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ORIGINAL ARTICLE

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Repair analysis of 4-hydroperoxycyclophosphamide-induced DNA interstrand crosslinking in the c-*myc* gene in 4-hydroperoxycyclophosphamide-sensitive and -resistant medulloblastoma cell lines

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Abstract Cyclophosphamide is one of the most active agents in the treatment of medulloblastoma. However, development of resistance to this alkylator frequently occurs and is the harbinger of tumor progression and death. In order to understand the biochemical basis of this resistance, we generated a panel of medulloblastoma cell lines in our laboratory that were resistant to 4-hydroperoxycyclophosphamide (4-HC). Previously, we have shown that elevated levels of aldehyde dehydrogenase and glutathione mediate cellular resistance to 4-HC. The present study was conducted to identify the third unknown mechanism mediating the resistance of cell line D283 Med (4-HCR) to 4-HC, testing the hypothesis that this resistance is mediated by an increased repair of DNA interstrand crosslinks (ICLs). The doses of 4-HC that produced a one- and two-log cell kill of D283 Med cells were 25 and 50 μ*M*, respectively, compared with values of 125 and 165 μ*M* in D283 Med (4-HCR), the resistant cell line. The formation and disappearance of 4-HC-induced DNA ICLs at the c-*myc* gene were subsequently studied by DNA denaturing/renaturing gel electrophoresis and Southern blot analysis. 4-HC-induced DNA ICLs in the c-*myc* gene exhibited a dose-dependent relationship. The percentage of the c-*myc* gene that was crosslinked was approximately 1–3% at a dose of 100 μ*M*. More than 50% of the DNA crosslinking in D283 Med (4-HCR) cells was removed by 6 h after drug treatment, whereas, in D283 Med cells, more than 90% of the DNA crosslinking was still present at 6 h. These findings suggest that the increased repair of DNA ICLs in D283 Med (4-HCR) may contribute significantly to its resistance to 4-HC.

Key words 4-Hydroperoxycyclophosphamide · c-*myc* · DNA interstrand crosslinks

Introduction

Cyclophosphamide is a widely used alkylating agent active against a broad spectrum of human tumors including medulloblastomas, leukemias, lymphomas, and carcinomas of the lung, breast, and cervix [3, 4, 9, 14, 27]. Cyclophosphamide is a prodrug that is activated by the cytochrome P-450 mixed function oxidase system in the liver [10]. Hydroxylation at this site produces 4-hydroxycyclophosphamide, which exists in a tautomeric equilibrium with aldophosphamide. Aldophosphamide generates the active alkylating metabolite phosphoramide mustard, although aldehyde dehydrogenase, which is replete in stem cells and intestinal mucosal cells, can detoxify aldophosphamide by conversion to carboxyphosphamide. DNA interstrand crosslinks (ICLs) induced by phosphoramide mustard are the critical lesions that produce tumor cytotoxicity [11]. Although 4-hydroxycyclophosphamide is too unstable for synthesis and in vitro studies, the preactivated cyclophosphamide derivative 4-hydroperoxycyclophosphamide (4-HC) is stable,

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and converts to 4-hydroxycyclophosphamide/aldophosphamide in solution [9].

A major problem in the treatment of cancer with chemotherapeutic agents, including alkylators, is that target cells develop resistance to the drug [9, 12, 28, 29]. The biochemical basis of this resistance is both multifactorial and incompletely characterized at present. Several mechanisms have been implicated in the cellular resistance to phosphoramide mustards. These include alterations in drug transport [7, 15, 25], increased glutathione-*S*-transferase activity [24, 31], elevated glutathione levels [1, 16], and aldehyde dehydrogenase-mediated detoxification of aldophosphamide [18, 19, 26].

Medulloblastoma cell lines, with both laboratory-generated or clinically acquired resistance to 4-HC, demonstrate increased levels of aldehyde dehydrogenase and glutathione [16]. However, one of the lines with laboratory-generated resistance, D283 Med (4-HCR), demonstrates an unknown mechanism of resistance. This D283 Med (4-HCR) demonstrates elevations in both aldehyde dehydrogenase and glutathione levels, but is crossresistant to phenylketocyclophosphamide, which is not metabolized by aldehyde dehydrogenase. In addition, this line is not sensitized to 4-HC by pretreatment following L-buthionine-*S,R*-sulfoximine-mediated depletion of glutathione.

The current studies were designed to test the hypothesis that the resistance of D283 Med (4-HCR) cells to 4-HC is mediated by an increased repair of DNA ICLs induced by 4-HC. DNA denaturing/renaturing gel electrophoresis and Southern blot analysis for gene-specific studies (using c-*myc*) were used to measure DNA ICL formation and repair induced by 4-HC in both drug-sensitive and -resistant cell lines. We now report an increased level of DNA repair in D283 Med (4-HCR) cells.

