Repair of O6-Methylguanine and O4-Methylthymine by the Human and Rat O6-Methylguanine-DNA Methyltransferases*

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In order to compare the ability of the human and rat O6-methylguanine-DNA methyltransferases (transferases) to repair in vitro O6-methylguanine (O6-MeGua) and O4-methylthymine (O4-MeThy) residues, which are two mutagenic DNA adducts formed by alkylating agents, we have purified both proteins to homogeneity. Gel electrophoresis of the proteins shows that the O4-MeThy repair is due to the transfer of the methyl group from the alkylated base to the transferase molecules. However, both proteins repair with different efficiencies the O6-MeGua and O4-MeThy residues present in alkylated DNA, poly[d(G-C)], poly[d(G-dC)], or in alkylated poly[d(A-T)] and poly[d(A-dT)], respectively. Reaction of both proteins with either methylated residues follows a second-order kinetics. The rate constants are 1 × 108 M⁻¹ min⁻¹ for both proteins acting on O6-MeGua and 4.8 × 106 or 1.8 × 106 M⁻¹ min⁻¹ for the rat or human protein acting on O4-MeThy, respectively. The activity of the mammalian transferases on O4-MeThy present in a poly[d(A-dT)] substrate is inhibited by double-stranded DNA.

Treatment with alkylating agents produces a variety of lesions in DNA (1). Among them, N2-methyladenine residues are considered as lethal lesions because they are a block to DNA replication (2), although these adducts are easily depurinated to form apurinic sites (3). O6-Methylguanine (O6-MeGua) and O4-methylthymine (O4-MeThy) residues cause base mispairing (4) and are believed to lead to mutagenesis and carcinogenesis (5). O6-MeGua and O4-MeThy are equally mutagenic in vitro, but it has been shown that O4-MeThy is strikingly more mutagenic than O6-MeGua in vivo (6).

In Escherichia coli, O6-MeGua and O4-MeThy residues are repaired by two DNA alkyltransferases (transferases), which transfer the alkyl group from the alkylated bases to one of their own cytoine residues, and are therefore suicide proteins (7). The Ada protein, encoded by the ada gene, is an inducible 39-kDa protein, which possesses two alkyl-acceptor cytoines repairing either O6-MeGua and O4-MeThy or the methylphosphotriesters (8). The Ogt protein, encoded by the ogt gene (9), is a 19-kDa non-inducible protein, which repairs O6-MeGua and O4-MeThy residues (10). A similar transferase activity exists in mammalian cells, and the cDNA encoding for this protein has been isolated from human (11–13), rat (14), and hamster cells (15). The proteins of different origins have the same active site and share a high percentage of homology (16).

Many results suggest that the cellular sensitivity to malignant transformation or tumor initiation by alkylating agents is correlated to the number of transferase molecules present in the cells (reviewed in Ref. 17). As O6-MeGua and O4-MeThy are the promutagenic lesions formed by this class of compounds, many attempts have been made to know whether the eukaryotic transferases were able to remove these two lesions from the cellular DNA. Several experiments failed to show the repair of O4-MeThy residues in mammalian cells (reviewed in Ref. 18). However, the affinity of the bacterial and mammalian transferases for O4-MeThy residues was recently suggested by incubating crude cell extracts with an oligonucleotide containing a single modified base (19), and the removal of O4-MeThy residues by a high amount of purified human transferase has also been reported (20).

The goal of our study was to determine whether the mammalian transferases were able to repair the O4-MeThy residues with the same efficiency as O6-MeGua. We have purified the transferases of human and rat origin and compared their activities on O6-MeGua and O4-MeThy residues present in various substrates. Our results show that the two eukaryotic proteins repair O4-MeThy residues in vitro and that the alkyl group is transferred to the acceptor protein. However, the rat and, especially, the human protein have a low affinity for these lesions.

EXPERIMENTAL PROCEDURES

Materials

(H)MNU (1.75 Ci/mmol) was from Amer sham Corp. DEAE-Sepha cel, Mono S HR5/5, poly(dA), and poly(dT) were from Pharmacia LKB Biotechnology, Inc. Calf thymus DNA, poly(dG-dC), poly(dG-C), make venom phosphodiesterase, and alkaline phosphatase were from Boehringer (Mannheim, Germany). The E. coli Ada protein (19-kDa fragment) was from Applied Genetics (Freeport, NY).

