Inhibition of gene-specific repair of alkylation damage in cells depleted of poly(ADP-ribose) polymerase

Tinna Stevnsner, Ruchuang Ding¹, Mark Smulson¹ and Vilhelm A. Bohr*

Laboratory of Molecular Genetics, National Institute of Aging, National Institutes of Health, 4940 Eastern Avenue, Baltimore, MD 21224 and ¹Department of Biochemistry and Molecular Biology, Georgetown University Medical School, 3900 Reservoir Road NW, Washington, DC 20007, USA

Received August 4, 1994; Revised and Accepted September 26, 1994

ABSTRACT

The role of the enzyme poly(adenosine diphosphate-ribose) polymerase (PADPRP) in DNA repair at the level of the gene was investigated with human HeLa cells in which PADPRP antisense transcripts are inducible with dexamethasone. After such induction, the cellular content of PADPRP is reduced by 90%. DNA damage and its repair was studied in the essential dihydrofolate reductase (DHFR) gene after exposure of the cells to either ultraviolet (UV) irradiation or the alkylating agent nitrogen mustard. The expression of the antisense construct had no effect on gene-specific repair of UV-induced pyrimidine dimers. In contrast, induced antisense cells were deficient in the gene-specific repair of nitrogen mustard-induced lesions. Dexamethasone itself did not inhibit gene-specific repair in control cells. Thus, PADPRP appears to participate in the gene-specific repair of alkylation damage, but not in the repair of UV-induced pyrimidine dimers. Clonal survival assays revealed that cells depleted of PADPRP showed an increased susceptibility to nitrogen mustard, supporting the notion that repair of essential genes is critical for cellular survival.

INTRODUCTION

Poly(adenosine diphosphate-ribose) polymerase (PADPRP) is an abundant nuclear enzyme that catalyzes the synthesis of poly(ADP-ribose) from nicotinamide adenine dinucleotide (NAD). The catalytic activity of PADPRP is strictly dependent on the presence of strand breaks in DNA (1,2), and the enzyme is thought to participate, along with other enzymes, in DNA repair, DNA replication, and other cellular processes (3,4). Ultraviolet (UV) irradiation (254 nm) induces the formation of pyrimidine dimers in DNA as well as a small number of strand breaks (5). In contrast, alkylating agents induce a substantial number of DNA strand breaks. It has therefore been suggested that PADPRP participates in the repair of alkylation damage but

not in the repair of UV-induced pyrimidine dimers. Consistent with this suggestion, 3-aminobenzamide (3-AB), which inhibits PADPRP, does not affect the repair of UV-induced damage in total genomic DNA or of the repair of pyrimidine dimers in the dihydrofolate reductase (DHFR) gene of Chinese hamster ovary (CHO) cells (6). However, 3-AB and benzamide, which have been used in many of the studies of the cellular roles of PADPRP, may not be specific for PADPRP (7).

Whereas classic DNA repair studies measured the repair events in the total genome, it has become evident that repair in individual genes can be a more important indicator for biological end points such as cellular survival (8). Recent studies have further suggested that the enzymology of gene-specific repair may differ from that of repair of the bulk of the genome (9), which occurs in the ~99% of the genome that is not transcribed. Because PADPRP is likely to contribute to some aspect of DNA repair, such as the restructuring of chromatin near the repair site, it is important to investigate whether this enzyme plays a role in gene-specific DNA repair.

Recently, a cell line was established from human HeLa-S3 cells in which the expression of PADPRP antisense transcripts is controlled by a glucocorticoid-inducible mouse mammary tumor virus promotor (10). Activity measurements and immunoblot analysis indicated that the concentration of this highly abundant nuclear enzyme was reduced to negligible levels during a 3 day period of antisense RNA induction. This antisense cell line provides a system in which it is possible to test directly, without the use of inhibitors, whether PADPRP participates in cellular processes such as total genomic DNA repair and gene-specific DNA repair. With the use of this system, depletion of PADPRP has been shown to result in a delay in overall genomic DNA strand break rejoining after exposure of cells to methyl methanesulfonate (MMS) (10). The observation that inhibition of repair was most marked at very early times after DNA damage may indicate a role for PADPRP in gene-specific repair, which occurs before bulk genome repair.

