

Cytochrome P450 expression and related metabolism in human buccal mucosa

Martin Vondracek, Zheng Xi, Pia Larsson¹, Valerie Baker², Katherine Mace³, Andrea Pfeifer³, Hans Tjälve¹, M.Teresa Donato⁴, M.José Gomez-Lechon⁴ and Roland C.Grafström⁵

Institute of Environmental Medicine, Karolinska Institutet, Box 210, S-171 77 Stockholm, ¹Department of Pharmacology and Toxicology, Swedish University of Agricultural Sciences, Box 573, S-751 23 Uppsala, Sweden, ²Unilever Research Laboratory, Colworth House, Sharnbrook, Bedfordshire, MK44 1LQ, UK, ³Nestle Research Center, CH-1000 Lausanne 26, Switzerland and ⁴Unit of Experimental Hepatology, University Hospital La Fe, E-46009 Valencia, Spain

⁵To whom correspondence should be addressed
E-mail: roland.grafstrom@imm.ki.se

Constituents in food and fluids, tobacco chemicals and many drugs are candidates for oral absorption and oxidative metabolism. On this basis, the expression of cytochrome P450 isozymes (CYPs) and the conversion of CYP substrates were analysed in reference to buccal mucosa. A RT-PCR based analysis of human buccal tissue from 13 individuals demonstrated consistent expression of mRNA for the CYPs 1A1, 1A2, 2C, 2E1, 3A4/7 and 3A5. CYP 2D6 was expressed in six out of the 13 specimens, whereas all samples were negative for 2A6 and 2B6. Serum-free monolayer cultures of the Siman virus 40 large T-antigen-immortalized SVpgC2a and the carcinoma SqCC/Y1 buccal keratinocyte lines expressed the same CYPs as tissue except 3A4/7 and 3A5 (SVpgC2a), and 2C, 2D6 and 3A4/7 (SqCC/Y1). Dealkylation of ethoxyresorufin and methoxyresorufin in both normal and transformed cells indicated functional 1A1 and 1A2, respectively. SVpgC2a showed similar activity as normal keratinocytes for both substrates, whereas SqCC/Y1 showed about 2-fold lower 7-ethoxyresorufin *O*-deethylation and 7-methoxyresorufin *O*-demethylation activities. SVpgC2a showed detectable and many-fold higher activity than the other cell types towards chlorzoxazone, a substrate for 2E1. Absent or minute catalytic activity of 2C9, 2D6 and 3A4 in the various cell types was indicated by lack of detectable diclofenac, dextromethorphan and testosterone metabolism (<0.2–0.5 pmol/min/mg). Metabolic activation of the tobacco-specific *N*-nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and the mycotoxin aflatoxin B₁ (AFB₁) to covalently bound adducts was indicated by autoradiographic analysis of both monolayer and organotypic cultures of SVpgC2a. In contrast, SqCC/Y1 showed lower or absent metabolic activity for these substrates. Finally, measurements of various non-reactive AFB₁ metabolites indicated rates of formation <0.1 pmol/min/mg in both normal and transformed cells. The results indicate presence of several CYPs of which some may contribute

to significant xenobiotic metabolism in human buccal epithelium. Notably, metabolic activation of AFB₁ was not previously implicated for oral mucosa. Further, the results show that CYP-dependent metabolism can be preserved or even activated in immortalized keratinocytes. Metabolic activity in SVpgC2a under both monolayer and organotypic culture conditions suggests that this cell line may be useful to pharmaco-toxicological and carcinogenesis studies.

Introduction

The cytochrome P450 (CYP) family of enzymes is responsible for oxidative metabolism of numerous endogenous and exogenous chemicals (1). The elimination of lipophilic chemicals is generally simplified following metabolism catalysed through CYPs, although chemicals may also be converted into reactive intermediates. Although the liver represents the major site for CYP-dependent metabolism, the extrahepatic tissues also express significant activity (2,3). Differential expression/activity of CYPs may possibly underlie the organ specificity of chemical carcinogens in various epithelia (4). Metabolism through CYPs in the oral mucosal lining could influence the uptake of drugs prescribed for oral/sublingual administration, and furthermore, may generate toxic and mutagenic products from procarcinogens in food, tobacco and other sources. The buccal mucosa is a primary site for oral cancer in high incidence areas (5). Moreover, the buccal mucosa represents the dominant surface area in direct contact with various agents, e.g. tobacco and betel quid.

Several observations have implied the existence of drug metabolism through CYPs in oral epithelium. Oral cancer associated to tobacco smoking and smokeless tobacco usage may involve CYP450-dependent activation of procarcinogens, including *N*-nitrosamines and polycyclic aromatic hydrocarbons (6,7). Further, exposure of laboratory animals to such carcinogens can induce oral squamous cell carcinoma (8). Cultured human oral keratinocytes metabolize the polycyclic aromatic hydrocarbon benzo[*a*]pyrene to DNA binding intermediates, notably at several-fold higher rates than oral fibroblasts (9). The tobacco-specific *N*-nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) is metabolized by α -hydroxylation and pyridine *N*-oxidation reactions to various metabolites and covalently bound intermediates in the epithelium of tissue explants and cultured normal keratinocytes from buccal mucosa (10). Although without an analysis of metabolic competency, human primary and papilloma virus oncogene-immortalized oral keratinocytes were shown to exhibit mRNA transcripts for several CYP450s involved in foreign compound metabolism (11).

