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Nucleotide excision repair genes as determinants of cellular sensitivity to cyclophosphamide analogs

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Abstract The objective of this study was to determine the relative importance of the first six complementation groups of the nucleotide excision repair cross-complementing genes (ERCC1–ERCC6) and the first complementation group of the X-ray repair cross-complementing genes (XRCC1), in the repair of DNA damage induced by the in vitro active cyclophosphamide (CP) derivatives 4-hydroperoxycyclophosphamide (4HC) and phosphorodiamidic mustard (PM). We compared the sensitivity of the wild-type CHO cell line, AA8, with that of the CHO mutant cell lines UV4 and UV20 (ERCC1$^-$), UV5 (ERCC2$^-$), UV24 (ERCC3$^-$), UV41 (ERCC4$^-$), UV135 (ERCC5$^-$), UV61 (ERCC6$^-$), and EM9 (XRCC1$^-$). Cell survival was determined using both growth inhibition and conventional clonogenic assays. The yield of DNA crosslinks in selected cell lines was determined using an ethidium bromide fluorescence assay. R*esults*: The rank ordering of sensitivity to both 4HC and PM, based on the combined survival data, was UV41/UV4/UV20 $\gg$ UV61/UV24/UV135/EM9 $\geq$ UV5 $\approx$ AA8. Thus mutations in the ERCC1 and ERCC4 genes impart a hypersensitivity to CP analogs. To confirm the importance of the ERCC1 gene for cellular resistance to 4HC and PM, UV20 cells were transfected with the human ERCC1 gene and subsequently exposed to 4HC and PM. The transfected cells displayed essentially wild-type resistance to both drugs. Furthermore, two interspecific hybrids derived from UV41, both of which retained the region of human chromosome 16 that harbors the ERCC4 gene, displayed essentially wild-type resistance to 4HC and PM, confirming the importance of ERCC4 for the repair of 4HC-induced DNA damage. When crosslinks were assayed after a 60-min treatment with 4HC or a 15-min treatment with PM, their yield paralleled the sensitivity of the cell lines to both drugs: UV41 cells showed markedly elevated levels of crosslinks, whereas AA8 and UV5 cells showed similar (low) levels of crosslinks. *Conclusions*: Our findings confirm the general pattern indicating that the ERCC1 and ERCC4 gene products are crucial for the repair of 4HC-induced DNA damage, while the other nucleotide excision repair genes examined are relatively unimportant. These data suggest that the hypersensitivity of ERCC1$^-$ and ERCC4$^-$ mutants to DNA crosslinking agents may reflect a defect in recombinational repair rather than nucleotide excision repair.

Key words Cyclophosphamide · 4-Hydroperoxycyclophosphamide · Phosphorodiamidic mustard · Chinese hamster ovary cells · DNA repair-deficient mutants · DNA crosslinks

Introduction

Cyclophosphamide (CP) and its analogs are widely used to treat many types of cancer [1]. Of particular importance is their use in high-dose conditioning therapy preceding bone marrow transplantation (BMT) for patients with hematological malignancies, including acute myeloid leukemia (AML) and chronic myeloid leukemia (CML), and solid tumors. Currently, almost 90% of all transplanted patients are conditioned with a CP-based regimen [2]. Unfortunately, clinical refractoriness to the drug prevents eradication of the disease in many patients. For example, when allogeneic BMT is used early in the course of AML or CML, the disease

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recurrence rate is quite low ($\leq 20\%$); however, when allogeneic BMT is used in advanced phases of these diseases, the recurrence rate typically increases to $\geq 60\%$ [2]. Such refractoriness is generally believed to be mediated by the development of drug resistance within the tumor cell population [3].

Several major mechanisms of cellular drug resistance have been identified for this class of agents in model systems. Of these, elevated aldehyde dehydrogenase (ALDH) activity [3, 4] is extremely important for resistance to CP analogs. Additional mechanisms common to CP and a variety of structurally similar bifunctional DNA-damaging agents include increased cellular glutathione (GSH) content and/or glutathione-S-transferase (GST) activity [5–12] and enhanced repair of drug-induced DNA lesions [13, 14]. A number of studies have demonstrated increased DNA repair capability in cell lines resistant to *cis*-diamminedichloroplatinum (*cis*-DDP) (reviewed in reference 14). Similarly, an enhanced removal of DNA crosslinks (DXLs) has been observed in lymphocytes from patients with melphalan-resistant chronic lymphocytic leukemia (CLL) [15, 16] and in a melphalan-resistant human breast cancer cell line [17]. An indication of the potential importance of specific nucleotide excision repair (NER) genes in clinical response to bifunctional DNA damaging agents comes from the observation that ERCC1 mRNA levels are increased two-to threefold in lymphocytes from patients with melphalan-resistant CLL [18]. Furthermore, variations in the levels of ERCC1 mRNA in tumor cells from ovarian cancer patients correlate well with response to *cis*-DDP-based therapy [19].

To better define the mechanisms conferring CP resistance in myeloid leukemia, we developed a panel of human CML cell lines with experimentally induced resistance to the in vitro active CP derivative, 4-hydroperoxycyclophosphamide (4HC). The characteristics of one of these lines, KBM7/B5-180[3], have been recently reported [20]; it is highly resistant to 4HC and significantly cross resistant to phosphorodiamidic mustard (PM), the ultimate cytotoxin derived from both CP and 4HC. The resistance of these CML cells to 4HC derives from two major mechanisms. Elevated ALDH is a major factor in their resistance to 4HC and accounts for most of the observed decrease in the DXL-induction efficiency in the resistant line. However, a significant component of its resistance to 4HC, and most of its resistance to PM, derives from an ability to tolerate drug-induced DXLs better than the parental cells [20]. KBM7/B5-180[3] cells do, in fact, appear to repair DXLs more rapidly than parental cells, as assayed by alkaline elution [21]. This observation, although subject to a variety of interpretations, interested us in determining whether these 4HC-resistant CML cells overexpress any of the genes known to be involved in the repair of CP-induced DNA damage. A literature survey indicated that, of the well-characterized NER genes (ERCC1-ERCC6), Chinese hamster ovary (CHO) mutants in complementation groups (cgs) 1 (such as UV4 and UV20; ERCC1$^-$) and 4 (such as UV41; ERCC4$^-$) are generally hypersensitive to DXL-inducing agents such as mitomycin C (MMC) and *cis*-DDP (22, 23). CP, when activated by hamster liver S9 supernatant fraction, appears to follow this trend; ERCC1$^-$ CHO mutants are about 20-fold more sensitive than wild-type cells, whereas ERCC2$^-$ lines show only marginally increased sensitivity [22]. However, the generality of this finding has recently been questioned by Kelner et al. [24], who reported that the sensitivity of repair-deficient CHO cells to Illudin S (which putatively exerts its cytotoxic effects by inducing DXLs) showed an unexpected pattern, with ERCC2$^-$ and ERCC3$^-$ mutants being the most sensitive to the drug.