Materials and methods

Cell culture and drug treatment

D283 Med is a human medulloblastoma-derived cell line established in our laboratory [13]. D283 Med (4-HCR) was made resistant to 4-HC as described previously [16]. The resistant line was treated three times with 4-HC every three passages before increasing the dose by 5 μ*M*. Cells used in this study were between passages 210 and 230. The resistant line was maintained by treating cells with 125 μ*M* 4-HC every three passages. Both cell lines were grown in suspension at 37°C in an atmosphere of 95% air/5% CO2 in improved modified Eagle's/zinc option medium containing 20% heat-inactivated fetal calf serum (Gibco, Grand Island, N.Y.) [13, 16].

Limiting dilution analysis

The cytotoxicity of 4-HC on either D283 Med or D283 Med (4-HCR) was determined by limiting dilution assays as previously described [16]. Exponentially growing cells were treated with 0, 6.25, 12.5, 25, 50, 100, 150, and 200 μ*M* 4-HC at 37°C for 1 h. The drug was removed, and the cells were seeded into 96-well tissue culture plates. Cells were incubated at 37°C in an atmosphere of 95% air/5% CO_2 for 12 days. The surviving fraction was determined by Spearman's analysis [20, 21].

Isolation of genomic DNA from tumor cells

Genomic DNA was isolated from cells following a protocol described previously [17]. Cells with or without drug treatment were pelleted and washed free of medium with ice-cold phosphate-buffered saline (pH 7.4). The cell pellet was dispersed by gentle vortexing and was lysed in a buffer containing 0.05 *M* $NaHCO_3/Na_2CO_3$, 1 m*M* EDTA, 0.5% *N*-lauryl sarcosine, and 0.3 mg/ml proteinase-K (pH 10.4). The cell lysate was incubated at 37°C for at least 3–4 h to digest proteins. The lysate was then deproteinized by phenol/chloroform extraction. The nucleic acids were precipitated by adding 0.2 volumes of 11 *M* ammonium acetate and 2.5 volumes of ice-cold 95% ethanol, and were resuspended in Tris-EDTA buffer (pH 8) at 4°C overnight.

DNA denaturing/renaturing gel electrophoresis and Southern blot analysis

This is a standard assay to detect DNA ICLs in a specific genomic region [17, 30]. Briefly, purified genomic DNA was digested with *Kpn*I at 37°C for 3 h and dissolved in 0.01 *M* Na_2HPO_4/NaH_2PO_4 (pH 7.0). An equivalent amount of the DNA sample isolated from cells treated with saline or 50 μ*M* 4-HC was denatured in 64% formamide dye, including 1 m*M* EDTA, 0.1% bromphenol blue, and 0.1% xylene cyanol, by heating at 65°C for 5 min. The denatured samples were quickly chilled on ice-salt slush (1 *M* $CaCl_2$, − 10°C) and loaded onto a 0.5% neutral agarose gel. A nondenatured DNA sample was loaded with 50% glycerol and 0.1% bromphenol blue. After completion of gel electrophoresis, the genomic DNA was transferred onto a GeneScreen Plus membrane. The membrane was hybridized with a ^{32}P-labeled human c-*myc* exon 1 probe (the *Xho*I-*Pvu*II fragment) at 42°C for 20 h in a solution containing 50% formamide, 10% dextran sulfate, 5 × saline-sodium-phosphate-EDTA (SSPE) (0.9 *M* NaCl, 50 m*M* Na_2HPO_4/NaH_2PO_4, pH 6.8, 1 m*M* EDTA), 1% sodium dodecyl sulfate (SDS), 1 × Denhardt's solution(0.02% Ficoll 400, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin Pentax Fraction V), and 250 μg denatured salmon sperm DNA. The blot was washed in 2 X SSPE, 0.1% SDS at room temperature for 20 min, then finally at 60°C for 30 min in 0.1 X SSPE and 0.1% SDS. The filter was exposed either to an X-ray film for several days at -80°C or to a PhosphoImage screen at room temperature. The quantitation of band intensities in each lane was determined by PhosphoImage scanning (Molecular Dynamics, Sunnyvale, Calif.). The percentage of crosslinked DNA formed in a sample was determined by dividing the amount of radioactivity in the double-stranded DNA band (cpm) by the total radioactivity detected in double- and single-stranded DNA bands (cpm).