Various drug substrates are available for characterization of the involvement of specific CYP450s, whereas metabolism of chemical carcinogens, e.g. NNK and aflatoxin B₁ (AFB₁), commonly involves several CYP450s (1,4). The possible metabolic conversion of AFB₁, a well-established hepato-

Abbreviations: AFB, aflatoxin B₁; AFL, aflatoxicol; AFM₁, aflatoxin M₁; AFP₁, aflatoxin P₁; AFQ₁, aflatoxin Q₁; CYP, cytochrome P450; EMA, elevated amino acids; HSM, high serum medium; NNK, *N*-nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; PBS, phosphate-buffered saline.

Table I. Primer sequences used in the analysis of cytochrome P450 (CYP)

CYP gene	Forward primer	Reverse primer	PCR fragment size (nt)
1A1	1190GGAGGCCTTCATCCTGGAGA	1485CCTCCCAGCGGGCAACGGTC	295
1A2	1112GGAGGCCTTCATCCTGGAGA	1391TCTCCCACTGGCCAGGACT	299
2A6	772CAACCAGCGCACGCTGGATC	1195CCAGCATAGGGTACACTTCG	423
2B6/7	843ACACAGTGAATTCAGCCACC	1132TGGTGTGTTGGGTGACAATG	289
2C	CCAGAGGTCACAGCTAAAGT	CCTGCTGAGAAAAGGCATGAA	344
2D6	883CCTGCGCATAGTGGTGGCTG	1236GCTTCTCCCAGACGGCCTCA	353
2E1	1029TGCCATCAAGGATAGGCAAG	1385AATGCTGCAAAATGGCACAC	356
3A4/7	1278TGACCCAAAGTACTGGACAG	1663CTATTCACAAAGTAATTGAG	385
3A5	1297TGACCCAAAGTACTGGACAG	1536TGAAGAAGTCCTTGCCTGTC	239

Table II. Expression of cytochrome P450 (CYP) subtypes in human buccal tissue and in the SV40 T-antigen-immortalized (SVpgC2a) and tumour (SqCC/Y1) buccal keratinocyte lines

Type of CYP	Expression by RT-PCR analysis ^a			
	Tissue	(n) ^b	SVpgC2a	SqCC/Y1
1A1	+	(13/13)	+	+
1A2	+	(13/13)	+	+
2A6	-	(0/13)	-	-
2B6	-	(0/13)	-	-
2C	+	(13/13)	+	-
2D6	+/-	(6/13)	+	-
2E1	+	(13/13)	+	+
3A4/7	+	(13/13)	-	-
3A5	+	(13/13)	-	+

^aRT-PCR reactions were conducted with actin primers as internal controls. Isolated RNAs from genetically engineered cell lines with stable expression of different CYP450 were used as positive controls as described in Materials and methods.

^bThe fraction of individuals which expressed the indicated type of CYP ($n = 13$).

Table III. Cytochrome P450 substrate metabolism in normal, SV40 T-antigen-immortalized (SVpgC2a) and tumor (SqCC/Y1) human buccal epithelial cells^a

Substrate	CYP isoform	Cell line		
		HOE (pmol/mg protein × min)	SVpgC2a (pmol/mg protein × min)	SqCC/Y1 (pmol/mg protein × min)
Ethoxyresorufin ^b	1A1	0.900 ± 0.036	1.020 ± 0.119	0.420 ± 0.055 ^d
Methoxyresorufin ^b	1A2	0.893 ± 0.071	0.990 ± 0.147	0.490 ± 0.055 ^d
Diclofenac ^c	2C9	<0.5	<0.5	<0.5
Dextromethorphan ^c	2D6	<0.4	<0.4	<0.4
Chlorzoxazone ^c	2E1	<0.5	14.6 ± 0.1	<0.5
Testosterone ^c	3A4	<0.2	<0.2	<0.2

^aResults are presented as the mean ± SEM from duplicate determinations in three separate experiments.

^bCells were incubated with the respective substrate for 1 h in serum-free growth medium (20). The respective media were analysed for dealkylated products by fluorescence spectroscopy.

^cCells were incubated with the respective substrate for 1, 6 and 24 h in serum-free and pituitary extract-free growth medium (20). The respective media were analysed by HPLC.

^dSignificantly different from normal cells ($P < 0.05$, ANOVA with Dunnett multiple comparisons test).

carcinogen (12), has not been reported for oral epithelium. The parallel assessment of CYP450 expression and related metabolism in cultured oral keratinocytes may provide informa-

tion as to the functionality of the xenobiotic-metabolizing CYP450s in oral mucosa. Since loss of ability for CYP450 metabolism is commonly associated with cell culture procedures (13), the associated analysis of oral tissue would provide a reference to the situation *in vivo*. Cytopathology associated with dental materials and usage of betel and tobacco was previously studied without characterization of the possible involvement of drug metabolizing enzymes in normal, Simian virus 40 T-antigen-immortalized (SVpgC2a) and tumor (SqCC/Y1) buccal keratinocytes (14). Thus, the aspect of loss or retention of normal (tissue-like) drug metabolism capacity in human oral keratinocyte lines requires further attention (10,14,15).

