric transcription factor and can stimulate or repress expression of target genes (Friedman et al., 1993). Induction of p53 occurs mainly by post-translational stabilization. In this regard, the MDM2 protein plays a central role in the targeting of p53 for degradation through the ubiquitin pathway (Haupt et al., 1997; Kubbutat et al., 1997). Stabilization of the p53 protein can thus be efficiently achieved through deregulation of the MDM2/p53 interaction. This occurs mainly by two mechanisms. The first one, presumably occurring following DNA damage, involves phosphorylation of the p53 protein by protein kinases like ATM, thereby preventing the p53/MDM2 interaction (Shieh et al., 1997; Banin et al., 1998; Canman et al., 1998). The second one, mainly occurring following aberrant growth signaling, involves E2F mediated expression of the p14^{ARF} gene and subsequent interaction of the p14^{ARF} protein with MDM2, thus also preventing the p53/MDM2 interaction (Kamijo et al., 1998; Sherr, 1998). Although these pathways seem to play a central role in the stabilization of the p53 protein, other studies have shown that p53 molecules lacking critical phosphorylation sites can also be stabilized following DNA damage and that oncogenic signaling in p14^{ARF} null

mutants can induce p53, thus indicating that additional mechanisms for p53 stabilization exist (Meek, 1999; Sherr and Weber, 2000).

Following stabilization, p53 can transactivate a number of target genes involved in cell cycle control and apoptosis. The mechanism whereby p53 mediate cell cycle arrest mainly involves transactivation of the cdk inhibitor p21 (el-Deiry, 1998). As discussed above, this protein inhibits the cdk proteins which are critical regulators of the cell cycle. p53 mediated upregulation of p21 therefore plays a major role in the growth inhibitory function of p53. More recently, another p53 target, the 14-3-3 σ protein, has been shown to be involved in cell cycle control especially in epithelial cells (Chan et al., 1999). While p53 mediated growth arrest is mainly dependent on its transactivating capacity, p53 mediated apoptosis is probably a more complex process and seems to occur by both transactivation of pro-apoptotic genes and repression of anti-apoptotic genes. Moreover, it has recently been suggested that p53 may transduce apoptotic signals through protein-protein interactions, e.g. through interaction with caspases (Ding et al., 1998). Several pro-apoptotic genes have been identified as targets for p53 transactivation including bax, CD95, redox related genes called PIGs, NOXA, APAF-1 and several others (Miyashita and Reed, 1995; Owen-Schaub et al., 1995; Polyak et al., 1997; Oda et al., 2000; Robles et al., 2001). p53 has also been shown to repress transcription of the anti-apoptotic gene Bcl-2 (Miyashita et al., 1994).

In addition, p53 also influences other cellular processes associated with its tumor suppressing properties, such as DNA-repair mechanisms and anti-angiogenic processes. The detailed mechanisms whereby p53 directs such different responses and how the individual cell is orchestrated to undergo a specific process remains unknown. In a recent review on the subject, the p53 protein was compared to a highly connected node in a network regulating tumor suppressing processes. In this regard, it may not be surprising that disruption of such a node can have serious effects on the total network function whereas disruption of less connected nodes may be of less importance (Vogelstein et al., 2000).

Death receptors

The death receptors are receptors that upon ligand binding induce apoptotic cell death. These proteins belong to the tumor necrosis factor (TNF) receptor superfamily and are characterized by two to five cysteine rich extracellular domains and an intracellular part containing a so called death domain. One of the most studied death receptor is the CD95 (APO-1/Fas) which is

expressed in many cell types including oral keratinocytes (Yoshioka et al., 1996; Muraki et al., 1997). Upon ligand binding, CD95 has been shown to oligomerize, preferentially into trimers, resulting in the assembly of the so called death-inducing signaling complex, the DISC. The DISC consists of the adaptor molecule Fas associated death domain-containing protein (FADD) and the FADD like ICE protease, caspase 8. According to the “induced proximity model” oligomerization of CD95 receptors and therefore also caspase 8 is sufficient to allow for the autoproteolytic cleavage of caspase 8, thus generating the active caspase (Salvesen and Dixit, 1999). This ultimately leads to a cascade of caspase activation events and cell death (discussed below).

