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Cytochrome P450 expression and related metabolism in human buccal mucosa

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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 B_1 (AFB_1) 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 AFB_1 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 AFB_1 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.

Abbreviations: AFB, aflatoxin B_1; AFL, aflatoxicol; AFM_1, aflatoxin M_1; AFP_1, aflatoxin P_1; AFQ_1, aflatoxin Q_1; CYP, cytochrome P450; EMA, elevated amino acids; HSM, high serum medium; NNK, *N*-nitrosamine 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone; PBS, phospate-buffered saline.

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 B_1 (AFB_1), commonly involves several CYP450s (1,4). The possible metabolic conversion of AFB_1, a well-established hepato-

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

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

[a]RT–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.
[b]The 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

[a]Results are presented as the mean ± SEM from duplicate determinations in three separate experiments.
[b]Cells 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.
[c]Cells 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.
[d]Significantly 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 information 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 AFB_1, 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 AFB_1 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 AFB_1 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/cm^2 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

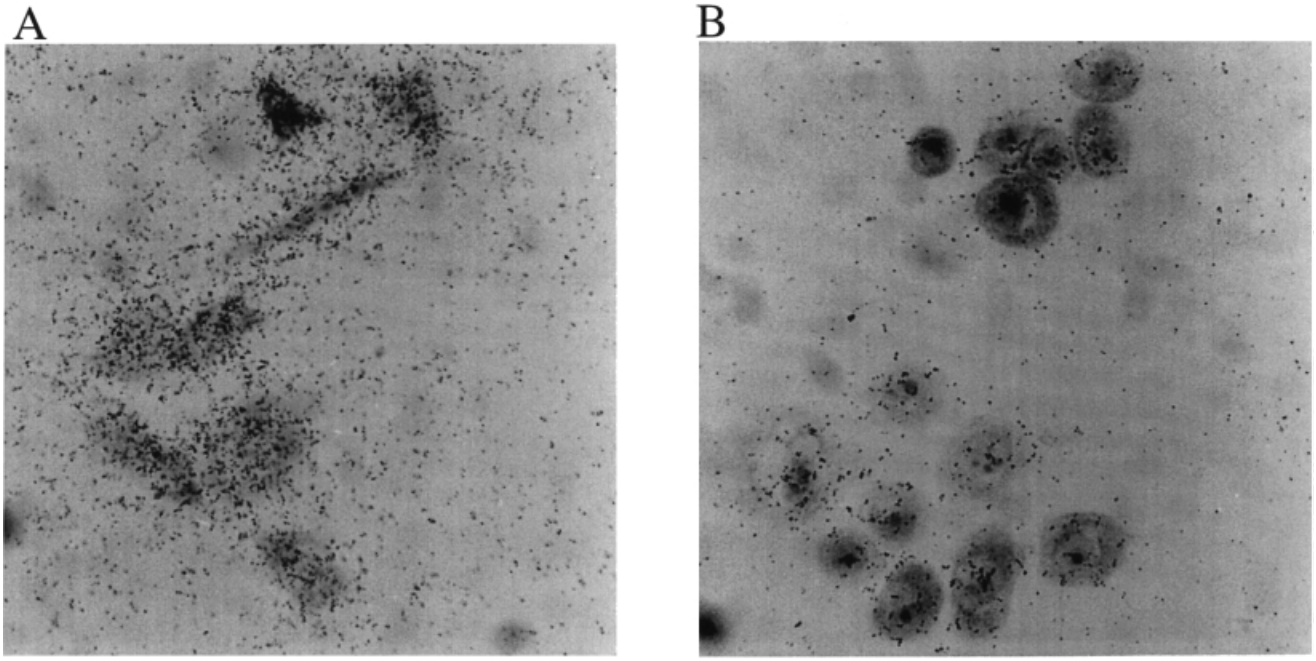

Fig. 1. Microautoradiograms of (**A**) SVpgC2a and (**B**) SqCC/Y1 exposed to 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in monolayer culture. The respective cell lines were grown on glass slides and exposed to 17 μM NNK for 24 h as described in Materials and methods. The metabolic activation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

