Potentiation of Carcinogen-induced Methotrexate Resistance and Dihydrofolate Reductase Gene Amplification by Inhibitors of Poly(adenosine diphosphate-ribose) Polymerase

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ABSTRACT

Poly(ADP-ribose)ylation of nuclear proteins is an immediate response of most eukaryotic cells to DNA strand breaks, as induced by carcinogen treatment. DNA amplification, on the other hand, can be induced in cell culture systems by chemical or physical carcinogens, too, reaching peak levels a few days after induction treatment. We have previously shown that 3-aminobenzamide, an inhibitor of poly(ADP-ribose)ylation, potentiates carcinogen-induced simian virus 40 DNA amplification in hamster cells which served as a short-term model system (Bürkle et al., Cancer Res., 47: 3632–3636, 1987). Here we report that those results can be extended to the development of methotrexate (MTX) resistance associated with dihydrofolate reductase (DHFR) gene amplification in a different hamster cell line. (a) Treatment with the alkylating carcinogen N-methyl-4-N'-nitro-N-nitrosoguanidine (MNNG) 3 days before selection with 350 nM MTX induced the MTX resistance frequency by 17- to 100-fold, as expected. Addition of 3-aminobenzamide (0.1 to 1 mM) before MNNG treatment further potentiated the frequency of MTX resistance by up to 5-fold in a dose-dependent manner, parallel to a potentiation of cytotoxicity. MTX resistance frequency was potentiated not only relative to the decrease in cell survival but also in absolute terms. The same potentiation occurred after cotreatment with benzamide (1 mM), another poly(ADP-ribose)ylation inhibitor, under conditions which precluded direct drug interactions. Benzamide, a noninhibitory analogue, had no effect on the MNNG-induced MTX resistance frequency. (b) Neither 3-aminobenzamide, nor benzamide, nor benzoic acid at 1 mM, respectively, had any effect on the spontaneous frequency of MTX resistance. (c) Individual MTX-resistant colonies were expanded to determine their DHFR gene copy number. The relative frequency of DHFR gene amplification was similar (14% versus 22%) whether clones were derived from cultures induced with MNNG alone or MNNG in the presence of 1 mM 3-aminobenzamide. We conclude that poly(ADP-ribose)ylation should act as a negative regulatory factor in the induction of DNA amplification, since inhibition of poly(ADP-ribose) polymerase potentiates both MNNG-induced simian virus 40 DNA amplification, as shown previously, and MNNG-induced MTX resistance associated with DHFR gene amplification, as shown in this paper.

INTRODUCTION

Poly(ADP-ribose)ylation of a variety of nuclear proteins is an immediate response of most eukaryotic cells to DNA strand breaks, as induced by carcinogen treatment. This posttranslational modification is catalyzed by poly(ADP-ribose) polymerase (EC 2.4.2.30; see Ref. 1 for review), an abundant nuclear enzyme (2). Upon binding to a DNA strand break the enzyme catalyzes the transfer of ADP-ribose residues from NAD+ to acceptor proteins, elongation to poly(ADP-ribose) chains, as well as branching of this polymer. The polymer is specifically degraded by poly(ADP-ribose) glycohydrolase and ADP-ribose protein lyase, and it undergoes a very high turnover under conditions of DNA breakage (3). Many in vitro and in vivo studies suggest that poly(ADP-ribose)ylation plays a role in DNA repair (e.g., Refs. 4–6) and possibly in DNA replication and differentiation, although the precise molecular mechanisms are still a matter of debate.

DNA amplification is a manifestation of genome instability which allows a cell to drastically overproduce a specific gene product (see Refs. 7–10 for review). DNA amplification appears to play a role in several aspects of the process of carcinogenesis (11–16). Furthermore, amplification of “drug resistance genes” represents an important mechanism of cytostatic drug resistance of tumor cells. Well-known examples are the amplification of the DHFR3 gene conferring MTX resistance and carbamyl-phosphate synthetase/aspartate transcarbamylase/dihydroorotase gene amplification conferring N-phosphonacetyl-L-aspartate resistance (7, 9, 10). Finally, amplification and overexpression of multidrug resistance genes (mdr) encoding drug transporter proteins can confer cross-resistance to a variety of nonrelated cytotoxic compounds, e.g., colchicine, doxorubicin, actinomycin D, or vinblastine (17). So far, no single molecular mechanism has been identified which could account for all the features of DNA amplification. Instead, a number of different mechanisms have been proposed, which may operate in different cell and selection systems or at different stages during the amplification process (for review, see Ref. 10).

