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POTENTIATION OF N-METHYL-N'-NITRO-N-NITROSOGUANIDINE-INDUCED O^6-METHYLGUANINE-DNA-METHYLTRANSFERASE ACTIVITY IN A RAT HEPATOMA CELL LINE BY POLY (ADP-RIBOSE) SYNTHESIS INHIBITORS

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The O^6-methylguanine-DNA-methyltransferase (transferase) activity in a rat hepatoma cell line (H4 cells) is enhanced as a response to DNA damaging agents. To study whether poly (ADP-ribosylation) is involved in this induction, the cells were treated with N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) that induces the transferase activity and stimulates poly (ADP-ribose) synthesis. Addition of poly (ADP-ribose) polymerase inhibitors enhanced the transferase increase induced by MNNG. The influence of the inhibitors on the transferase induction was dose and time-dependent. The results suggest that poly (ADP-ribose) is involved in the induction of this protein.

We have previously shown that treatment with a single dose of an alkylating agent (1), or with other DNA-damaging agents (2), increased the number of O^6-methylguanine-DNA-methyltransferase (transferase) molecules in a rat hepatoma cell line (H4 cells). This protein seems to play an important role in the cell sensitivity to alkylating agents (3), and specially to the chloroethyl-N- nitrosoureas used in cancer therapy, as it prevents the formation of inter strand DNA cross-links by these compounds (4). Therefore, induction of the transferase activity in tumor cells might be of importance in the development of resistance to alkylating drugs.

However, the mechanism underlying the transferase increase is not yet understood. A common feature between the various

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ABBREVIATIONS MNNG: N-methyl-N'-nitro-N-nitrosoguanidine; O^6-meGua: O^6-methylguanine; 3-AB: 3-aminobenzamide; 3-MB: 3-methoxybenzamide; 6-AN: 6-aminonicotinamide; MNU: N-methyl-nitrosourea.

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treatments which induce the transferase activity in H4 cells is the formation of DNA single strand breaks, which are formed either directly during the treatment, or during the repair of DNA damage. A response to DNA single strand breaks in mammalian cells is the synthesis of poly (ADP-ribose) (5), which ribosylates different proteins (6) and seems to play a role in DNA repair (7) and transformation (8).

In this study, we investigated a possible role of poly (ADP-ribose) in the induction of the transferase activity after treatment with an alkylating agent. The cells were treated with MNNG which is known to stimulate the poly (ADP-ribose) synthesis (9-10) and with different poly (ADP-ribose) synthesis inhibitors (11). Results show that inhibition of MNNG-stimulated poly (ADP-ribose) synthesis results in an enhanced induction of transferase in the cells.

MATERIALS AND METHODS

Cell culture. H4 cells (derived from a rat hepatoma)(2) were grown in Dulbecco's medium supplemented with 5% fetal calf serum, 5% horse serum, streptomycin (50 µg/ml), penicillin (50 IU/ml), in a 5 % CO2 atmosphere. The doubling time was about 15 hours and the plating efficiency ranged between 85 and 90 %.

Drug treatment. MNNG, obtained from Sigma Chemical Company, was dissolved in 0.1 M Na acetate (pH 4.0) and added at appropriate concentration in the culture medium. Poly (ADP-ribose) synthesis inhibitors (3-aminobenzamide, 3-methoxybenzamide and 6-aminonicotinamide) and 3-aminobenzoic acid were obtained from Sigma Chemical Company. They were dissolved in the culture medium.

Measurement of poly (ADP-ribose) synthesis. Exponentially growing cells were treated for 1 hour with 10 µM MNNG, with or without 2 mM 3-AB. The poly (ADP-ribose) synthesis was measured by incubating the cells with [^{3}H]-NAD and measuring the radioactivity incorporated in the acid-precipitable material, as already described (12).

Determination of the O^6-methylguanine-DNA-methyltransferase activity. Cells were trypsinized, washed in PBS and disrupted by sonication (10^8 cells / ml)in a buffer containing 70 mM Hepes (pH 7.6), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10 % glycerol and proteases inhibitors (antipain, leupeptin and aprotinin, 20 µg / ml each). Cell extracts were centrifuged at 12000 g for 10 min at 4°C. The transferase activity was determined by incubating aliquots of the supernatant with [^{3}H]MNU-treated DNA, prepared as already described (13), and measuring the disappearance of O^6-meGua from this substrate. Briefly, the incubation mixture contained in a final volume of 100 µl, 70 mM Hepes pH 7.6, 1 mM EDTA, 1 mM dithiothreitol, 5 µg of [^{3}]MNU-treated DNA (100 fmoles of O^6-meGua) and increasing amounts of cell extracts. After 20

minutes at 37°C, the substrate was acid hydrolyzed, and the remaining O^6-meGua was measured by HPLC, as already described 1(2).

