Increased removal of 3-alkyladenine reduces the frequencies of hprt mutations induced by methyl- and ethylmethanesulfonate in Chinese hamster fibroblast cells

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ABSTRACT
We have previously reported the isolation of mammalian cell lines expressing the 3-methyladenine DNA glycosylase I (tag) gene from E.coli. These cells are 2–5 fold more resistant to the toxic effects of methylating agents than normal cells (15). Kinetic measurements of 3-methyladenine removal from the genome in situ show a moderate (3-fold) increase in Tag expressing cells relative to normal as compared to a high (50-fold) increase in exogenous alkylated DNA in vitro by cell extracts. Excision of 7-methylguanine is as expected, unaffected by the tag* gene expression. The frequency of mutations formed in the hypoxanthine phosphoribosyl transferase (hprt) locus was investigated after methylmethanesulfonate (MMS), ethylmethanesulfonate (EMS), N-nitroso-N-methylurea (NMU) and N-nitroso-N-ethylurea (NEU) exposure. Tag expression reduced the frequency of MMS and EMS induced mutations to about half the normal rate, whereas the mutation frequency in cells exposed to NMU or NEU is not affected by the tag* gene expression. These results indicate that after exposure to compounds which produce predominantly N-alkylations in DNA, a substantial proportion of the mutations induced is formed at 3-alkyladenine residues in DNA.

INTRODUCTION
Simple monofunctional alkylating agents have strong toxic, mutagenic, and carcinogenic effects which are thought to be a direct consequence of their interaction with DNA (1, 2). The spectrum of alkylation products formed in DNA depends on the reactivity and type of the alkylating agent (3, 4) and different lesions have different biological implications (5–7). An important factor determining the biological effects of alkylation is the ability of cells to remove the various alkylation products before the DNA is being used as a template during DNA replication (6). The primary enzymes involved in such repair are the alkyltransferases for dealkylation of O6-guanine and O4-thymine and the alkylbase DNA glycosylases for excision of N-alkylated purines and O2-alkylated pyrimidines (8–11).

The damaging effect of O6-guanine methylation (m6G) has been extensively studied in mammalian cells whereas much less is known about the effects of other alkylation products such as 3-alkyladenine, which is known to be cytotoxic as well as premutagenic in bacteria (12–14). Recently, mammalian cells with increased capacity for 3-methyladenine m3A removal were described (15–18). We constructed Chinese hamster V79 fibroblast (RJKO) cells and murine haemopoietic stem (FDCP1) cells expressing the E.coli 3-methyladenine DNA glycosylase I (tag) gene (15). The Tag enzyme is specific for repair of 3-alkyladenine (19), although a very low activity for the repair of 3-methylated guanine has been reported (20). These tag+ expressing cell lines are more resistant to the cytotoxic effects of methylating agents than the corresponding parent cell lines and the protecting effect was more pronounced in the more sensitive murine cells (15). It was concluded that m3A excision is a limiting step in cell survival after alkylation exposure. This conclusion was supported by the independent investigation of Habraken and Laval (18) showing that the alkylation sensitive Hamster mutant irs1 cells became more alkylation resistant after transfection with the E.coli alkA+ gene or the rat 3-methyladenine-DNA-glycosylase gene (APDG). The conclusion was further supported by the observation that glial cell lines which are resistant to haloethylnitrosourea have 2–3 fold higher 3-methyladenine DNA glycosylase activity than the corresponding sensitive cells (21). In contrast, the overexpression of the human N-methylpurine-DNA-glycosylase gene (MPG) in Chinese hamster ovary (CHO) cells did not result in increased resistance to methylating treatment (17). This apparent discrepancy may indicate a possible cell type-specific or a DNA repair enzyme-specific effect of increased rate of m3A removal.

In this communication it is shown that the Tag expressing RJKO cell line shows more efficient excision of m3A from its chromosomal DNA than control cells. Furthermore, it is shown that ethyl methanesulfonate (EMS) as well as methyl methanesulfonate (MMS) induced mutations in the hprt gene were reduced in cells expressing the tag gene.

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MATERIALS AND METHODS
Mammalian cell cultures and DNA hybridisation
The RJKO derivate cell lines (22) were maintained as described (15). Clone 1 cells represents the cells transfected by the tag+ retrovirus vector (pBK 204) and clone 13 cells transfected by the parent vector (pZipNeoSV(X)1; ref. 21). Southern blot analysis was carried out as described in Sambrook et al. (23) using a random primed [32P]-labelled tag gene (24) fragment as probe.