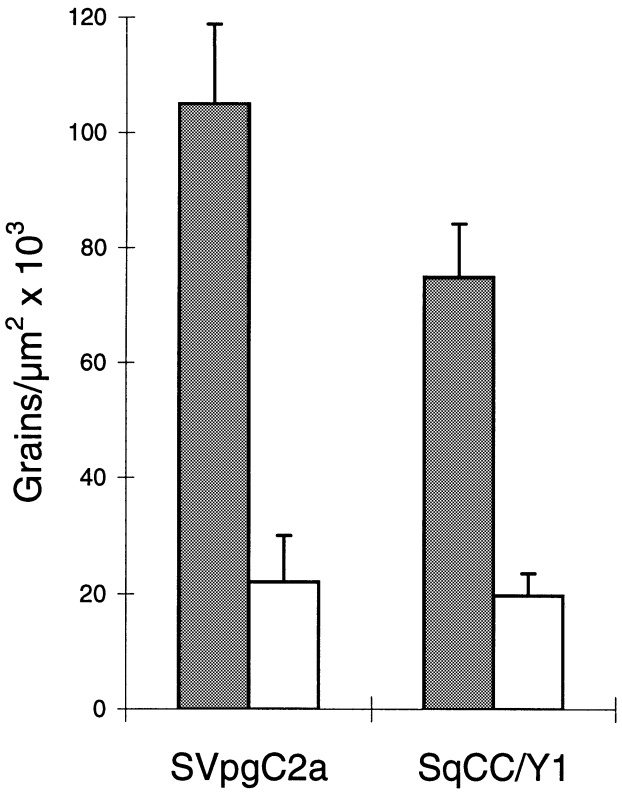

Fig. 2. Assessment of metabolic activation of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in SVpgC2a and SqCC/Y1. Autoradiograms, as depicted in Figure 1, were analysed by microscopic counting of the number of silver grains covering the cell surface areas. Background was determined from assessment 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 ± 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/cm^2 reach 75% confluency at 4–6 days; normal fibroblasts seeded at 7×10^3 cells/cm^2 reach 100% confluency at 5–7 days; the SVpgC2a cells seeded at 4.1×10^3 cells/cm^2 reach 100% confluency at 5 days; and the SqCC/Y1 cells seeded at 1×10^4 cells/cm^2 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/cm^2 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× 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 Ca^{2+}) 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 AFB_1).

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 µl consisting of 2 µl cDNA, 20 mM Tris–HCl pH 8.55, 16 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 150 µg/ml bovine serum albumin, 200 µM 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 µl 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 µM ethoxyresorufin, 10 µM methoxyresorufin, 250 µM diclofenac, 400 µM chlorzoxazone, 100 µM dextromethorphan or 250 µM testosterone in EMA followed by collection of the respective medium. The cultures exposed to ethoxyresorufin and methoxyresorufin were exposed to 10 µM dicumarol to inhibit conjugation of the dealkylated product. Medium samples from the incubations with 250 µM diclofenac, 400 µM chlorzoxazone, 100 µM dextromethorphan and 250 µM 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 6β-hydroxylation were measured by HPLC analysis (13,28–30).

