Strategic down-regulation of DNA polymerase β by antisense RNA sensitizes mammalian cells to specific DNA damaging agents

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ABSTRACT

Previously, mouse NIH 3T3 cells were stably transfected with human DNA polymerase β (β -pol) cDNA in the antisense orientation and under the control of a metallothionein promoter [Zmudzka, B.Z. and Wilson, S.H. (1990) *Som. Cell Mol. Gen.*, 16, 311-320]. To assess the feasibility of enhancing the efficacy of chemotherapy by an antisense approach and to confirm a role for β -pol in cellular DNA repair, we looked for increased sensitivity to DNA damaging agents under conditions where β -pol is down-regulated in the antisense cell line. Such a sensitization is anticipated only where β -pol is rate-limiting in a DNA repair pathway. A number of agents were tested: *cis*-diamminedichloroplatinum II (cisplatin); 1,3-bis(2-chloroethyl)-1-nitrosourea (BCNU); ionizing radiation and the radio-mimetic drug bleomycin; the bifunctional alkylating agents nitrogen mustard and L-phenylalanine mustard (melphalan); the monofunctional alkylating agent methyl methane sulfonate (MMS) and ultraviolet (UV) radiation. In the cases of cisplatin and UV radiation, a significant enhancement of cytotoxicity was observed. Damage as a result of both of these agents is thought to be repaired by the nucleotide excision repair (NER) pathway. The results suggest that, in this cell line, β -pol is involved in and is rate-limiting in NER. We propose that down-regulation of β -pol by antisense approaches might be used to enhance the cytotoxic effects of cisplatin and other DNA damaging chemotherapeutic agents.

INTRODUCTION

Many of the chemotherapeutic agents most commonly used in the clinic such as alkylating agents, cisplatin and ionizing radiation are known to damage DNA (1-5). The cytotoxicity of these agents, as well as UV radiation, is thought to result from the formation of various DNA adducts (1,2,4-6). One of the most important factors in the determination of sensitivity to a particular agent may be the DNA repair capacity of the cell (7). Many cell

lines and tumors become resistant to drug treatment both *in vitro* and *in vivo* as a result of enhanced DNA repair, and in certain instances, elevated levels of an enzyme involved in DNA repair have been observed (8-11). Conversely, where DNA repair enzymes are deficient (e.g. in *Xeroderma pigmentosum*), cells are hypersensitive to specific types of DNA damage (12,13). Therefore, any mechanism able to suppress DNA repair efficiency might increase the effectiveness of chemotherapy.

Repair of damaged DNA is a sequential, multi-enzyme process (14,15). In general, for excision repair to occur, the damaged site is first excised, double-stranded DNA is then resynthesized on the single-stranded template by DNA polymerase(s), and finally, the gap is sealed by DNA ligase. DNA polymerases are therefore essential for repair of most types of DNA damage and it is possible that down-regulation of a DNA polymerase might sensitize cells to DNA damaging agents including those used for cancer chemotherapy.

DNA polymerase β is the smallest of the five known eukaryotic cellular DNA polymerases and, unlike other polymerases, it lacks additional catalytic activities such as exo- or endo-nuclease (16). It can conduct processive 'gap-filling' synthesis on short gapped single-stranded DNA templates (<6 nucleotides), a reaction consistent with 'short-patch' synthesis required for DNA repair (17). Inhibitor studies in permeabilized cell systems have implicated β -pol in DNA synthesis associated with repair after exposure to many agents: nitrosoureas, UV radiation, bleomycin and ionizing radiation (18-21). Other polymerases (α - or δ -pol) have also been implicated in repair synthesis (20,22-25). Perhaps the only conclusion to be drawn from these types of studies is that the degree to which each polymerase is involved depends upon the agent producing the damage, the conditions of the assay and the level of damage produced. Nevertheless, it has generally become assumed that β -pol fills shorter gaps (26,27), while the other polymerases have been suggested to carry out synthesis of longer stretches (28). Consistent with its role in short-patch repair, β -pol has recently been demonstrated to be required for G:U base excision repair in an *in vitro* assay system (29). The possibility exists that polymerases may act sequentially: another polymerase first carries out repair synthesis on a longer gap (e.g. after excision of UV damage), but then ceases and β -pol fills in

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the resulting short gap (28,30). DNA polymerase β could, therefore, be involved in both 'short' and 'long' patch repair and would be an ideal candidate for DNA repair down-regulation studies.