Before evaluating repair gene expression in CML cells, we therefore felt that it was important to establish whether the pattern of sensitivity of repair-deficient cells to these CP analogs did in fact parallel that suggested by Hoy et al. [22]. In the present study we characterized the sensitivity of a panel of seven repair-deficient CHO mutant lines for their sensitivity to 4HC and PM. Both of these drugs exhibit direct cytotoxicity in vitro without the necessity for prior activation. 4HC itself has been used widely for purging malignant cells from remission marrow in autologous BMT (e.g. references 25–27), and PM is the ultimate DXL-inducing metabolite of both CP and 4HC. PM shows two important differences from 4HC/CP in that its activity is independent of ALDH and it does not generate acrolein, itself a cytotoxic and DNA-damaging agent, as well as a potential inhibitor of NER [28], which are potentially confounding factors in mechanistic studies. We also examined the sensitivity of this panel of cell lines to *cis*-DDP, since their response to this drug has been previously characterized [22, 23].

Materials and methods

Drugs

4HC was provided by Dr. S. Rowley at the Fred Hutchinson Cancer Center, Seattle, Wash. PM was provided by Dr. R.F. Struck at the Southern Research Institute in Birmingham, Ala., and by the Chemical Synthesis Branch of the NCI, Bethesda, Md. *cis*-DDP was purchased from Bristol Laboratories, Syracuse, N.Y. Stock solutions were prepared by dissolving the drugs in phosphate-buffered saline (PBS; GIBCO, Grand Island, N.Y.) immediately before treatment.

Cell lines and culture conditions

The characteristics of the NER$^-$ mutants used in this study, UV4 and UV20 (ERCC1$^-$), UV5 (ERCC2$^-$), UV24 (ERCC3$^-$), UV41 (ERCC4$^-$), UV135 (ERCC5$^-$), and UV61 (ERCC6$^-$), have been described previously [29, 30], as has the X-ray sensitive EM9 (XRCC1$^-$) mutant [31]. These mutants were all derived from the wild-type CHO AA8 line. The so-called 41XP hybrids were obtained

by fusing UV41 cells with human xeroderma pigmentosum fibroblasts [32]. Unidirectional segregation of the human chromosomes when these initial hybrids were cultured in the presence of MMC resulted in the selection of alkylating agent-resistant hybrids that had hamster phenotype and retained only human chromosome 16, or at least the p13.13-p13.2 region thereof, that contains the complementing human ERCC4 gene. We examined two 41XP hybrids – designated 41XP3542-2-20 and 41XP2991-2-03 – that retained region p13.13-p13.2 of human chromosome 16. As controls in these experiments, we examined two additional hybrids – designated 41XP2991-2-17 and 41XP3542-2-24 – that retained a similar fragment of chromosome 16 but not the ERCC4 gene [32].

Cell lines (except for 41XP hybrids) were maintained as attached monolayer cultures in T-75 flasks (Corning Lab Ware, Corning, N.Y.) in McCoy's 5A medium (modified, with L-glutamine, without sodium bicarbonate; GIBCO) supplemented with 15% fetal bovine serum (FBS), penicillin (50 units/ml), and streptomycin (50 μg/ml) (all from Sigma, St. Louis, Mo.). 41XP hybrids were similarly maintained in DMEM (GIBCO) supplemented with 10% FBS, L-glutamine, and sodium bicarbonate. Cells were passaged every 2–3 days to ensure exponential growth. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Cell suspensions were prepared by exposing the cultures to 1 ml 0.05% trypsin (GIBCO) for 6 min at 37°C; trypsin activity was terminated by the addition of 3 ml fresh supplemented growth medium.

Transfection of UV20 cells with human ERCC1

A full-length ERCC1 cDNA clone (pSVL5E) was provided by Dr. J. Hoeijmakers. The insert was excised by digestion with EcoRI and PvuII, blunt-ended by treatment with Klenow fragment, ligated to BstX1 linkers [33], and cloned into the BstX1 site of the pCDM8-neo eukaryotic expression vector under the control of the CMV promoter. About 10 μg of the resulting purified pCD-ERCC1 plasmid was added to two 100-mm Petri dishes, each containing about 10^6 UV20 ($ERCC1^-$) cells, which were then subjected to calcium phosphate-mediated transfection [34]. The calcium phosphate was washed off with PBS-5 m*M* EDTA on the following morning, and the cells were re-fed with normal supplemented growth medium. About 48 h later, medium containing 400 μg/ml G418 was added to the cultures to select for neo^+ transfectants. The cultures (henceforth referred to as UV20-$ERCC1^+$) were detached 12 days later by exposure to trypsin and further subcultured in T-25 flasks (Corning) in supplemented growth medium containing 200 μg/ml G418. The cultures were then expanded until a sufficient cell density was obtained such that the cells could be tested for their sensitivity to 4HC and PM using a clonogenic assay, as described below.