Results

Cytotoxicity studies

D283 Med cells were relatively sensitive to 4-HC compared with D283 Med (4-HCR) cells (Fig. 1). The doses of 4-HC that produced a one- and two-log kill of D283 Med were 25 and 50 μ*M*, respectively, compared with values of 125 and 165 μ*M* in D283 Med (4-HCR).

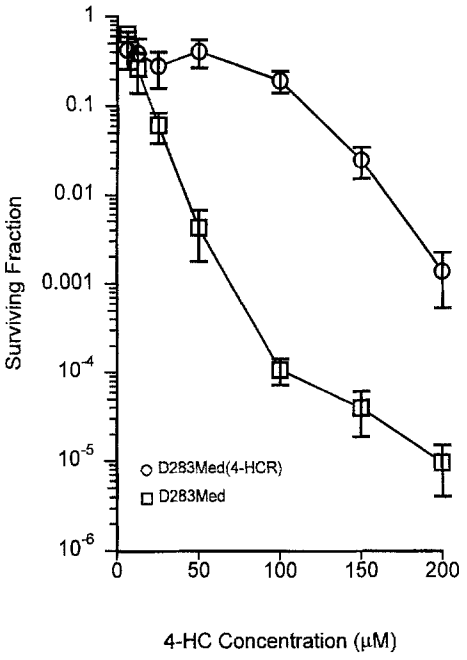

Fig. 1 Survival of D283 Med and D283 Med (4-HCR) cells following exposure to various concentrations of 4-HC for 1 h.After drug treatment 4-HC was washed out and D283 Med and D283 Med (4-HCR) cells were seeded into 96-well tissue culture plates for limiting dilution analysis. *Circles and Squares* represent the mean of at least three independent experiments, and *bars* indicate the standard deviation

DNA ICL formation and disappearance in the c-*myc* gene

*Kpn*I digestion of the genomic DNA from either drug-sensitive D283 Med or drug-resistant D283 Med (4-HCR) cell lines gave rise to a 14-kb band on the Southern blot after hybridization with the c-*myc* exon 1. The pattern of *Eco*RI digestion of the same genomic DNA on the blot was consistent with prior studies showing the rearrangement of c-*myc* in D283 Med (data not shown) [5]. As depicted in Fig. 2, the formation of DNA ICLs in the c-*myc* gene was dose dependent. Approximately 1–3% of the c-*myc* gene was crosslinked at the 100-μ*M* dose in the drug-sensitive cells. The amount of DNA interstrand crosslinking in the c-*myc* gene formed in the drug-resistant line was similar to that in the drug-sensitive line. A concentration of 50 μ*M* 4-HC was used to study the repair kinetics in both the sensitive and resistant lines.

The repair kinetics of DNA ICLs induced by 4-HC in the c-*myc* gene was monitored by measuring the amount of DNA interstrand crosslinking remaining in the gene after drug removal. Cells were postincubated in fresh medium for various times (0, 1, 2, 3, 4, 6, 12, and 24 h) after drug treatment. The initial DNA crosslinking formed immediately after 50-μ*M* treatment was 1.52 ± 0.81% (mean ± SD, $n = 3$) in the drug-sensitive line and 1.22 ± 0.62% (mean ± SD, $n = 3$) in the drug-resistant line. More than 50% of the DNA crosslinking