In the study of different cell types with respect to CD95 mediated apoptosis signaling, two different signaling pathways have been identified. Following activation of pro-caspase 8, the active form can directly cleave and activate a main apoptosis executor, caspase 3. Alternatively, active caspase 8 can cleave the pro-apoptotic Bcl-2 family protein Bid (discussed below) which sequentially translocates to the mitochondria and induces release of cytochrome c (Li et al., 1998; Luo et al., 1998; Yin et al., 1999). Cytochrome c normally plays a critical role in the respiratory chain during cellular aerobic respiration. However, upon cytochrome c release into the cytosol, the ced-4 like protein APAF-1 binds to cytochrome c followed by an energy dependent conformational change of the complex ultimately leading to APAF-1 oligomerization, recruitment of caspase 9 and thus generation of the apoptosome (Li et al., 1997). Subsequent activation of caspase 9 then leads to apoptosis. Release of cytochrome c from mitochondria has also been demonstrated in non-receptor mediated apoptotic pathways and is now almost considered a universal feature of apoptotic cell death. Importantly, both the CD95 receptor gene itself and the apoptosome-protein APAF-1 gene have been shown to be regulated by the p53 protein (Muller et al., 1998; Robles et al., 2001). Moreover, although CD95 mediated apoptosis is clearly operative in oral keratinocytes, the relative involvement of the two CD95 mediated apoptotic pathways has not been elucidated.

The Bcl-2 family

As discussed above, several apoptotic pathways other than CD95 mediated apoptosis involve the mitochondria. Such pathways are mainly regulated by members of the Bcl-2 family. The proteins belonging to this family can be divided into three groups. The first group consists of anti-apoptotic proteins that contain four Bcl-2 homology (BH) domains where BH1, BH2 and BH4 seem to be involved in anti-apoptotic activity, e.g. Bcl-2 and Bcl-X_L (Yin et al., 1994;

Hirota et al., 1999). The second and third groups contain pro-apoptotic proteins, where the second group includes multidomain proteins such as Bax, Bak and Bok, whereas the third group comprises BH3 domain only-proteins e.g. Bid and Bim. The relative ratio of these proteins has been suggested to determine the sensitivity of the cell to undergo apoptosis from various stimuli (Oltvai and Korsmeyer, 1994; Tsujimoto and Shimizu, 2000). In this regard, members of the Bcl-2 family can, via their BH domains, form heterodimers and thereby regulate their respective anti- or pro-apoptotic features. A classical example is heterodimer formation between Bcl-2 and Bax (Oltvai et al., 1993). Furthermore, the recently reported three dimensional structures of Bax, Bcl-2 and Bid, proteins representing both pro- and anti-apoptotic groups, indeed revealed that the conserved BH domains are located on the protein surface (Chou et al., 1999; McDonnell et al., 1999; Suzuki et al., 2000; Petros et al., 2001).

Although the molecular mechanisms whereby these proteins execute their function is still a matter of controversy, their key function seems to be regulation of cytochrome c release from mitochondria, thus promoting formation of the apoptosome and caspase activation. Upon various apoptotic stimuli, the cytosolic proteins Bax and Bid translocate to the mitochondria, sequentially leading to release of cytochrome c (Wolter et al., 1997; Luo et al., 1998). This release can be inhibited by Bcl-2 and Bcl-X_L (Yang et al., 1997; Finucane et al., 1999). The mechanism whereby Bcl-2 proteins promote or inhibit cytochrome c release is likely to involve formation of pores in the outer mitochondrial membrane. Two major mechanisms have been proposed. Either the pro-apoptotic proteins themselves generate pores by homo- and heteropolymerization or the pro-apoptotic proteins trigger opening of the mitochondrial permeability transition pore (PTP). However, since some recent studies have suggested that the mitochondrial structure remains intact long after cytochrome c release, the involvement of the PTP pore must be considered with care (Martinou et al., 1999; von Ahsen et al., 2000). Moreover, the activation/deactivation mechanisms for the Bcl-2 proteins themselves are quite complex processes and are likely to involve both transcriptional regulation and post-translational mechanisms such as proteolytic cleavage and phosphorylation (Fadell et al., 1999).

Caspases

Both receptor- and mitochondria-mediated apoptotic pathways involve downstream caspase activation cascades. At least 14 different caspases have been identified in mammals and 8 of them play an important role in apoptosis. The caspases are expressed in most cell types and are

inactive pro-enzymes that need proteolytic cleavage to exert their catalytic activity. The apoptotic caspases can generally be divided into initiators and effectors. The initiators e.g. caspase 8 and 9, present in the DISC and apoptosome respectively, are enzymes that cleave and activate downstream effector caspases. In contrast, the effectors are responsible for the cleavage of various cellular targets (Shi, 2002). All caspases are cysteine proteases with a strong preference for cleavage of the peptide bond C-terminal to an aspartate (Earnshaw et al., 1999). The cleavage of pro-caspases results in heterodimerization of the cleavage products followed by dimerization of such heterodimers. Thus, active caspases function as tetramers (Liang and Fesik, 1997). Initiator caspases have autocatalytic activity and are, according to the induced proximity model, activated during clustering (Salvesen and Dixit, 1999). These enzymes subsequently cleave downstream effectors. The effectors in turn cleave a number of cellular substrates, including cytoskeletal proteins (e.g. lamins and actin), proteins involved in DNA repair (e.g. PARP) and cell cycle regulating proteins (e.g. p21) (Tewari et al., 1995; Takahashi et al., 1996; Levkau et al., 1998). One important effector is caspase 3. This enzyme has been shown to be responsible for cleavage of several cellular targets thereby generating apoptotic hallmarks such as DNA fragmentation and plasma membrane blebbing (Sahara et al., 1999; Wolf et al., 1999; Sebbagh et al., 2001).