Survival curve analysis

For the clonogenic assay, cells were set at 10^6 per flask in T-25 flasks 24 h before the drug treatment. The cells were washed with PBS and treated with either 4HC for 60 min, PM for 15 min, or *cis*-DDP for 60 min in serum-free medium at 37°C. The drug was then removed, and the cells were washed with PBS and detached by exposure to trypsin. Known numbers of cells were plated at various dilutions in supplemented growth medium in six-well plates and incubated for 8 days. Cells were stained with 0.5% gentian violet, and colonies of more than 50 cells were counted. The surviving fraction was defined as the ratio of the plating efficiencies of drug-treated cells to untreated control cells.

For the growth inhibition assay, 10^6 cells/flask were plated in T-25 flasks 24 h before the drug treatment. The cells were washed with PBS and treated with the drug for 60 min (4HC) or 15 min (PM), as described above. The drug was then removed, and the cells washed with PBS and detached by exposure to trypsin. Cells (5×10^4) in a total volume of 4 ml of supplemented growth medium were seeded in triplicate in 60-mm Petri dishes and incubated under optimal growth conditions for 72, 84, or 96 h, depending on the cell line. The cells were washed with PBS, detached by exposure to trypsin, and the number of cells was determined using a Coulter counter (Coulter, Hialeah, Fl.). The growth inhibition data were expressed as a pseudo surviving fraction, defined as the growth in the drug-treated cultures relative to untreated controls, and were plotted versus drug concentration.

All survival data were analyzed using the linear quadratic model:

$$-\ln SF(C) = \alpha C + \beta C^2$$

where $SF(C)$ is the surviving fraction at a drug concentration C, and α and β are constants, using Scientific Graphpad (Scientific LOGICS, Cupertino, Calif.). Relative sensitivities were determined as the ratios of isoeffective drug concentrations.

Determination of DNA cross-link induction

DXLs were determined using a modification [17] of the ethidium bromide (EB) fluorescence assay described by De Jong and colleagues [35]. An advantage of this method is that it allows the rapid and simultaneous characterization of multiple cell lines. Briefly, after treatment with 4HC or PM as described above, the cells were detached by exposure to trypsin and resuspended in PBS. Cells (5×10^5) were combined with 200 μl lysis buffer (4 *M* NaCl; 50 m*M* KH_2PO_4; 10 m*M* EDTA; 0.1% Sarkosyl; pH 7.2; plus 20 μl RNase, 2 mg/ml) and incubated for 16 h at 37°C. Heparin (25 μl; 500 IU/ml) was added, and the lysate was incubated for 20 min at 37°C. The lysate was combined with 3 ml of a solution containing EB (10 μg/ml), 20 m*M* K_2HPO_4, and 0.4 m*M* EDTA (pH 11.8). The DNA was denatured by boiling for 5 min followed by rapid cooling to room temperature. The samples were kept in the dark until analysis. EB fluorescence was determined using an Aminco SPF-125 spectrofluorometer ($\lambda_{excitation} = 525$ nm; $\lambda_{emission} = 580$ nm). The percentage of crosslinked DNA in the treated cells (C_t) was calculated as:

$$C_t = (f_t\text{-}f_c)/(1\text{-}f_c) \times 100\%$$

where f_t and f_c are the fluorescence fraction for drug-treated and untreated control cells, respectively, calculated as the ratio of the fluorescence intensities with and without heat denaturation (35). Salmon sperm DNA was used to generate a control response curve.

Results

Survival of CHO cells exposed to 4HC

Survival curves for wild-type AA8 cells and the seven mutant cell lines following treatment with 4HC, obtained using the growth inhibition and clonogenic assays, are shown in Fig. 1A and B, respectively. The rank ordering of the sensitivity of the mutants to 4HC based on the data in Fig. 1A was UV41/UV4/UV20 $\gg$ UV61/UV24/UV5/UV135/EM9 $>$ AA8. Thus UV41 ($ERCC4^-$) and UV4/UV20 ($ERCC1^-$) cells were by far the most sensitive mutants. Using the clonogenic survival assay, the partial ranking was: UV41/UV4/UV20 $\gg$ UV5 $>$ AA8. The same ranking of sensitivities was thus obtained using the growth inhibition and clonogenic survival assays. The relative sensitivities of the wild-type and mutant cell lines to 4HC are further compared in Table 1. It should be noted that,

