Carcinogenesis vol.18 no.10 pp.1889–1895, 1997

O^6-Methylguanine-DNA methyltransferase activity in human buccal mucosal tissue and cell cultures. Complex mixtures related to habitual use of tobacco and betel quid inhibit the activity *in vitro*

Yun Liu[1,4], Suzanne Egyhazi[2], Johan Hansson[2], Sumati V.Bhide[3], Prabha S.Kulkarni[1,5] and Roland C.Grafström[1,6]

[1]Institute of Environmental Medicine, Karolinska Institutet, S-171 77 Stockholm, [2]Institute of Oncology-Pathology, Karolinska Hospital, S-171 76 Stockholm, Sweden and [3]Cancer Research Institute, Tata Memorial Center, Parel, 400 012 Bombay, India

Present addresses: [4]Department of Preclinical Oral Science, Karolinska Institutet, S-141 04 Huddinge, Sweden and [5]University of Miami, PO Box 016960, Miami, FL 33101, USA

[6]To whom correspondence should be addressed at: Division of Experimental Carcinogenesis, Institute of Environmental Medicine, Karolinska Institutet, Box 210, S-171 77 Stockholm, Sweden

Extracts prepared from tissue specimens of normal, non-tumourous human buccal mucosa, and cultured buccal epithelial cells and fibroblasts, exhibited O^6-methylguanine-DNA methyltransferase (MGMT) activity by catalysing the repair of the premutagenic O^6-methylguanine lesion in isolated DNA with rates of 0.2 to 0.3 pmol/mg protein. An SV40 T antigen-immortalized buccal epithelial cell line termed SVpgC2a and a buccal squamous carcinoma line termed SqCC/Y1, both of which lack normal tumour suppressor gene *p53* function, exhibited about 50 and 10% of the MGMT activity of normal cells, respectively. The normal, experimentally transformed and tumourous buccal cell types showed MGMT mRNA levels which correlated with their respective levels of MGMT activity. Exposure of buccal cell cultures to various organic or water-based extracts of products related to the use of tobacco and betel quid, decreased both cell survival (measured by reduction of tetrazolium dye) and MGMT activity (measured subsequently to the exposures in cellular extracts). Organic extracts of bidi smoke condensate and betel leaf showed higher potency than those of tobacco and snuff. An aqueous snuff extract also decreased both parameters, whereas an aqueous areca nut extract was without effect. The well-established sulph-hydryl-reactive agent Hg^{2+}, a corrosion product of dental amalgam, served as a positive control and decreased MGMT activity following treatment of cells within a range of 1–10 μM. Taken together, significant MGMT activities were demonstrated in buccal tissue specimens and in the major buccal mucosal cell types *in vitro*. Lower than normal MGMT activity in two transformed buccal epithelial cell lines correlated with decreased MGMT mRNA and lack of functional p53. Finally, *in vitro* experiments suggested the potential inhibition of buccal mucosal MGMT activity by complex mixtures present in the saliva of tobacco and betel nut chewers.

Introduction

O^6-Methylguanine-DNA methyltransferase (MGMT*) repairs premutagenic O^6-methylguanine lesions induced in DNA by alkylating agents (reviewed in 1–3). MGMT acts both as a transferase and as an acceptor for the alkyl group by forming S-alkylcysteine at the catalytic site of the protein (1–3). This reaction leads to the restoration of intact DNA concomitant with the functional inactivation of MGMT. The level of MGMT expression has a decisive role in the protection against toxic, mutagenic and carcinogenic effects of alkylating agents as demonstrated by clinical and experimental studies (1–3). For example, targeted expression or complete disruption of MGMT in mice, promotes resistance or increases sensitivity, respectively, to the insult from treatment with alkylating agents (4,5).

The basis for MGMT expression and its regulation is only partly understood. The terms Mer^+ and Mer^- (Mer indicates methyl repair) have been used to define phenotypes which are proficient or deficient in MGMT activity, respectively (6,7). Various human normal and tumorous tissues and cell types exhibit different MGMT contents, and considerable inter-individual variation is observed when a given tissue is examined (1,2). Although tumours in general are Mer^+, partial loss or absence of MGMT is found in 20–30% of tumour cell lines when compared to their normal, non-transformed counterparts (1–3,6,7). Immortalization of human fibroblasts by transfection of the SV40 T antigen (SV40T) gene increases the frequency of the negative phenotype (8,9). Exposure of laboratory animals and certain cell lines to alkylating and other, usually DNA-damaging, chemicals or physical agents, may cause up to six-fold increases in MGMT (1–3). Recent studies with mice identified that expression of the p53 tumour suppressor gene is necessary for induction of MGMT by ionizing radiation, although the constitutive expression of MGMT was independent of p53 status (10). The role of p53 in MGMT regulation is unclear and possibly species-specific, since in cultures of normal and tumorous human cells, transient over-expression of wild type (wt), but not mutant, p53 protein, could suppress activities related to transcription or translation of MGMT (11).

Epidemiological studies have shown a causal relationship between the use of tobacco, or betel quid with tobacco and oral cancer (12–14). The habitual use of these and related products, such as snuff, bidi and areca nut, naturally involves a direct and frequent exposure of primarily the buccal mucosal lining to many potentially toxic compounds (12,13). Tobacco smoke, tobacco and areca nut contain various mutagenic and carcinogenic N-nitroso compounds, of which some, such as the tobacco-specific carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone, can be metabolized by the oral mucosal epithelium to alkylating intermediates and aldehydes (15). The possible importance of unrepaired O^6-alkylguanine lesions in human oral carcinogenesis, is indicated from the evidence that frequent mutational inactivation of p53 in oral squamous

***Abbreviations:** MGMT, O^6-methylguanine-DNA methyltransferase; SV40 T, SV40 T antigen; MTT, 1-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; FBS, foetal bovine serum; HBS, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid-buffered saline solution.

carcinomas most often occurs by G:C to A:T transitions (16). Collectively, these findings imply that the habitual use of the above-mentioned products are likely to involve a significant formation of O^6-alkylguanine in the oral mucosa.