In a previous report, we have described the isolation of mouse NIH 3T3 cell lines stably transfected with human β -pol cDNA in the antisense direction and under the control of a metallothionein promoter (31). This antisense cell line has been used in the current experiments to determine the effect of sudden down-regulation of β -pol on the cellular sensitivity to a variety of DNA damaging agents, a sensitization effect is anticipated only where β -pol is rate-limiting in the DNA repair pathway. The aim of our study was to explore the feasibility of enhancing the efficacy of chemotherapy by an antisense mechanism and, because of the method used, we would also identify a role for β -pol in DNA repair in the intact cell. We found an increased sensitivity to cisplatin and UV radiation in the antisense cells under conditions where β -pol is down-regulated. This study confirms that β -pol plays a critical role in cellular DNA repair and is a suitable target for chemosensitization by antisense gene therapy approaches.

MATERIALS AND METHODS

DNA damaging agents

Cisplatin (Platinol-AQ) and BCNU (BiCNU) were obtained from Bristol Laboratories, Evansville, IN. Bleomycin (Blenoxane) was from Mead Johnson, Princeton, NJ. Nitrogen mustard (mechlorethamine: Mustargen) was purchased from Merck, Sharp & Dohme, West Point, PA and L-phenylalanine mustard (melphalan) and MMS from Sigma, St Louis, MO.

Cell culture

NIH 3T3 cells (non-transfected cells) were maintained in Dulbecco's modified Eagle medium with 10% dialyzed fetal bovine serum (Hyclone, Logan, UT). NIH 3T3 cells stably transfected with β -pol in the antisense orientation (antisense cells) were routinely passaged in the same medium supplemented with 0.5 μ M methotrexate (MTX: Sigma). The transfected β -pol vector also contained the mouse DHFR gene and the original selection procedure in MTX resulted in amplification of the inserted DNA. Cell lines were routinely tested and found to be free of mycoplasma.

Immunoblotting

Actively growing antisense cells, both control and Zn^{2+} induced (65 μ M for 5 days), were used for analysis of β -pol protein levels. Cell monolayers were washed twice with ice-cold phosphate buffered saline and the cells isolated by scraping. The cells were suspended in a buffer containing 150 mM NaCl, 10 mM Tris pH 7.5, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 1 mM EDTA and the protease inhibitors phenylmethylsulfonyl-fluoride (1 mM) and aprotinin (2.7 μ g/ml) and then rapidly lysed by freeze-thawing three times in a dry ice bath. This lysate was centrifuged at 8000 g for 5 min. The supernatant fraction (20 μ g protein) was separated by electrophoresis in a 12.5% SDS-polyacrylamide gel and proteins in the gel were transferred electrophoretically to a nitrocellulose membrane. Equal loading of samples and transfer to nitrocellulose was verified by staining the membrane with Coomassie Brilliant Blue. The membrane was first blocked in 5% skimmed milk for 1 h and β -pol was measured by

incubating with a mouse anti- β -pol monoclonal antibody 18S (32) and then with an antibody to mouse IgG conjugated to horseradish peroxidase (HRP; Amersham, Arlington Heights, IL). Immobilized HRP activity was detected by enhanced chemiluminescence (ECL; Amersham), following the directions outlined by the manufacturer. Biotinylated protein molecular weight markers were also purchased from Amersham. The level of β -pol in each sample was determined from integrated densitometry signals of the β -pol bands as measured using Millipore BioImage Visage Software on a Sun Sparc Station II Workstation (BioImage Products, Ann Arbor, MI). Calibration curves (concentration versus integrated optical density) were set up by electrophoresing known amounts of pure recombinant rat β -pol and then probing with monoclonal antibody 18S as described above (32).

Cytotoxicity assays

It was necessary to determine DNA damaging agent sensitivity by growth inhibition assays since the antisense cells do not clone efficiently under the growth conditions required for down-regulation of β -pol. For Zn^{2+} induction, antisense cells were plated at a density of 1×10^5 /175 cm² flask and pretreated with MTX medium containing $ZnCl_2$ (Sigma) at a concentration of 65 μ M for 4 days. On day 4, cells were harvested and seeded in 6-well dishes (5000 cells/well) in Zn^{2+} containing medium without MTX. The antisense cells were exposed to DNA damaging agents 24 h after seeding in 6-well dishes. Experiments were undertaken in parallel to determine the effect of Zn^{2+} exposure (65 μ M for 5 days) on the sensitivity of non-transfected NIH 3T3 cells to the same agents.