There is ample evidence from cell culture studies that the amplification of authentic cellular genes (e.g., drug resistance genes) or integrated viral sequences can be greatly induced by chemical or physical carcinogens (18–25), cytostatic agents (24, 25), hypoxic conditions (26, 27), and certain viruses (28, 29). Apparently trans-acting protein factors play a role in this induction phenomenon (25, 30, 31). After induction, amplification usually requires many hours up to several days to develop and become detectable.

We initially studied a possible role of poly(ADP-ribose) synthesis in DNA amplification in a SV40-transformed Chinese hamster cell line (CO 60) (23) that amplifies the integrated SV40 sequences after carcinogen treatment. Our results showed that inhibition of carcinogen-stimulated poly(ADP-ribose) synthesis by 3AB was correlated with a 2- to 6-fold potentiation of inducible DNA amplification in CO 60 cells (32). This led us to the hypothesis that poly(ADP-ribose)ylation may act as an endogenous negative regulatory factor in the induction of DNA amplification. Since amplification was assayed as overall increase of SV40 sequences in a short-term assay and since carcinogen induction was associated with a relatively high toxicity, it was important to verify that the observed effect occurred in living, i.e., clonogenic cells. The system we chose to address this question was the induction of DHFR amplification in CHO

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3 The abbreviations used are: DHFR, dihydrofolate reductase; 3AB, 3-aminobenzamide; Bz, benzamide; BzA, benzoic acid; CHO, Chinese hamster ovary cells; EF, enhancement factor; FRG, Federal Republic of Germany; LD50, 50% lethal dose; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; MTX, methotrexate; PE, plating efficiency; 1× SSC, 150 mM sodium chloride and 15 mM sodium citrate; SDS, sodium dodecyl sulfate; SV40, simian virus 40.
cells, mediating MTX resistance (9, 18-22, 26). This system can further show whether the amplification of authentic cellular genes is modulated by inhibitors of poly(ADP-ribosylation) in the same fashion as the amplification of integrated SV40 sequences. Here we describe that, indeed, inhibitors of poly(ADP-ribosylation) potentiate the frequency of MNNG-induced MTX resistance and, in parallel, DHFR gene amplification.

MATERIALS AND METHODS

Cells. CHO-9 cells were a kind gift of Dr. B. Kaina, Heidelberg, FRG, and were routinely maintained in a 1:1 mixture of Dulbecco's minimal essential medium and Ham's F10 (without thymidine and hypoxanthine; Biochrom, Berlin, FRG) supplemented with 100 units of penicillin, 100 µg/ml of streptomycin, 2 mm glutamine, and 10% heat-inactivated fetal calf serum (Biochrom) at 37°C in an atmosphere of 5% CO₂. Cells were free of Mycoplasma contamination.

Drug Treatment and MTX Selection. The protocol we used is outlined in Fig. 1. CHO-9 cells were seeded in 150-cm² culture flasks at 2 x 10⁶ for controls or 4 x 10⁶ for MNNG treatment. The next day, cultures were first given benzamide or derivatives (3AB, Bz, or BzA sodium salt; Sigma, Munich, FRG) followed by MNNG addition (Serva, Heidelberg, FRG) to this medium 1 h later ("pretreatment"). In some experiments the order of drug treatment was reversed, as indicated. In this case cultures were first treated with MNNG for 1 h, whereupon medium was removed, followed by addition of fresh medium containing benzamide or derivatives ("posttreatment"). After 3 days without further medium change, cultures were trypsinized and replated for determination of PE and, in parallel, the rate of MTX resistance. For plating efficiency, 500 or 2000 cells (controls/carcinogen treatment) were plated in triplicate onto 10-cm Petri dishes in medium containing 10% dialyzed fetal calf serum. On Day 11 after induction, these cultures were fixed and stained with Meyer's hemalum (Merck, Darmstadt, FRG, and were routinely maintained in a 1:1 mixture of Dulbecco's minimal essential medium and Ham's F10 (without thymidine and hypoxanthine; Biochrom, Berlin, FRG) supplemented with 100 units of penicillin, 100 µg/ml of streptomycin, 2 mm glutamine, and 10% heat-inactivated fetal calf serum (Biochrom) at 37°C in an atmosphere of 5% CO₂. Cells were free of Mycoplasma contamination.