We have now examined the effect of induction of PADPRP antisense transcripts on the gene-specific repair of pyrimidine

*To whom correspondence should be addressed

dimers and of nitrogen mustard (HN2)-induced alkylation damage. UV represents a prototype DNA damage for which there is maximal understanding of the repair process. UV irradiation (254 nm) introduces two main photoproducts into the DNA, the pyrimidine dimer and the 6–4 photoproducts. Both are mutagenic, but the pyrimidine dimer is by far the most frequent, representing ~80% of the total lesions. Our studies therefore involve the measurement of this lesion. PADPRP is more likely to be involved in the repair of alkylation damage, and for those experiments we used HN2 as a prototype alkylator.

MATERIALS AND METHODS

Materials

Bis(2-chloroethyl)methylamine (nitrogen mustard, HN2) was obtained through the Developmental Therapeutics Program (Division of Cancer Treatment, National Cancer Institute) and was dissolved immediately prior to use in 0.1 M HCl. Dexamethasone was obtained from Sigma and dissolved in 75% ethanol. [³H]Thymidine (>80 Ci/mmol) and [α -³²P]dCTP (3000 Ci/mmol) were from DuPont-New England Nuclear, and restriction endonuclease *Hind*III was from Boehringer Mannheim.

Probe

The genomic probe for detection of a 22 kb *Hind*III fragment of the human DHFR gene has been described previously (11). The probe was labeled with [³²P] by random multiprimer labeling.

Cell culture

HeLa-S3 cells transfected with the pMAMneo construct (control cells) or the PADPRP antisense pMAMneo construct (PADPRP-as cells) have been described in detail previously (10). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μ g/ml). The assay for detection of gene-specific repair has been described previously (12). In brief, exponentially growing cells were incubated for 3 days with 0.3 μ Ci/ml [³H]thymidine and PADPRP antisense transcripts were induced on the 3rd day by the addition of 1 μ M (final) dexamethasone to the medium. The cells were then subcultured and incubated in isotope-free medium with or without 1 μ M dexamethasone for another 2 days.

Colony formation—survival assay

The survival of antisense and control cells after exposure to various doses of HN2 was assessed by colony-forming ability. Cells (10^6 – 2×10^6) were incubated in T-150 flasks (Falcon) in the presence or absence of 1 μ M dexamethasone for 48 h, exposed to trypsin, and plated in triplicate at densities of from 500 to 5×10^5 cells/10 cm dish. The cells were further incubated in the presence or absence of 1 μ M dexamethasone for an additional 24 h, washed once with phosphate-buffered saline, and treated with HN2 at various concentrations at 37°C for 30 min. Cells were then washed twice with phosphate-buffered saline and grown in medium without HN2 for 2–3 weeks, until colonies had formed. The cells were fixed and stained with Giemsa stain (Sigma), and the number of colonies containing >40 cells was determined. Plating efficiency controls for both antisense and control cells untreated with HN2 were performed in parallel. Plating efficiency was 40–60%.

Analysis of pyrimidine dimer repair in the DHFR gene

Prior to UV irradiation the medium was removed from the prelabeled cells. The cells were then UV irradiated (254 nm) at 15 J/m². After irradiation, the cells for the 0 h (no repair) time point were lysed immediately, whereas those for later repair time points were incubated in culture medium with or without dexamethasone, supplemented with 10 μ M bromodeoxyuridine and 1 μ M fluorodeoxyuridine in order to density-label the replicated DNA after damage. After cell lysis, high molecular weight DNA was isolated and digested with *Hind*III. The parental DNA was separated from the replicated DNA on CsCl equilibrium gradients. Portions (10 μ g) of each parental DNA sample were treated with T4 endonuclease V or with endonuclease buffer alone, and the DNA samples were resolved on 0.5% alkaline agarose gels, transferred to membranes, and hybridized to the specific probe for the DHFR gene (Southern blots). Autoradiograms of the membranes were quantitated by densitometry or with a Betascope analyzer. The relative intensities of parallel bands representing full-length fragments were compared to determine adduct frequency as previously described (12).

