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Biochemistry

Cisplatin-resistant cells express increased levels of a factor that recognizes damaged DNA

(cancer treatment/DNA repair/drug resistance/xeroderma pigmentosum)

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ABSTRACT **Cancer treatment with the drug cisplatin is often thwarted by the emergence of drug-resistant cells. To study this phenomenon, we identified two independent cellular factors that recognize cisplatin-damaged DNA. One of the two factors, designated XPE binding factor, is deficient in complementation group E of xeroderma pigmentosum, an inherited disease characterized by defective repair of DNA damaged by ultraviolet radiation, cisplatin, and other agents. Human tumor cell lines selected for resistance to cisplatin showed more efficient DNA repair and increased expression of XPE binding factor. These results suggest that XPE binding factor may be responsible, at least in part, for the development of cisplatin resistance in human tumors and that the mechanism may be increased DNA repair.**

Drugs used in cancer chemotherapy may be extremely effective in killing tumor cells, but relapses occur all too often due to the subsequent development of drug resistance in the tumor cells. One model for this phenomenon is the development of methotrexate resistance *in vitro*. The level of resistance may increase 100- to 1000-fold when cells are grown in the presence of methotrexate and can be due to many mechanisms, including amplification or mutations of the gene for dihydrofolate reductase (*dhfr*) (1). A second model is the simultaneous development of resistance to multiple drugs, including adriamycin, actinomycin D, vinca alkaloids, and podophyllotoxins (2). Again the level of resistance may be very high and has been shown to arise from amplification or mutations of multidrug resistance (*mdr*) genes that code for membrane glycoproteins involved in drug efflux (3, 4).

However, neither the *dhfr* nor *mdr* gene is involved in the development of resistance to a number of therapeutically important drugs that form DNA adducts, including agents such as nitrogen mustard, cyclophosphamide, mitomycin C, and cisplatin. Tissue culture models indicate that the level of resistance to such agents is limited. For example, cell lines grown in cisplatin exhibit only 5- to 50-fold increased resistance (5). There has been indirect evidence that resistance is mediated at least in part by DNA repair (6), but evidence for the increased expression of specific DNA repair proteins has been lacking because the biochemistry of DNA repair in humans has been poorly understood.

To study resistance to cisplatin, we reasoned that the repair of damaged DNA must depend on cellular factors that are capable of recognizing the site of damage. Such factors can be identified by an extension of the gel electrophoresis binding assay utilizing damaged DNA as the probe for analyzing cell extracts (7).

This report describes two independent cellular factors that recognize cisplatin-damaged DNA. One was detected by a cisplatin-damaged DNA probe, appeared to recognize only that form of damaged DNA, and was designated as cisplatin-crosslinked DNA (CCD) binding factor. The other was detected by a UV-damaged DNA probe, also bound to cisplatin-damaged and single-stranded DNA in competition experiments, was deficient in xeroderma pigmentosum (XP) cells from complementation group E, and was designated as XPE binding factor. When human tumor cells were selected for resistance to cisplatin, they showed increased expression of XPE but not CCD binding factor and, concomitantly, showed more efficient DNA repair in a transfection assay.

MATERIALS AND METHODS

Cell Lines. The HeLa cell line was kindly provided by M. Dieckmann and P. Berg (Stanford University). The HT1080 human fibrosarcoma cell line (CCL 121, ATCC) was kindly provided by A. Ganesan and P. Hanawalt (Stanford University). The XP cell lines GM02250A (group A), GM03021A (group G), GM02415 (group E), and GM02450D (group E) were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). The last two cell lines were the only cells from XP complementation group E available from the Cell Repository and were derived from two individuals who were second cousins (8). All cells were grown in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum.

Resistant cells were obtained by growing HeLa and HT1080 cells in stepwise increasing concentrations of cisplatin to produce resistant cell lines. Drug was added to the medium 4 hr after trypsinization and with each change of the medium. The resistant cells represent pooled colonies of surviving cells.

Assays for Cisplatin and UV Resistance. To determine cisplatin resistance, each of two independent experiments was done in quadruplicate. The cells were seeded into 96-well microtiter plates, allowed to grow in the presence of different doses of cisplatin for 72 hr, and then assayed for cell number by the colorimetric signal produced by cleavage of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (9). The plates were read in a Microelisa reader (Dynatech MR 580).

To determine UV resistance, each of two experiments was done in duplicate. The cells were seeded onto 85-mm tissue culture plates, exposed to UV radiation from a germicidal lamp at a fluence of 2.24 J/m^2 per sec, allowed to grow for 72 hr, and then assayed for cell number by measurement of nucleic acid content after alkaline lysis (10).

Gel Mobility Shift Assay. Nuclear extracts were made as described (11). The DNA fragment f148 was the 148-base-pair *Hin*dIII–*Pvu* II fragment from the 5′ region of the bacterial chloramphenicol acetyltransferase (CAT) gene (12). The DNA was gel purified, end-labeled with Klenow fragment, and

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Abbreviations: CAT, chloramphenicol acetyltransferase; CCD binding factor, cisplatin-crosslinked DNA binding factor; r_f, molar ratio of platinum to nucleotide phosphates; XP, xeroderma pigmentosum.