Strains

The pKT 100 plasmid carrying the human transferase cDNA (obtained from Dr. S. Mitra) was grown in E. coli JM 107 (11). The pcDNA II plasmid carrying the rat transferase cDNA (14) was from laboratory stocks and was grown in E. coli KT 233 (oda-`-, ogt-`). The rat hepatoma cells (H4 cells) (21) were from laboratory stocks.

Determination of the Transferase Activity

Removal of O6-MeGua Residues—The reaction mixture contained (in a final volume of 100 μl) 70 μM Hepes, pH 7.6, 5 μM EDTA, 1 μM dithiothreitol, (H)MNU-treated calf thymus DNA (corresponding to
400 fmol of O6-MeGua prepared as described (22) and increasing amounts of protein. After 20 min at 37 °C, the samples were supplemented with authentic markers and acid-hydrolyzed. The N7-methylguanine and O6-MeGua remaining in the substrate were separated by HPLC, using a C18 Bondapack column (22), and quantified by liquid scintillation spectroscopy. The same protocol was used to measure the removal of O6-MeGua residues from [3H]MNU-treated poly(dG·dC) or poly(d(G·C)). The incubation mixtures contained also about 400 fmol of O6-MeGua.

Removal of O4-MeThy Residues—[3H]MNU-treated poly(dA·dT) was prepared by incubating poly(dT) with [3H]MNU (1.75 Ci/mol) for 3 h at 37 °C. After extensive dialysis, it was annealed with an equimolar amount of poly(dA) (23). This substrate (corresponding to 400 fmol of O4-MeThy) was incubated in 70 mm Hepes, pH 7.6, 6 mM EDTA (final volume 120 μl), with increasing amounts of protein, for 45 min at 37 °C. It was then hydrolyzed to nucleosides by sequential incubation with DNase I, snake venom phosphodiesterase, and alkaline phosphatase (23). After addition of authentic markers, the alkylation products (O6-MeGua, N7-MeThy, O4-MeThy, and phosphotriesters) were separated by HPLC using a C18 Bondapack column eluting with 24% methanol/H2O at a flow rate of 0.8 ml/min (24). They were quantified by scintillation spectroscopy.

Protein Gel Electrophoresis

[3H]MNU-treated poly(dA·dT) (corresponding to 2400 cpm of O4-MeThy) was incubated (final volume 100 μl) with the E. coli, rat, or purified human transferases or with ammonium sulfate precipitated E. coli transferases. The protein separation was then done in 12.5% SDS-polyacrylamide gels (25). The gels were fixed in methanol/acetic acid, soaked in ENHANCE (DuPont NEN), then exposed to x-ray films.

Purification Protocol

The rat transferase was purified from the E. coli KT 233 expressing the rat transferase cDNA (14). Exponentially growing bacteria (1 liter of LB medium) were harvested by centrifugation, washed in SSC, and disrupted by sonication at 0 °C in the presence of proteases inhibitors. The purification steps were as described by Koike et al. (20) for the human transferase and were, successively: ammonium sulfate precipitation, DEAE-Sephacel chromatography, and Mono S column chromatography. The human transferase was purified from E. coli JM 107 transfected with the pKT 100 plasmid (11). The purification steps were as described (20).

RESULTS

Purification of the Rat Transferase—The proteins present after each purification step were detected by gel electrophoresis (Fig. 1). The purification (Table I) was about 870-fold with a 40% yield. Gel electrophoresis of the Mono S fractions containing the transferase activity showed a single band of protein (Fig. 1) with a molecular mass of about 23 kDa, in agreement with the 22.2 kDa calculated from the cDNA sequence (14).

The purified human transferase showed also a single band in gel electrophoresis (data not shown).