For the cisplatin, BCNU, bleomycin, nitrogen mustard, melphalan and MMS studies, cells (triplicate wells) were treated for 1 h at 37°C in a 10% CO₂ incubator with serial dilutions of the drugs in medium without MTX or Zn^{2+} . The cell monolayers were then washed once with Hanks' balanced salt solution (HBSS) and 2 ml fresh medium ($\pm Zn^{2+}$) was added. For UV irradiation (254 nm, UVC), cell monolayers were washed twice with HBSS, then exposed to UV radiation (fluorescence 37.5 μ W/cm²) for the required length of time. Medium ($\pm Zn^{2+}$, 2 ml) was then added to each well. For γ irradiation, cells in the presence of the seeding medium, were exposed to a ¹³⁷Ce source in a Mark I irradiator model 68 (J.L. Shepherd and Associates) at a dose rate of 7.33 Gy/min for varying lengths of time. After irradiation, the cells were washed once with HBSS and 2 ml fresh medium ($\pm Zn^{2+}$) was added to each well.

For each experiment, cells were counted after ~1 week, when 'control' non-treated cells were 80% confluent. Cells numbers were determined by a nuclear lysis procedure (33). Results are expressed as the number of damaging agent-treated cells relative to the number of cells in control wells (% control growth). For each agent, the dose that inhibited growth to 37% of control values (D_{37}) was determined. Statistical comparisons of D_{37} values were done by a paired-sample *t*-test ($\alpha = 0.005$).

RESULTS

Down-regulation of β -pol after zinc induction

In the previously published characterization of the antisense cell line, it was shown that growth in medium containing $ZnCl_2$ (65 μ M) resulted in a change in morphology of the cells and a decreased doubling time (31). Similar effects were seen in the current study, with no observable changes in control non-trans-

Figure 1. Immunoblot analysis showing β -pol protein levels in uninduced and Zn^{2+} induced (65 μM for 5 days) antisense 3T3 cells. Samples of cell lysates (20 μg protein) or 2 ng of purified recombinant rat polymerase (β -pol) were subjected to 12.5% SDS-PAGE, transferred electrophoretically to nitrocellulose membrane and then probed with anti- β -pol 18S monoclonal antibody or nonspecific IgG (uninduced cells only).

fectected NIH 3T3 cells under the same conditions. Previous activity gel analysis of the antisense cells showed a decrease in β -pol level after Zn^{2+} induction [5 days, 65 μM : (31)]. The effectiveness of the expression vector was confirmed in this series of experiments by immunoblotting using a newly characterized monoclonal antibody (18S) raised against purified recombinant rat β -pol (32). The level of β -pol in cell lysates, as determined from integrated optical densitometry signals, was found to be 3–4-fold lower in Zn^{2+} induced antisense cells than in parallel cultures without Zn^{2+} induction (Fig. 1). These results are consistent with the earlier measurements using activity gels and indicate that the level of β -pol protein is significantly decreased after Zn^{2+} pretreatment.

Effect of zinc induction on the cytotoxicity of DNA damaging agents

We hypothesized that if β -pol is rate-limiting in DNA repair occurring upon exposure to a DNA damaging agent, then in cell lines where β -pol could be down-regulated by an antisense mechanism, there would be an increased sensitivity to this agent. In these experiments, down-regulation in the antisense cells could be achieved by Zn^{2+} induction and, therefore the cytotoxicity of each DNA damaging agent was compared in Zn^{2+} induced and non-induced antisense cells. If sensitization was observed, it was necessary to demonstrate that Zn^{2+} pretreatment did not also enhance cytotoxicity in non-transfected 3T3 cells, and therefore

that the sensitization following Zn^{2+} induction was a specific and presumably β -pol related effect.

With non-transfected cells, neither Zn^{2+} pretreatment only, nor Zn^{2+} treatment before and after exposure to the drug resulted in enhanced growth inhibition by cisplatin (Fig. 2A) and the mean D_{37} values obtained ($\pm \text{Zn}^{2+}$ induction, $n = 4$) were not significantly different from each other. After a 1 h exposure to cisplatin, the D_{37} value obtained in the uninduced antisense cell line was $12.9 \pm 1.4 \mu\text{M}$ (mean $\pm$ S.E., $n = 7$). Zn^{2+} induction (both pretreatment only and treatment before and after cisplatin exposure) resulted in a small but highly significant (paired-sample *t*-test, $P < 0.005$) enhancement of cytotoxicity (D_{37} of 8.0 ± 0.4 and $7.0 \pm 0.3 \mu\text{M}$ respectively). The complete survival curves are shown in Figure 2B.