NNK and AFB_1 metabolism

Aflatoxin B_1 generally labelled with thymidine [G-^{3}H]AFB_1 (18 Ci/mmol) in methanol was obtained from Moravek Biochemicals (Brea, CA) and purified by reverse-phase LC as described (31). Carbonyl-^{14}C-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone ([^{14}C]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 µM [^{14}C]NNK and 0.3 µM [^{3}H]AFB_1, 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 [^{14}C]NNK and [^{3}H]AFB_1. 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 µm) 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 toludine 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 AFB_1 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 µM [^{3}H]AFB_1 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 M_1 (AFM_1), aflatoxin P_1 (AFP_1), aflatoxicol (AFL), aflatoxin Q_1 (AFQ_1) and the *endo*- and *exo*-GSH–AFB_1 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

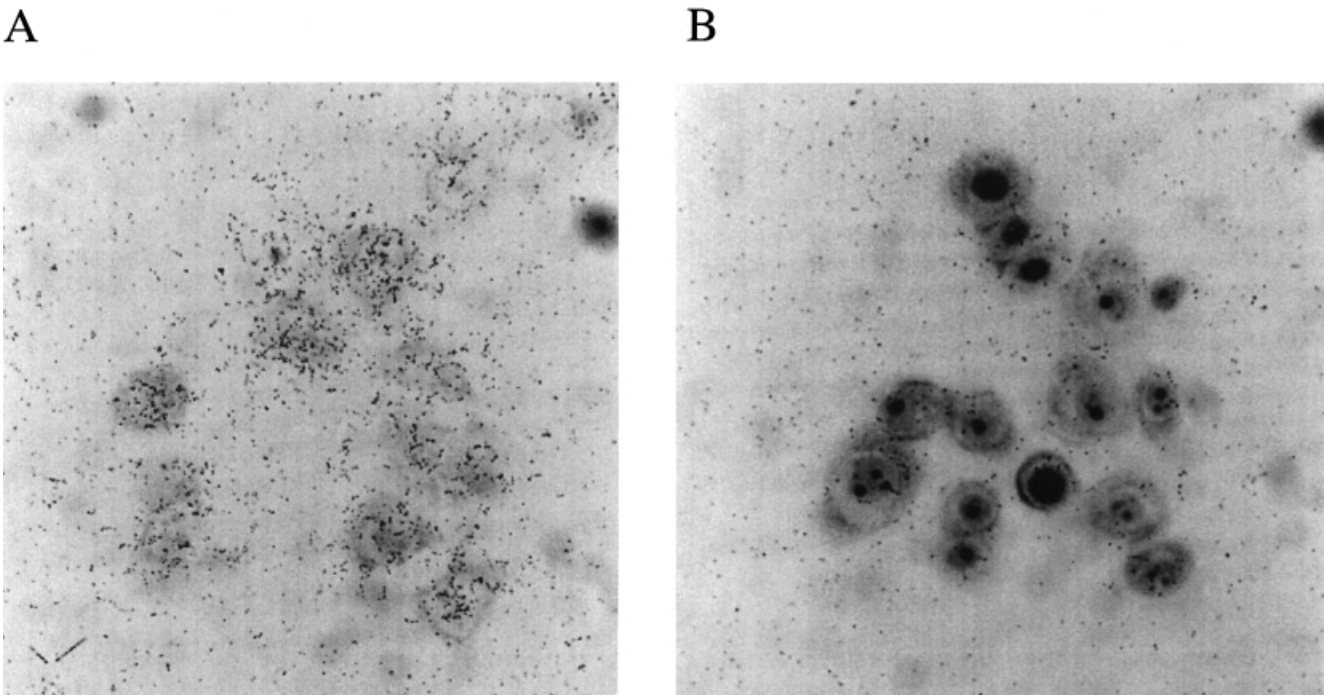

Fig. 3. Microautoradiograms of (**A**) SVpgC2a and (**B**) SqCC/Y1 exposed to AFB_1 in monolayer culture. The respective cell lines were grown on glass slides and exposed to 0.3 µM [^{3}H]AFB_1 for 24 h as described in Materials and methods. The metabolic activation of AFB_1 is indicated by the presence of silver grains over the respective cell surface areas. Original magnification ×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 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).