In this study, tissue specimens, normal keratinocytes and the cell lines SVpgC2a and SqCC/Y1 were included to simultaneously analyse expression and function of xenobiotic metabolizing CYP450s in human buccal epithelium. Presence of CYP1A1, 1A2, 2A6, 2B6, 2C, 2D6, 2E1, 3A4/7 and 3A5 was determined by RT-PCR in efforts to complement and substantiate previous work (11). The metabolism of drug substrates with specificity towards CYP1A1, 1A2, 2C9, 2D6, 2E1 and 3A4, i.e. ethoxyresorufin, methoxyresorufin, diclofenac, dextromethorphan, chlorzoxazone and testosterone, respectively, was analysed *in vitro*. Further, the possible metabolic activation of two chemical carcinogens in tobacco and food, NNK and AFB₁, respectively, was analysed by incubation of both monolayer and organotypic cultures with radiolabelled compounds. An autoradiographic analysis previously applied for detection of carcinogen adducts in buccal explant cultures (10) was complemented by measurement of AFB₁ metabolites by HPLC (16). The results extend CYP450 expression in primary oral keratinocytes to intact (non-cultured) tissue and immortalized and malignant cell lines, indicate that certain CYP450s are functional in oral epithelium, and suggest that AFB₁ is a possible oral carcinogen that can contribute to the carcinogenicity of tobacco.

Materials and methods

Monolayer cultures

Human buccal tissue was obtained from non-cancerous patients undergoing maxillofacial surgery with approval of the Karolinska Institute ethical committee. Primary cultures of normal keratinocytes were obtained after incubation of tissue specimens with 0.17% trypsin in phosphate-buffered saline solution (PBS) at 4°C for 18–24 h, and seeding of the cells at 5×10^3 cells/cm² on fibronectin/collagen-coated dishes in Epithelial medium with elevated amino acids (EMA) (17). For inclusion in organotypic cultures, outgrowths of fibroblasts were derived from tissue explants maintained in high serum medium (HSM) (18). Fibroblast cell lines were subsequently transferred and grown as in low serum medium (LSM) (18). The immortal cell line SVpgC2a, derived by transfection and stable integration of the SV40 T-antigen gene to human

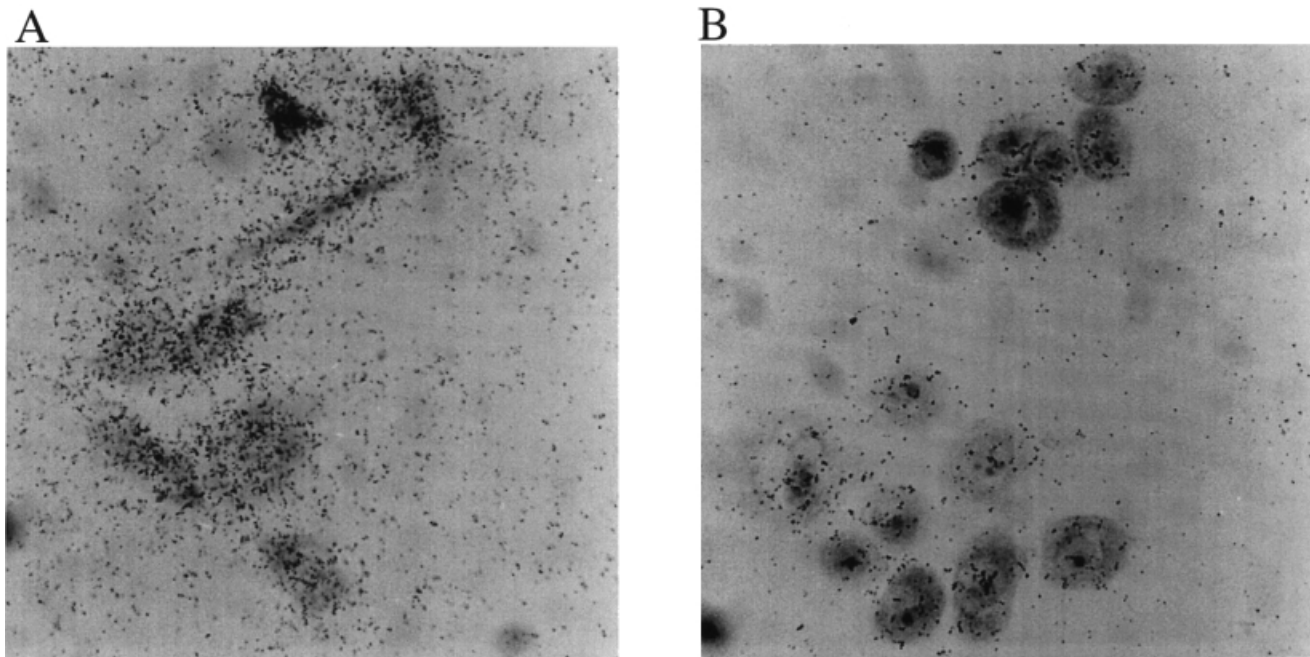


Fig. 1. Microautoradiograms of (A) SVpgC2a and (B) SqCC/Y1 exposed to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in monolayer culture. The respective cell lines were grown on glass slides and exposed to 17 μM NNK for 24 h as described in Materials and methods. The metabolic activation of NNK is indicated by the presence of silver grains over the respective cell surface areas. Original magnification $\times 200$.