RESULTS AND DISCUSSION

We have previously shown that treatment of H4 cells with a single dose of MNNG (10 µM) induces the transferase activity which is maximum 48 hours after the treatment (1). To test whether poly (ADP-ribosylation) is involved in the induction of this protein, 3-AB, an inhibitor of the poly (ADP-ribose) polymerase, was added in the culture medium at 5 mM for 7 hours after the MNNG treatment (Fig. 1). Addition of 3-AB enhanced the transferase activity induced by MNNG, whereas cell treatment with 3-AB alone did not show any detectable modification of the constitutive level of this activity. The number of transferase molecules per cell ,calculated from the linear part of the curves, was 54000±3500, 53600±4000, 137500± 5500 and 361200±7600 for control, 3-AB, MNNG and MNNG plus 3-AB treated cells, respectively.

To study the influence of various incubations times with 3-AB on MNNG-induced transferase increase, we used a lower (2 mM) 3-AB concentration to avoid non-specific side effects of this compound(14). The cells were incubated for 1 hour with 10 µM

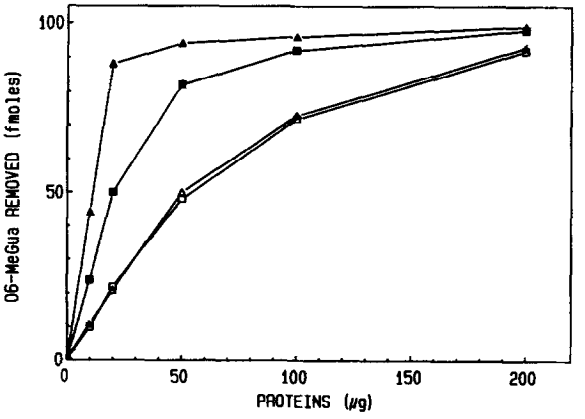

Fig.1 Removal of O^6-MeGua from alkylated DNA by H4 cell extracts.
[^{3}H]MNU-treated DNA was incubated with extracts from control cells (□ ------ □), cells treated for 7 hours with 3-AB (5 mM) (△ ------ △),for 1 hour with MNNG (10 µM) (■ -------- ■) or for 1 hour with 10 µM MNNG then for 7 hours with 5mM 3-AB (▲ -------- ▲).

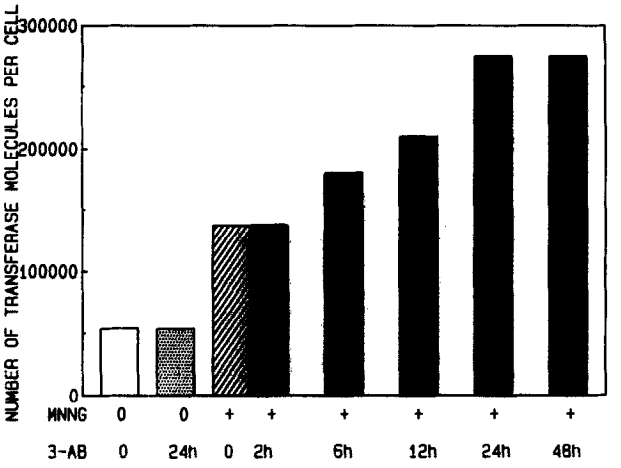

Fig.2 Number of transferase molecules after 1 hour MNNG-treatment and increasing exposure times to 3-AB.
H4 cells were incubated for 1 hour with 10 µM MNNG, rinsed, and grown for the indicated times in the presence of 2 mM 3-AB. In each case, the transferase activity was measured 48 hours after the MNNG treatment.

MNNG,then for 2 to 48 hours with 2 mM 3-AB, and in each case the transferase activity was determined 48 hours after the MNNG treatment. Results (Fig.2) show that at least a 6 hours incubation period with 3-AB is required to enhance the MNNG-induced increase, and that the maximum enhancement is obtained after a 24 hours exposure to the inhibitor. These results also show that the influence of 3-AB is dose - dependent, as the maximum number of transferase molecules per cell, after treatment with 2 mM 3-AB, was 275000±8600.