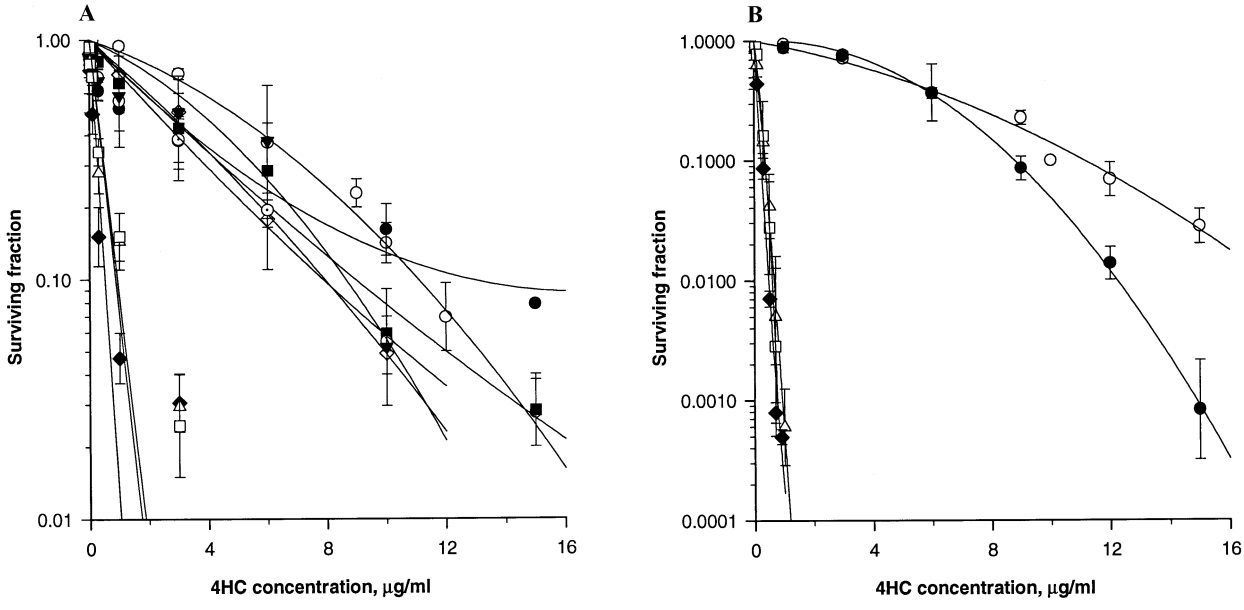

Fig. 1A, B Survival of wild-type AA8 (○), UV4 (□), UV20 (△), UV5 (●), UV24 (■), UV41 (◆), UV135 (◇), UV61 (⊙), and EM9 (▽) cells following treatment with 4HC at 37°C for 60 min. Survival was assayed by either (**A**) growth inhibition or (**B**) clonogenic assay

Table 1 Relative sensitivity factors of nucleotide excision repair-deficient (NER$^-$) CHO mutants to 4HC, PM and *cis*-DDP (*n/d* not determined)

Cell Line	Genotype	cg	Relative sensitivity factor[a] 4HC	PM	*cis*-DDP
AA8	Wild-type		1	1	1
UV4	ERCC1$^-$	1	30.1[b]	42.4[b]	n/d
			10.0[c]	n/d	
UV20	ERCC1$^-$	1	31.5[b]	22.3[b]	50
			10.5[c]	n/d	
UV5	ERCC2$^-$	2	1.27[b]	1.27[b]	7
			0.88[c]	1.04[c]	
UV24	ERCC3$^-$	3	1.25[c]	2.02[c]	7
UV41	ERCC4$^-$	4	49.3[b]	22.6[b]	50
			16.4[c]	45.1[c]	
UV135	ERCC5$^-$	5	1.40[c]	1.57[c]	10
UV61	ERCC6$^-$	6	1.48[c]	2.15[c]	n/d
EM9	XRCC1$^-$		1.30[c]	1.42[c]	0.8
41XP hybrids					
2991-2-03	*h*ERCC4$^+$[d]		1.09[b]	0.85[b]	n/d
3542-2-20	*h*ERCC4$^+$		1.43[b]	0.92[b]	n/d
2991-2-17	ERCC4$^-$		32.7[b]	32.7[b]	n/d
3542-2-24	ERCC4$^-$		42.5[b]	42.3[b]	n/d
UV20-ERCC1$^+$	*h*ERCC1$^+$[z]		1.36[b]	1.19[b]	n/d

[a]Defined as the ratio of the drug concentrations required to reduce the surviving fraction to 0.1 in wild-type (AA8) cells and in the cell line under comparison
[b]Based on clonogenic survival data
[c]Based on growth inhibition data
[d]*h* refers to the complementing human gene

where available, the relative sensitivity factors based on clonogenic survival data are more representative than those based on growth inhibition data because the latter assay begins to lose its resolution at surviving fractions below about 0.05.

Survival of CHO cells exposed to PM and *cis*-DDP

Similar data for PM are shown in Fig. 2A, B and in Table 1. The rank order of sensitivity of the mutants to PM based on the combination of growth inhibition and

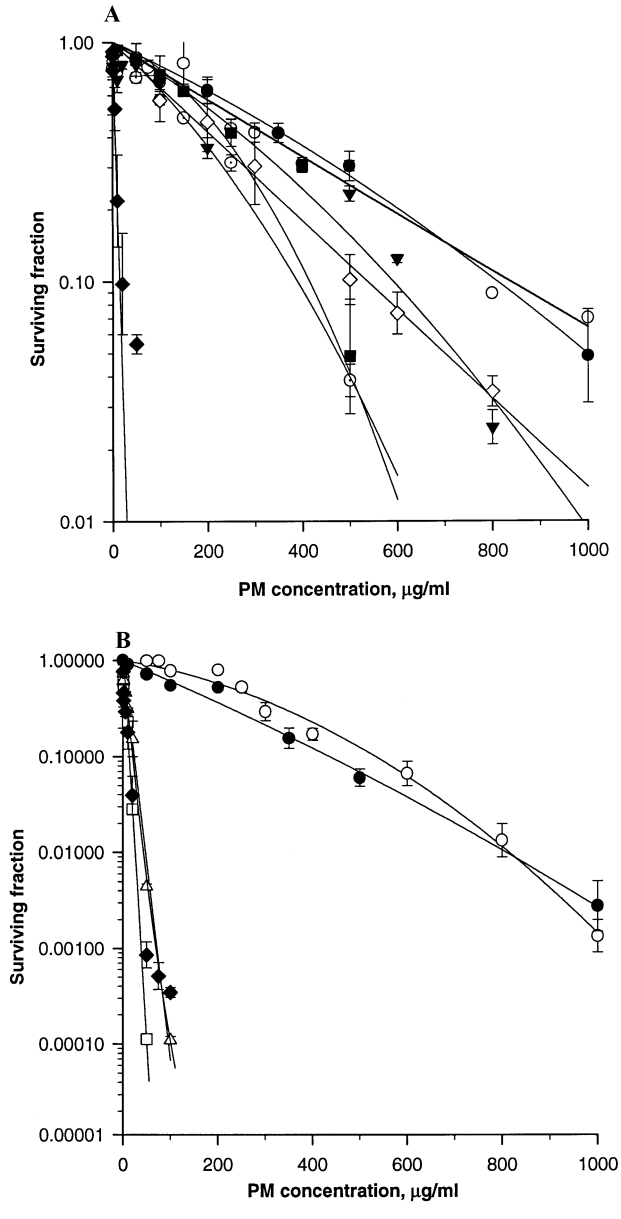

Fig. 2A, B Survival of wild-type AA8 (○), UV4 (□), UV20 (△), UV5 (●), UV24 (■), UV41 (◆), UV135 (◇), UV61 (⊙), and EM9 (▽) cells following treatment with PM at 37°C for 15 min. Survival was assayed by either (**A**) growth inhibition or (**B**) clonogenic assay

clonogenic survival data was UV41/UV4/UV20≫ UV61/UV24 ≥ UV135/EM9 > UV5/AA8. This is essentially similar to the ranking following 4HC exposure.