Mercury is a potential toxicant that is released and ionized in the saliva from corrosion of dental amalgam (17,18). Tobacco use may as well involve exposure to mercury, because of the environmental presence of this compound and the resulting pollution of tobacco crops (19). The well-documented thiol-reactivity of the mercuric ion (Hg^{2+}) underlies its binding to cellular macromolecules and its interference in many cellular functions (17,18). Partly purified MGMT was strongly inhibited by exposure to metal ions such as Hg^{2+} (20,21). The underlying mechanism presumably involves the reaction with one or several protein thiols in MGMT, including the thiol group of $cysteine_{145}$ at the acceptor site (1). Alkylating agents or aldehydes may also inactivate MGMT by a similar mechanism (22–25). Therefore, the possibility should be considered that the complex mixtures present in the saliva of tobacco and betel quid users contain reactive compounds which may affect O^6-methylguanine repair in oral mucosa.

To our knowledge, no information exists on the presence and regulation of MGMT in human oral mucosa. We have now investigated the expression of MGMT in specimens of buccal mucosa, and cultures of both normal epithelial cells and fibroblasts from this tissue. Also studied are two buccal epithelial cell lines which do not exhibit normal p53 function. SVpgC2a is a SV40T-immortalized cell line in which wt p53 is inactivated by complexing with SV40T (26). Derived from a buccal squamous carcinoma, the SqCC/Y1 cell line produces no p53 protein from a single rearranged p53 allele with two missense mutations (26,27). Together, normal buccal epithelial cells and these transformed cell lines constitute a two-step model of oral carcinogenesis that involves early loss of p53 function under the identical serum-free culture conditions (28). These experimental systems were compared in regards to MGMT protein and mRNA levels. Finally, we also evaluated if complex mixtures from tobacco, tobacco smoke and betel quid can influence MGMT, using Hg^{2+} concurrently as a positive control for inhibition of MGMT.

Materials and methods

Materials

Calf thymus DNA alkylated with [^{3}H]N-methyl-N-nitrosourea was used as substrate for MGMT and obtained from Dr I.Nilsen (Institute of Medical Biology, University of Tromsö, Norway). The substrate contained 64 pmol O^6-methylguanine/mg DNA. Human MGMT cDNA, excised from the plasmid pHM 14 (29), was obtained from Dr P.Karran (Imperial Cancer Research Fund, Clare Hall Laboratories, England). The aqueous and organic snuff extracts were obtained from Dr M.Curvall (Swedish Tobacco Company, Stockholm, Sweden), and prepared as described (30). The aqueous areca nut extract was obtained from Dr H.Bartsch (German Cancer Research Center, Heidelberg, Germany), and prepared as described (31). The extracts from tobacco, bidi smoke condensate and betel leaf were prepared as previously described (32,33). Deoxycytidine 5′-[α-^{32}P]triphosphate (3000 Ci/mmol) was from Amersham Sweden AB (Solna, Sweden). Klenow polymerase and enolase cDNA were from Boehringer-Mannheim Biochemica Scandinavia AB (Bromma, Sweden). 1-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and 1-[4,5-dimethylthiazol-2-yl]-3,5-diphenylformazan were from Sigma Chemical Co. (St Louis, MO). Mercury chloride was from E.Merck (Darmstadt, Germany). Media M 199 powder and foetal bovine serum (FBS) were from Gibco BRL Life Technologies (Lab Design AB, Lidingö, Sweden). MCDB 153 medium was prepared from various stock solutions and used to make EMA, a standardized serum-free medium suitable for culture of both normal and transformed buccal epithelial cell types (28,34). The growth medium for fibroblasts, LSM, is a 1:1 mixture of MCDB 153 and M 199 supplemented with various growth factors and 1.25% FBS (35).

Cell cultures

Human buccal tissue was obtained from non-cancerous patients undergoing maxillo-facial reconstructive surgery. Tissue specimens were only accepted from individuals which had declared themselves as non-tobacco users. Cultures of normal epithelial cells were obtained after incubation of tissue specimens with 0.17% trypsin in calcium-free 4-(2-hydroxyethyl)-1-piperazine-ethane-sulphonic acid-buffered saline solution (HBS) at 4°C for 18–24 h (34). The specimen was held with forceps and the epithelium was then collected into the solution by scraping of the epithelial side with another forceps. The resulting mixture was carefully triturated several-fold, and the resultant suspension of small aggregates and single cells was collected, rinsed, pelleted and resuspended in EMA (34). Primary cultures of epithelial cells, at ~75% confluency and fibroblast outgrowths from explant cultures (35), respectively, were dissociated using trypsin (0.25%), ethyleneglycol-bis-(β-aminoethyl ether) N,N,N′,N′-tetra-acetic acid (EDTA, 0.02%) and polyvinylpyrrolidone (1%) in HBS, and the cells were pelleted and resuspended in their respective growth media. Epithelial cells were inoculated and passaged on fibronectin/collagen-coated dishes at 5×10^3 cells/cm^2, whereas the fibroblasts were transferred at the same density on regular non-coated dishes (34,35). The immortal cell line SVpgC2a, derived by transfection of the SV40T gene to human buccal epithelial (26), and the buccal carcinoma cell line SqCC/Y1, were both grown and transferred using the similar culture conditions and methods as for normal epithelial cells (28). The normal cell types were used in passages 1–5, the SvpgC2a line in passages 36–40 and the SqCC/Y1 line in passages 95–100.