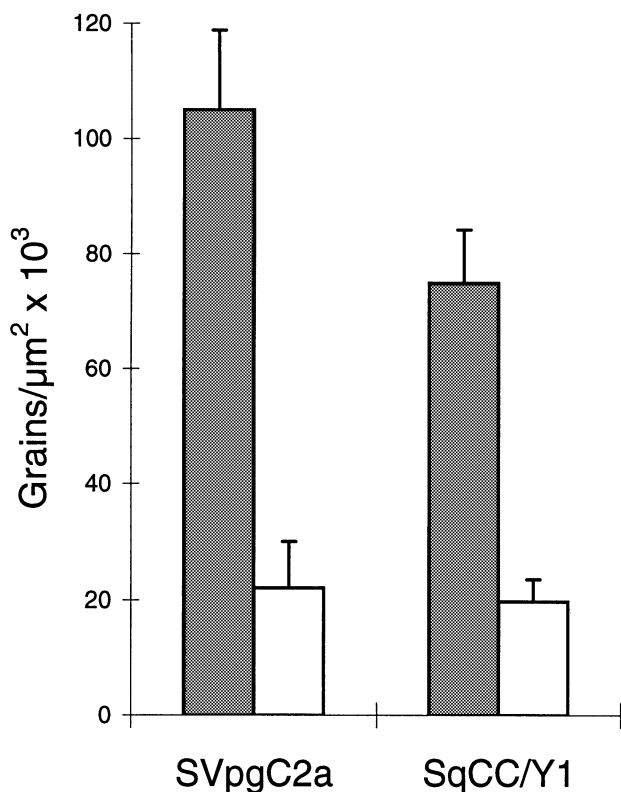


Fig. 2. Assessment of metabolic activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in SVpgC2a and SqCC/Y1. Autoradiograms, as depicted in Figure 1, were analysed by microscopic counting of the number of silver grains covering the cell surface areas. Background was determined from assessment of the number of grains covering an adjacent, cell-free surface area of the same size. A minimum of 500 cells was scored in each experiment. The results are mean \pm SEM from three separate experiments. For both cell types, the number of grains was significantly increased over the cell surface areas as compared with background (Student's *t*-test; $P < 0.05$). ■, Cell surface areas; □, background.

buccal keratinocytes (19), and the buccal carcinoma cell line SqCC/Y1 were both cultured in EMA. All cell lines were grown on regular non-coated Petri dishes. The protocols (seeding density and time required to reach the preferred states of confluency) utilized for cell passage were as follows: normal keratinocytes seeded at 5×10^3 cells/cm² reach 75% confluency at 4–6 days; normal fibroblasts seeded at 7×10^3 cells/cm² reach 100% confluency at 5–7 days; the SVpgC2a cells seeded at 4.1×10^3 cells/cm² reach 100% confluency at 5 days; and the SqCC/Y1 cells seeded at 1×10^4 cells/cm² reach 90% confluency at 5 days (14). Normal keratinocytes were used in first passage, fibroblasts in passages 3–5 (for the generation of organotypic cultures, see below), the SVpgC2a line in passages 59–64, and the SqCC/Y1 line in passages 115–120. The preparation procedure and compositions of the various growth media were recently summarized (20). For the RT-PCR and metabolic assessment of CYPs in the respective keratinocyte lines in monolayer culture (see below), the cells were seeded at 2.5×10^3 /cm² and incubated in 60 mm dishes in EMA for 3–4 days, assuring that proliferative subconfluent cultures were analysed.

Organotypic cultures

Organotypic epithelia of the SVpgC2a and SqCC/Y1 cells were regenerated *in vitro* as recently described (21). Briefly, type I collagen was extracted from rat-tail tendons (22) and gels prepared on ice by mixing a 4 mg/ml collagen solution (in 0.1% acetic acid) with $10 \times$ Hank's balanced salt solution and serum to a ratio of 8:1:1 and a pH of 7.2 to 7.4. Gels were fabricated with fibroblasts at a density of 10^5 cells/ml (23) and polymerized by incubating 1 ml of the mixture to each of 24 wells at 37°C for 60 min. Thereafter, collagen gels were equilibrated for 18 h in EMA. The SVpgC2a and SqCC/Y1 cells were suspended at 3×10^5 cells/ml in EMA before being seeded onto collagen gels. After 24 h at 37°C and the formation of a confluent monolayer, the gels were transferred to stainless-steel grids. Each grid was placed in a 60 mm dish and the medium (containing 1 mM Ca²⁺) added to a level that positioned the epithelial monolayer at the air-liquid interface. The cultures were then incubated for 10 days with medium changes at 2 day intervals for generation of the organotypic structures utilized in the metabolism studies (NNK and AFB₁).

RT-PCR analysis

Extraction of RNA from the oral tissue samples (snap frozen in liquid nitrogen at the time of surgery) and the cell lines was carried out using the RNeasytotal RNA purification system (Qiagen AG, Basel, Switzerland). The synthesis of cDNA and the PCR reactions of single-stranded cDNA from 7.5 μg total RNA was performed with the First Strand cDNA Synthesis kit (Boehringer Mannheim, Rotkreuz, Switzerland) using oligo d(T)₁₅ primers synthesized by