In agreement with previous data for other bifunctional DNA-damaging agents such as *cis*-DDP [22], the X-ray-sensitive mutant line EM9 ($XRCC1^-$) showed minimal cross-sensitivity to either 4HC or PM (Figs. 1, 2). In further agreement with previous data for *cis*-DDP [22, 23], $ERCC1^-$ and $ERCC4^-$ mutants were hypersensitive to this agent, whereas $ERCC2^-$, $ERCC3^-$ and $ERCC5^-$ mutants showed a modest (although significant) sensitivity to *cis*-DDP (Table 1).

DNA crosslinking in wild-type and mutant cells exposed to 4HC

Figure 3A, B shows the yield of DXLs in the AA8, UV5, and UV41 cell lines following treatment with various concentrations of 4HC and PM, respectively. These three cell lines were chosen to represent the wild-type strain (AA8), a drug-hypersensitive mutant strain (UV41), and a relatively insensitive mutant strain (UV5) as a negative control. It is apparent that the level of induced DXLs paralleled the sensitivity of the cell lines to the drug; UV41 cells showed significantly elevated levels of DXLs at all concentrations of 4HC or PM that were examined; AA8 and UV5 cells showed

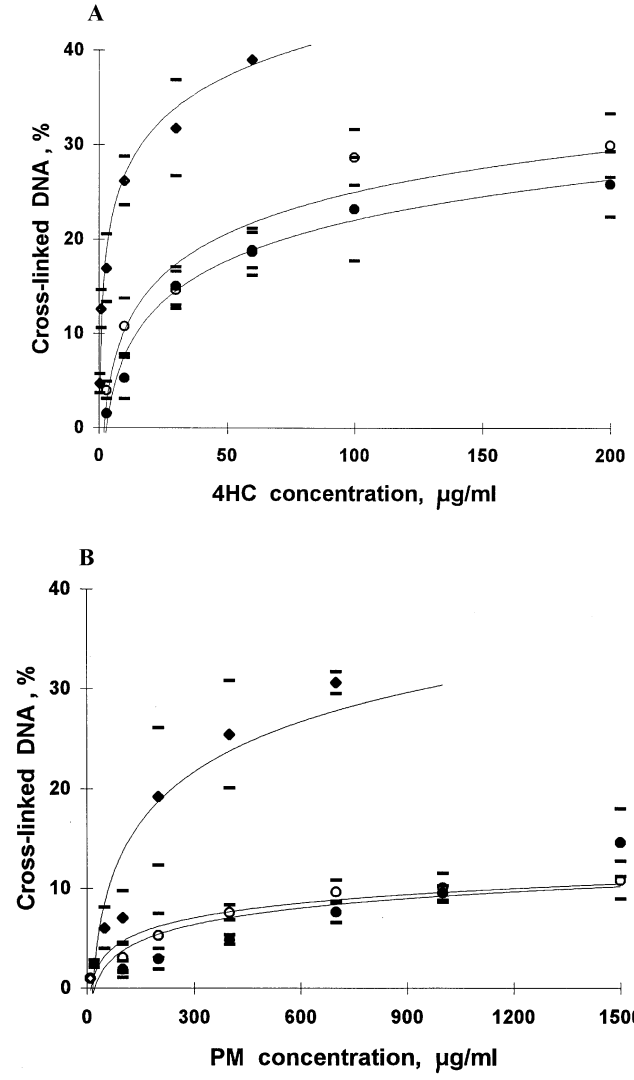

Fig. 3A, B Level of DNA crosslinking detected in the wild-type AA8 (○), UV5 (●), and UV41 (◆) cells as measured by the ethidium bromide fluorescence assay immediately after a 60-min treatment with various concentrations of either (**A**) 4HC or (**B**) PM. Values are the average ± SD of four separate experiments for each cell line

similar, low levels of DXLs after treatment with either 4HC or PM.

Sensitivity of UV41-derived interspecific hybrid cells to 4HC and PM

Figure 4A, B shows the survival curves for four different 41XP hybrids following treatment with 4HC and PM, respectively. Both of the hybrids that retained 16p13.13-p13.2, 41XP2991-2-03 and 41XP3542-2-20, showed close to wild-type resistance to both drugs, although it should be noted that the 41XP3542-2-20 hybrid showed a slightly increased sensitivity to 4HC at concentrations above 10 μg/ml. In contrast, the two hybrids, 41XP2991-2-17 and 41XP3542-2-24, in which 16p13.13-p13.2 was deleted showed UV41-like hypersensitivity to both drugs.