In summary, programmed cell death by apoptosis is a complex cellular suicide process involving several steps and mediators. The explosive growth in our understanding of this process is far more complex than is outlined here and the above text is merely a brief introduction to the most relevant apoptosis regulators for this study. Furthermore, although the vast progress in apoptosis research is quite impressive, there is still a need for intensive research in order to elucidate further details about the process and its implications in pathological conditions.

Aim of the present study

The overall aim of the present study was to characterize the expression of biotransformation enzymes and to study smokeless tobacco toxicity with emphasis on programmed cell death in human oral epithelium.

The specific sub-aims were to:

- characterize the expression and function of cytochrome P450 isoenzymes in oral tissue specimens and cultured cells, including the comparison of normal, immortalized and malignant keratinocyte cell lines
- establish standardized methodology for transcript profiling (termed StaRT-PCR), involving quantitative measurement of mRNA levels
- determine transcripts qualitatively and quantitatively for different classes of biotransformation enzymes, including cytochrome P450 isoforms as well as those involved in conjugation reactions and detoxification of reactive oxygen, using StaRT-PCR and microarray analyses
- study oral lesions caused from usage of smokeless tobacco with special reference to programmed cell death and the expression of two apoptosis-related genes, namely p53 and Bcl-2
- study influences of smokeless tobacco extract in cultured oral keratinocytes, including processes regulating tissue homeostasis, and expression of p53 and Bcl-2
- establish a protocol implicating p53-mediated apoptosis utilizing mitomycin C
- assess the influence of STE on apoptosis induction, both by exposure to mitomycin C and transforming growth factor- β , as well as through activation of the CD95 receptor
- compare toxicity of smokeless tobacco extract in normal, p53-proficient versus malignant, p53-deficient oral keratinocyte lines
- compare the toxicity exerted by Swedish and US smokeless tobacco extracts

Results and discussion

Metabolic competence of oral tissue and cultured cells

Expression of CYP450-forms in oral keratinocytes and tissue specimens (Paper I and III)

Studies in papers I and III were designed to characterize the expression of various biotransformation enzymes in oral tissue specimens and cultured cells. Initial experiments using semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) revealed almost identical expression of CYP450 isoenzymes in oral tissue specimens as previously reported for primary cultures of oral keratinocytes (Farin et al., 1995). Accordingly, oral tissue specimens and normal oral keratinocytes were found to express CYP1A1, 1A2, 2C, 2D6, 2E1 and 3A forms. Notably, expression of the highly polymorphic CYP2D6 gene was detected in 6 of the 13 tissue specimens analyzed. Concomitant analysis of SV40T antigen-immortalized (SVpgC2a) and malignant (SqCC/Y1) oral keratinocytes demonstrated loss of the various CYP3A forms in immortalized cells and loss of CYP2C, 2D6 and 3A4/7 transcripts in malignant cells. Moreover, subsequent studies described in paper III, in which normal and SVpgC2a cells were analyzed by a recently developed quantitative RT-PCR method (StaRT-PCR, discussed below) surprisingly demonstrated comparatively higher levels of CYP1A1, 1B1 and epoxide hydrolase (EPX1) transcripts in SVpgC2a as compared to normal cells. This analysis also added NADPH oxidoreductase (POR), the aryl hydrocarbon (Ah-) receptor and Ah-receptor nuclear translocator (ARNT) to the initial study. Thus, the overall analysis of CYP450 transcripts and related factors indicated that the expression profiles of oral tissue specimens and normal oral keratinocytes are very similar. Moreover, the SVpgC2a cell line retained expression of a majority of the transcripts and even showed elevated expression of some transcripts i.e. CYP1A1 and 1B1. In contrast, the malignant SqCC/Y1 cell line had lost expression of many CYP450 forms.