Preparation of tissue and cell extracts for MGMT assay

Specimens of human buccal mucosa, obtained as above, were immediately frozen at –70°C. Cell cultures at ~75% confluency were washed once with HBS, removed from the dishes with a rubber policeman into 0.5 ml HBS at 4°C and then pelleted by centrifugation at 800 ***g***. The samples were washed twice at 4°C with HBS and once with assay buffer, i.e. 50 mM Tris-HCl, pH 7.8, containing 1 mM dithiothreitol and 0.1 mM EDTA, and the cells were stored frozen as a pellet at –70°C until analysis. For preparation of extracts, the tissue and cell samples were thawed and subsequently homogenized in 0.2 ml assay buffer with a Dounce homogenizer. The samples were then sonicated four times for 10 s with intermittent cooling for 1 min on ice, and centrifuged at 8000 ***g*** for 30 min at 4°C. The supernatants were then collected and stored at –70°C until analysis.

Assay of MGMT activity

Transferase activity was assayed as described by Myrnes *et al*. (36). Tissue and cell extracts were incubated with 11.5 μg ^{3}H-methylated-DNA substrate at 37°C in assay buffer (see above) for 20 min; a reaction period found to be sufficient for transfer of the radio-labelled methyl group to the MGMT molecules in the extract (36). An extract prepared from rat liver was used as a positive control in each set of experiments. The reactions were stopped by addition of trichloro-acetic acid (TCA) to a final concentration of 5%. To remove remaining ^{3}H-labelling in DNA by hydrolysis, the mixtures were incubated at 80°C for 30 min followed by cooling for 5 min on ice. The incubates were then collected onto circular Whatman GFC filters (2.5 cm), using a Millipore 12-well filter holder apparatus. Following three washes with 10 ml of 5% TCA, the filters were analysed for protein-bound radioactivity. The protein content of the tissue and cell extracts was determined according to Bradford (37). Calculation of MGMT activity was performed as described (36) and the number of transferase molecules in cell cultures estimated by relating the specific MGMT activity to the cell counts obtained with a hemocytometer. Student's *t*-test was used to evaluate differences ($P < 0.05$) between the various cell types. For assay of the influences of various complex mixtures and Hg^{2+}, buccal fibroblasts were exposed as described for the survival assay (see below) and the MGMT activity then measured as above.

Northern blot analysis of MGMT-specific mRNA expression

Total RNA was extracted from the various cell lines after lysis of the cells with 0.1 M sodium acetic acid solution (pH 5.2) containing 5 mM EDTA and 0.5% sodium dodecyl sulphate, followed by phenol extraction and alcohol precipitation using standard procedures. Aliquots of RNA (20 μg) were electrophoresed on a 1% agarose gel with 0.7% formaldehyde, followed by transfer to a nitrocellulose filter. Hybridization with ^{32}P-labelled MGMT cDNA probe was carried out overnight in a solution containing 41% formamide, 10% dextran sulphate, 4×SSC buffer (1×SSC: 150 mM NaCl plus 15 mM sodium citrate, pH 7.0), 1×Denhardt's solution and 5 mM Tris-HCl (pH 7.5) at 44°C. The filters were washed at 54°C in 0.1×SSC and 0.1% sodium dodecyl sulphate, and then exposed to MP hyperfilm (Amersham). The nitrocellulose filter was stripped in a rehybridization solution consisting of 5 mM Tris-HCl (pH 8.0) containing 0.2 mM EDTA, 0.05% sodium pyrophosphate decahydrate, 0.002% BSA, 0.002% Ficoll and 0.02% polyvinylpyrrolidone

Table I. O^6-Methylguanine-DNA methyltransferase (MGMT) in human buccal tissue and cultured normal, transformed and tumourous buccal cells[a]

Types of sample	Number of cases	MGMT activity		Expression of MGMT mRNA
		fmol/mg[b]	Molecules/cell[b]	Ratio of MGMT/Enolase[c]
Tissue	14	245 ± 13	ND[d]	ND
Epithelial cells	6	232 ± 23	18,900 ± 2900	1.0
Fibroblasts	3	295 ± 59	21,400 ± 7200	1.2
SV40 T-antigen immortalized epithelial cells (SVpgC2a)		117 ± 10[e]	5600 ± 1200[e]	0.4
Carcinoma cells (SqCC/Y1)		36 ± 15[e]	2200 ± 950[e]	0.1

[a]The activity of MGMT was assayed using preparation of tissue and cell extracts and methylated DNA as described in Materials and methods, and the results based on protein content and determination of cell numbers.
[b]The results are expressed as mean ± SEM. The results in the immortalized and carcinoma cells were obtained from at least four determinations in separate experiments. The protein contents of epithelial cells, fibroblasts, the SVpgC2a and the SqCC/Y1 cell lines were 0.30 ± 0.14, 0.26 ± 0.06, 0.18 ± 0.04 and 0.18 ± 0.08 mg/10^6 cells, respectively.
[c]Equal amounts of total RNA isolates from the various cell lines were size separated by gel electrophoresis and hybridized to a labeled MGMT probe. The expression of MGMT-specific mRNA was normalized to enolase-specific mRNA and the expression ratios determined by computerized densitometry. The data given are mean values of two separate experiments. Normal epithelial cells were given an arbitrary value of 1.0.
[d]Not determined.
[e]Significantly different ($P < 0.05$) from normal tissue epithelial cells.

for 1 h at 65°C. To standardize MGMT expression against a house-keeping gene, the nitrocellulose filter was then rehybridized with a ^{32}P-labelled enolase probe and then processed as described above. The relative amounts of MGMT or enolase mRNA, respectively, were determined using a computerized densitometer (Ultrascan XL, Pharmacia-Upjohn, Stockholm, Sweden).