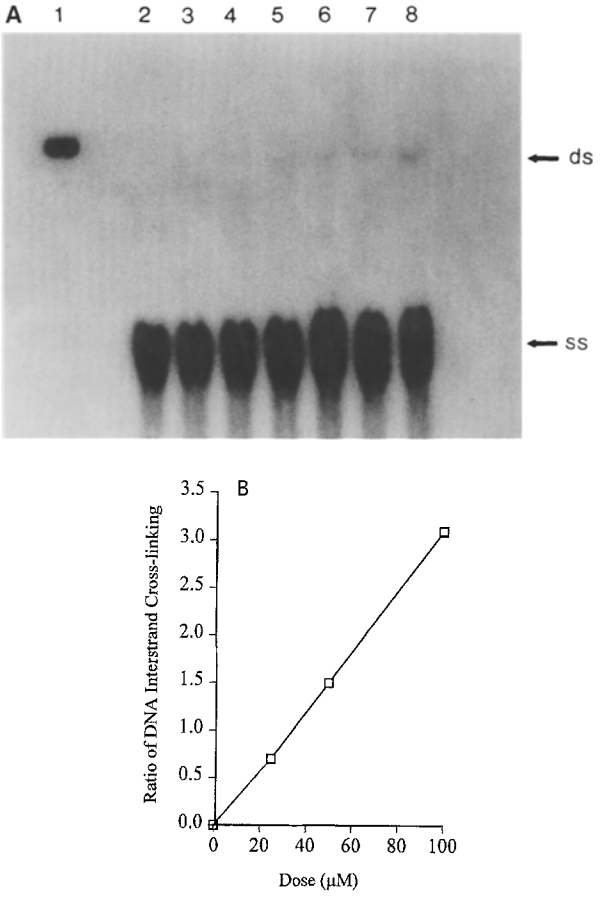

Fig. 2A,B Detection of 4-HC-induced DNA ICLs in the c-*myc* gene immediately after drug treatment for 1 h in the D283 Med cell line.Genomic DNA was isolated from control (*lanes 1* and *2*) or 4-HC-treated cells (3.125, 6.25, 12.5, 25, 50, and 100 μ*M in lanes 3 to 8*, respectively) and digested with *Kpn*I restriction enzyme. **A** Restricted DNA was loaded on a 0.5% agarose gel in either native (*lane* 1) or denatured conditions (*lanes* 2 to 8). After gel fractionation, the genomic DNA was blotted, and hybridized with the ^{32}P-labelled c-*myc* probe. The autoradiogram was obtained by exposing the filter to an X-ray film for a week at -80°C. The positions of double- or single-stranded (*ds* or *ss*) DNA are indicated at the right-hand side of the picture. Each lane contained 10 μg DNA except the native control lane, which was loaded with 2 μg of DNA. **B** Quantitation of the amount of DNA ICL formation was determined by dividing the radioactivity in the double-stranded band by the total radioactivity in the same lane. The actual percentage of DNA interstrand crosslinking formed for 25, 50, and 100 μ*M* is plotted. The double-stranded material was too light to be scanned in cells treated with 12.5 μ*M* or of lower 4-HC at concentration

was removed by 6 h after drug treatment in the resistant cells, while more than 90% still remained in the sensitive line (Fig. 3). DNA ICLs were not measurable 12 h after drug treatment in either the parent or resistant cell lines (data not shown).

Discussion

Cellular resistance to cyclophosphamide is mediated by multiple factors [9, 27, 28]. Several mechanisms are

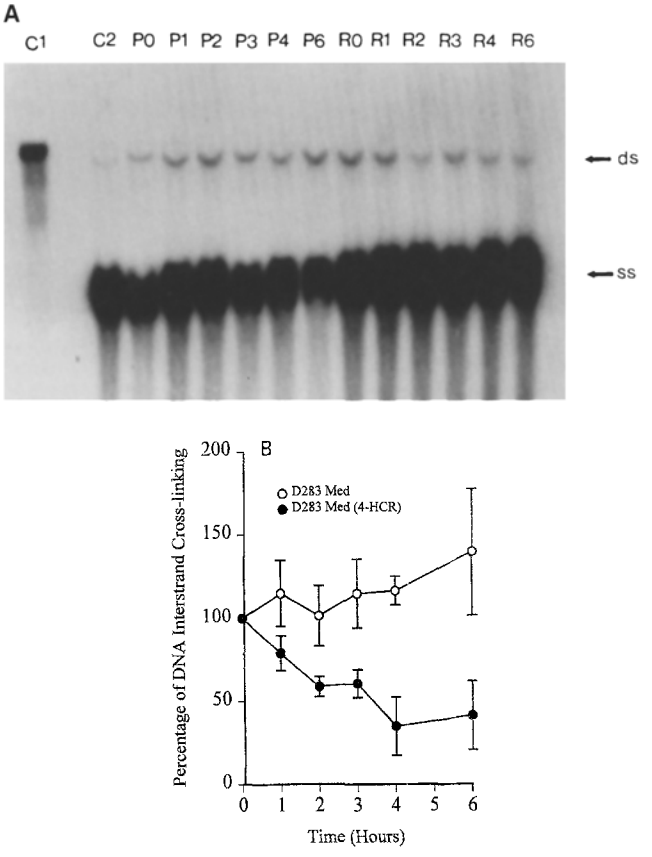

Fig. 3A,B Disappearance of 4-HC-induced DNA ICLs in the c-*myc* gene after 1 h of drug treatment in D283 Med and D283 Med (4-HCR).Drug-sensitive or -resistant cells were treated with 50 μ*M* 4-HC for 1 h. Genomic DNA was isolated from cells postincubated in fresh medium for different lengths of time. **A** *C1* is the native control and *C2* is the denatured control of the untreated sample. *P0*, *P1*, *P2*, *P3*, *P4*, and *P6* are the samples isolated from the parental line, and *R0*, *R1*, *R2*, *R3*, *R4*, and *R6* are those isolated from the drug-resistant line at 0, 1, 2, 3, 4, or 6 h after drug treatment. The samples were processed in the same way as described in the legend of Fig. 2. The autoradiogram was developed 5 days after exposure. Positions of double- or single-stranded (*ds* or *ss*) DNA are indicated at the right-hand side of the picture. **B** The percentage of the crosslinked gene remaining after different postincubation times was calculated by using the initial amount of crosslinking (at 0 h) of the corresponding line as the reference. (*bars*, SEM, $n = 3$)