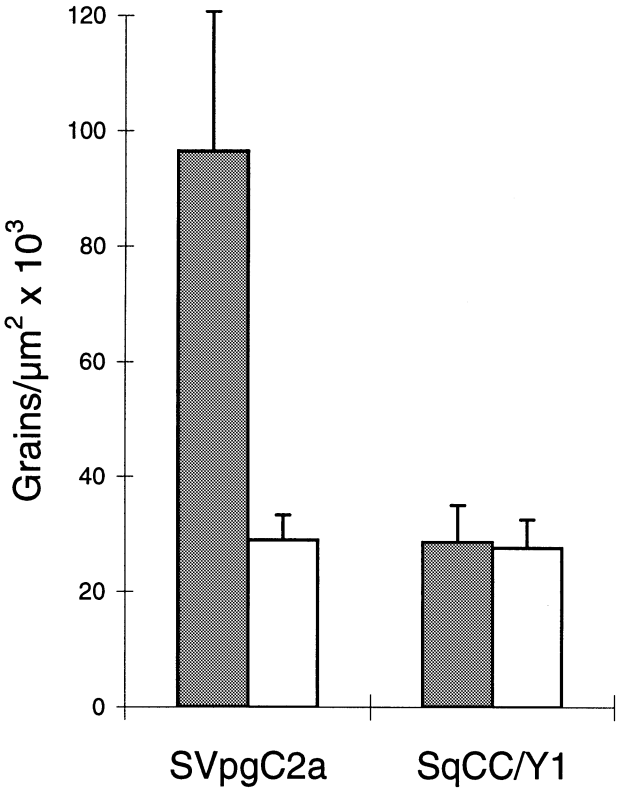

Fig. 4. Assessment of metabolic activation of AFB_1 in SVpgC2a and SqCC/Y1. Autoradiograms, as depicted in Figure 3, were analysed by microscopic counting of the number of silver grains covering the cell surface areas. Background was determined from assessment of the number of grains covering an adjacent, cell-free surface area of the same size. A minimum of 500 cells was scored in each experiment. The results are mean ± 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.

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-hydroxydiclophenac, *O*-demethylation of dextromethorphan and 6β-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 AFB_1 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 AFB_1 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 AFB_1 (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