Experiments were designed to determine the changes of the transferase activity with time, in H4 cells treated with MNNG and 3-AB. The cells were incubated for 1 hour with MNNG, then with or without 3-AB for 24 hours, rinsed and grown for different time lenghts. Results (Fig. 3) show that after exposure to MNNG alone, the number of transferase molecules is maximum after 48 hours, then decrease to reach the control value after 4 days. When the cells are treated with both MNNG and 3-AB, the maximum increase occurs also after 48 hours, but the number of transferase molecules per cell decreases more slowly and 6 days after the treatment it remains about 3-fold higher than in non-treated cells.

This enhancement of the transferase activity induced by MNNG was also observed in cells incubated for 24 hours with two

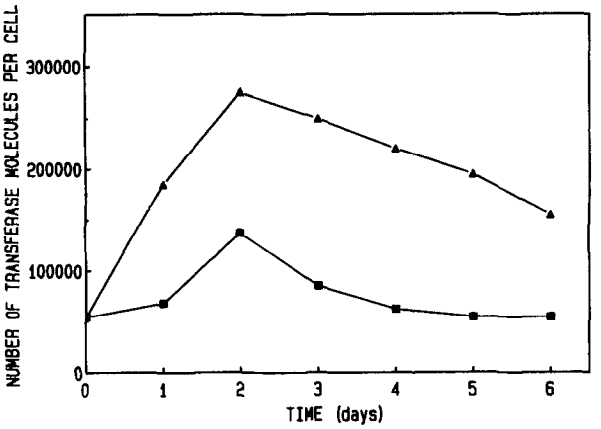

Fig.3 Kinetics of the appearance of transferase molecules in H4 cells after MNNG treatment, in the presence or absence of 3-AB.

Cells were treated for 1 hour with 10 µM MNNG (time 0), then grown for 24 hours with (▲ ------- ▲) or without (■------■) 2 mM 3-AB. The transferase activity was measured at the indicated times after MNNG treatment.

other poly (ADP-ribose) synthesis inhibitors, 3-MB (2.5 mM) and 6-AN (1 mM) (Table 1). However, the analogue 3-aminobenzoic acid, which does not inhibit poly (ADP-ribosylation), did not modify the number of transferase molecules in MNNG-treated cells. At these concentrations, the inhibitors alone were not toxic for the cells, but they increased the MNNG cytotoxicity (Table 1). They also inhibit the formation of poly (ADP-ribose) in H4 cells in response to MNNG treatment: the amount of [^{3}H]NAD incorporated in the acid-

Table 1. Influence of different poly (ADP-ribose)synthesis inhibitors on cell survival and MNNG-induced transferase activity

CELL TREATMENT	CELL SURVIVAL	NUMBER OF TRANSFERASE MOLECULES PER CELL
None	100	54000 ± 3500
3-aminobenzamide (2 mM)	98	53600 ± 4000
MNNG (10µM)	45	137500 ± 5500
MNNG+3-aminobenzamide(2mM)	30	275000 ± 8600
MNNG+3-methoxybenzamide(2.5mM)	28	286200 ± 7400
MNNG+6-amononicotinamide(1 mM)	31	278400 ± 6600
MNNG+3-aminobenzoic acid(2mM)	45	133800 ± 4400

H4 cells were incubated for 1 hour with 10 µM MNNG, rinsed, then grown for 24 hours in the presence of the inhibitors. 48 hours after the MNNG tretament, the transferase activity was measured using increasing amounts of cell extracts, or cells were plated for survival.

precipitable fraction of 2.5 x 10^5 cells corresponded to 310, 1050 and 180 cpm in control, MNNG (10 µM, 1 hour) and MNNG plus 3-AB (2 mM) treated cells, respectively.

It has been shown that MNNG-induced DNA amplification in a SV-40 transformed cell line was enhanced by 3-AB (15). However, we have evidence (data not shown) that the transferase increase in H4 cells is not related to DNA amplification. Poly (ADP-ribosylation) has numerous biological effects (reviewed in 16 and 17) and the mechanism through which the transferase is increased remains to be elucidated.

ACKNOWLEDGMENTS

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