Assay of cell survival

Survival was assayed with the colorimetric MTT reduction assay in fibroblasts as previously described (38,39). The cells were seeded on 100 mm dishes at 1×10^4 cells/cm^2 in LSM and incubated for 24 h. The medium was removed and the cells were exposed to Hg^{2+} (1 h) or to each complex mixture (3 h) in chemically defined LSM (without FBS and thiols, i.e. cysteine), at the indicated concentrations. These exposure times were identical to those used in our previous studies of Hg^{2+} and various extracts (28,39). Solvent controls, i.e. dimethyl sulphoxide or ethanol up to 0.1% v/v, were found to insignificantly influence the MTT reduction and MGMT activities. Following the exposures, the cells were washed once with HBS before incubation in LSM containing 50 μg/ml MTT for 2 h. The cells were again washed once with HBS followed by addition of 2-isopropanol containing 0.04 N HCl for 15 min to dissolve the MTT formazan. The lysate was collected, centrifuged and the absorbance was determined at 560 nm (38).

Results

The presence of MGMT in extracts prepared from normal, non-tumourous tissue and several types of cultured cells from human buccal mucosa was investigated (Table I). Tissue specimens from 14 individuals exhibited a mean MGMT activity of 0.2 pmol/mg protein. The activities from these individuals varied in a uni-modal fashion from 40 to 385 fmol/mg, indicating a 10-fold inter-individual variation (Figure 1). Cultures of epithelial cells and fibroblasts from normal tissue exhibited MGMT activities that were similar to those in the tissue specimens (Table I). Fibroblasts showed somewhat higher, although not significantly different, activity and number of transferase molecules per cell than epithelial cells. The ranges of MGMT levels in epithelial cells and fibroblasts corresponded to 220–260 and 222–412 fmol/mg, respectively. In contrast, the SV40T-immortalized epithelial cell line SVpgC2a, and the squamous carcinoma line SqCC/Y1, showed about 50 and 10%, respectively, of the mean activity of epithelial cell lines derived from normal tissue (Table I). Northern blot hybridization of total RNA isolates from the various cell cultures demonstrated that all cell types contained MGMT mRNA transcripts of the same size, i.e. slightly below 1.0 kb (not shown). The levels of MGMT mRNA differed among the cell lines in a manner similar to their MGMT activities, utilizing enolase as a constitutively expressed reference gene (40) (Table I).

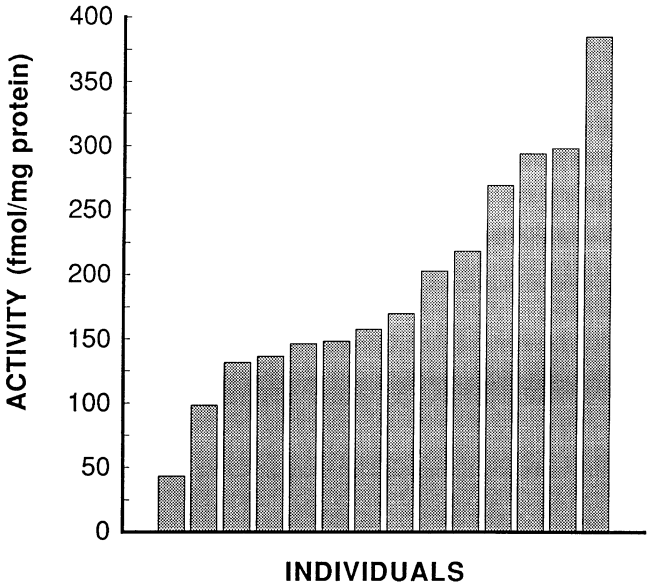

Fig. 1. MGMT activity of individual specimens of human buccal mucosa. The activities of the extracts prepared from 14 tissue specimens were assayed as described in Material and methods.

To investigate the MGMT activity following exposure to various complex mixtures obtained from tobacco and betel quid ingredients, the cytotoxicity of various organic and aqueous extracts derived from such products was initially determined using the MTT assay. This viability assay reflects mitochondrial integrity and depends on succinate dehydrogenase activity, which, like MGMT, contains a thiol residue that is critical for enzyme function (38). Due to the large number of cells required, ~10^9, buccal fibroblasts were utilized in both sets of assays. In the tested concentration range of 3 μg/ml to 1 mg/ml, exposure of the cells for 3 h to extracts of tobacco, bidi smoke condensate, snuff and betel leaf decreased the cell viability in a dose-dependent manner, whereas an areca nut extract was without significant effect (Figure 2). The concentrations required to decrease the MTT

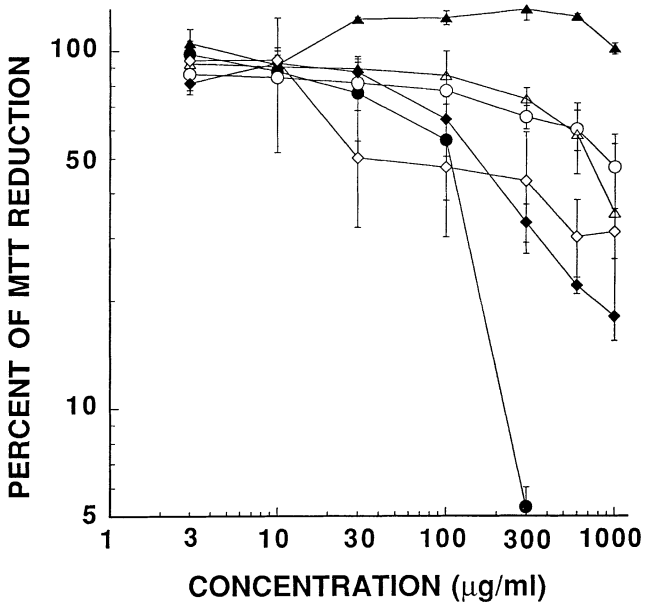

Fig. 2. Cell survival of human buccal fibroblasts following exposure to various complex mixtures related to the use of tobacco and betel quid. The cell survival was assayed by MTT reduction as described in Materials and methods. The amounts added of each of the various extracts were in μg/ml, except for the aqueous snuff extract, which was added in μl/ml. Data are expressed as mean ± SEM of three separate experiments with duplicate dishes in each experiment. (●) Snuff aqueous extract, (○) snuff organic extract, (◆) betel leaf organic extract, (◇) bidi smoke condensate, (▲) areca nut aqueous extract, (△) tobacco organic extract.