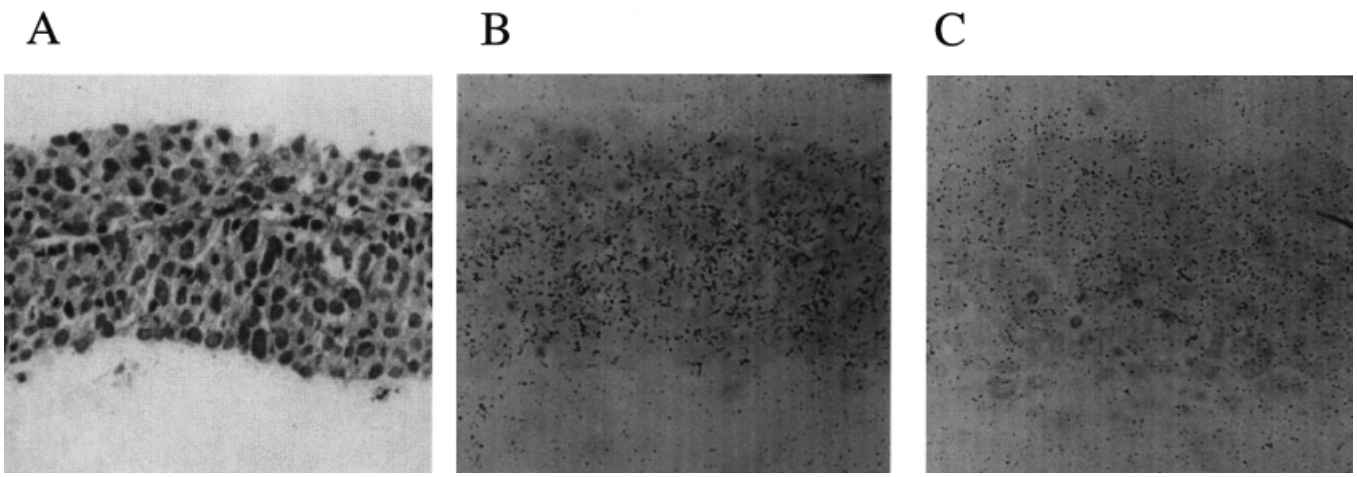

Fig. 5. Microautoradiogram showing metabolic activation of NNK and AFB_1 in SVpgC2a in organotypic culture. (**A**) A tissue equivalent was formed by co-culture of SVpgC2a with buccal fibroblasts embedded in a collagen lattice as described in Materials and methods. The cultures were exposed for 24 h to (**B**) 17 μM [^{14}C]NNK and (**C**) 0.3 μM of ^{3}H-AFB_1, 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 ×200.

the monolayer and organotypic cultures to 90°C abolished the labelling for both substrates (not shown). Finally, non-reactive AFB_1 metabolites, including AFM_1, AFP_1, AFL and the *endo*- and *exo*-glutathione AFB_1 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 AFB_1 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 $\geqslant$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 AFB_1 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 AFB_1, 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 AFB_1. The lack of detectable AFB_1 metabolism in SqCC/Y1 in fact implied that conversion of methoxyresorufin (discussed above) was primarily catalysed through 1A1. Although non-reactive AFB_1 metabolites could not be identified in the present study, the possible bioactivation of AFB_1 may be highly relevant in oral cancer causation. AFB_1 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 AFB_1 (45,46). The possibility that AFB_1 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 AFB_1 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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References

1. Guengerich,F.P., Hosea,N.A., Parikh,A., Bell-Parikh,L.C., Johnson,W.W., Gillam,E.M. and Shimada,T. (1998) Twenty years of biochemistry of human P450s: purification, expression, mechanism and relevance to drugs. *Drug Metab. Dispos.*, **26**, 1175–1178.