CYP450-dependent metabolism in cultured oral keratinocytes (Paper I)

Further studies were performed to assess if transcription of the various CYP450 genes resulted in catalytically active enzymes. Metabolism studies using typical CYP450 substrates generally revealed low levels or absence of CYP450 activity. SVpgC2a cells showed similar 7-ethoxyresorufin O-demethylation (EROD) and 7-methoxyresorufin O-deethylation (MROD) activities as normal oral keratinocytes whereas SqCC/Y1 showed about 2-fold lower activities. These activities were in a similar range as previously observed in non-induced human

hepatocytes and skin keratinocytes (Roberts et al., 1993; Raffali et al., 1994), and indicated the presence of functional CYP1A1 and 1A2 forms. However, with the exception of chlorzoxazone, a substrate for CYP2E1, which was significantly metabolized in the SVpgC2a cells, metabolism of substrates corresponding to the CYP2C9, 2D6 and 3A4 forms was not detected in any of the three cell types. Thus, proteins corresponding to the 2C9, 2D6 and 3A4 transcripts may either be non-functional or absent in these cells. Although it can not be ruled out that a functional CYP1A1 enzyme may have contributed to the metabolism of methoxyresorufin (Lewis, 1996), the results indicated that CYP1A2 is present and functional in oral keratinocytes. Moreover, similar dealkylation rates in SVpgC2a and normal cells, and the significant hydroxylation of chlorzoxazone in SVpgC2a cells indicated that some CYP450 enzymes are maintained or even elevated during the process of immortalization.

Activation of pro-carcinogens in cultured oral keratinocytes (Paper I)

On the basis that cultured oral keratinocytes express several CYP450 transcripts and to some extent also display the corresponding enzymatic activity, their potential capacity for activating the potent pro-carcinogens aflatoxin B₁ and NNK was tested in SVpgC2a and SqCC/Y1 cells. Application of an autoradiographic technique based on incubations of cultured cells with radiolabeled substrates i.e. [G-³H]-aflatoxin B₁ and [¹⁴C]-NNK, revealed covalently bound metabolites of both aflatoxin B₁ and NNK in SVpgC2a cells. In contrast, SqCC/Y1 cells were only able to metabolize NNK. Furthermore, SVpgC2a cells, but not SqCC/Y1 cells were able to metabolize both pro-carcinogens in organotypic cultures. Using a similar technique, previous studies from our laboratory demonstrated that normal oral keratinocytes are able to metabolize NNK when grown in monocultures as well as in explant cultures (Liu et al., 1993). The activation of aflatoxin B₁ in SVpgC2a cells was initially interpreted as being due to an active CYP1A2 enzyme. However, subsequent studies in paper III indicated that this was a consequence of active CYP1B1. Collectively, the analysis of mRNA expression, substrate conversion and carcinogen activation, indicated that normal and immortalized oral keratinocytes exhibit similar metabolic capacities whereas the malignant SqCC/Y1 cell line is likely to be less metabolically active. Although non-reactive metabolites of aflatoxin B₁ were not identified in the culture medium of SVpgC2a cells, detection of covalently bound aflatoxin B₁ metabolites in these cells implied that aflatoxin B₁ could be a possible oral carcinogen. In this regard, tobacco crops from areas with a high incidence of oral cancer have been reported to contain fungal contamination and presence of aflatoxin B₁ (Varma et al., 1991; el-Maghraby and Abdel-Sater, 1993; Warke et al., 1999).

Transcript profiling by StaRT-PCR (Paper II)

Paper II describes an interlaboratory evaluation of a recently developed technique for gene expression measurements. The methodology was originally developed in 1998 by Willie and colleagues at the Medical College of Ohio, Toledo, USA and is based on competitive RT-PCR reactions combined with the usage of standardized mixes of competitive templates (CTs) (Willey et al., 1998). The ground-breaking idea is to prepare unique CTs for each gene. With the only exception of a short deletion, the respective CTs are designed to consist of identical sequences as the native target DNA. Several such CTs are subsequently mixed at defined concentrations to generate standardized CT mixes. To enable a comparison between various mixes, a house-keeping CT, e.g. β -actin, is also included in each mix. Finally, these CT mixes are used in competitive PCR reactions. The technique allows for quantitative gene expression measurements and the obtained data can be directly compared between different genes as well as different samples. The studies in paper II were designed to evaluate the reproducibility of this technique between different laboratories. Consequently, a single cDNA sample was sent to four different laboratories located in the United States, the Netherlands and Sweden and analyzed in a blinded fashion. A summary of the results from each laboratory demonstrated a high and consistent reproducibility for the methodological procedures. Thus, in this study, a method for standardized and quantitative RT-PCR analysis was established and shown to allow for reliable inter- and intra-laboratory comparisons of gene expression data. The technique was termed StaRT-PCR.