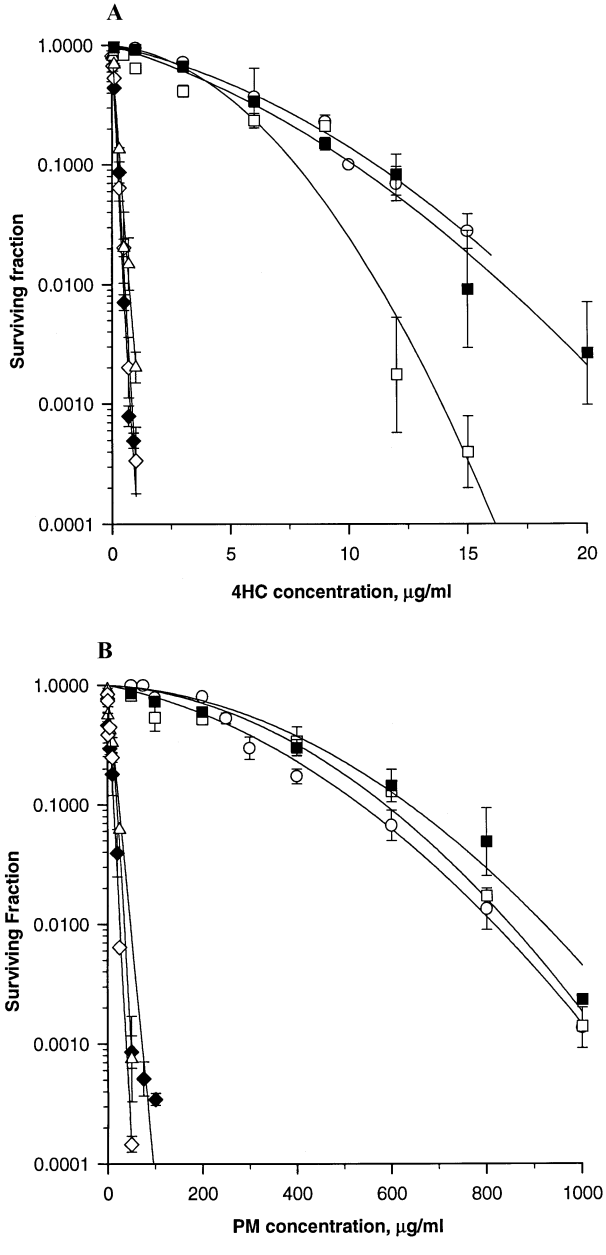

Fig. 4A, B Survival of wild-type AA8 (○), UV41 (●), and of four different UV41/XP hybrids following treatment with either (**A**) 4HC at 37°C for 60 min or (**B**) PM at 37°C for 15 min. The 41XP2991-2-03 (■) and 41XP3542-2-20 (□) hybrids retain the human chromosome region 16p13.13-p13.2, whereas the 41XP2991-2-17 (△) and 41XP3542-2-24 (◇) hybrids do not. Survival was assayed by clonogenic assay

Sensitivity of UV20-derived ERCC1-transfected cells to 4HC and PM

Figure 5A, B shows the survival curves for wild-type AA8 and UV20 (ERCC1$^-$) cells treated with 4HC and PM; also shown are the survival curves for the UV20-ERCC1$^+$ cultures obtained after transfection of UV20 cells with the human ERCC1 cDNA. The sensitivity of these UV20-ERCC1$^+$ cells approximated that of the wild-type AA8 line. The untransfected UV20 cells showed the anticipated hypersensitivity to these drugs (see also Figs. 1, 2). Thus, over expression of the human ERCC1 gene in UV20 cells restored almost wild-type resistance to both 4HC and PM.

Discussion

Clinical resistance to CP analogs is commonly believed to have a cellular basis [3]. The best-characterized mechanism for the development of cellular resistance to CP analogs involves upregulation of one or more isoforms of the ALDH family of enzymes that oxidize aldophosphamide (an intermediate metabolite of both CP and 4HC) to the nontoxic carboxyphosphamide, in preference to the generation of the cytotoxic species, PM [3]. Elevated ALDH activity does in fact appear to be of paramount importance for the CP-resistant phenotype of some rodent and human tumor cell lines in vitro, as evidenced by the direct demonstration of elevated ALDH levels and by the complete reversal of resistance by ALDH inhibitors [4, 36–40]. In contrast, a 4HC-resistant murine KHT tumor line shows no elevation of ALDH; rather, its resistance appears to be based entirely in an elevation of GSH/GST [10, 41]. In other tumor models, ALDH appears to play a significant but only partial role in the CP-resistant phenotype [42, 43]. Indeed, in two recent detailed investigations of CP analog resistance in human tumor cell lines, medulloblastoma [44] and KBM-7/B5-180^3 CML [20], resistance has been found to be multifactorial, with ALDH being a major but nonetheless partial component. In both of these studies a temporal component of the CP-resistant phenotype was suspected. In the case of KBM-7/B5-180^3 CML cells the temporal component appears to be as important as the upregulation of

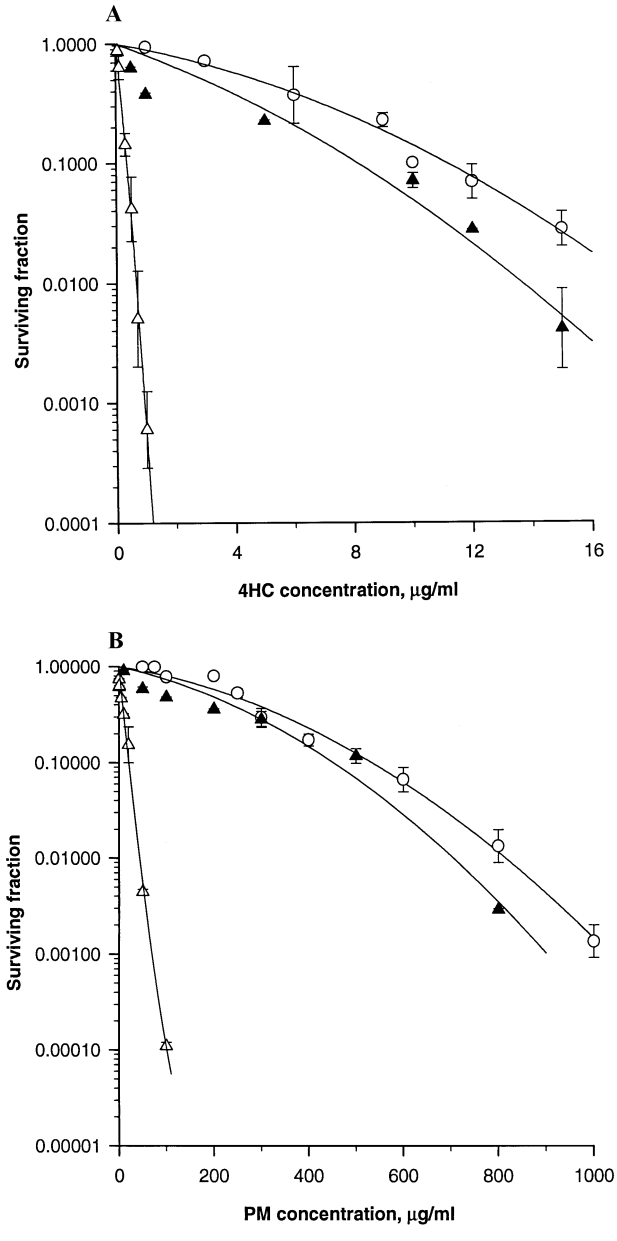

Fig. 5A, B Survival of AA8 (○), UV20 (△), and UV20-ERCC1$^+$ (▲) cultures exposed to (**A**) 4HC at 37°C for 60 min or (**B**) PM at 37°C for 15 min