2. Oesch,F., Glatt,H. and Schimassmann,H. (1977) The apparent ubiquity of epoxide hydratase in rat organs. *Biochem. Pharmacol.*, **26**, 603–607.
3. Guengerich,F.P. and Mason,P.S. (1979) Immunological comparison of hepatic and extrahepatic cytochromes P-450. *Mol. Pharmacol.*, **15**, 154–164.
4. Guengerich,F.P. and Shimada,T. (1998) Activation of procarcinogens by human cytochrome P450 enzymes. *Mutat. Res.*, **400**, 201–213.
5. Jussawalla,D.J. and Deshpande,V.A. (1971) Evaluation of cancer risk in tobacco chewers and smokers: an epidemiologic assessment. *Cancer*, **28**, 244–252.
6. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Human: Tobacco Habits Other than Smoking: Betel-quid and Acreanut Chewing; and some Related Nitrosamines (1985). International Agency for Research on Cancer, Lyon, pp. 209–223.
7. IARC Monographs on the Evaluation of Carcinogenic Risk of Chemicals to Human: Tobacco Smoking (1985). International Agency for Research on Cancer, Lyon, pp. 267–285.
8. Rivenson,A., Hoffmann,D., Prokopczyk,B., Amin,S. and Hecht,S.S. (1988) Induction of lung and exocrine pancreas tumors in F344 rats by tobacco-specific and Areca-derived *N*-nitrosamines. *Cancer Res.*, **48**, 6912–6917.
9. Autrup,H., Seremet,T., Arenholt,D., Dragsted,L. and Jepsen,A. (1985) Metabolism of benzo[*a*]pyrene by cultured rat and human buccal mucosa cells. *Carcinogenesis*, **6**, 1761–1765.
10. Liu,Y., Sundqvist,K., Belinsky,S.A., Castonguay,A., Tjälve,H. and Grafström,R.C. (1993) Metabolism and macromolecular interaction of the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in cultured explants and epithelial cells of human buccal mucosa. *Carcinogenesis*, **14**, 2383–2388.
11. Farin,F.M., Bigler,L.G., Oda,D., McDougall,J.K. and Omiecinski,C.J. (1995) Expression of cytochrome P450 and microsomal exposide hydrolase in cervical and oral epithelial cells immortalized by human papillomavirus type 16 E6/E7 genes. *Carcinogenesis*, **16**, 1391–1401.
12. Jackson,P. and Groopman,J. (1999) Aflatoxin and liver cancer. *Baillieres Best Pract. Clin. Gastroenterol.*, **13**, 545–555.
13. Gomez-Lechon,M.J., Donato,T., Ponsoda,X., Fabra,R., Trullenque,R. and Castell,J.V. (1997) Isolation, culture and use of human hepatocytes in drug research. In Castell,J. and Gomez-Lechon,M. (eds) *In vitro Methods in Pharmaceutical Research.* Academic Press, New York, pp. 129–154.
14. Grafström,R.C., Norén,U.G., Zheng,X., Elfwing,Å. and Sundqvist,K. (1997) *Growth and Transformation of Human Oral Epithelium In Vitro. Advances in Cancer Research: Risk and Progression Factors in Carcinogenesis,* Vol. 143. Springer-Verlag, Heidelberg, pp. 275–306.
15. Grafström,R.C. (1990) Carcinogenesis studies in human epithelial tissues and cells *in vitro*: emphasis on serum-free culture conditions and transformation studies. *Acta Physiol. Scand.*, **592** (suppl.), 93–133.
16. Hoogenboom,L.A.P., Tulliez,J., Gautier,J.-P. Coker,R.D., Melcion,J.-P., Nagler,M.J., Polman,T.H.G. and Delort-Laval,J. Absorbtion, distribution and excretion of aflatoxin-derived ammoniation products in lactating cows. *Food Addit. Contam.*, in press.
17. Sundqvist,K., Kulkarni,P., Hybbinette,S.S., Bertolero,F., Liu,Y. and Grafström,R.C. (1991) Serum-free growth and karyotype analyses of cultured normal and tumorous (SqCC/Y1) human buccal epithelial cells. *Cancer Commun.*, **3**, 331–340.
18. Liu,Y., Arvidson,K., Atzori,L., Sundqvist,K., Silva,B., Cotgreave,I. and Grafström,R.C. (1991) Development of low- and high-serum culture conditions for use of human oral fibroblasts in toxicity testing of dental materials. *J. Dent. Res.*, **70**, 1068–1073.