Transcript profiling of detoxification enzymes in oral keratinocytes (Paper III)

Based on the initial analysis of various CYP450 forms in cultured oral keratinocytes and the finding that immortalized cells exhibit similar or even elevated metabolic activities as compared to normal cells, transcript levels for several enzymes involved in various detoxification reactions were determined in normal and SVpgC2a cells. A combination of the microarray gene chip technique and StaRT-PCR was used to “profile” expression of such enzymes in normal keratinocytes from two donors and in SVpgC2a cells. As discussed above, the expression levels of various CYP450 forms were found to be quite low in both normal and immortalized cells. In contrast, transcripts for POR, the Ah-receptor and ARNT were generally expressed at higher levels in both cell types. Moreover, significantly elevated levels of CYP1A1, 1B1 and the Ah-receptor in SVpgC2a cells as compared to normal cells indicated that long term culturing may serve to induce the Ah-receptor pathway. On the other hand, increased levels of these transcripts may be coupled to phenotypic alterations associated with

immortalization. In this regard, elevated expression of CYP1B1 has previously been reported in a number of tumors although without the assessment of oral epithelium (Murray et al., 1997). Transcripts for enzymes involved in conjugation reactions were generally detected at higher levels as compared to CYP450 transcript levels. Two forms of sulfotransferases were detected without significant alterations between normal and immortalized cells. Epoxide hydrolase, previously detected in primary oral keratinocytes (Farin et al., 1995), was not detected in normal cells whereas low non-quantifiable levels were detected in immortalized cells. The glucuronosyltransferase (UGT2B) as well as microsomal glutathione transferase (MGST1) included in the analysis were found at lower levels in immortalized cells as compared to normal cells. Various forms of glutathione transferases were detected in both cell types with the exception of GSTT1 that was absent in normal cells. Thus, the analysis indicated that most transcripts for enzymes involved in conjugation reactions were present in both normal and immortalized oral keratinocytes. Furthermore, the transcripts for enzymes involved in detoxification of reactive oxygen, i.e. catalase, superoxide dismutase and glutathione peroxidase, were detected at relatively high levels in both cell types. Consequently, the qualitative and quantitative assessment of transcripts for enzymes involved in various detoxification reactions indicated a substantial capacity for conjugation and reactive oxygen detoxification reactions, whereas CYP450 dependent metabolism was likely lower. In agreement with the studies in paper I, the comparison between normal and immortalized oral keratinocytes indicated relatively few alterations in gene expression. The few noted differences are potentially of a compensatory nature and may reflect efforts to maintain an overall detoxification capacity. However, some of the differences may mirror changes associated with an altered, immortalized phenotype, e.g. CYP1B1. Given that the relative expression of enzymes involved in phase I and II reactions was not previously studied in cultured oral cells, the studies in paper III identified several transcripts not previously reported for oral keratinocytes, e.g. CYP1B1 and MGST1. Moreover, the results obtained by StaRT-PCR were in good agreement with the microarray results although the latter technique was found to be up to 100-fold less sensitive. Microarray techniques may therefore serve as valuable tools in the screening of changes in gene expression among a large number of genes. Our results however, underline the importance for alternative, more accurate techniques for confirmation of results obtained from microarrays.

Smokeless tobacco toxicity in oral epithelium

Smokeless tobacco toxicity in cultured oral keratinocytes (Paper IV)

While several studies have addressed the correlation between oral cancer and smokeless tobacco usage, few studies have investigated the mechanisms underlying smokeless tobacco toxicity in human oral keratinocytes. On this basis, the toxicity exerted by a smokeless tobacco extract (STE) prepared from Scandinavian moist snuff was studied in normal oral keratinocytes. Using a 3 h exposure protocol followed by up to 45 h of cellular recovery, experiments were designed to indicate the relative involvement of non-PCD and PCD in the toxicity exerted by STE. With trypan blue as a marker for loss of membrane integrity, morphological hallmarks as indicators of apoptosis and expression of involucrin as a marker for differentiation, STE was shown to reduce net growth and induce cell death primarily by necrosis in a dose dependent manner in cultured oral cells. In contrast, only minute increases of apoptosis were detected throughout the STE concentration range. Moreover, expression of involucrin was not altered by exposure to STE. These results agree with previous studies where other types of STE were shown to induce primarily non-PCD although those studies used a different exposure protocol and also reported elevated levels of apoptosis (Bagchi et al., 1997; Bagchi et al., 1999). Thus, toxicity exerted by STE is likely to involve primarily non-programmed mechanisms of death in cultured oral keratinocytes.

p53 associated apoptosis in cultured oral keratinocytes (Paper IV)