ALDH, and it has been further suggested that this may involve accelerated repair of DXLs [21]. Although a convincing demonstration of an involvement of DNA repair activity in the CP-resistant phenotype is lacking, experimental and clinical data for other bifunctional DNA alkylating agents, such as melphalan and *cis*-DDP, do indicate that repair may be important for the development of tumor cell resistance (for example, references 13–19).

Before further evaluating the specific nature of the putative repair component of the KBM-7/B5-180[3] phenotype, we decided to use an in vitro model system to better define which repair pathways would likely be involved in CP resistance development. A survey of the existing literature revealed surprisingly little about the involvement of specific DNA repair genes in the removal of DNA lesions induced by CP and its analogs. In fact, the only report that we could find was the study by Hoy et al. [22] suggesting that activated CP is a "typical" bifunctional alkylating agent inasmuch as ERCC1$^-$ mutants, but not ERCC2$^-$ mutants, are hypersensitive to the drug. Many of the genes whose products mediate the repair of damage to genomic DNA have now been identified. At least 11 cgs of NER/ERCC genes and 7 XP cgs (plus a variant group) have been identified to date [45]; there is some (although incomplete) overlap between these two categories [46]. In addition, at least eight X-ray repair cgs [47] have been identified. A variety of additional gene products may be involved in the repair of DXLs. For example, for *cis*-DDP, in addition to the NER genes, important roles for DNA polymerases [48, 49], thymidylate synthase cycle genes [50], and various damage-recognition factors (for example, references 51, 52) have also been described.

In the present study we examined the sensitivity of various NER$^-$ mutants to the cytotoxic effects of 4HC and PM. These CP analogs are both active in vitro. Indeed, PM is the ultimate DNA alkylating moiety of both CP and 4HC. As regards bifunctional alkylation products, these three drugs should therefore exhibit the same dependency on DNA repair enzymes. However, the effects of PM, unlike, those of 4HC and CP, are independent of ALDH activity. Furthermore, PM, in contrast to 4HC, does not generate acrolein, which is both a cytotoxic and a DNA-damaging agent, while producing very different types of lesions from PM (for example, reference 53). In addition to the possibility that the true pattern of sensitivity to activated CP or 4HC could be affected by an additional confounding sensitivity of the cells to acrolein, the formation of acrolein could also affect the activity of enzymes involved in the repair of PM-induced DXLs. This situation, however, appears unlikely since the repair of PM-induced DNA damage in human lymphocytes does not appear to be susceptible to modulation even by high levels of exogenous acrolein [28]. Still, some caution must be applied to the interpretation of these data since acrolein, which is a highly reactive molecule, may be effective as a repair inhibitor only if it is generated at the site of DNA alkylation, rather than added to the extracellular medium. Assigning the relative importance of different genes based on the response to 4HC could also be obscured by secondary alterations resulting from the original 'brute force' mutagenesis procedure [29] or by the contribution to cytotoxicity by monofunctional adducts or other lesions derived from acrolein. However, the observation that 4HC and PM exhibited the same pattern of cytotoxicity towards this panel of NER$^-$ lines (Table 1) suggests that acrolein does not contribute significantly to the observed effects.

Two of the six NER genes characterized here, ERCC1 and ERCC4, appear to be particularly important for the repair of DNA damage induced by a wide variety of bifunctional alkylating agents [22]. Hoy et al. [22] noted that mutants in cg1 ($ERCC1^-$) and cg4 ($ERCC4^-$) are hypersensitive to the lethal effects of agents such as *cis*-DDP that produce DXLs, whereas mutants such as UV5 in cg2 ($ERCC2^-$) are only moderately sensitive to these drugs, indicating the significance of the ERCC1 and ERCC4 proteins in the repair of DNA damage caused by *cis*-DDP and other bifunctional DNA-damaging agents. In marked contrast, the sensitivity of repair-deficient CHO cells to the DXL-inducing drug Illudin S shows an unusual pattern, with ERCC2 and ERCC3 DNA helicase-deficient mutants being highly sensitive to the drug [24]. The results of the present study with both 4HC and PM indicate a "normal" response, with members of cg1 and cg4 showing a hypersensitive response to these CP derivatives. In fact, Hoy et al. [22] divided DXL-inducing agents into two classes: class 1, including melphalan and activated CP, produce a hypersensitive response in $ERCC1^-$ lines, whereas $ERCC2^-$ lines show no increased sensitivity; class 2, including *cis*-DDP and MMC, produce a hypersensitive response in $ERCC1^-$ lines and a modest response in $ERCC2^-$ lines. Based on the data in Table 1, neither 4HC nor PM evoked a hypersensitive response other than in cg1/cg4 mutants (and would thus be assigned to class 1, along with activated CP), while our data obtained with *cis*-DDP (Table 1) confirm the anticipated class-2 response for this drug inasmuch as mutants in cgs 2, 3, and 5 did show a weak response.

The specific involvement of the ERCC4 gene in 4HC cytotoxicity was confirmed by examining the restoration of the resistance to 4HC in a series of interspecific hamster/human hybrid cell lines in which a known human chromosome (or fragment thereof) complements the defective hamster repair gene. These 41XP hybrids were generated as part of the successful effort to map the ERCC4 gene to 16p13.13-p13.2 [32]. As shown in Fig. 4, both of the hybrids that retained 16p13 (i.e. ERCC4) were highly resistant to 4HC and PM. In contrast, both hybrids that lacked 16p13 showed UV41-like hypersensitivity to these drugs. Thus, a human gene that segregates concordantly with ERCC4 is uniquely capable of reversing the hypersensitivity of UV41 cells to 4HC and PM. The specific involvement of ERCC1 was similarly confirmed by the restoration of wild-type resistance to 4HC/PM in UV20 cells transfected with a full-length human ERCC1 cDNA (Fig. 5). Thus, the human ERCC1 and ERCC4 genes reversed the hypersensitivity of UV20 and UV41 cells, respectively, to CP analogs.