19. Kulkarni,P.S., Sundqvist,K., Betsholtz,C., Höglund,P., Wiman,K.G., Zhivotovsky,B., Bertolero,F., Liu,Y. and Grafström,R.C. (1995) Characterization of human buccal epithelial cells transfected with the simian virus 40 T-antigen gene. *Carcinogenesis*, **16**, 2515–2521.
20. Nilsson,J.A., Zheng,X., Sundqvist,K., Liu,Y., Atzori,L., Elfwing,Å., Arvidson,K. and Grafstrom,R.C. (1998) Toxicity of formaldehyde to human oral fibroblasts and epithelial cells: influences of culture conditions and role of thiol status. *J. Dent. Res.*, **77**, 1896–1903.
21. Hansson,A., Bloor,B.K., Haig,Y., Morgan,P.R., Ekstrand,J. and Grafström,R.C. (2001) Expression of keratins in normal, immortalized and malignant oral epithelia in organotypic culture. *Oral Oncol.*, in press.
22. Fusenig,N. (1994) In Leigh,I., Lane,E. and Watt,F. (eds) *Epithelial-mesencuchymal Interactions Regulate Keratinocyte Growth and Differentiation in vitro*, *The Keratinocyte Handbook.* Cambridge University Press, Cambridge, pp. 71–94.
23. Tomakidi,P., Breitkreutz D., Fusenig,N.E., Zoller,J., Kohl,A. and Komposch,G. (1998) Establishment of oral mucosa phenotype *in vitro* in correlation to epithelial anchorage. *Cell Tissue Res.*, **292**, 355–366.
24. Mace,K., Gonzalez,F.J., McConnell,I.R., Garner,R.C., Avanti,O., Harris,C.C. and Pfeifer,A.M. (1994) Activation of promutagens in a human bronchial epithelial cell line stably expressing human cytochrome P450 1A2. *Mol. Carcinog.*, **11**, 65–73.
25. Mace,K., Bowman,E.D., Vautravers,P., Shields,P.G., Harris,C.C. and Pfeifer,A.M. (1998) Characterisation of xenobiotic-metabolising enzyme expression in human bronchial mucosa and peripheral lung tissues. *Eur. J. Cancer*, **34**, 914–920.
26. Prough,R.A., Burke,M.D. and Mayer,R.T. (1978) Direct fluorometric methods for measuring mixed function oxidase activity. *Methods Enzymol.*, **52**, 372–377.
27. Donato,M.T., Gomez-Lechon,M.J. and Castell,J.V. (1993) A microassay for measuring cytochrome P450IA1 and P450IIB1 activities in intact human and rat hepatocytes cultured on 96-well plates. *Anal. Biochem.*, **213**, 29–33.
28. Bort,R., Ponsoda,X., Carrasso,E. and Gomez-Lechon,M. (1996) Metabolism of acecofenac in humans. *Drug Metab. Disp.*, **24**, 834–841.
29. Peter,R., Bocker,R., Beaune,P.H., Iwasaki,M., Guengerich,F.P. and Yang,C.S. (1990) Hydroxylation of chlorzoxazone as a specific probe for human liver cytochrome P-450IIE1. *Chem. Res. Toxicol.*, **3**, 566–573.
30. Kronbach,T., Mathys,D., Gut,J., Catin,T. and Mayers,U. (1997) High-performance liquid chromatographic assays for bufuralol 1'-hydrolase, debrisoquine 4-hydroxylase and dextromethorphan *O*-demethylase in microsomes and purified P-450 isozymes of human liver. *Anal. Biochem.*, **162**, 24–32.
31. Larsson,P., Pettersson,H. and Tjälve,H. (1989) Metabolism of aflatoxin B1 in the bovine olfactory mucosa. *Carcinogenesis*, **10**, 1113–1118.
32. Larsson,P. and Tjälve,H. (1992) Binding of aflatoxin B1 metabolites in extrahepatic tissues in fetal and infant mice and in adult mice with depleted glutathione levels. *Cancer Res.*, **52**, 1267–1277.
33. Ingelman-Sundberg,M., Oscarson,M. and McLellan,R.A. (1999) Polymorphic human cytochrome P450 enzymes: an opportunity for individualized drug treatment. *Trends Pharmacol. Sci.*, **20**, 342–349.
34. Tsyrlov,I.B., Goldfarb,I.S. and Gelboin,H.V. (1993) Enzyme-kinetic and immunochemical characteristics of mouse cDNA-expressed, microsomal and purified CYP1A1 and CYP1A2. *Arch. Biochem. Biophys.*, **307**, 259–266.