The cytotoxicity of UV radiation in non-transfected 3T3 cells was not changed by Zn^{2+} induction (data not shown). There was no significant sensitization in the antisense cells after only Zn^{2+} pretreatment, but prolonged Zn^{2+} exposure, before and after UV irradiation, did enhance UV-induced growth inhibition (Fig. 2C). The D_{37} values obtained, 10.5 ± 0.2 and $5.9 \pm 0.6 \text{ J/m}^2$ (mean $\pm$ S.E., $n = 6$) for control and Zn^{2+} treated antisense cells respectively were significantly different ($P < 0.005$) and the mean sensitization observed was 1.8-fold, a value identical to that obtained for cisplatin.

Pretreatment with Zn^{2+} did not significantly increase MMS cytotoxicity in the antisense cells (Fig. 2D). The D_{37} values obtained in Zn^{2+} uninduced and induced cells were 2.0 ± 0.05 and $1.7 \pm 0.14 \text{ mM}$ respectively for a 1 h exposure. Zn^{2+} induction was similarly without effect on the cytotoxicity of any other of the DNA damaging agents tested (BCNU, bleomycin, nitrogen mustard, melphalan and γ radiation; data not shown).

Because of the method used for the cytotoxicity assays (i.e. growth inhibition assays), it was necessary to show that the demonstrated sensitization to cisplatin or UV irradiation was as a result of enhanced cell kill, and not merely a consequence of a change in cellular growth rate following exposure to the DNA damaging agents. Control and Zn^{2+} induced 3T3 and antisense cells were treated with the appropriate D_{37} dose of cisplatin or UV radiation, under the same conditions as used in the cytotoxicity experiments, and then the cell growth rate was determined by daily cell counts. For every treatment condition, the treated cells grew at the same rate as undamaged cells (data not shown). These data suggest that sensitization of cells to cisplatin and UV irradiation is a result of enhanced cell killing by these agents following Zn^{2+} induction.

DISCUSSION

There are numerous reports implicating β -pol and the other DNA polymerases in the various pathways of mammalian DNA repair (18–29). Many of these studies have utilized enzyme inhibitors and *in vitro* systems (purified enzyme, cell extracts or permeabilized cells), often under non-physiological conditions. Therefore, although there is much indirect evidence for the role of β -pol in DNA repair, the importance of β -pol *in vivo* is still uncertain.

If one of the key determinants of cellular sensitivity to DNA damaging agents is the efficiency of repair of DNA damage, then when repair of a lethal DNA lesion is compromised, for example by down-regulating an enzyme involved in the rate-limiting step, the end result would be sensitization of the cell to the damaging agent. In this study, under conditions where β -pol is suddenly down-regulated by antisense RNA expression, increased sensi-

Figure 2. Effect of zinc induction (65 μ M for 5 days) on the sensitivity of non-transfected NIH 3T3 cells to a 1 h exposure to cisplatin (A). Effect of zinc induction on the sensitivity of antisense cells to (B) cisplatin, (C) UV irradiation and (D) MMS (1 h exposure). Cells were pretreated, plated and dosed as described in Materials and Methods and cell growth was determined by nuclei counts when control non-drug treated wells were ~80% confluent. The results represent the mean $\pm$ S.E. for four to seven independent experiments. Control (●): No Zn^{2+} treatment; + Zn (■): Zn^{2+} pretreatment only (65 μ M, 5 days); + Zn + Zn (◆): Zn^{2+} treatment before and after exposure to DNA damaging agent.

tivity to cisplatin and UV radiation was observed. The most straightforward explanation of these results is that down-regulation of β -pol suppresses DNA repair and enhances cytotoxicity in the antisense cell line. We are currently developing other model cell lines in which to further study the effects of β -pol down-regulation. Recent data suggest that a non-catalytic domain of β -pol can be used as a potential inhibitor of intracellular β -pol and may provide another means for sensitizing cells to therapeutically relevant DNA damaging agents (34).