Excision of N-methylpurines
Cell extracts (15) were assayed for DNA glycosylase activity as described by Riazuddin and Lindahl (25) with minor modifications (19). The substrate used was DNA alkylated by [3H-me]dimethylsulphate (3.95 Ci/mmol) to a specific activity of 400,000 dpm/µg and reaction mixtures contained 30 ng DNA and 10 µg protein extract. Excised bases were analysed by reverse phase HPLC as described (19). Excision of N-methylpurines from the chromosomal DNA was assayed by plating growing RJKO cells at 1 × 10^6 cells/80 cm² flask 1 day prior to treatment. Each flask received single 30 min exposures of 10 µCi/ml of N-[3H]methyl-N-nitrosourea (16.4 Ci/mmol, Amersham) in 5 ml of serum free medium without G418. Cells were harvested by trypsinisation at time 0, 3 and 5 hours after treatment, and lysed by suspending in 500 µl 1×SSC, 2% SDS followed by freeze/thawing. Lysates were treated with RNaseA (0.5 mg/ml-Sigma) for 30 min and subsequently with Proteinase K (1 mg/ml-Sigma) for 30 min at 37°C, extracted twice with SSC saturated phenol neutralised with 0.2 volumes of 1M Tris – HCl, pH 8.0. DNA was ethanol precipitated (2xvol. ethanol, 0.3 M NaAc), washed once with 70% ethanol and resuspended in 20 µl 0.1 M HCl with cold m3A and m7G as markers. Alkylated purines were released by hydrolysis at 70°C for 30 min, and analysed as above.

Assay of mutagenicity
Mutation frequency at the hprt locus was measured as previously described (26) with minor modifications. Briefly, cells at 1 × 10^6 cells/80 cm² culture flask were exposed to MMS, EMS, NMU or NMU, at equitoxic doses (7), in MEM without serum and replenished with fresh medium. After 7 days of incubation, without G-418, cells were subcultured in 14 cm petri-dishes at 4 × 10^4 cells for counting viable cells and at 2 × 10^5 (for EMS, NMU and NMU) or 1 × 10^6 (for MMS) for selecting mutants. Selective growth of hprt mutants was obtained by the addition of 10 µg/ml of 6-thioguanine. Cells were stained after 6 days for counting of viable cells and after 8 days for the mutants.

RESULTS
Properties of Chinese hamster V79 cells expressing the E.coli tag gene
The isolation of RJKO cells which stably express the E.coli tag+ gene has been described previously (15). Southern blot analysis was carried out to verify the presence and estimate the copy number of the integrated tag+ gene sequences. Cellular DNA was extracted and restricted with AccI which cleaves in the middle of the tag gene (24). For clone 1 cells hybridisation to a fragment of 2 kb was observed and found to be approximately 10 times stronger than hybridisation to another smaller band of 0.6 kb (Fig. 1). These results are interpreted as the stronger band representing multiple repeated copies of the tag gene within the retroviral vector and the weak band reflecting one of the regions flanking the repeats which includes half of a single copy tag gene. It is hence estimated that clone 1 cells contain approximately 5 copies of the E.coli tag gene. This value corresponds well to the independent estimate made from comparison of the hybridisation signal to those obtained with known amounts of plasmid standards (Fig. 1).

Previous analysis of cell extracts indicated that tag+ transfected cells had about 10-fold increase in DNA glycosylase release of alkylated bases from dimethylsulphate treated DNA. For a better evaluation of the relative increase in 3-methyladenine DNA glycosylase activity excision of m3G and m7A was analysed individually in a kinetic experiment (Fig. 2A). As expected, the rate of m7G excision was identical for tag+ expressing cells (clone 1), and control cells (clone 13). However, the excision rate of m3A by extracts of clone 1 cells were about 50 times faster than for clone 13 cells.

To compare the effect on m3A excision from the genome in situ with that obtained with extracts, clone 1 and clone 13 cells were exposed to [3H]-NMU for 30 minutes and the DNA was analysed for remaining alkylation damage as a function of incubation time after exposure. To eliminate variations caused by differences in the amounts of DNA recovered, m3A excision rates were evaluated as ratios of m3A to m7G. This normalisation was justified from the observation of similar m7G excision by cell extracts from both cell types. After 5 hours of incubation 3 times more m3A is remaining in DNA of clone 13 cells than in the Tag expressing cells (clone 1), implying that tag gene expression does increase m3A repair capacity, although not as much as the extract measurements would indicate (Fig. 2).