Repair of O6-MeGua and O4-MeThy Residues by the Purified Rat and Human Transferases—The relative activity of the two eukaryotic transferases on O6-MeGua and O4-MeThy residues was measured by incubating the proteins with [3H]MNU-treated DNA or [3H]MNU-treated poly(dA·dT). To circumvent problems of interpretation owing to technical differences, [3H]MNU of the same specific activity was used to alkylate both substrates and the incubation mixtures contained the same amount of either O6-MeGua or O4-MeThy residues. Both proteins remove the O6-MeGua residues with the same efficiency from alkylated DNA, poly(dG·dC), or poly(dA·dT) (Fig. 2, A and B, and Table II). However, a much lower number of O4-MeThy residues was removed by the same amounts of proteins, particularly in the case of human transferase (Fig. 2) (e.g. after 45 min of incubation, about 3.5- and 20-fold less O4-MeThy than O6-MeGua residues were removed by 10 ng of rat or human protein, respectively). The removal of O4-MeThy from alkylated poly(dA·dT) or poly(dA·dT) was identical (Table II).

Using protein concentrations sufficient to repair about 50% of each alkylated base, the O6-MeGua removal from alkylated DNA or from alkylated poly(dG·dC) was completed in less than 2 min. However, the O4-MeThy repair by the human protein acting on poly(dA·dT) was much slower (Fig. 3). A plot of ln(S0/SP)/P0/S as a function of time (Fig. 3, inset) was linear, showing that the reaction between the human protein and the methylated poly(dA·dT) follows a second-order kinetics. A similar kinetics was observed with the rat protein (not shown). The
Activity of the rat and human transferases on 06-MeGua and 04-MeThy residues

|                | Human transferase | Rat transferase |
|----------------|-------------------|-----------------|
| Activity** on 06-MeGua present in: |                  |                 |
| DNA            | 31,400            | 26,700          |
| Poly(dG-dC)    | 30,600            | 25,200          |
| Poly(dG-C)     | 30,800            | 25,400          |
| Activity** on 04-MeThy present in: |                  |                 |
| Poly(dA-dT)    | 1,540             | 7,890           |
| Poly(dA-T)     | 1,420             | 7,850           |

* The activity (pmol of alkylated base repaired/1 mg of protein/45 min) was calculated from data of the linear part of the curves obtained by incubating increasing amounts of protein with the different substrates.

FIG. 3. Time dependence of the 06-MeGua and 04-MeThy repair. MNU-treated DNA (A), MNU-treated poly(dG-dC) (C), or MNU-treated poly(dA-dT) (E) were incubated for increasing time lengths with the human transferase. For details see "Experimental Procedures." Data were plotted (insert) according to the second order rate equation where P1 and S1 are the protein and 06-MeThy concentrations at time zero, and P1 and S1 the concentrations at time t, respectively.

rate constant was calculated as $1 \times 10^6$ M$^{-1}$ min$^{-1}$ for the rat or human protein acting on 06-MeGua residues present in alkylated DNA or poly(dG-dC). It was $4.8 \times 10^6$ and $1.8 \times 10^6$ M$^{-1}$ min$^{-1}$ for the rat and human transferases acting on poly(dA-dT), respectively.

It should be noted that, under our experimental conditions, the same amount of E. coli Ada protein (19-kDa fragment) removed 75 and 62% of the 06-MeGua or 04-MeThy residues present in alkylated DNA or poly(dA-dT), respectively. Both residues were removed at the same rate, and their total removal was performed in about 2 min, in agreement with previously reported data (8).

Transfer of the Methyl Group from 04-MeThy to the Acceptor Proteins—This transfer was checked by gel electrophoresis of the proteins (Fig. 4). A band of about 23 kDa was observed when the purified rat or human transferases were incubated with the alkylated poly(dA-dT) (Fig. 4, lanes b and c). As a control, the polymer was incubated with the E. coli Ada protein (19-kDa fragment) (Fig. 4, lane a). However, when the same amount of substrate was incubated with crude H4 cell extract (Fig. 4, lane d), no radioactive protein was detected in the gel, although the transfer of the methyl group from 06-MeGua could be detected when a lower amount of cell extract was incubated with $[^3H]$MNU-treated DNA (26).

It should be noted that no significant repair of the 02-MeThy or of the phosphotriesters was observed with the rat or the human protein under our experimental conditions (data not shown).