Analysis of alkali-labile sites induced by HN2

Antisense and control cells were labeled with [³H]thymidine and induced with dexamethasone as described above. Prior to treatment with HN2, the medium was removed and replaced with medium containing 1% fetal bovine serum with or without 1 μ M dexamethasone. After 30 min, 100 μ M HN2 was added to the medium for 30 min. Cells were then lysed immediately or after repair incubation in medium, with or without dexamethasone, supplemented with 10 μ M bromodeoxyuridine and 1 μ M fluorodeoxyuridine. After cell lysis, high molecular weight DNA was isolated and digested to completion with *Hind*III. Parental DNA was separated from replicated DNA by CsCl equilibrium gradient centrifugation. Duplicate portions of each DNA sample were depurinated at all heat-labile alkylated bases and then hydrolyzed in alkali to cleave the DNA at apurinic sites as previously described in detail by Wasserman *et al.* (13). The DNA samples were then subjected to electrophoresis in 0.5% alkaline agarose gels, Southern transferred, and hybridized to the specific probe for the DHFR gene. Band intensities were quantitated with a densitometer or a Betascope analyzer. The average frequency of apurinic sites in the DHFR gene was calculated from the fraction of DNA in the band from the treated sample compared to that from the untreated sample. The average number of apurinic sites/DNA fragment (N) was calculated from: $N = -[\ln(\text{fraction of molecules free of apurinic sites})]$.

RESULTS

Effect of depletion of PADPRP on cell survival

The repair efficiency in specific genes in many instances correlates with cell survival (8). We therefore investigated the effect of induction of PADPRP antisense transcripts on the survival of the cells after exposure to HN2. Control and antisense HeLa cells were incubated in the presence or absence of dexamethasone (1 μ M) for 72 h as described in Materials and Methods. Dexamethasone was subsequently removed from the cultures and the cells were incubated with HN2 (0–20 μ M) for 30 min. Cells were then incubated in the absence of HN2 for 2–3 weeks, until colonies contained >40 cells. The number of

Figure 1. Effect of dexamethasone on the cytotoxicity of HN2 to control cells and antisense cells. Cells were incubated in the absence or presence of 1 μ M dexamethasone for 72 h, and then treated with various concentrations of HN2 for 30 min at 37°C. Cell survival was determined by a colony-forming assay. Values represent the mean $\pm$ SEM of at least three independent experiments. As, PADPRP antisense cells; Con, control cells. (+) Dex, presence of 1 μ M dexamethasone; (-) Dex, absence of 1 μ M dexamethasone.

colonies were then counted, and the results are shown in Fig. 1. Antisense cells exposed to dexamethasone were markedly more sensitive to HN2 than either control cells (incubated in the absence or presence of dexamethasone) or antisense cells not exposed to dexamethasone; dexamethasone-treated antisense cells yielded approximately one tenth the number of colonies observed for the other groups at 10 and 20 μ M N2. Dexamethasone-treated and non-treated control cells showed a similar resistance to HN2, indicating that dexamethasone had no effect on the survival of control cells. These results are consistent with our previous observation that reduced PADPRP expression inhibits DNA repair in the overall genome (10). They are also consistent with our previous data showing that the MMTV promoter is not 'leaky' in the antisense cell line, as no increased susceptibility to HN2 was observed in uninduced antisense cells relative to control cells.

Effect of depletion of PADPRP on the repair of UV-induced pyrimidine dimers

Analysis of the effect of the induction of antisense PADPRP transcripts on the gene-specific DNA repair of UV-induced pyrimidine dimers is shown in Table 1, Fig. 2 and Fig. 4A. PADPRP antisense cells were incubated in the absence or presence of 1 μ M dexamethasone for 72 h, exposed to 15 J/m²

Table 1. Initial damage frequency of UV-induced pyrimidine dimers and of nitrogen mustard-induced alkali-labile sites in the DHFR gene of PADPRP antisense cells

Lesion	Initial lesion frequency per 10 kb	
	(-) Dexamethasone	(+) Dexamethasone
UV pyrimidine dimers	1.61 $\pm$ 0.12	1.47 $\pm$ 0.10
HN2 alkali-labile sites	1.34 $\pm$ 0.26	1.69 $\pm$ 0.02

Data are means $\pm$ SEM of several gels from at least 3 individual experiments with each damaging agent.