That cg1 and cg4 mutants are uniquely hypersensitive to DXL-inducing agents may be the result of a defect in recombinational repair rather than NER, analogous to the repair/recombination defect in yeast *rad1* and *rad10* mutants [45, 54]. The congruence of the phenotypes of cg1 and cg4 mutants still can be rationalized by reference to NER, however. In NER, the ERCC1 and ERCC4(XPF) proteins are believed to form a heterodimer that interacts with the XPA damage-recognition protein, forming a complex endonuclease that incises the damaged DNA strand 5′ to a bulky lesion, while the ERCC5(XPG) protein makes the 3′ incision [55–58]. It is possible that this same heterodimeric endonuclease is involved in the recombinational repair of DXLs but without a requirement for the additional accessory proteins that are involved in NER.

As regards DNA damage, the yield of DXLs in AA8, UV5, and UV41 cells following treatment with 4HC or PM paralleled the sensitivity of the cell lines to the respective drug (Fig. 3). This was somewhat surprising because these data were obtained soon after a 60-min (4HC) or 15-min (PM) treatment with the drug. It is well known from studies using alkaline elution that DXLs induced by activated CP and CP analogs with in vitro activity require a period of about 4 h for full development in a variety of mammalian cell lines, which is presumed to reflect the conversion of mono- to di-adducts [10, 21, 53, 59, 60]. Therefore, only after a significant period under repair-permissive conditions should the levels of DXLs in wild-type and mutant cells be different, assuming that the hypersensitivity of the latter is due to an inability to repair DXLs. Not only were marked differences between AA8 and UV41 cells discernible soon after treatment, but also the EB-DXL assay did not detect DXLs in either cell line when it was performed with cells that were incubated for 4 h at 37°C after drug exposure. A similar paradox can be seen in studies of melphalan resistance reported by Panasci and colleagues, also using the EB-DXL assay. These authors found that for parental and melphalan-resistant MCF-7 human breast cancer cells the level of DXLs peak after about 4 h and decrease thereafter, but more rapidly for the resistant line [17]. In contrast, when they applied the same assay to lymphocytes from untreated CLL patients or from patients with melphalan-resistant CLL, a very different time course was observed: whereas DXL levels in cells from untreated patients continued to increase up to 24 h after a 35-min drug treatment, DXL levels in cells from the melphalan-resistant group were maximal immediately after treatment and declined thereafter, not unlike the present data with CHO cells [16]. As with the 4HC/PM data discussed above, the time-course of melphalan-induced DXLs measured by alkaline elution in both cultured cells and rodent models generally peaks about 12 h after the termination of drug treatment (for example, references 61–63).

Based on a consensus of these alkaline elution data, in some instances the EB-DXL assay produces data consistent with expectation, whereas in other cases the kinetics are atypical. Thus, although the measured

yields of DXLs in these CHO lines paralleled the cytotoxicity data under the described experimental conditions, we are somewhat uncertain about the general validity of this assay. One possibility is that the relatively mild cell lysis conditions (as regards deproteinization) associated with the EB-DXL assay results in the detection of a significant level of DNA-protein crosslinks, in addition to DXLs, and that these lesions are formed and repaired much more rapidly than DXLs (or they may be relatively unstable). It is also possible that alterations in the chromatin structure of cells associated with the acquisition of the drug-resistant or -sensitive phenotype modulates not only the quantity but also the structure and stability of the drug-induced DNA crosslinks [14] and that these alterations are differentially manifested in different assay systems.

There are several clinical implications of these data. Firstly, it should be remembered that in mutants such as UV41 the repair gene is severely defective and thus is essentially "forced" to be rate limiting for the repair of PM adducts. To what extent differences in ERCC1 and/or ERCC4 expression that may be encountered among/within primary tumors would affect their sensitivity to CP analogs is unknown. The ERCC4 gene has only recently been cloned [64], and there is no information yet as to its variation in tumors. Secondly, if the exact mechanism(s) for repair of PM adducts can be elucidated, this information could be potentially used in two ways: (1) to circumvent resistance or prevent it from arising at all; and (2) to develop ways to transfect specific genes into normal bone marrow progenitors, thereby enabling patients to better tolerate intensive chemotherapy without appreciable myelosuppression. Analogous studies of MDR-1 gene transfection as a means to increase marrow resistance to chemotherapy are already under way [65, 66].

In summary, the observation that ERCC1$^-$ and ERCC4$^-$ lines are hypersensitive to the cytotoxic effects of 4HC and PM, whereas ERCC2$^-$ lines are minimally sensitive, confirms the general trend reported by others [22]. In addition, ERCC3$^-$, ERCC5$^-$, and ERCC6$^-$ mutant lines show minimal sensitivity to these drugs. This hypersensitivity of cg1 and cg4 mutants to crosslinking agents might reflect a recombinational repair defect in addition to the NER defect, reminiscent of the shared repair/recombination defect in yeast *rad1* and *rad10* mutants ([54]; Dr. L.H. Thompson, personal communication). Clearly, it will be interesting to examine the expression of the ERCC1 and ERCC4 genes in cell lines with experimentally induced resistance to CP analogs, such as the 4HC/PM-resistant KBM-7/B5-180[3] CML cell line [20] and CP-resistant medulloblastoma lines [44], bearing in mind the possible DNA-repair component of their CP-resistant phenotype. Ultimately, if positive, we would propose to extend these studies to an examination of CP-resistant primary leukemic cells from patients undergoing conditioning therapy before marrow/stem cell transplantation, with a correlation to clinical treatment outcome.

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