Inhibition of the 04-MeThy Repair by Double-stranded DNA—Crude extracts from rat (H4 cells) (Fig. 4) or human (LICH cells) hepatoma cells (18) fail to show O4-MeThy repair. To obtain experimental conditions similar to those previously used with the cell extracts, the activity of the purified rat transferase was measured in the presence of crude cell extracts or of purified cellular DNA. The removal of O4-MeThy residues decreased to less than 10% in the presence of cellular extracts (200 mg of proteins) and dramatically decreased when cellular DNA was added in the incubation medium, whereas the activity on 06-MeGua was not significantly modified under these conditions using as substrate either MNU-treated DNA or poly(dG-dC) (Fig. 5). A similar decreased transferase activity on O4-MeThy in the presence of DNA was observed with the purified human protein (data not shown). It should be stressed that the DNA concentrations used in the present experiments are in the range of the amounts of DNA contained in the cellular extracts previously used to examine the O4-MeThy repair (Fig. 4) (18).

DISCUSSION

The purified transferases from human and rat origin are able to repair in vitro the O4-MeThy residues present in an alkylated poly(dA-dT). However, the two proteins have a low affinity for these residues. The rate constant for the human and rat transferases acting on O4-MeGua residues at 37 °C was $1 \times 10^9$ M$^{-1}$ min$^{-1}$, in good agreement with the value reported by Chan et al. (27), who determined this value by extrapolation of data obtained at lower temperatures. However the rate constants for the rat and, especially, the human transferases acting on O4-MeThy present in poly(dA-dT) were much lower: $4.8 \times 10^6$ and
1.8 x 10^5 m^{-1} min^{-1}, respectively. Few results have provided evidence that O^4-MeThy was repaired in mammalian cells. The removal of O^4-MeThy from a poly(dA-dT) substrate by monkey liver extracts was suggested by Becker and Montesano (28), but it was not shown whether this repair was due to a transferase activity. More recently the transfer of the methyl group from O^4-MeThy to the human transferase has been shown (30). No comparative data were available, however, on the relative repair of O^6-MeGua and O^4-MeThy by the eukaryotic transferases. Therefore our results, which show that the repair of O^4-MeThy is due to the actual transfer of the methyl group to the acceptor human or rat protein, are the first to compare the relative activities of these two purified proteins on O^6-MeGua and O^4-MeThy residues and to determine the rate of the reactions.

The O^4-MeThy repair was measured using as substrates alkylated poly(dA-dT) and poly(dA-T), because alkylation of DNA produces a very low amount of O^4-MeThy, representing 0.06% of the total alkylation (29). However, it is known that a B-conformation is a general structure to alternating pyrimidine-purine polymers (30). Furthermore, the two proteins remove O^6-MeGua residues with the same efficiency from alkylated DNA, poly(dG-dC), or poly(dG-C)], and, in the same experimental conditions, the 19-kDa fragment of the E. coli Ada protein removes O^4-MeThy residues from poly(dA-dT) as efficiently as O^6-MeGua from DNA. Therefore the two eukaryotic transferases and the E. coli protein have different abilities to repair O^4-MeThy, although they share the same active site (16). There is also a high degree of homology between the rat and the human transferases (14); nevertheless, these two proteins have different activities on O^4-MeThy residues. However, inhibition of the transferase activity on poly(dA-dT) by double-stranded DNA may suggest that the protein does not bind well to polymers consisting of only A and T residues, and that the activity for O^4-MeThy in normal DNA could be higher than in poly(dA-dT). This inhibition by DNA can also explain why many experiments failed to demonstrate O^4-MeThy repair by cell extracts containing large amounts of cellular DNA (18).

If we assume that these results can be extrapolated to the in vivo situation, the higher activity of the human protein on O^6-MeGua suggests that O^4-MeThy could be seldom repaired, because the transferase present in the cells might be preferentially used to repair O^6-MeGua residues. The different activities of the human and rat proteins on O^6-MeGua and O^4-MeThy, and the higher transferase inducibility in rat cells by DNA damage (31), would also suggest that the O^4-MeThy residues are less efficiently repaired in human cells. However, human cells of Mer+ phenotype possess a higher number of transferase molecules than rodent cells (32), and the overall repair capacity for O^4-MeThy might be similar in the cells of different origin. The different activity of the rat transferase on O^6-MeGua and O^4-MeThy residues could in part explain the different rates of removal of these two adducts, which have been measured in rat hepatocytes in vivo (33), although we cannot exclude the possibility that another repair mechanism exists in vivo for the removal of O^4-MeThy adducts.