Figure 2. Effect of induction of PADPRP antisense transcripts on the formation and repair of UV-induced pyrimidine dimers in the DHFR gene. Antisense cells were incubated in the presence of 1 μ M dexamethasone for 72 h before exposure to UV irradiation (254nm, 15J/m²) (Dex.) or mock-treated before UV irradiation (No dex.). The cells were then lysed immediately or allowed to repair their DNA for 3, 8 or 24 h. High molecular weight DNA was isolated, digested to completion with *Hind*III, treated or not treated with T4 endonuclease V (indicated by +; no treatment by -), and subjected to Southern blot analysis with a DHFR gene-specific probe as described in Materials and Methods. Autoradiograms from representative Southern blots are shown.

UV light (254 nm) and then either lysed immediately or allowed to repair their DNA for 8 or 24 h. DNA samples were run in parallel with or without treatment with the T4 endonuclease and the resulting membrane was probed for the DHFR gene. Fig. 2 shows a representative autoradiogram of a Southern analysis used for the calculations in Table 1 and Fig. 4A. The appearance of a strong DHFR band in the T4 endonuclease-treated lanes at 24 h indicates that considerable repair has taken place. The data are based on several gels from each of 3 independent experiments. When the PADPRP antisense cells were treated with dexamethasone [thus reducing the PADPRP levels by 90% (10)] prior to UV irradiation, a slightly lower frequency of pyrimidine dimers were formed in the DNA compared to cells not treated with dexamethasone. However, dexamethasone treatment had no significant effect on the rate of repair of pyrimidine dimers. Thus, depletion of PADPRP did not affect the rate of repair of pyrimidine dimers in the DHFR gene.

Effect of depletion of PADPRP on the repair of HN2-induced alkali-labile sites

We incubated control and antisense cells in the absence or presence of 1 μ M dexamethasone for 72 h before treatment with 100 μ M HN2 at 37°C for 30 min. The cells were then either lysed immediately or allowed to repair their DNA for 8 or 24 h. The formation and repair of alkylated bases were measured by removal of alkylated purine bases, alkaline hydrolysis and Southern analysis as described in Materials and Methods. Representative quantitative Southern blots of dexamethasone

Figure 3. Effect of induction of PADPRP antisense transcripts on the formation and repair of HN2-induced alkali-labile sites in the DHFR gene in control and PADPRP antisense cells. Cells were incubated with 1 μ M dexamethasone for 72 h before exposure to 100 μ M HN2 for 30 min. The cells were then lysed immediately or allowed to repair their DNA for 3, 4, 8 or 24 h. High molecular weight DNA was isolated, digested to completion with *Hind*III, and exposed to heat and alkali as described in Materials and Methods. Autoradiograms of representative Southern blots with a DHFR gene-specific probe are shown. All samples were analyzed in duplicate. Treatment of the cells with HN2 is indicated by + and no HN2 treatment is indicated by -. The first 4 lanes are controls and represent DNA from cells not treated with HN2 that was (lanes 3 and 4) or was not (lanes 1 and 2) subjected to heat treatment to remove purine bases.

induced antisense and control cells is shown in Fig. 3. At 0 h there is substantial damage in the DHFR gene as visualized by the light density in the 0 h lanes compared to the control lanes. The repair results for induced and non-induced antisense cells, based on three independent experiments, are shown in Table 2 and Fig. 4B. Dexamethasone treatment slightly increased the initial frequency of HN2-induced adducts in PADPRP antisense cells, although this difference did meet the level of statistical significance (Table 2). At all time points there was less repair of the alkali-labile sites in the dexamethasone-treated cells than in the non-treated cells. This indicates that PADPRP does play a role in the gene-specific repair of HN2-induced alkali-labile sites.

To rule out the possibility that dexamethasone itself had an inhibitory effect on DNA repair, we measured the effect of dexamethasone on the repair of HN2-induced DNA damage in control cells. At all time points except 2 h, the extent of repair was actually slightly higher in the dexamethasone-treated cells than in the non-treated cells (Table 2).

DISCUSSION

Activation of PADPRP requires DNA single- or double-strand breaks (14). Furthermore, cells treated with the PADPRP inhibitor 3-AB are sensitized to agents that generate strand breaks in DNA (15). Thus, PADPRP has been thought to participate in DNA repair, and several recent studies support this notion

Figure 4. Effect of PADPRP depletion on the repair of UV-induced pyrimidine dimers (A) and of nitrogen mustard-induced lesions (B) in the DHFR gene. Cells were incubated in the absence or presence of dexamethasone before and after DNA damage. Data are means of several gels from at least 3 individual biological experiments. Bars represent $\pm$ SEM.