Table II. Effects of various complex mixtures on the activity of MGMT in human buccal fibroblasts[a]

Mixtures	Concentration (μg/ml)	MGMT activity[b] (% of control)
Organic extracts		
Betel leaf extract	130[c]	56 ± 5
	320[d]	52 ± 10
Snuff extract	900[c]	69 ± 13
	1000[e]	47 ± 9
Bidi smoke condensate	100[c]	73 ± 6
	600[d]	58 ± 2
Tobacco extract	700[c]	80 ± 3
	1000[e]	66 ± 13
Aqueous extracts		
Snuff extract	100[c]	74 ± 16
	150[d]	62 ± 7
Areca nut extract	1000[e]	123 ± 3

[a]The MGMT activity was measured in cellular extracts prepared subsequently to exposure of the cells to each extract for 3 h.
[b]The results were obtained by subtraction of the activity found with each corresponding solvent control. The organic solvents, dimethyl sulfoxide or ethanol up to 0.1% (v/v), or H_2O, influenced the MGMT activity <10%. The results are mean ± SEM of three separate experiments, each with duplicate dishes. The MGMT activity of the non-exposed fibroblasts corresponded to 254 fmol per mg protein.
[c]The concentration which caused ~50% inhibition of MTT reduction.
[d]The concentration which caused ~70% inhibition of MTT reduction.
[e]The highest tested concentration of 1 mg/ml was used, since this extract did not decrease the levels of MTT reduction to 50 or 70%, respectively, at this or lower levels.

activity to 50% in these cells were ~100, 130, 700 and 900 μg/mg of the organic bidi, betel leaf, tobacco and snuff extracts, respectively, and ~100 μl/ml of an aqueous snuff extract.

To study the effects of the complex mixtures on MGMT activity, the concentrations (up to the maximal tested level of 1 mg/ml) which decreased survival to 50 and 30% of the control values, respectively, were compared. The exposure protocol followed was identical to that used in the MTT assay, although the MGMT activity was subsequently assayed in cellular extracts as described in Materials and methods. The various complex mixtures decreased the MGMT activity to differing degrees (Table II). At 50% survival, the organic extract from betel leaf was relatively the most potent on a concentration basis, followed in descending order by the organic snuff, bidi and tobacco extracts. At 30% survival or at the highest tested extract concentration of 1 mg/ml, all organic extracts had further decreased the MGMT activity. The aqueous extract of snuff also reduced the MGMT activity at the corresponding toxicity levels (100 and 150 μl/ml, respectively), whereas the areca nut extract was without effect up to the highest tested level. The various extracts decreased MTT reduction to a higher extent than the MGMT activity, although the level of difference varied.

Finally, the influence of Hg^{2+} on the MGMT activity of buccal fibroblasts was also studied. This agent served as a positive control, since direct addition of this metal ion to partly purified MGMT preparations from human placenta or to rat liver extracts, was previously shown to inhibit MGMT activity in a range of 20–500 μM (20,21). Exposure of cells to Hg^{2+} decreased the MGMT activity in a dose-related manner, with a 50% inhibitory concentration already at 9 μM (Figure 3). For comparative reasons, our previously obtained results on the effect of Hg^{2+} on MTT reduction are included (39), demonstrating that survival is decreased to around 10% at the onset of MGMT inhibition (Figure 3).

Discussion

Through the habitual use of tobacco and betel quid, the buccal mucosa is exposed to many N-nitroso compounds that may be converted to alkylating intermediates (12–14,28). Therefore, the ability of this tissue to repair alkylation damage in DNA may be of outmost importance in preventing associated pathological changes. The present study demonstrated that buccal mucosa have MGMT activity similar to esophagus, stomach, small intestine, colon and lung (41,42), whereas the liver exhibits about four-fold higher activity, and the brain as well as the mammary gland show about three-fold lower activity (41,43,44). Furthermore, the 10-fold inter-individual variation in MGMT content noted in this study is within the range of 4- to 60-fold variations previously demonstrated in other human tissues (24,41–44). Early passage cultures of normal buccal epithelial cells and fibroblasts also showed similar mean MGMT activities as the tissue specimens. Collectively these results indicate that the major cell types of the buccal mucosa are able to remove O^6-alkylguanine lesions, although the data also indicate interindividual variations. The presence and ranges of MGMT in different persons may be applied to predicting individual tissue susceptibility to the adverse effects of alkylating agents, whether of general environmental origin or specifically administered in cancer therapy (1–3).

Comparisons of normal, SV40T-immortalized and tumorous buccal epithelial cells under the identical serum-free culture

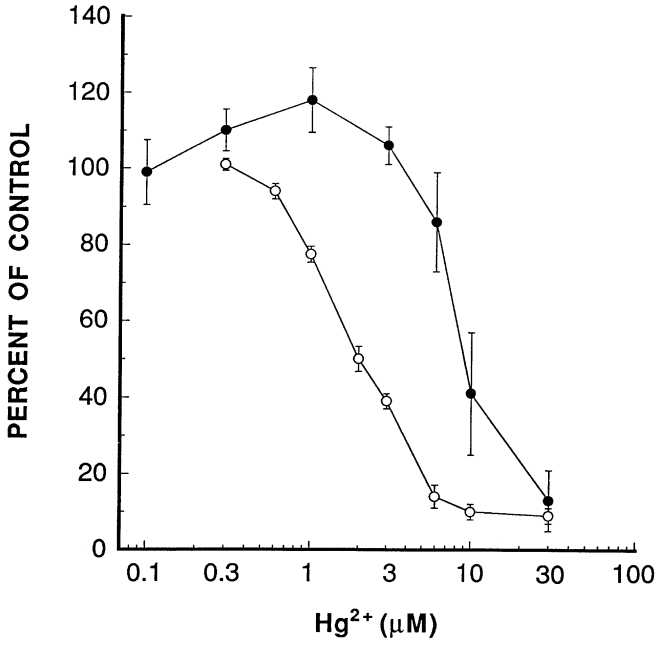

Fig. 3. Cell survival and MGMT activity of human buccal fibroblasts exposed to Hg^{2+}. Cell viability was determined by the MTT assay. The MGMT activity was analysed in extracts, prepared after the cellular exposure to Hg^{2+}, as described in Materials and methods. The results were expressed as mean ± SEM, and derived from three separate experiments with duplicate dishes in each experiment. (●) MGMT activity; (○) MTT reduction.