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REFERENCES

1. Singer, B., and Grunberger, D. (1985) Molecular Biology of Mutagens and Carcinogens, Plemun Press, New York
2. Boiteux, S., Huisman, O., and Laval, J. (1984) EMBO J. 3, 2569-2573
3. Drinkwater, N. R., Miller, E. C., and Miller, J. A. (1980) Biochemistry 19, 5067-5092
4. Swann, P. F. (1990) Mutat. Res. 233, 81-94
5. Saffhill, R., Margison, G. P., and O’Connor, P. J. (1985) Biochem. Biophys. Acta 820, 111-145
6. Desanjh, M. K., Singer, B., and Eismann, J. M. (1991) Biochemistry 30, 7077-7093
7. Lindahl, T., Demple, B., and Roberts, P. (1982) EMBO J. 1, 1359-1363
8. McCarthy, T. V., Karran, P., and Lindahl, T. (1984) EMBO J. 3, 545-550
9. Potter, P. M., Wilkinson, M. C., Fitton, J., Carr, F. J., Brennand, J., Cooper, D. P., and Margison, G. P. (1987) Nucleic Acids Res. 15, 9177-9193
10. Robeck, G. W., Cozens, S., Carroll, P., and Samson, L. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3039-3043
11. Tano, K., Shiota, S., Cellier, J., Foote, R. S., and Mitra, S. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 686-690
12. Rydberg, B., Spurr, N., and Karran, P. (1990) J. Biol. Chem. 265, 9563-9569
13. Hayakawa, H., Koike, G., and Sekiguchi, M. (1990) J. Mol. Biol. 213, 739-747
14. Robeh-Karton, I., and Laval, F. (1991) Biochem. Biophys. Res. Commun. 177, 597-602
15. Rafferty, J. A., Elder, R. H., Watson, A. J., Cawkwell, L., Potter, F. M., and Margison, G. P. (1992) Nucleic Acids Res. 20, 1891-1896
16. Lang, C., Nakamura, T., Nakato, Y., Sakami, K., Hayakawa, H., and Sekiguchi, M. (1992) Carcinogenesis 13, 837-843
17. Pegg, A. E. (1990) Cancer Res. 50, 6110-6129
18. Bresnick, E., Dohan, M. E., Fraenkel-Conrat, H., Hall, J., Karran, P., Laval, F., Margison, G. P., Montesano, R., Pegg, A. E., Potter, P. M., Singer, B., Swenberg, J. A., and Yarasch, D. B. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 1762-1767
19. Sasanfar, M., Desanjh, M. K., Eismann, J. M., and Laval, F. (1991) J. Biol. Chem. 266, 2767-2771
20. Koike, G., Makita, H., Takeda, H., Hayakawa, H., and Sekiguchi, M. (1990) J. Biol. Chem. 265, 14754-14762
21. Laval, F., Little, J. B. (1977) Radiat. Res. 71, 571-578
22. Boiteux, S., and Laval, F. (1985) Carcinogenesis 6, 805-809
23. McCarthy, T. V., and Lindahl, T. (1986) Nucleic Acids Res. 13, 2683-2698
24. Weinfield, M., Drake, A. F., Saunders, J. K., and Paterson, M. C. (1985) Nucleic Acids Res. 15, 7067-7077
25. Lasnilli, U. K. (1970) Nature 227, 680-685
26. Laval, F. (1992) in Induced Effects of Genotoxic Agents in Eukaryotic Cells (Rosman, T., ed) pp. 39-50, Hemisphere Publishing Corp., Washington, DC
27. Chan, C. L., Wu, Z., Ciardelli, T., Statagatu, A., and Brennich, E. (1993) Arch. Biochem. Biophys. 1, 193-200
28. Becker, R. A., and Montesano, R. (1985) Carcinogenesis 6, 313-317
29. Dolan, M. E., and Pegg, A. E. (1985) Carcinogenesis 6, 1611-1614
30. Lonchosissoff, G. P., Butler, P. J. G., and Klag, A. (1981) J. Mol. Biol. 149, 745-760
31. Leffebre, P., and Laval, F. (1985) Cancer Res. 45, 57-5705
32. Gerson, S. L., Trej, J. E., Miller, K., and Berger, N. A. (1986) Carcinogenesis 7, 745-749
33. Belinsky, S. A. White, C. M., Boucheron, J. A., Richardson, P. C., Swenberg, J. A., and Anderson, M. (1986) Cancer Res. 46, 1280-1284