Exposure of cells to cisplatin primarily induces DNA-interstrand cross-links and to a lesser extent, DNA-intrastrand and DNA-protein cross-links (35). Intrastrand cross-links between neighboring guanine bases have been implicated as the cytotoxic lesion because the *trans* isomer, which can react with DNA but cannot form this cross-link, is therapeutically inactive. When cells are irradiated with UV light, the two major DNA adducts formed are cyclobutane pyrimidine dimers and (6-4) photopro-

ducts in a ratio of ~3:1 (6). The UV sensitivity of cells generally correlates better with the capacity to repair (6-4) photoproducts than cyclobutane dimers (6). Cell lines derived from *Xeroderma pigmentosum* patients and which are deficient in NER are hypersensitive to both UV radiation and cisplatin (36). Conversely, cisplatin resistant mammalian cell lines are also more resistant to UV radiation (37). In addition, the *E.coli* NER system (UvrABC) recognizes DNA damage caused by both cisplatin and UV irradiation (38). It is clear that DNA damage as a result of both cisplatin and UV exposure share the common feature that they are recognized and repaired by the NER pathway (12). Therefore, the data presented here suggest that, in our experimental cell line, β -pol is involved in and is rate-limiting in NER of cisplatin and UV lesions. The results of the present study do not exclude the possibility that β -pol is also important in repair pathways other than NER in this experimental cell line, but only that it may not be rate-limiting. These experiments also do not

suggest involvement or eliminate the other DNA polymerases in the various repair pathways.

It is interesting that Zn^{2+} pretreatment alone is sufficient to sensitize antisense cells to cisplatin, whereas prolonged Zn^{2+} exposure is required in order for sensitization to UV. This implies that for sensitization to UV to occur, β -pol must remain down-regulated for a longer period of time. Following UV irradiation, 50–70% of cyclobutane pyrimidine dimers have been shown to be removed from the DNA of normal human fibroblasts within 24 h (39). The (6–4) photoproducts are removed more efficiently with 70% being removed during the first 2–3 h after irradiation (40). It seems possible therefore, that both types of UV photoproducts contribute to cytotoxicity in the antisense cell line. After exposure of cells to cisplatin, there is a fast initial removal of more than half of all the cisplatin–DNA adducts with removal completed in 4.5 h (12). This is in agreement with the requirement for a shorter time of β -pol down-regulation.

There was no apparent sensitization in the antisense line to any other of the agents studied, all of which form DNA lesions repaired by pathways other than NER. After treatment with chloroethyl-nitrosoureas, alkyl groups can be removed from the O^6 position of guanine in repair-competent NIH 3T3 cells by O^6 -alkylguanine DNA-alkyltransferase, thus blocking the formation of cross-links (41,42). Exposure to the bi-functional alkylating agents results in cross-linking of DNA which correlates with cytotoxicity (2). Base excision repair is initiated by specific DNA glycosylases (43). Irradiation with ionizing radiation produces a number of lesions in cells such as DNA–protein cross-links, base alterations and DNA single- and double-strand breaks (5). There is evidence that it is the latter lesion that lead to cell killing and repair is thought to involve recombination (5). Treatment with bleomycin can also result in DNA double-strand breaks (4).

In addition to its role in DNA repair, β -pol may also function in DNA replication in some systems since it can substitute for Pol I during DNA replication in *E.coli* (44) and is required for the conversion of single-stranded to double-stranded DNA in *Xenopus* oocytes (45). It has been demonstrated recently that, of all the polymerases tested, only β -pol was able to perform translesion synthesis on a substrate containing a single cisplatin adduct (46). This implicates β -pol as the only polymerase able to bypass a cisplatin adduct during DNA replication and suggests another mechanism whereby cells could become sensitized to cisplatin following down-regulation of β -pol.

An association between cisplatin sensitivity and β -pol level has been noted previously. Elevated levels of β -pol mRNA have been found in several cisplatin-resistant cell lines *in vitro* (10,47) and β -pol gene amplification has been observed in patient tumors that have failed cisplatin therapy (10). However, other workers were not able to detect changes in β -pol (either basal levels or following cisplatin exposure) in their cisplatin resistant cell lines and concluded that β -pol is not involved in the cisplatin-resistant phenotype (48). In addition, dideoxythymidine, an inhibitor of β -pol, could not sensitize cells to cisplatin (48).

In summary, under conditions where β -pol is down-regulated, we observed an enhancement of the cytotoxicity of both cisplatin and UV radiation in our experimental cell line. This is the first example, to our knowledge, of strategic down-regulation of an excision repair enzyme in mammalian cells. The modest down-regulation afforded by antisense RNA expression (~3-fold) is associated with sensitization of cells to specific DNA damaging agents by a similar magnitude. Based upon these encouraging

results, particularly for cisplatin which is a widely used anticancer agent, we plan to more fully characterize the effects of down-regulation of β -pol in tumor cell lines. Finally, we propose that it may be possible to enhance the cytotoxic effects of cisplatin and other DNA damaging chemotherapeutic agents by an antisense gene therapy approach.

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