Genset (Paris, France); the sequences and respective PCR product size are shown in Table I. Amplification reactions were conducted in a final volume of 50 μ l consisting of 2 μ l cDNA, 20 mM Tris-HCl pH 8.55, 16 mM $(\text{NH}_4)_2\text{SO}_4$, 2.5 mM MgCl_2 , 150 μ g/ml bovine serum albumin, 200 μ M each deoxynucleotide triphosphate (Boehringer Mannheim), 50 pmol each forward and reverse primer and 1.5 U *Taq* DNA polymerase (Biotaq; Bioprobe systems, Montreuil sous Bois, France). The reaction mixtures were heated for 2 cycles to 98°C for 1 min, 60°C for 2 min and 72°C for 2 min and then cycled 28 times through a 1 min denaturation step at 94°C, a 1 min annealing step at 60°C and a 2 min extension step at 72°C in a DNA Thermal Cycler apparatus (BioConcept, Allschwil, Switzerland). These conditions were used for all amplifications except with the CYP3A4/7 primers where the annealing step was performed at 50°C. All reactions were conducted with actin primers as internal controls. Isolated RNA from genetically engineered cell lines with stable expression of the respective CYP450s was used as a positive control in each RT-PCR experiment (24,25). An aliquot of 10 μ l from each reaction was separated on a 2% agarose gel and visualized by ethidium bromide staining. All PCR products were of the correct size ensuring that the results reflected amplification of cDNA and not genomic DNA.

Metabolism of various CYP substrates

Established methods were used to assess the metabolic activity of different CYPs in monolayer cultures of the normal, SVpgC2a and SqCC/Y1 cells (13,26–30). Briefly, the cells were incubated for up to 24 h with 10 μ M ethoxyresorufin, 10 μ M methoxyresorufin, 250 μ M diclofenac, 400 μ M chlorzoxazone, 100 μ M dextromethorphan or 250 μ M testosterone in EMA followed by collection of the respective medium. The cultures exposed to ethoxyresorufin and methoxyresorufin were exposed to 10 μ M dicumarol to inhibit conjugation of the dealkylated product. Medium samples from the incubations with 250 μ M diclofenac, 400 μ M chlorzoxazone, 100 μ M dextromethorphan and 250 μ M testosterone were incubated with β -glucuronidase/arylsulfatase for 2 h at 37°C before the metabolism analysis (13). Resorufin formed during 7-ethoxyresorufin *O*-deethylation and 7-methoxyresorufin *O*-demethylation assays was quantified fluorimetrically (27). The products from diclofenac 4' hydroxylation, chlorzoxazone 6-hydroxylation, dextromethorphan *O*-demethylation and testosterone 6 β -hydroxylation were measured by HPLC analysis (13,28–30).

NNK and AFB₁ metabolism

Aflatoxin B₁ generally labelled with thymidine [³H]AFB₁ (18 Ci/mmol) in methanol was obtained from Moravek Biochemicals (Brea, CA) and purified by reverse-phase LC as described (31). Carbonyl-¹⁴C-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (¹⁴C]NNK; 29.9 mCi/mmol) was obtained from Chemsyn

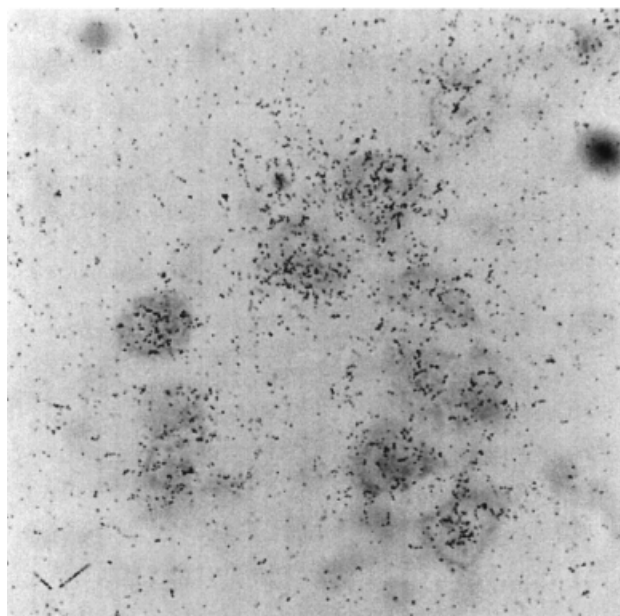
Science Laboratories (Lenexa, KS). For assessment of metabolic activation by autoradiography, the SVpgC2a and SqCC/Y1 cell lines were grown in EMA to 50–80% confluency on glass slides and then exposed for 24 h to 17 μ M [¹⁴C]NNK and 0.3 μ M [³H]AFB₁, respectively. Notably, these concentrations optimize the detection of bound radioactivity, i.e. higher levels of radioactivity increase background, and thus, decrease the sensitivity of the assay (32). Similarly, organotypic cultures were incubated for 24 h submerged in medium with identical concentrations of [¹⁴C]NNK and [³H]AFB₁. Subsequently, the monolayer cultures were fixed in 4% phosphate-buffered formaldehyde solution and then covered with Kodak NTB-2 emulsion. The organotypic cultures were fixed in phosphate-buffered formaldehyde, dehydrated in an ethanol series and embedded in Technovit 7100 (Heraeus Kulzer GmbH, Wehrheim, Germany). Sections (2 μ m) were then prepared and mounted on glass slides, followed by exposure to emulsion as above. Exposure of the slides was carried out for 2–4 weeks at 4°C, followed by photographic development, fixation and staining with toluidine blue dye. The extensive extractions in various media during the fixation procedures remove unbound radioactive material, and the autoradiograms therefore show only covalently bound radioactive material (32). More than 3-fold increases in the number of silver grains covering the cell or tissue surface areas, as compared with background, are indicative of significant conversion of the substrates to covalently bound adducts. Formation of AFB₁ metabolites was assessed in normal and SVpgC2a cells by HPLC. Cells were grown to 80% confluency and then exposed to 0.3 or 1.0 μ M [³H]AFB₁ as above. Diethylether extracts of the medium were evaporated under nitrogen flow and the residue was dissolved in 50:50 (v/v) methanol/water and analysed by HPLC (16). Medium was also incubated for 1 h at 37°C with β -glucuronidase and then extracted and analysed as above. The HPLC separation included aflatoxin M₁ (AFM₁), aflatoxin P₁ (AFP₁), aflatoxicol (AFL), aflatoxin Q₁ (AFQ₁) and the *endo*- and *exo*-GSH-AFB₁ conjugates using authentic standards as references; the level of detection corresponded to a metabolic rate of ≥ 0.1 pmol/mg/min for each metabolite (16).