Based on the fact that the tumor suppressor protein p53 plays a central role in the protection against development of various cancers, the well established cancer therapeutic and DNA-damaging agent mitomycin C (MMC) was evaluated in the search for a p53-associated apoptosis control in cultured oral cells. The amount of p53 protein in MMC-exposed oral keratinocytes was analyzed by immunocytochemical analysis followed by computerized image analysis. Apoptosis was assessed by morphological hallmarks as well as by parallel FACS analysis. Using an identical protocol as for the STE exposure experiments, exposure to MMC (1 and 10 µg/ml) was shown to reproducibly reduce net growth, induce expression of the p53 protein and cause apoptotic cell death in oral keratinocytes. Although it can not be ruled out that MMC activates apoptosis by several mechanisms, the results demonstrated that our conditions and methods reproducibly allow for the assessment of p53 induction and apoptosis in cultured oral keratinocytes. Moreover, MMC was previously shown to induce p53-

dependent apoptosis in both tumor-derived and normal keratinocytes (Merlo et al., 1995; Xu et al., 1995). Thus, based on our results and previous studies, MMC is likely to constitute a useful positive control for evaluation of STE toxicity, apoptosis and p53 induction in cultured oral keratinocytes.

Expression of apoptosis related genes in oral keratinocytes exposed to STE (Paper IV)

Although some studies do not correlate expression of the tumor suppressor protein p53 to lesions caused by smokeless tobacco usage (Ibrahim et al., 1996; Merne et al., 2002), several other studies have demonstrated significantly increased levels of this tumor suppressor in tobacco related lesions (Field et al., 1994; Wood et al., 1994; Brennan et al., 1995; Wedenberg et al., 1996). Expression of p53 was therefore investigated in cultured oral cells exposed to various concentrations of STE. Oral keratinocyte cultures from five donors were exposed to STE with a resulting variable increase in the expression of p53. Three cell lines showed clear upregulation of p53 although without an obvious dose-effect relationship while two cell lines showed small changes in p53 expression. Importantly, using exposure to MMC as a positive control, all cell lines demonstrated the ability to stabilize p53 upon exposure to DNA damaging agents. Moreover, none of the cell lines underwent apoptotic cell death following exposure to STE whereas MMC exposure consistently induced apoptosis. Thus, it appears that oral keratinocytes from different donors respond differently to STE induced toxicity as demonstrated by differential expression of the tumor suppressor protein p53. Furthermore, using one “p53 responsive” culture, StaRT-RCR analysis of transcript levels for p21 and bax did not demonstrate any significant alteration of these transcripts following exposure to STE thus indicating that stabilized p53 did not mediate transcriptional activation of these genes. In contrast, transcript levels of the anti-apoptotic gene Bcl-2 were slightly elevated in STE exposed oral cells. Overall, the results agreed with previous studies where some investigators correlated p53 expression to smokeless tobacco usage whereas others did not (Wood et al., 1994; Ibrahim et al., 1996; Wedenberg et al., 1996; Merne et al., 2002). However, slight increases of Bcl-2 transcripts in STE exposed oral keratinocytes indicated that smokeless tobacco may have anti-apoptotic effects.

Inhibition of apoptosis in cultured oral keratinocytes (Paper IV)

Based on the known inhibitory effect on apoptosis by the major tobacco alkaloid nicotine (Wright et al., 1993; Maneckjee and Minna, 1994; Heusch and Maneckjee, 1998), and the increased levels of Bcl-2 transcripts in STE exposed oral keratinocytes, we next evaluated the

influence of STE on MMC induced apoptosis. Pre-exposure of oral keratinocytes to non-toxic doses of STE was shown to markedly reduce MMC induced apoptosis. The cells were washed twice between the various treatments to ensure minimal interaction of the agents in the growth medium. A similar protocol using post-exposure of STE to MMC treated cells showed similar decreases in apoptosis frequencies, indicating that STE did not reduce the availability of MMC to the cells but rather affected the apoptotic process. Based on earlier nicotine toxicity studies from our laboratory (Liu et al., 1993) and the known content of nicotine in STE, similar experiments were performed using nicotine instead of STE. The results showed a similar degree of apoptosis inhibition and thus indicated that nicotine may in part account for the inhibitory effect exerted by STE. The results suggest that low and relatively non-toxic doses of STE inhibit apoptosis. These results may appear to contradict previous studies where STE was shown to induce apoptosis (Bagchi et al., 1999; Mangipudy and Vishwanatha, 1999). However, those studies were performed using relatively high and toxic doses of STE. In this regard, recent studies using organotypic cultures of oral keratinocytes have in fact shown that high doses of STE decrease keratinocyte proliferation whereas low doses stimulate proliferation (Wang et al., 2001). It should be noted that the various STE and nicotine doses used in our experiments are comparable to nicotine concentrations in saliva of snuff users (Curwall M, Swedish Match Snuff Division, personal communication).