For parallel determination of MTX resistance 5 x 10⁶ cells (except stated otherwise) were plated in triplicate onto 10-cm Petri dishes in medium containing 10% dialyzed fetal calf serum and 350 nM MTX (Sigma). The LD₉₀ of MTX for this cell line was determined to be 22 nM (data not shown). In these cultures, MTX-containing medium was changed on Days 6, 9, 12, and 16. On Days 21 to 24 cultures were fixed and stained for colony counting or, alternatively, used to determine DHFR gene amplification in individual colonies.

Determination of DHFR Copy Number. Individual MTX-resistant colonies were randomly picked, transfected to microwell dishes, and expanded in selection medium (350 nM MTX). Approximately 10⁶ cells of each clone were processed for DNA slot blot hybridization as described (32). In brief, total genomic DNA was extracted, RNA was hydrolyzed by alkali, and DNA was sucked onto nitrocellulose filters. Slot blots were prehybridized in 50% formamide, 5x SSC, 5x Denhardt's solution, 50 mm sodium phosphate buffer, pH 6.5, 100 µg/ml of tRNA, and 0.1% SDS at 50°C for 30 min, followed by hybridization under the same conditions for 2 days with a single-stranded ³²P-labeled RNA probe (33) for a hamster DHFR intron sequence (DHFR-B in Ref. 34; kindly provided by Dr. H. Cedar, Jerusalem, Israel). Filters were washed with four changes of 1x SSC, 0.1% SDS at 70°C and autoradiographed for 5 days. Specific hybridization of the RNA probe under these conditions was verified on a Southern blot of CHO DNA (data not shown). As a control for the exact amount of cellular DNA bound to the filter, blots were subsequently rehybridized overnight at 42°C (without prior stripping of the DHFR probe) with ³²P-labeled total CHO DNA (35) as probe, washed, and autoradiographed for 16 h. Given the large difference in hybridization intensities between the DHFR and the CHO probe, residual signals of the former hybridization were negligible. Densitometry of the autoradiographs, normalization of densitometer readings according to appropriate reconstitution experiments, and determination of amplification factors (DHFR gene copy numbers of the MTX-resistant clones relative to those of MTX-sensitive control CHO-9 cells) were done as described (32). The criterion for amplification was an amplification factor of 2 or more.

RESULTS

DHFR gene amplification conferring resistance to the cytotoxic drug MTX is one of the most extensively studied systems of inducible amplification of a cellular gene (7, 9, 10, 18-22, 26). The experimental protocol we followed is similar to the one described by Kleinberger et al. (19) and is outlined in Fig. 1. CHO-9 cells were treated with MNNG, an alkylating carcinogen, with or without addition of inhibitors of poly(ADP-ribosylation). Three days later, when the frequency of amplification reaches its maximum (19), cultures were trypsinized and replated into medium containing 350 nM MTX to select for MTX-resistant cells which overexpress the DHFR gene, possibly due to DHFR gene amplification. To measure the cytotoxic effects of the different induction treatments, plating efficiencies were assayed in parallel. Table 1 shows that MNNG treatment (2 µM) alone leads to a large reduction of plating efficiency as compared with untreated controls, while the MTX resistance frequency is increased by 92-fold (EF), in agreement with earlier reports (18-22). MNNG treatment is known to induce a transient stimulation of poly(ADP-ribose) polymerase activity and an increased turnover of the polymer within minutes.
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utes, lasting up to hours (3, 36). To see how an inhibition of poly(ADP-ribosyl)ation would influence the MTX resistance rate, cells were incubated with increasing concentrations of 3AB, a competitive poly(ADP-ribose) polymerase inhibitor (37). It is important to note that we used inhibitors at only 1 mM or lower to avoid nonspecific effects occurring at higher concentrations (38, 39). Under these conditions, however, enzyme inhibition may be partial only (32). When 3AB is added 1 h before MNNG treatment, there is a dose-dependent decrease in plating efficiency below the level induced by MNNG alone (Table 1), as expected (4, 5). By contrast, the MTX resistance frequency is further increased by up to 5-fold (Table 1). Colonies from MNNG/3AB-treated cultures displayed a very similar size distribution compared with MNNG-treated controls and were homogeneously distributed on the dishes, making it highly unlikely that satellite colony formation accounted for the observed effect. Furthermore, we can exclude that the increase in MTX resistance frequency is due to a selective killing of nonamplifying cells, since the total yield of MTX-resistant clones derived from the cultures treated on Day 0 was clearly higher after combined induction with MNNG/1 mM 3AB as compared with MNNG alone (732 versus 400 clones in the experiment shown in Table 1). Thus there is not only a relative, but also an absolute enhancement of resistance induction by 3AB cotreatment. In a separate experiment, 3AB shows a similar dose-related potentiation of the MTX resistance frequency after induction with 1 µM MNNG (Fig. 2A).