Results

Expression of CYP450s in human buccal tissue and cultured human buccal keratinocyte lines

Presence of mRNA transcripts for the CYPs 1A1, 1A2, 2A6, 2B6, 2C, 2D6, 2E1, 3A4/7 and 3A5 was determined using RT-PCR (Table II). Analysis of tissue specimens obtained from 13 individuals demonstrated expression of these mRNAs with the exception of 2A6 and 2B6. The analysis of epithelium separated from connective tissue by micro-dissection of frozen

A



B

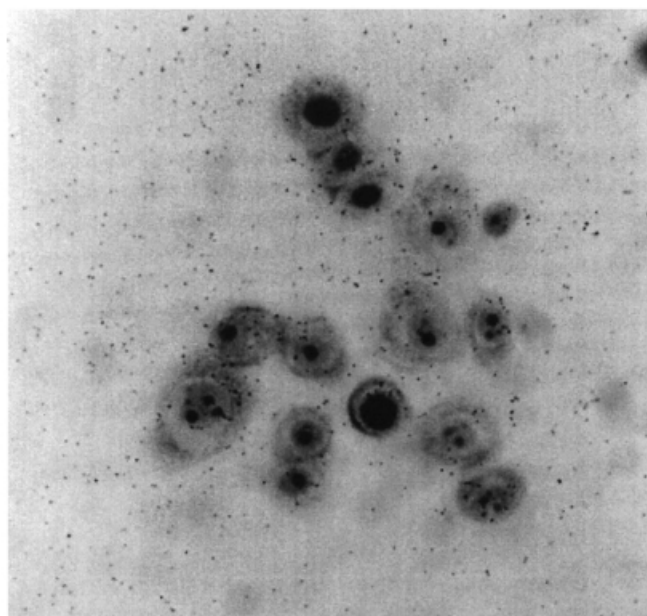


Fig. 3. Microautoradiograms of (A) SVpgC2a and (B) SqCC/Y1 exposed to AFB₁ in monolayer culture. The respective cell lines were grown on glass slides and exposed to 0.3 μ M [³H]AFB₁ for 24 h as described in Materials and methods. The metabolic activation of AFB₁ is indicated by the presence of silver grains over the respective cell surface areas. Original magnification $\times 200$.

sections gave identical results as for the intact specimen for two individuals (not shown). Only six of the 13 individuals expressed 2D6. SVpgC2a expressed transcripts for 1A1, 1A2, 2C, 2D6 and 2E1. SqCC/Y1 expressed transcripts for 1A1, 1A2, 2E1 and 3A5. As for tissue, transcripts of 2A6 and 2B6

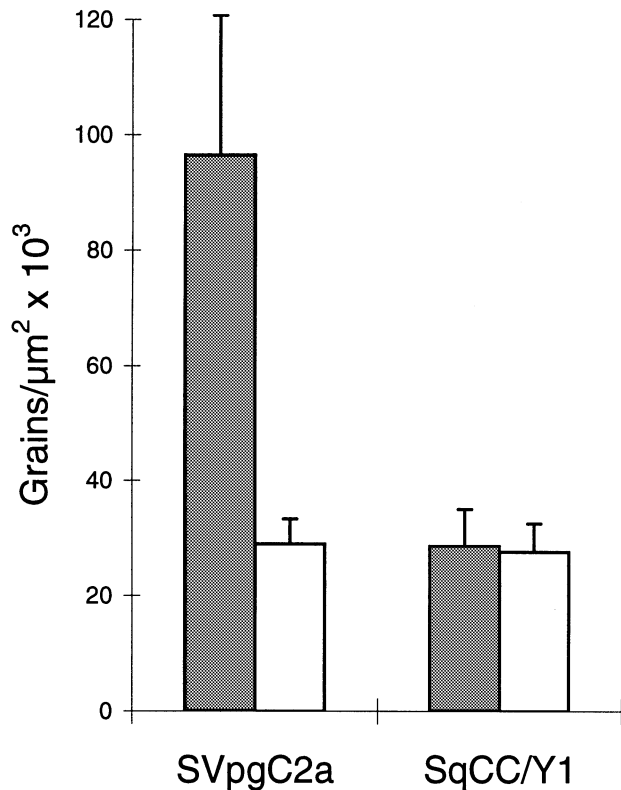


Fig. 4. Assessment of metabolic activation of AFB₁ in SVpgC2a and SqCC/Y1. Autoradiograms, as depicted in Figure 3, were analysed by microscopic counting of the number of silver grains covering the cell surface areas. Background was determined from assessment of the number of grains covering an adjacent, cell-free surface area of the same size. A minimum of 500 cells was scored in each experiment. The results are mean \pm SEM from three separate experiments. For SVpgC2a, the number of grains was significantly increased over the cell surface areas as compared with background (Student's *t*-test; *P* < 0.05). ■, Cell surface areas; □, background.

were not detected in the cell lines (Table II). Analysis of normal buccal keratinocyte cultures from two individuals demonstrated a CYP pattern identical to tissue (not shown).