Assessment of snuff induced oral lesions (Paper IV)

To study the various effects of smokeless tobacco *in vivo*, tissue specimens from normal oral mucosa, SDL and OLP were analyzed for mitotic and apoptotic index, lymphocyte infiltration, and p53 and Bcl-2 expression. OLP specimens were included as a reference lesion on the basis of its well documented expression of p53 and elevated levels of apoptosis in basal keratinocytes (Dekker et al., 1997; Bloor et al., 1999). Using morphological criteria for scoring of apoptosis and mitosis frequencies, the mitotic index in SDL was found to be significantly elevated as compared to normal tissue. In contrast, the apoptotic frequencies were very similar. In OLP specimens the apoptosis frequencies were, as expected, significantly elevated as compared to normal mucosa and SDL. Moreover, scoring of sub-epithelial infiltration of lymphocytes as an index of inflammation in the respective lesions, confirmed the generally held view that both SDL and OLP lesions are associated with inflammation (Axell, 1993; Sugerman et al., 2000). Furthermore, immunohistochemical analysis of p53 expression revealed scattered p53 positive cells in approximately half of the normal cases whereas almost all SDL specimens showed this pattern. Moreover, four SDL specimens showed relatively

frequent p53 staining and one showed strong staining. Similarly, most of the normal specimens showed scattered Bcl-2 positive cells whereas almost all SDL specimens expressed Bcl-2 at a higher frequency. OLP specimens showed consistently strong staining for p53 in the basal layer whereas only four specimens showed scattered Bcl-2 positive cells. Thus, the results suggest that lesions associated with habitual usage of snuff showed increased expression of the tumor suppressor p53, whereas apoptosis frequencies were similar to those found in normal tissue. Elevated levels of the anti-apoptotic protein Bcl-2 in SDL indicated that smokeless tobacco usage may have anti-apoptotic effects. Moreover, the OLP specimens served in their expected role as a p53 positive apoptosis control. Although it can not be ruled out that p53 may have functions other than to induce apoptosis in SDL, the overall *in vivo* results agreed with the results from cultured oral keratinocytes. Thus, exposure to low doses of smokeless tobacco may variably upregulate the tumor suppressor p53 as well as the anti-apoptotic Bcl-2 protein, and inhibit induction of apoptosis in human oral epithelium.

Influences of STE on caspase activity (Paper IV)

Since apoptosis induction by p53 has recently been suggested to involve mechanisms other than transactivation and transrepression of various p53 target genes, influences of STE on caspase 3 activity was investigated (Chandler et al., 1997; Fuchs et al., 1997; Ding et al., 1998; Liu et al., 2000). Although small but significant increases in caspase 3 like activity were detected in oral keratinocytes exposed to MMC, hematopoietic Jurkat cells, where caspase activity has been closely linked to induction of apoptosis were chosen as a model system. Upon treatment of Jurkat cells with anti CD95-antibody or staurosporin, the cells showed typical apoptosis morphology and highly increased activity of caspase 3 as measured by the cleavage of DEVD-AMC. However, upon pre-treatment of these cells with STE, the cleavage of the substrate peptide significantly decreased as compared to the respective positive control, notably without inducing significant toxicity to the cells. Moreover, subsequent experiments in paper V demonstrated that CD95-mediated apoptosis was significantly inhibited also in oral keratinocytes pre-exposed to STE. These results thus indicate that STE can inhibit apoptosis in a different cell system and from stimuli other than MMC. Moreover, they suggest that one mechanism whereby smokeless tobacco can inhibit apoptosis is through inhibition of caspases.

Smokeless tobacco toxicity in cultured malignant oral keratinocytes (Paper V)

To further assess the toxicity exerted by STE and to compare the responses of normal and malignant oral keratinocytes, toxicity experiments similar to those initially described for normal oral cells were performed on malignant (SqCC/Y1) cells. Using the sensitive colony forming efficiency assay, SqCC/Y1 cells were shown to be at least ten-fold more resistant to STE toxicity than normal cells. Moreover, from measurements of trypan blue uptake as a marker for necrosis, morphological hallmarks as indicators of apoptosis and expression of involucrin as a marker for differentiation, SqCC/Y1 cells were found to be similar to normal cells, primarily undergoing necrosis following STE exposure. The higher resistance of SqCC/Y1 to STE induced toxicity was further confirmed by these experiments. The slight increases in apoptosis frequencies noted at relatively high concentrations of STE in both cell lines were likely independent of a functional p53 protein, since SqCC/Y1 cells carry a single mutated p53 allele (Reiss et al., 1992). The results agree with previous observations from our laboratory where SqCC/Y1 cells were shown to be relatively resistant to several factors that inhibited growth or induced cell death (Sundqvist et al., 1991). Furthermore, a higher resistance of tumor cells to smokeless tobacco toxicity may promote selective expansion of tumor cells in the oral mucosa of smokeless tobacco users.