UV-irradiated (UV-f148) or crosslinked with cisplatin (Pt-f148). DNA was irradiated with 5000 J/m^2 from a UV germicidal lamp.

DNA was usually damaged with cisplatin as described (13) by incubating DNA with cisplatin in Pt buffer (3 mM sodium chloride/1 mM sodium phosphate, pH 7.4) at 37°C for 18–24 hr in the dark. After the incubation, sodium chloride was added to 0.1 M and the cisplatin-crosslinked DNA was precipitated with 2 volumes of ethanol. The cisplatin concentration varied between 0.3 μM and 30 μM. The DNA concentration was 0.1 mg/ml (300 μM nucleotide phosphate). The molar ratio of platinum to nucleotide phosphate at the onset of the incubation, r_f, varied between 0.001 and 0.10. Under these conditions, the molar ratio of bound platinum to nucleotide phosphate, r_b, reaches a steady-state level of $\approx$0.8 r_f after 18–24 hr.

Cisplatin-crosslinked probe DNA was prepared under somewhat different conditions because of the small amount of DNA involved: 5 ng of end-labeled f148 was incubated with 7 ng of cisplatin in 25 μl of Pt buffer. These conditions produced a molar ratio of bound platinum to nucleotide phosphate of $\approx$0.08, as determined by comparison of the mobility shift in an 8% polyacrylamide gel with DNA damaged by cisplatin under the standard conditions described above.

The probe DNA (0.2 ng) was incubated with nuclear extracts and resolved by electrophoresis on a nondenaturing polyacrylamide gel as described (7). Incubations included 1000 ng of the alternating copolymer poly(dI-dC)·poly(dI-dC) to mask the effect of nonspecific DNA binding proteins.

Transfection of Cisplatin-Crosslinked DNA. DNA was transfected by electroporation as described (14). A 1080-μF capacitor was charged to 250 V and then discharged through a 1-ml cell suspension containing 2 × 10^6 cells and 15 μg of cisplatin-crosslinked pRSVcat DNA (13). After 72 hr, cell extracts were prepared and assayed for CAT activity by the acetylation of [^{14}C]chloramphenicol (12). The reaction products were extracted with ethyl acetate, separated by thin-layer chromatography, autoradiographed, and quantified by counting the spots in a scintillation counter. The equivalence of transfection efficiencies for different arms of a given experiment was verified by cotransfecting 15 μg of a second, undamaged marker plasmid pRSVgpt and assaying for transient expression of xanthine-guanine phosphoribosyltransferase by the conversion of [^{14}C]xanthine to xanthine monophosphate (15).

RESULTS

An end-labeled DNA fragment was incubated with HeLa nuclear extract and resolved by electrophoresis. When the DNA probe was damaged with either UV radiation or cisplatin and incubated with extract, it migrated with decreased mobility in the gel (Fig. 1, lanes 1 and 6, respectively). In separate experiments, no mobility shift was seen with undamaged DNA probe or in the absence of extract. The bands of shifted mobility were sensitive to Pronase and resistant to RNase A and thus appeared to represent protein–DNA complexes.

The UV-irradiated probe was shifted to bands B1 and B2, whereas the cisplatin-crosslinked probe was shifted to band B. Bands B1/B2 and band B had different mobilities, indicating that each DNA probe detected at least one independent protein factor.

To measure the affinity of the nuclear factors for different forms of DNA, the incubations were done in the presence of competitor DNA in various forms. UV-irradiated, cisplatin-crosslinked, and single-stranded DNA but not intact double-stranded DNA competed for binding in bands B1 and B2 (Fig. 1, lanes 2–5). Thus, the binding activity in bands B1 and B2

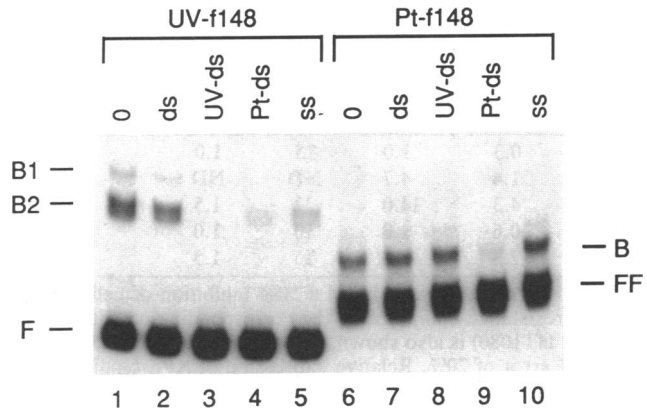

FIG. 1. Two distinct cellular factors that bind to damaged DNA. The positions of the free probes UV-f148 and Pt-f148 are denoted by F and FF, respectively. Cisplatin damage causes the probe (FF) to migrate with decreased mobility compared to the UV-irradiated probe (F). Protein–DNA complexes are indicated by bands of shifted mobility, B1, B2, and B. Incubations were done with 1 μg of extract for the UV-irradiated probe and 0.5 μg of extract for the cisplatin-crosslinked probe in the presence of 200 ng of unlabeled competitor M13mp18 DNA that was either intact double-stranded (ds), UV-irradiated (UV-ds), cisplatin-crosslinked (Pt-ds), or single-stranded (ss).