CYP-dependent metabolism in vitro

Metabolism of substrates used to characterize CYP1A1, 1A2, 2C9, 2D6, 2E1 and 3A4, i.e. ethoxyresorufin, methoxyresorufin, diclofenac, dextromethorphan, chlorzoxazone and testosterone, respectively, was studied (Table III). Based on 1 h incubations, normal keratinocytes showed similar EROD and MROD activities as SVpgC2a whereas SqCC/Y1 showed lower activities. SVpgC2a showed significant chlorzoxazone 6-hydroxylation. In contrast, normal and SqCC/Y1 cells did not exhibit activity for this substrate. Oxidation of diclofenac to 4-hydroxydiclofenac, *O*-demethylation of dextromethorphan and 6 β -hydroxylation of testosterone was not detected in the three cell types. Normal keratinocytes from three donors showed similar negative results following incubation periods for up to 24 h (not shown).

Analysis of bound NNK and AFB₁ metabolites in the SVpgC2a and SqCC/Y1 cell lines

Autoradiographic technique was used to evaluate the ability of the SVpgC2a and SqCC/Y1 to metabolize the carcinogens NNK and AFB₁ (in radiolabelled forms) to reactive intermediates that would covalently bind to cellular constituents. Monolayer cultures of both cell lines showed significant conversion of NNK, i.e. about 5- and 4-fold higher numbers of silver grains were present on the cell surface areas of SVpgC2a and SqCC/Y1, respectively (Figures 1 and 2). SVpgC2a also converted AFB₁ to reactive intermediates whereas SqCC/Y1 showed a lack of AFB₁ metabolism (Figures 3 and 4). For both agents, active metabolism was associated with uniform labelling of the cells. A regenerated organotypic epithelium of the SVpgC2a cells metabolized NNK and AFB₁ (Figure 5) whereas the SqCC/Y1 cells showed lack of activity in organotypic culture (not shown). The metabolically active SVpgC2a cells form a homogeneous multi-layered epithelium composed of basaloid cells (Figure 5A) (21). The SVpgC2a epithelium showed uniform distribution of silver grains, present at about 5-fold higher levels than background for both carcinogens (Figure 5B and C). Brief heating of

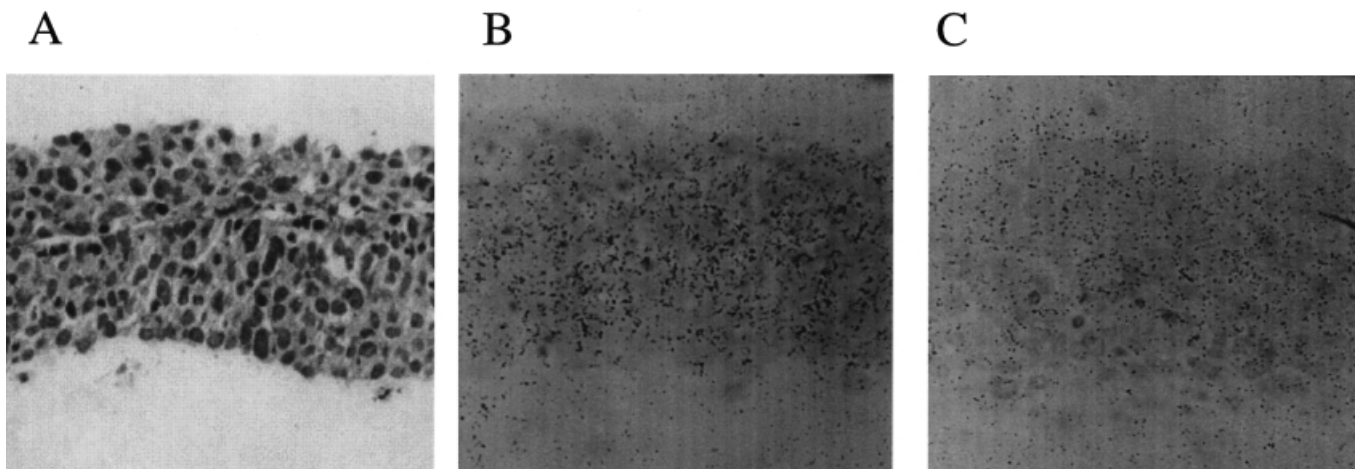


Fig. 5. Microautoradiogram showing metabolic activation of NNK and AFB₁ in SVpgC2a in organotypic culture. (A) A tissue equivalent was formed by co-culture of SVpgC2a with buccal fibroblasts embedded in a collagen lattice as described in Materials and methods. The cultures were exposed for 24 h to (B) 17 μ M [¹⁴C]NNK and (C) 0.3 μ M of ³H-AFB₁, respectively, and histological sections processed for autoradiography. Separate experiments showed about 5-fold increased number of grains over background for the respective agents. Original magnification \times 200.

the monolayer and organotypic cultures to 90°C abolished the labelling for both substrates (not shown). Finally, non-reactive AFB₁ metabolites, including AFM₁, AFP₁, AFL and the *endo*- and *exo*-glutathione AFB₁ conjugates, were not detected in the incubation medium of monolayer cultures of SVpgC2a or cultures from two normal donors (not shown).