We wanted to exclude the possibility that the observed effect results from direct interactions between 3AB and MNNG which could play a role when both drugs are present simultaneously. Since it is known that MNNG damages DNA very rapidly and is labile in culture medium (data not shown), we reversed the order of drug addition in a separate set of experiments. Fig. 2, B and C, shows that the same potentiation effect occurs if 3AB is added in fresh medium 1 h after MNNG treatment (Fig. 2C; asterisk), at a time when most of the MNNG has already been inactivated (data not shown). Bz, another potent inhibitor of poly(ADP-ribosyl)ation, has a very similar enhancing effect on MNNG-induced MTX resistance when applied 1 h after MNNG addition (Fig. 2, D and E, asterisks). Surprisingly, addition of Bz before MNNG consistently results in an inhibition by about 50% of MNNG-induced MTX resistance (Fig. 2E and data not shown), an effect we never observed with 3AB (Table 1; Fig. 2, A and B). Since, in addition, the well-known cytotoxic effect of Bz and MNNG was almost absent under the conditions of Bz preaddition (data not shown), we interpret this result as a consequence of some direct interaction of Bz and MNNG. This finding may add to the list of nonspecific effects of poly(ADP-ribosyl)ation inhibitors (38–40).

As a specificity control we used BzA, a structural analogue of Bz which does not inhibit poly(ADP-ribosyl)ation (37). BzA posttreatment (1 mM) has no effect on MNNG-induced MTX resistance (Fig. 2E). Likewise, 3AB and Bz as well as BzA had no effect on the MTX resistance frequency in the absence of MNNG treatment (Fig. 2F). This is in line with the fact that these compounds are nontoxic, nonmutagenic, and noncarcinogenic on their own at the concentrations used in this study. Viewed together, inhibition of poly(ADP-ribosyl)ation by 3AB or Bz under conditions which preclude a direct drug interaction with MNNG is correlated with a potentiation of the MNNG-induced MTX resistance frequency.

To confirm that MTX resistance was indeed associated with DHFR gene amplification individual colonies from MNNG- or MNNG/3AB-induced cultures were randomly picked, expanded under MTX selection, and analyzed by quantitative DNA slot blot hybridization with a hamster DHFR intron probe (34, 41, 42) in conjunction with total CHO DNA as a control probe for the amount of cellular DNA applied to the filter (22). Slot blot autoradiograms are shown in Fig. 3. We found DHFR gene amplification in 2 of 14 clones obtained after induction with MNNG (2 µM) alone and in 4 of 18 clones after combined induction with MNNG (2 µM)/3AB (1 mM). Thus the relative frequency of DHFR amplification among resistant clones is roughly similar without or with 3AB coinduction (14% and 22%, respectively). We thus infer that, along with the potential of MTX resistance frequency, 3AB potentiates the frequency of DHFR amplification to the same extent.