oxazaphosphorine-nonspecific, such as elevations of glutathione [1, 16] or glutathione-*S*-transferase [24, 31], which also detoxify alkylating agents such as melphalan and cisplatin. Other mechanisms are oxazaphosphorine-specific, such as the increase in aldehyde dehydrogenase, which converts aldophosphamide to the nonalkylating moiety carboxyphosphamide [18, 19]. The role of DNA repair in mediating cellular resistance to cyclophosphamide has not, to our knowledge, been precisely demonstrated [8]. This could be due to the difficulties in detecting phosphoramide mustard-induced DNA ICLs. 4-HC-induced DNA ICLs in the overall genome were not reproducibly detected by an ethidium bromide assay, which has been demonstrated to quantitate, in the whole genome, DNA ICLs induced by several other DNA crosslinking agents [2]. Using this ethidium bromide assay, we demonstrated a dose-dependent relationship with nitrogen mustard (data not shown), which produces a high percentage of DNA interstrand crosslinking, suggesting that differences in the degree of DNA interstrand crosslinking produced by 4-HC and other alkylators may underlie the lack of sensitivity of the ethidium bromide assay for the detection of 4-HC-induced DNA ICLs.

DNA denaturing/renaturing gel electrophoresis and Southern blot analysis offer an alternative way to study DNA ICL formation and repair in tumor cells [30], and this approach was subsequently used for our 4-HC studies. Our results indicate similar peak 4-HC-induced DNA ICL formation in the c-*myc* genes of D283 Med and D283 Med (4-HCR), but more rapid removal of the DNA ICLs in D283 Med (4-HCR), and they suggest that this repair is an additional mechanism of resistance of D283 Med (4-HCR) to 4-HC. To our knowledge, this is the first time that increased repair of DNA ICLs has been implicated in resistance to 4-HC.

Recent studies have shown that increased DNA ICL repair activity at the gene level is inversely related to the sensitivity of cells to DNA crosslinking agents. Lymphoblastoid cell lines derived from Fanconi's anemia patients that are hypersensitive to mitomycin C are deficient in the removal of mitomycin C-induced DNA ICLs in ribosomal RNA genes [23]. In two laboratory-generated cisplatin-resistant human ovarian carcinoma cell lines, a marked increase in DNA ICL repair activity in three different genes has been observed. Interestingly, the DNA ICL repair activity at the overall genome was comparable in both sensitive and resistant cells studied [32]. These data demonstrate that DNA ICL repair activity at the gene level correlates better with cell survival than does repair activity at the overall genome level, although the different assays used (alkaline elution for total genome, and denaturation/renaturation gel electrophoresis with Southern blot for specific genes) preclude absolute proof of this. This phenomenon is consistent with the notion that mammalian cells preferentially repair DNA damage in genes that are essential for cell survival [6, 22]. In addition, this study points out the importance of investigating DNA ICL damage in individual genes and the value of this approach in delineating DNA repair to mediate cellular resistance. Our studies in the formation and repair of 4-HC-induced DNA ICLs in the c-*myc* genes of D283 Med and D283 Med (4-HCR) strengthen the notion that increased DNA ICL repair at the gene level contributes to cellular resistance.

The choice of target gene(s) for gene-specific crosslink studies is problematic. C-*myc* was chosen for the current studies because it is rapidly transcribed. However, c-*myc* is rearranged in D283 Med and amplified in the majority of permanent cell lines or transplantable xenografts derived from medulloblastoma, and these alterations may be associated with a very adverse

prognosis [5]. It is, therefore, not clear if c-*myc* is particularly appropriate for alkylator-induced cross-link studies or if any rapidly transcribed gene, such as a ribosomal RNA gene, would be equally sensitive for evaluation of 4-HC-induced DNA ICL formation and repair [23]. Nevertheless, c-*myc*-specific DNA ICL analysis allowed the demonstration of enhanced DNA ICL repair in D283 Med (4-HCR), which was not possible with the ethidium bromide DNA-binding assay. The precise nature of this enhanced repair remains speculative, and future studies will address the specific repair enzymes that potentially mediate these processes, as well as the importance of DNA ICL formation and repair in c-*myc* versus other genes.

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