Discussion

The oral mucosa is exposed to numerous chemicals as portal of entry, and substantial capacity for biotransformation could be expected. In this regard, the demonstration of transcripts for several CYPs in normal primary and immortalized oral keratinocytes (11) provided a major incentive for the present study. The current analyses in buccal mucosa included additional CYP transcripts and the potential for alterations during the *in vivo* to *in vitro* transition. Moreover, metabolism of selected CYP substrates and the chemical carcinogens NNK and AFB₁ were studied. The results extend the previously implicated expression pattern for oral CYPs, provide evidence for translation/activity of some but not all of the transcribed CYPs, and finally imply that SV40 T-antigen-immortalization of keratinocytes may preserve or even activate the capacity for xenobiotic metabolism.

RT-PCR analysis of 13 buccal tissue specimens indicated consistent presence of several CYP mRNA, including 1A1, 1A2, 2C, 2E1, 3A4/7 and 3A5, and 2D6 in six of the cases (cf. Table II). The result adds members of the 2C and 3A families to the CYPs previously implied from studies of primary oral keratinocytes (11). The 2D6 gene is highly polymorphic in the Scandinavian population (33). However, the 2D6 primers were designed to include polymorphic variants, implying that the results reflected absent or low (non-detectable) expression in some individuals. Normal keratinocytes showed an identical transcript pattern to tissue, and the transformed cell lines, separately or together, reflected the expression of all CYPs except 3A4/7. The 2A6 and 2B6 forms were not detected in the tissue specimens or the cell lines (Table II). Previous characterizations of primary oral keratinocytes implied lack of 2A6 and variable presence of 2B6 (11), implying the possibility of inter-individual variations and/or differential sensitivity of the PCR reactions. The CYP patterns (and the metabolism results discussed below) in SVpgC2a and SqCC/Y1 support the fact that CYP expression may be variably altered during the long-term growth associated with immortalization and malignant transformation.

Typical CYP substrates (35) were applied for assessment of metabolic activity *in vitro* (Table III). The detection of oxidative metabolism for three of six substrates indicated low amounts of some mRNAs or selective formation/degradation of the corresponding proteins. Similar dealkylation rates for ethoxyresorufin and methoxyresorufin in normal and immortalized oral keratinocytes likely reflected the preservation of 1A1 and 1A2 in SVpgC2a, although it is known that 1A1 may also dealkylate methoxyresorufin (34,35). Activities in a similar range as in oral keratinocytes were previously reported for epidermal keratinocytes and for hepatocyte lines of human origin (36,37). The fact that chlorzoxazone was metabolized at ≥ 30 -fold higher rates in the SVpgC2a cells as compared with normal and SqCC/Y1 cells (where no substrate conversion was detected) indicated that immortalization may also activate the capacity for drug metabolism. Thus, the results argue against the general notion that long-term culture will eradicate

drug-metabolizing activity. Although some CYP activity is clearly indicated for oral epithelium, the lack of detectable activity of 2C, 2D and 3A in fact suggests that oral mucosa may be a useful site for delivery of drugs to the systemic circulation; these CYP families metabolize >95% of all of the currently used human drugs (38).

The application of sensitive autoradiographic technique demonstrated metabolic activation of both the tobacco-specific carcinogen NNK and the mycotoxin AFB₁ in monolayer and organotypic cultures of SVpgC2a whereas the activity was lower or absent in SqCC/Y1 (Figures 1–5). The 1A1, 1A2, 2B6 and 3A4 forms are major metabolizers of NNK, but 2A6, 2E1, 2D6, 2C (i.e. 2C9) and 3A5 also show activity (35,39–41). Correspondingly, 1A2, 2A6 and 3A4 are major metabolizers of AFB₁, but 3A5 also shows activity (35,40,42–44). Consequently, all of the CYPs indicated by RT-PCR in SVpgC2a could have contributed to NNK metabolism whereas 1A2 was the likely activator of AFB₁. The lack of detectable AFB₁ metabolism in SqCC/Y1 in fact implied that conversion of methoxyresorufin (discussed above) was primarily catalysed through 1A1. Although non-reactive AFB₁ metabolites could not be identified in the present study, the possible bioactivation of AFB₁ may be highly relevant in oral cancer causation. AFB₁ is generated by the fungus *Aspergillus flavus*, and the storage of tobacco crops and betel nut commonly involves contamination with this fungus and presence of AFB₁ (45,46). The possibility that AFB₁ metabolism contributes to the overall genetic damage in buccal mucosa of tobacco and betel nut chewers should therefore be considered.

In summary, the combined analysis of mRNA transcripts and metabolic activity indicated a limited capacity for oxidative metabolism in the buccal mucosa. In agreement with previous work (9,47), different classes of chemical carcinogens may anyway undergo metabolism to reactive intermediates in the epithelium. The SVpgC2a cell line may serve in both monolayer and organotypic culture for metabolic studies at one standardized and serum-free culture condition since it showed similar or even higher activities as normal keratinocytes subcultured only once in order to enable metabolic assessments. Finally, the results identify AFB₁ as a possible oral carcinogen. Overall, the present study focused on defining 'basal levels' of CYP activity at culture conditions previously applied for assessments of tobacco and betel nut toxicity (10,14,48,49). Future studies may consider addressing the inducibility of CYPs and the expression of enzymes that conjugate and detoxify the reactive intermediates potentially formed through CYPs in oral mucosa.

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