conditions, indicated that the MGMT activities of both of the transformed cell lines were much lower than both the mean value and the narrow range of activities noted in cultures of normal epithelial cells. In agreement with other studies (1–3,49), the MGMT activity correlated to the levels of MGMT mRNA in both normal and transformed buccal cell cultures, indicating the importance of transcription rates for functional MGMT activity. Although p53 has been implicated in MGMT regulation (10,11,45), the possible connection with lower MGMT activity in the transformed buccal lines remains unclear. Selection for the Mer^- phenotype during prolonged culture of certain cell lines has previously been indicated (2,3,46), and may explain the loss of MGMT in the continuous buccal lines. Recent studies of several SV40T-transfected human fibroblast lines indicated that expression of the Mer^--phenotype coincided with immortalization following crises, and that the extended culture of the various lines *per se*, did not result in loss of MGMT (9). Other studies showed that exposure of oral epithelial cells, immortalized *in vitro* by papilloma virus genes, underwent further malignant transformation following exposure to alkylating agents, whereas normal cells showed resistance to the corresponding treatment (47). Although many factors influence the probability of cellular transformation (28,48), information about MGMT expression would be useful in such studies.

Several complex mixtures related to tobacco use and betel quid chewing caused cytotoxicity in buccal fibroblasts as shown by decreased MTT reduction. The extracts of both bidi smoke and betel leaf were relatively more toxic than the snuff and tobacco extracts. Notably, the MGMT activity was influenced in a similar, although less pronounced, manner by these extracts. Whereas the areca nut extract did not influence the MTT or MGMT activities, the positive control, Hg^{2+}, at μM levels inhibited both activities in a dose-dependent manner. The results agree with a requirement of thiol residue modification for inactivation of both MTT and MGMT, since Hg^{2+}, in contrast to the areca nut extract, is a highly thiol-reactive agent that efficiently depletes the cellular content of glutathione in buccal cell cultures (35,39,50,51). Various thiol-reactive aldehydes, found both in smokeless tobacco and in tobacco smoke, were previously shown to both decrease cellular thiols and inhibit MGMT activity in human cell cultures, using the similar exposure protocol as in the present study (23,25). Moreover, alkaloids and certain N-nitroso compounds present in tobacco and/or betel quid have demonstrated thiol-reactivity in cultured human buccal epithelial cells (28,50,51), and could have contributed to the inhibitory actions of the complex mixtures demonstrated in fibroblasts. Inhibition of MGMT activity by Hg^{2+} occurred only at highly cytotoxic levels, where the cellular content of free protein thiols would be significantly decreased (39). Therefore, the significance of MGMT inhibition by agents with extreme thiol-reactivity, such as Hg^{2+}, should be viewed with caution, since vital cellular functions involving numerous proteins are likely to be simultaneously affected. Clearly, future studies of enzymes other than MGMT may clarify the specificity of the inhibitory actions of chemicals or complex mixtures.

In conclusion, this study demonstrated significant MGMT activity in buccal mucosa and its major cell types, of critical importance in the protection of this potential target tissue against cell killing and carcinogenesis induced by alkylating agents. Decreased MGMT mRNA and protein in transformed buccal epithelial cells coincided with compromised p53 function, indicating that mechanistic studies are needed to clarify a possible association. Finally, *in vitro* experiments indicated the possible inhibition of MGMT by the habitual use of tobacco and betel quid. The release and absorption of aqueous or lipid-soluble reactive chemicals from these sources may potentially reduce the number of functional MGMT molecules in buccal mucosa and, therefore, increase the risk of oral cancer associated with the concomitant exposure to alkylating agents.

Acknowledgements

We are grateful to Drs H.Bystedt and L.von Konow at the Department of Maxillofacial Surgery, Karolinska Hospital, Stockholm, for providing surgical specimens, and Ms Å.Elfwing for technical assistance. We also thank Dr I.Cotgreave for constructive comments on the manuscript. This investigation was supported by grants from the Swedish Council for Forestry and Agricultural Research, the Swedish Cancer Society, the Swedish Tobacco Company and the Swedish National Board of Laboratory Animals.

References

1. Pegg,A.E. (1990) Mammalian O^6-alkylguanine-DNA alkyltransferase: regulation and importance in response to alkylating carcinogenic and therapeutic agents. *Cancer Res.*, **50**, 6119–6129.
2. Mitra,S. and Kaina,B. (1993) Regulation of repair of alkylation damage in mammalian genomes. *Prog. Nucl. Acids Res. Mol. Biol.*, **44**, 109–142.
3. Pegg,A.E., Dolan,M.E. and Moschel,R.C. (1995) Structure, function, and inhibition of O^6-alkylguanine-DNA alkyltransferase. *Prog. Nucl. Acid Res. Mol. Biol.*, **51**, 167–221.
4. Becker,K., Dosch,J., Gregel,C.M., Martin,B.A. and Kaina,B. (1996) Targeted expression of human O^6-methylguanine-DNA methyltransferase (MGMT) in transgenic mice protects against tumour initiation in two-stage skin carcinogenesis. *Cancer Res.*, **14**, 3239–3244.
5. Tsuzuki,T., Sakumi,K., Shiraishi,A., *et al.* (1996) Targeted disruption of