Table 2. Initial damage frequency and repair of HN2-induced alkali-labile sites in the DHFR gene of PADPRP control cells

Repair time (h)	Repair (%)	
	(-) Dexamethasone	(+) Dexamethasone
2	8	8
4	25	28
8	33	44
24	42	49
Initial lesion frequency	1.39	2.18

Data are means of several gels from one biological experiment.

(10,16,17). Most studies of the role of PADPRP in DNA repair have analyzed the repair of total genomic DNA. However, DNA damage and repair processes differ for different regions of the genome, and important biological end points such as survival may be better correlated with repair of essential genes than with repair of the general, overall genome (9,18–23). Therefore, we have investigated the role of PADPRP in DNA repair at the level of the gene.

With the use of a cell line in which the expression of PADPRP can be controlled by induction of antisense PADPRP transcripts, we have been able to study specifically whether PADPRP plays a role in the repair of different types of DNA adducts without

the use of enzyme inhibitors. We have shown that depletion of PADPRP does not affect the repair of pyrimidine dimers in the actively transcribed DHFR gene. This result is in agreement with our previous observations with 3-AB as an inhibitor (20), and other researchers have reported no effect of PADPRP inhibitors on overall genome DNA repair (5). In contrast to the results for UV-induced damage, we have shown that depletion of PADPRP decreases the rate of repair of alkali-labile sites induced in the DHFR gene by HN2. This observation is also consistent with previous data suggesting a role for PADPRP in the repair of alkylation damage in total genomic DNA (10,17). Furthermore, depletion of PADPRP renders cells more sensitive to HN2 (Fig. 4), indicating that gene-specific repair capacity and PADPRP activity are important factors for cell survival.

There has been much interest in understanding the possible role of PADPRP in nucleotide excision repair (NER). On the basis of results with a human cell-free system, Satoh and Lindahl have suggested that unmodified PADPRP binds to DNA strand breaks, and auto-poly(ADP-ribosylation) of the bound PADPRP then induces its release and allows access of DNA repair enzymes to the lesion (24). Thus, PADPRP may function as a temporary protector of breaks introduced in the DNA. Alternatively, binding of PADPRP may be a signal for the repair processes to initiate. In a more recent study, Satoh *et al.* (16) showed that a PADPRP-depleted cell extract repaired DNA strand breaks efficiently, suggesting that PADPRP plays an incidental, and possibly even a negative role, in DNA excision repair. We tested the model originally proposed by Satoh and Lindahl (24) with PADPRP deletion mutants (25). Mutants with an intact N-terminal DNA binding domain, which were therefore capable of binding to DNA strand breaks in the *in vitro* assay, inhibited DNA repair. However, the inhibition exerted by mutants with deletions in the NAD binding active site domain or the automodification domain was not alleviated by NAD. A PADPRP mutant with a deletion in the DNA binding domain did not inhibit DNA repair. Thus, the behavior of these mutants was consistent with the model proposed by Satoh and Lindahl. Another model has been proposed that suggests that PADPRP modifies histones and thereby opens chromatin structure and allows access of the DNA repair machinery to the lesion (26–28). Our current data showing that depletion of PADPRP decreases the gene-specific repair rate for alkylation damage is consistent with this model.

It is unlikely that PADPRP exerts its effects on DNA repair via an interaction with the transcription–repair complex (29) which appears to be associated with RNA polymerase II-transcribed genes. We have previously shown that the strand bias of repair, which is thought to reflect transcription–repair coupling, is more marked for the repair of pyrimidine dimers than for the repair of HN2-induced alkylation damage (30). If PADPRP affected this coupling, we would expect to detect a greater inhibition of the repair of UV-induced damage than of the repair of HN2-induced damage in PADPRP-depleted cells. The absence of such a bias supports the notion that PADPRP may increase the accessibility of other enzymes to chromatin.

ACKNOWLEDGEMENTS

T.S. gratefully acknowledges travel grants from the Dagmar Marshalls Foundation, the Neye Foundation and the 'Fonden til Lægevidenskabens Fremme'. This work was supported in part by a grant to M.S. CA25344 from the National Cancer Institute.

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