35. Lewis,D.F.V. (1996) *Substrate Specificity and Metabolism. Cytochromes P450: Structure, Function and Mechanism.* Taylor and Francis, Bristol, pp. 115–167.
36. Raffali,F., Rougier,A. and Roguet,R. (1994) Measurement and modulation of cytochrome-P450-dependent enzyme activity in cultured human keratinocytes. *Skin Pharmacol.*, **7**, 345–354.
37. Roberts,E.A., Xie,Z.W., Yang,S. and Lipa,J. (1993) Inducibility of enzyme activities associated with the cytochrome P-450 1A family, ethoxyresorufin *O*-deethylase and methoxyresorufin *O*-demethylase in human hepatocyte lines derived from normal liver tissue. *Drug Metab. Dispos.*, **21**, 56–61.
38. Evans,W.E. and Relling,M.V. (1999) Pharmacogenomics: translating functional genomics into rational therapeutics. *Science*, **286**, 487–491.
39. Smith,T.J., Guo,Z., Gonzalez,F.J., Guengerich,F.P., Stoner,G.D. and Yang,C.S. (1992) Metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone in human lung and liver microsomes and cytochromes P-450 expressed in hepatoma cells. *Cancer Res.*, **52**, 1757–1763.
40. Penman,B.W., Chen,L., Gelboin,H.V., Gonzalez,F.J. and Crespi,C.L. (1994) Development of a human lymphoblastoid cell line constitutively expressing human CYP1A1 cDNA: substrate specificity with model substrates and promutagens. *Carcinogenesis*, **15**, 1931–1937.
41. Crespi,C.L., Penman,B.W., Gelboin,H.V. and Gonzalez,F.J. (1991) A tobacco smoke-derived nitrosamine, 4-(methylnitrosamino)-1-(3- pyridyl)-1-butanone, is activated by multiple human cytochrome P450s including the polymorphic human cytochrome P4502D6. *Carcinogenesis*, **12**, 1197–1201.
42. Aoyama,T., Yamano,S., Guzelian,P.S., Gelboin,H.V. and Gonzalez,F.J. (1990) Five of 12 forms of vaccinia virus-expressed human hepatic cytochrome P450 metabolically activate aflatoxin B1. *Proc. Natl Acad. Sci. USA*, **87**, 4790–4793.
43. Gallagher,E.P., Wienkers,L.C., Stapleton,P.L., Kunze,K.L. and Eaton,D.L. (1994) Role of human microsomal and human complementary DNA-expressed cytochromes P4501A2 and P4503A4 in the bioactivation of aflatoxin B1. *Cancer Res.*, **54**, 101–108.
44. Kranendonk,M., Carreira,F., Theisen,P., Laires,A., Fisher,C.W., Rueff,J., Estabrook,R.W. and Vermeulen,N.P. (1999) *Escherichia coli* MTC, a human NADPH P450 reductase competent mutagenicity tester strain for the expression of human cytochrome P450 isoforms 1A1, 1A2, 2A6, 3A4 or 3A5: catalytic activities and mutagenicity studies. *Mutat. Res.*, **441**, 73–83.

45. Varma,S.K., Verma,R.A. and Jha,A.K. (1991) Ecotoxicological aspects of aspergilli present in the phylloplane of stored leaves of chewing tobacco (*Nicotiana tobaccum*). *Mycopathologia*, **113**, 19–23.
46. Warke,R.G., Kamat,A.S. and Kamat,M.Y. (1999) Irradiation of chewable tobacco mixes for improvement in microbiological quality. *J. Food Prot.*, **62**, 678–681.
47. Autrup,H. (1986) Metabolism of polycyclic hydrocarbons in human target tissues. In Hoffman,D. and Harris,C.C. (eds) *Mechanisms of Tobacco Carcinogenesis*, Vol. 23. Branbury Report, Cold Spring Harbor, pp. 259–271.
48. Sundqvist,K., Liu,Y., Nair,J., Bartsch,H., Arvidson,K. and Grafström,R.C. (1989) Cytotoxic and genotoxic effects of areca nut-related compounds in cultured human buccal epithelial cells. *Cancer Res*., **49**, 5294–5298.
49. Sundqvist,K. and Grafström,R.C. (1992) Effects of areca nut on growth, differentiation and formation of DNA damage in cultured human buccal epithelial cells. *Int. J. Cancer*, **52**, 305–310.

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