the DNA repair methyltransferase gene renders mice hypersensitive to alkylating agent. *Carcinogenesis*, **17**, 1215–1220.
6. Day,R.S., Ziolkowski,C.H.J., Scudiero,D.A., Meyer,S., Lubiniecki,A.S., Girardi,A.J., Galloway,S.M. and Bynum,G.D. (1980) Defective repair of alkylated DNA by human tumour and SV40-transformed cell strains. *Nature*, **288**, 724–727.
7. Yarosh,D.B. (1985) The role of O^6-methylguanine-DNA methyltransferase in cell survival, mutagenesis and carcinogenesis. *Mutat. Res.*, **145**, 1–16.
8. Green,M.H.L., Karran,P., Lowe,J.E., Preisley,A., Arlett,C.F. and Mayne,L. (1990) Variation in the loss of O^6-methylguanine-DNA methyltransferase during immortalization of human fibroblasts. *Carcinogenesis*, **11**, 185–187.
9. Harris,L.C., von Wronski,A.M., Venable, C.C., Remack, J.S., Howell, S.R. and Brent, T.P. (1996) Changes in O^6-methylguanine-DNA methyltransferase expression during immortalization of cloned human fibroblasts. *Carcinogenesis*, **17**, 219–224.
10. Rafferty,J.A., Clarke,A.R., Sellappan,D., Santibanez Koref,M., Frayling.I.M. and Margison,G.P. (1996) Induction of murine O^6-alkylguanine-DNA-alkyltransferase in response to ionising radiation is p53 gene dose dependent. *Oncogene*, **12**, 693–697.
11. Harris,L.C., Remack,J.S., Houghton,P.J. and Brent,T.P. (1996) Wild-type p53 suppresses transcription of the human O^6-methylguanine-DNA methyltransferase gene. *Cancer Res.*, **56**, 2029–2032.
12. International Agency for Research on Cancer (1985) *Evaluation of the Carcinogenic Risk of Chemicals to Humans. Tobacco Smoking.* Vol. **38**, Lyon.
13. International Agency for Research on Cancer (1985) *Evaluation of the Carcinogenic Risk of Chemicals to Humans. Tobacco Habits Other Than Smoking; Betel-Quid and Areca-Nut Chewing and Some Related Nitrosamines.* Vol. 37, Lyon.
14. Thomas,S. and Wilson,A. (1993) A quantitative evaluation of the aetiological role of betel quid in oral carcinogenesis. *Oral Oncol. Eur. J. Cancer*, **29B**, 265–271.
15. Liu,Y., Sundqvist,K., Belinsky,S.A., Castonguay,A., Tjälve,H. and Grafström,R.C. (1993) Metabolism and macromolecular interaction of the tobacco-specific N-nitrosamine 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone in cultured explants and epithelial cells of human buccal mucosa. *Carcinogenesis*, **14**, 2383–2388.
16. Greenblatt,M.S., Bennett,W.P., Hollstein,M. and Harris,C.C. (1994) Mutations in the p53 tumour suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res.*, **54**, 4855–4878.
17. World Health Organization (1991) *Environmental Health Criteria 118: Inorganic Mercury.* World Health Organization, Geneva, pp. 35–46.
18. Department of Health and Human Services, Public Health Services (1993) *Dental Amalgam: A Scientific Review and Recommended Public Health Service Strategy for Research, Education and Regulation.* Department of Health and Human Services, Public Health Services, Washington, D.C.
19. Nadkarni,R.A. (1974) Some considerations of metal content of tobacco products. *Chem. Industry*, **7**, 693–694.
20. Scicchitano,D.A. and Pegg,A.E. (1987) Inhibition of O^6-alkylguanine-DNA-alkyltransferase by metals. *Mutat. Res.*, **192**, 207–210.
21. Bhattacharyya,D., Boulden,A.M., Foote,R.S. and Mitra,S. (1988) Effect of polyvalent metal ions on the reactivity of human O^6-methylguanine-DNA methyltransferase. *Carcinogenesis*, **9**, 683–685.
22. Pegg,A.E., Wiest,L., Foote,R.S., Mitra,S. and Perry,W. (1983) Purifications and properties of O^6-alkylguanine-DNA methyltransferase from rat liver. *J. Biol. Chem.*, **258**, 2327–2333.
23. Krokan,H., Grafström,R.C., Sundqvist,K., Esterbauer,H. and Harris,C.C. (1985) Cytotoxicity, thiol depletion and inhibition of O^6-methylguanine-DNA methyltransferase by various aldehydes in cultured human bronchial fibroblasts. *Carcinogenesis*, **6**, 1755–1759.
24. Espina,N., Lima,V., Lieber,C.S. and Garro,A.J. (1988) In vitro and *in vivo* inhibitory effect of ethanol and acetaldehyde on O^6-methylguanine-DNA methyltransferase. *Carcinogenesis*, **9**, 761–766.
25. Grafström,R.C., Dypbukt,J.M., Sundqvist,K., Atzori,L., Nilsen,I., Curren,R.D. and Harris,C.C. (1994) Pathobiological effects of acetaldehyde in cultured human epithelial cells and fibroblasts. *Carcinogenesis*, **15**, 985–990.
26. 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.
27. Reiss,M., Brash,D.E., Munoz-Antonia,T., Simon,J.A., Ziegler,A., Vellucci,V.F. and Zhou,H.-L. (1992) Status of p53 tumour suppressor gene in human squamous carcinoma cell lines. *Oncol. Res.*, **4**, 349–358.
