O6-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

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Extracts prepared from tissue specimens of normal, non-tumourous human buccal mucosa, and cultured buccal epithelial cells and fibroblasts, exhibited O6-methylguanine-DNA methyltransferase (MGMT) activity by catalysing the repair of the premutagenic O6-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 extract also decreased both parameters, whereas an aqueous areca nut extract was without effect. The well-established sulph-hydryl-reactive agent Hg2+, a corrosion product of dental amalgam, served as a positive control and decreased MGMT activity following treatment of cells with 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

O6-Methylguanine-DNA methyltransferase (MGMT+) repairs premutagenic O6-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-(methylNitosamino)-1-(3-pyridyl)-1-butaneone, can be metabolized by the oral mucosal epithelium to alkylating intermediates and aldehydes (15). The possible importance of unrepaired O6-alkylguanine lesions in human oral carcinogenesis, is indicated from the evidence that frequent mutational inactivation of p53 in oral squamous
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 \( \text{O}^2\text{H}\)-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 (\( \text{Hg}^{2+} \)) 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 \( \text{Hg}^{2+} \) (20,21). The underlying mechanism presumably involves the reaction with one or several protein thiols in MGMT, including the thiol group of cystein(145) at the acceptor site (I). 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 \( \text{O}^2\text{H}\)-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, SVpC2a 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 \( \text{Hg}^{2+} \) concurrently as a positive control for inhibition of MGMT.

Materials and methods

Materials

Calf thymus DNA alkylated with \( [\text{H}]\text{H} \)-methyl-N-nitrosourea was used as substrate for MGMT and obtained from Dr J.Nilsen (Institute of Medical Biology, University of Tromsö, Norway). The substrate contained 64 pmol \( \text{O}^2\text{H}\)-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 extract was obtained from Dr H.Bartsch (German Cancer Research Center, Heidelberg, Germany), and prepared as described (30). The aqueous acetone 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). Deoxyctydde 5'-[\text{H}]3'-phosphate (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 cells (28,34). The growth medium for fibroblasts, L929, is a 1:1 mixture of MCDB 153 and M 199 supplemented with various growth factors and 1.25% FBS (35).
and the cells were exposed to Hg\textsuperscript{2+} in a manner similar to their MGMT mRNA levels. The levels of MGMT mRNA differed among the cell lines in a manner similar to their MGMT mRNA transcripts of the same size, i.e. slightly below 1.0 kb (not shown). 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.

<table>
<thead>
<tr>
<th>Types of sample</th>
<th>Number of cases</th>
<th>MGMT activity (fmol/mg protein)</th>
<th>Expression of MGMT mRNA</th>
<th>Ratio of MGMT/Enolase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tissue</td>
<td>14</td>
<td>245 ± 13</td>
<td>ND\textsuperscript{a}</td>
<td>ND\textsuperscript{a}</td>
</tr>
<tr>
<td>Epithelial cells</td>
<td>6</td>
<td>232 ± 23</td>
<td>18,900 ± 2,900</td>
<td>1.0</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>3</td>
<td>295 ± 59</td>
<td>21,400 ± 7,200</td>
<td>1.2</td>
</tr>
<tr>
<td>SV40 T-antigen immortalized epithelial cells (SVpgC2a)</td>
<td>117 ± 10\textsuperscript{a}</td>
<td>5600 ± 1,200\textsuperscript{a}</td>
<td>0.4</td>
<td></td>
</tr>
<tr>
<td>Carcinoma cells (SqCC/Y1)</td>
<td>36 ± 15\textsuperscript{c}</td>
<td>2200 ± 950\textsuperscript{c}</td>
<td>0.1</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{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.

\textsuperscript{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\textsuperscript{6} cells, respectively.

\textsuperscript{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 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 SV40 T-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).

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\textsuperscript{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 for 1 h at 65°C. To standardize MGMT expression against a house-keeping gene, the nitrocceullose filter was then rehybridized with a \textsuperscript{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-Uijfijn, Stockholm, Sweden).

**Results**

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**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\textsuperscript{4} cells/cm\textsuperscript{2} in LSM and incubated for 24 h. The medium was removed and the cells were exposed to Hg\textsuperscript{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\textsuperscript{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).

**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.
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 bidi, bidi leaf organic extract, (○) bidi smoke condensate, (▲) areca nut aqueous extract, (△) tobacco organic extract.

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

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

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 H2O, 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.

For comparative reasons, our previously obtained results on the effect of Hg2+ 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 utmost 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 O6-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
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 cell lines was previously indicated. Selection for the Mer– phenotype during prolonged culture of certain cell lines has previously been indicated as a possible association. Finally, in vitro experiments indicated that mechanistic studies are needed to clarify a possible association. 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. 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.

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.Elfving for technical assistance. We also thank 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


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 Hg²⁺, 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 Hg²⁺ 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 Hg²⁺, 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.

Fig. 3. Cell survival and MGMT activity of human buccal fibroblasts exposed to Hg²⁺. Cell viability was determined by the MTT assay. The MGMT activity was analysed in extracts, prepared after the cellular exposure to Hg²⁺, 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.
The DNA repair methyltransferase gene renders mice hypersensitive to alkylating agent. 
Carcinogenesis, 17, 1215–1220.

Y. Liu et al.


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