Inhibition of receptor-mediated apoptosis in oral keratinocytes (Paper V)

To extend the previous observation that STE significantly inhibited MMC-induced apoptosis in cultured keratinocytes, the effect of STE on receptor mediated apoptosis, i.e. TGF- β and CD95-mediated apoptosis, was studied. Since the malignant SqCC/Y1 cells were previously shown to be resistant to both CD95 and TGF- β mediated apoptosis they were not included in this analysis (unpublished observation). Both anti-CD95 and TGF- β treatments resulted in elevated frequencies of apoptosis in normal cells. Using an identical pre-exposure protocol as that previously employed for the experiments on MMC-induced apoptosis, STE was shown to significantly reduce the levels of apoptosis from the anti-CD95 and TGF- β positive controls. Thus, smokeless tobacco is likely to inhibit apoptosis induced via several pathways including those from DNA damaging agents like MMC and stimulation of various cellular receptors like the CD95 and TGF- β receptors. Moreover, inhibition of CD95 mediated apoptosis may be coupled to inactivation of caspases as was observed in Jurkat cells.

Toxicity comparison of North American and Scandinavian STE (Paper V)

Based on recent studies demonstrating considerable differences between smokeless tobacco products from North America and Scandinavia (Brunnemann et al., 2002), as well as substantial variations in extract preparation procedures, a comparison between North American and Scandinavian STE was carried out. For this analysis, a commercially available brand of North American moist snuff was compared to the Scandinavian moist snuff product used in our previous experiments. The exposure protocol employed was identical to the one utilized in our previous smokeless tobacco experiments. Both extracts reduced net growth of normal oral keratinocytes without significant differences between them. In addition, no significant increase in apoptosis frequencies was observed at the various concentrations tested. However, when exposing the cells to STE for periods longer than 3 hours, significant levels of apoptosis were detected although again without differences between the two extracts. Moreover, under these exposure conditions, the majority of the cells had lost membrane integrity and stained positive for trypan blue. Thus, the results implied minor or no differences between the two extracts in terms of direct toxicity, suggesting that the various results obtained from experiments using Scandinavian STE may also be applicable to other types of smokeless tobacco products.

Conclusions

In this study, expression and to some extent function of various biotransformation enzymes were investigated in oral epithelium and cultured cells. The comparative expression analysis of cytochrome P450 enzymes, conjugation enzymes and reactive oxygen detoxification enzymes in tissue specimens as well as normal and immortalized keratinocyte lines generally demonstrated a similar expression pattern of these enzymes, whereas malignant keratinocytes were found to be less metabolically active. The results thus indicate that both normal oral keratinocytes and the SVpgC2a cell line constitute valuable *in vitro*-model systems for the exploration of oral disease processes. The capacity of cultured oral keratinocytes to activate tobacco specific nitrosamines and the mycotoxin aflatoxin B₁ further underlines the usefulness of these cell lines in various toxicity studies, including smokeless tobacco related toxicity studies. From another point of view, expression profiling and substrate conversion analysis of various detoxification enzymes provided interesting insights concerning the multi-step process of oral cancer development. In this regard, the immortalized SVpgC2a cell line may be viewed as a model for pre-malignant oral lesions. Thus, although this cell line resembles normal oral keratinocytes with regards to detoxification capacities, the finding that certain enzymes, e.g. CYP1B1 and 2E1, are expressed at comparatively higher levels may indicate that such differences are of importance during malignant transformation of oral cells.

Subsequent employment of cultured normal oral keratinocytes in smokeless tobacco toxicity studies and assessment of biopsies from smokeless tobacco induced oral lesions revealed that high doses of smokeless tobacco are likely to exert simple, toxic effects with minimal or no involvement of programmed cell death mechanisms. Involvement of the tumor suppressor protein p53 in smokeless tobacco related toxicity was variably indicated from both *in vivo* and *in vitro* observations. An association between p53 stabilization and induction of apoptosis was however not observed. In contrast, low doses of smokeless tobacco were shown to inhibit p53 associated apoptosis. Furthermore, a similar effect was observed by the major tobacco alkaloid nicotine. The results thus suggest that exposure to smokeless tobacco may impede cellular processes of critical importance in the regulation of normal tissue homeostasis as well as in the protection against cancer development. The snuff dippers lesion has traditionally been described as a hyper-proliferative lesion with increased thickening accompanied by hyperkeratosis. The results from this study imply that inhibition of apoptosis may be of

importance in smokeless tobacco related pathology. Moreover, the results suggest that various tobacco products may exert adverse health effects through nicotine, a finding of potential importance also in other tobacco related diseases.

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