28. Grafström,R.C., Norén,U.G., Zheng,X., Elfwing,Å. and Sundqvist,K. (1997) Growth and transformation of human oral epithelium *in vitro*. In Muller-Hermelink,H.K., Neumann,H.G. and Dekant,W. (eds) *Risk and Progression Factors in Carcinogenesis. Recent Results of Cancer Research*, vol. 143. Springer-Verlag, Heidelberg, pp. 275-306.
29. Rydberg,B., Spurr,N. and Karran,P. (1990) cDNA cloning and chromosomal assignment of the human O^6-methylguanine-DNA methyltransferase. cDNA expression in *Escherichia coli* and gene expression in human cells. *J. Biol. Chem.*, **265**, 9563–9589.
30. Jansson,R., Romert,L., Magnusson,J. and Jenssen,D. (1991) Genotoxicity testing of extracts of a Swedish moist oral snuff. *Mutat. Res.*, **261**, 101–115.
31. Nair,U.J., Floyd,R.A., Nair,J., Bussachini,V., Fresen,M. and Bartsch,J. (1987) Formation of reactive oxygen species and 8-hydroxy-deoxyguanosine in DNA *in vitro* with betel quid ingredients. *Chem.-Biol. Interactions*, **63**, 157–169.
32. Shirname,L.P., Menon,M.M. and Bhide,S.V. (1984) Mutagenicity of betel quid and its ingredients using mammalian test systems. *Carcinogenesis*, **5**, 501–503,
33. Padma,P.R., Lalitha,V.S., Amonkar,A.J. and Bhide,S.V. (1989) Anticarcinogenic effect of betel leaf extract against tobacco carcinogens. *Cancer Lett.*, **45**, 195–202.
34. Sundqvist,K., Kulkarni,P., Hybbinette,S.S., Bertolero,F., Liu,Y. and Grafström,R.C. (1991) Serum-free growth and karyotype analysis of cultured normal and tumourous (SqCC/Y1) buccal epithelial cells. *Cancer Commun.*, **3**, 331–340.
35. 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.
36. Myrnes,B., Nordstrand,K., Giercksky,K.E., Sjunneskog,C. and Krokan,H. (1984) A simplified assay for O^6-methylguanine-DNA methyltransferase activity and its application to human neoplastic and non-neoplastic tissues. *Carcinogenesis*, **5**, 1061–1064.
37. Bradford,M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248–254.
38. Denizot,F. and Lang,R. (1986) Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. *J. Immunol. Meth.*, **13**, 271–277.
39. Liu,Y., Cotgreave,I., Atzori,L. and Grafström,R.C. (1992) The mechanism of Hg^{2+} toxicity in cultured human oral fibroblasts: the involvement of cellular thiols. *Chem.-Biol. Interactions*, **85**, 69–78.
40. Giallongo,A., Feo,S., Moore,R., Croce,C.M. and Showe,L.C. (1986) Molecular cloning and nucleotide sequence of a full-length cDNA for human α-enolase. *Proc. Natl Acad. Sci.*, **83**, 6741–6745.
41. Myrnes,B., Giercksky,K.E. and Krokan,K. (1983) Interindividual variation in the activity of O^6-methylguanine-DNA methyltransferase and uracil-DNA glycosylase in human organs. *Carcinogenesis*, **4**, 1565–1568.
42. Grafström,R.C., Pegg,A.E., Trump,B.F. and Harris,C.C. (1984) O^6-alkylguanine-DNA alkyltransferase activity in normal human tissues and cells. *Cancer Res.*, **44**, 2855–2857.
43. Pegg,A.E., Roberfroid,M., von Bahr,C., Foote,R.S., Mitra,S., Braesil,H., Likhachev,A. and Montesano,R. (1982) Removal of O^6-methylguanine from DNA by human liver fractions. *Proc. Natl. Acad. Sci. USA*, **79**, 5162–5165.
44. Wiestler,O., Kleihues,P. and Pegg,A.E. (1984) O^6-Alkylguanine-DNA methyltransferase activity in human brain and brain tumours. *Carcinogenesis*, **5**, 121–124.
45. Russell,S.J., Ye,Y.-W., Waber,P.G., Shuford,M., Schold,S.C., Jr and Nisen,P.D. (1995) p53 Mutations, O^6-alkylguanine DNA alkyltransferase activity, and sensitivity to procarbazine in human brain tumours. *Cancer*, **75**, 1339–1342.
46. Karran,P., Stephenson,C., Macpherson,P., Cairns-Smith,S. and Priestley,A. (1990) Coregulation of the human O^6-methylguanine-DNA methyltransferase with two unrelated genes that are closely linked. *Cancer Res.*, **50**, 1532–1537.
47. Kim,M.S., Shin,K.-H., Baek,J.-H., Cherrick,H.M. and Park,N.-H. (1993) HPV-16, Tobacco-specific N-nitrosamine, and N-methyl-N′-nitro-N-nitrosoguanidine in oral carcinogenesis. *Cancer Res.*, **53**, 4811–4816.
48. 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.*, **140** (suppl. 592), 93–133.
49. Kroes,R.Å. and Erickson,L.C. (1995) The role of mRNA stability and transcription in O^6-methylguanine DNA methyltransferase (MGMT) expression in Mer^+ human tumour cells. *Carcinogenesis*, **16**, 2255–2257.
50. Sundqvist,K., Liu,Y., Nair,J., Bartsch,B., 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.
51. Grafström,R.C., Jernelöv,M.I., Dypbukt,J.M., Sundqvist,K., Atzori,L. and Zheng,A. (1996) Aldehyde toxicity and thiol redox state in cell cultures

from human aerodigestive tract. In Mohr,U., Adler,K.B., Dungworth,D.I., Harris,C.C., Plopper,C.G. and Saracci,R. (eds) *Correlation's Between In Vitro and In vivo Investigations in Inhalation Toxicology.* ILSI Press, Washington D.C., pp. 319–336.

Received on February 11, 1997; revised on May 8, 1997; accepted on June 11, 1997