**DISCUSSION**

In this paper we show that two inhibitors of poly(ADP-ribosyl)ation, 3AB and Bz, potentiate the MNNG-induced MTX resistance frequency under conditions which preclude direct interference of the inhibitor with MNNG. In the absence of MNNG treatment, i.e., in the absence of DNA strand breaks and consequent activation of poly(ADP-ribose) polymerase, the inhibitors have no effect on MTX resistance.

Three different mechanisms of MTX resistance have been recognized so far, all of which are induced by carcinogen treatment: (a) alterations of MTX transport; (b) DHFR gene amplification and consequent overexpression; and (c) DHFR mutations leading to a reduced enzyme affinity for MTX. Apparently there is a window of MTX concentrations to select for each of these mechanisms, transport alterations being favored at low, DHFR amplification at moderate, and mutations at high selection pressure, respectively (9, 21, 43). Determination of DHFR gene copy numbers in our MTX-resistant cell clones showed that the relative frequency of DHFR gene amplification was approximately 20%, irrespective of whether cultures had been induced with MNNG alone or with MNNG in the presence of 3AB. This implies that, along with the potentiation of the MTX resistance frequency, the frequency of
DHFR amplification is potentiated to the same extent. Apparently, alternative resistance mechanisms, which accounted for the resistant phenotype in the majority of our clones but have not been characterized in this work, were potentiated likewise. The relative frequency of DHFR amplification events in our experiments was lower than the about 50% described in a previous study. The amplification factor of controls (MTX-sensitive CHO-9 cells) is 1 by definition. A, MTX-resistant clones obtained after induction with 2 μM MNNG; B, clones obtained after induction with 2 μM MNNG in the presence of 1 mM 3AB. contr., control.

This work is an extension of an earlier study on amplification of integrated SV40 DNA in CO 60 cells where we have found that 3AB potentiates MNNG-induced DNA amplification measured directly by quantitative slot-blot hybridization in a short-term assay (32). The results reported here are in good agreement with our previous observations and furthermore reveal that the potentiating effect of poly(ADP-ribosyl)ation inhibitors exists independently of SV40 functions and is of biological relevance since it is assayed in clonogenic cells.

Recently a loss of amplified c-myc oncogene sequences was described after treatment of HL-60 cells with 7.5 mM Bz or other drugs including dimethyl sulfoxide (44). These findings, however, differ from ours in two respects. (a) A cell system was used in which amplified sequences are present constitutively, whereas we induced de novo amplification. (b) High inhibitor concentrations were utilized to trigger the loss of amplified sequences which may affect metabolic pathways other than poly(ADP-ribosyl)ation (38). This is further detailed in a recent report showing in vitro as well as in intact cells that Bz concentrations below 1 mm already inhibit poly(ADP-ribose) polymerase, whereas higher concentrations inhibit mono(ADP-ribose) transferases in addition (39). Therefore, the specificity of the reported loss of c-myc amplification by Bz is not clear.

Our results are suggestive of a negative regulatory role for poly(ADP-ribosyl)ation in the induction process of DNA amplification. Since 3AB and Bz are effective only under conditions of DNA damage and consequent stimulation of poly(ADP-ribose) polymerase and since they potentiate the frequency not only of DHFR gene amplification but also of other MTX resistance mechanisms, it is likely that poly(ADP-ribosyl)ation exerts this role by controlling DNA repair or other processes involved in the cellular recovery from DNA damage (4–6). Inhibitors of poly(ADP-ribosyl)ation are known to interfere with DNA repair and could thus further enhance cellular responses to DNA damage such as DNA amplification. Furthermore, these inhibitors aggravate cell cycle disturbances which occur after DNA damage, leading to a prolongation of S-phase and to an accumulation of cells at the G2-M boundary (45). Such cell cycle disturbances, on the other hand, are supposed to be critical for the induction of DNA amplification (9).

To avoid the use of chemical inhibitors and their inherent side effects and to formally prove our hypothesis of a negative regulatory role of poly(ADP-ribosyl)ation in carcinogen-induced DNA amplification, we are currently establishing a molecular genetic approach to modulate cellular poly(ADP-ribose) metabolism. We hope that this will lead to a clearer understanding of the role(s) of poly(ADP-ribosyl)ation in cell physiology as well as in the multistep process of carcinogenesis.

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