Suppression of alkylation induced mutagenicity by tag gene expression
To assess the effects of increased m3A repair on mutation induction mutation frequencies at the hprt locus were measured after exposure to MMS and NMU and after exposure to the corresponding ethylating agents, EMS and NEU (Fig. 3). The spontaneous hprt mutation frequencies were similar for clone 1 and clone 13 cells and always below 4 × 10⁻⁶ (results not shown). When cells were exposed to MMS or EMS, the induced mutation frequencies for the tag+ gene-expressing cells were lower than for clone 1 cells.
only half that observed for control cells (Fig. 3A, C). When cells were exposed to NMU or NEU, the mutation frequencies were much higher and similar for clone 1 and clone 13 cells (Fig. 3B, D). Taken together, these results indicate that alkyladenine represents a significant premutagenic lesion after MMS and EMS exposure, but does not contribute to the overall mutation frequency induced by nitrosoureas, which produce more O-alkylations in DNA.

DISCUSSION

The presence of repeated copies of E.coli tag gene sequences under control of a retroviral long terminal repeat promoter in the genome of Chinese hamster V79 cells results in efficient expression of m3A DNA glycosylase I activity. A 50-fold increase is observed in the rate of m3A excision from alkylated DNA by cell extracts in vitro (Fig. 2A). However, only a moderate increase in the rate of m3A excision from the genome can be observed (Fig. 2B). This difference may reflect that the prokaryotic Tag enzyme, which is lacking a nuclear localisation signal, only to a limited extent diffuses across the nuclear membrane. It could also be that Tag have reduced activity on DNA within chromatin relative to free DNA. Nevertheless, the increased rate of m3A excision from the genome is sufficiently high to increase cellular resistance to the toxic effects of MMS and NMU (15) and also to reduce the mutagenic effects of N-alkylating agents (Fig. 3). The mutation frequency observed after MMS exposure is reduced to half the normal value by Tag expression, suggesting that half of the mutations induced in normal cells are formed at the sites of m3A in DNA. However, on a molar basis m3A is nevertheless much less potent than m6G in promoting mutations. Considering that the relative yield of m3A to m6G by MMS exposure is 40:1 (3, 4) and assuming that the remaining mutations in Tag expressing cells are formed at m6G residues it can be estimated that m3A is 40 times less potent than m6G as a premutagenic lesion. This is further confirmed by the results of the NMU experiments for which the mutation frequency is the same irrespective of Tag expression. After NMU exposure the ratio of m3A to m6G is about 2:1 (3, 4) and the formation of m3A will in this case not contribute significantly to the overall mutation frequency. Previous reports have shown that nitrosourea produces a high frequency of GC → AT transitions (27-30), as expected from mispairing of 6-methylguanine with thymine (31-33), and that adenine modifications do not contribute substantially to the total mutagenicity effect.

The results of the mutation experiments with the ethylating agents are analogous to those for methylations. The EMS induced mutation frequency is reduced whereas the NEU induced mutations are unaffected by Tag expression. The EMS effect accords with previous enzymatic studies showing that Tag can excise 3-ethyladenine from DNA (25). EMS is known to produce a much higher level of 6-alkylguanine than MMS, nearly to the same extent as NMU. Nevertheless, Tag expression reduces EMS mutagenicity, but has no effect on NMU induced mutations, suggesting that 3-ethyladenine has a higher mutagenic potential than m3A. This is indirectly supported by the observation that mutations at AT base pairs are relatively frequent after exposure to NEU and N-ethyl-N′-nitro-N-nitrosoguanidine, in spite of a high proportion of 6-ethylguanine relative to 3-ethyladenine (27, 30).

In one experiment, a higher (and quite toxic) concentration of EMS (5 mg/ml) was used for the mutagenesis experiment (data not shown). It appeared that the effect of Tag expression on the mutation frequency decreased with such treatment. This can be explained by the formation of high levels of 6-ethylguanine which will exceed the capacity for transferase repair, so that 6-ethylguanine then contributes more to the total mutation frequency. These results may correspond to those of Yang et al. (34) obtained with N-methyl-N′-nitro-N-nitrosoguanine treated diploid human fibroblasts. In a low dose group they found that
GC → AT mutations contributed only to 19% of the total base substitutions, whereas the majority (84%) of base substitutions identified among the high dose group were GC → AT transitions.

In spite of the fact that Tag expression reduces EMS induced mutations there is no effect of Tag expression on EMS cytotoxicity, in contrast to results obtained with MMS (data not shown). These results indicate that 3-ethyladenine repair is not limiting for survival and perhaps that persistent 3-ethyladenine unlike m3A can be handled by the nucleotide excision repair system. Nucleotide excision repair is functioning on a longer time scale than base excision repair and persistent lesions may therefore also more frequently be converted into mutations.

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