was capable of recognizing multiple forms of damaged DNA. By contrast, only cisplatin-crosslinked DNA competed for binding in band B (Fig. 1, lanes 7–10). We designated this latter binding activity as cisplatin-crosslinked DNA (CCD) binding factor. Note that bands B1 and B2 were not detected by the Pt-f148 DNA probe, probably because cisplatin-crosslinked DNA had lower affinity for the factor(s) in B1/B2 than for CCD binding factor (data not shown).

The binding activity in bands B1 and B2 was notably absent in cells from an XP patient in complementation group E (7). Furthermore, cells from an affected second cousin of the first group E patient contained no binding activity even when the gel was heavily overexposed (Fig. 2). Thus, the binding activity in bands B1 and B2, designated XPE binding factor, appears to be involved in the pathogenesis of XP group E. By contrast, CCD binding factor was present in every complementation group tested.

As a tissue culture model for the development of drug resistance, human cell lines were grown in stepwise increasing doses of cisplatin. At each step the surviving cells were

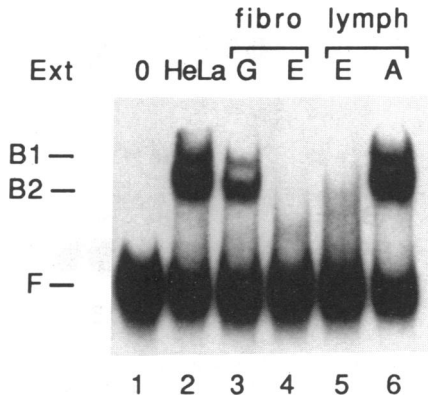

FIG. 2. Binding activity is missing in XP cells from complementation group E. UV-irradiated f148 DNA was incubated with 2 μg of nuclear extract (Ext) made from HeLa cells and XP cells from groups G, E, and A. The cells were lymphoblastoid (lymph) or primary fibroblasts (fibro). No binding activity was detected in the two XP group E cell lines.

Table 1. Drug-resistant cell lines

Cell line	Cisplatin ID_{50}, μg/ml	Cisplatin Relative resistance	UV ID_{50}, J/m²	UV Relative resistance	XPE binding factor
HeLa	0.3	1.0	15	1.0	1.0
HeLa-R1	1.4	4.7	ND	ND	4.0
HeLa-R3	4.3	14.0	22	1.5	4.3
HT1080	0.6	1.0	10	1.0	1.0
HT-R2	2.3	3.8	15	1.5	2.7

ID_{50} is the dose that produced a 50% inhibition of cell growth. Relative resistance compared to the corresponding parent cell line (HeLa or HT1080) is also shown. Measurements of the ID_{50} had an estimated error of 20%. Relative expression of XPE binding factor was determined by scanning densitometry of the gel in Fig. 3. ND, not determined.

pooled and assayed for resistance to cisplatin. HeLa cells, originally derived from a cervical carcinoma, were grown sequentially in cisplatin at 0.3 μg/ml, 0.6 μg/ml, and 1.2 μg/ml for a period of at least 1 month for each step. The surviving cells from the first and third steps, HeLa-R1 and HeLa-R3, were found to be 4.8 and 14 times more resistant than the parent HeLa line (Table 1). Similarly, HT1080 cells, derived from a human fibrosarcoma, were grown in cisplatin at 0.6 μg/ml and 1.2 μg/ml. The cells from the second step, HT-R2, were found to be 3.8 times more resistant than the parent HT1080 line.

When the cell lines were assayed for growth following UV irradiation, the resistant lines were found to have acquired increased resistance to UV about 1.5 times that of the respective parent lines (Table 1). The resistance to UV irradiation was modest and not as great as it was for cisplatin.

Since XP cells are defective in the repair of UV- and cisplatin-induced lesions, we reasoned that the resistant cells might show increased expression of factors involved in the corresponding DNA repair pathway. Extracts were made from parent and resistant cell lines and assayed for expression of XPE binding factor (Fig. 3). CCD binding factor was assayed as well. When HeLa and HT1080 cells were grown in the presence of cisplatin, the surviving cells expressed 2.7- to 4.3-fold increased levels of XPE binding factor (Table 1). By contrast, CCD binding factor was unaffected. The increase in XPE binding factor was not an isolated phenomenon, since it was observed in two different cell lines derived from different human tumors.

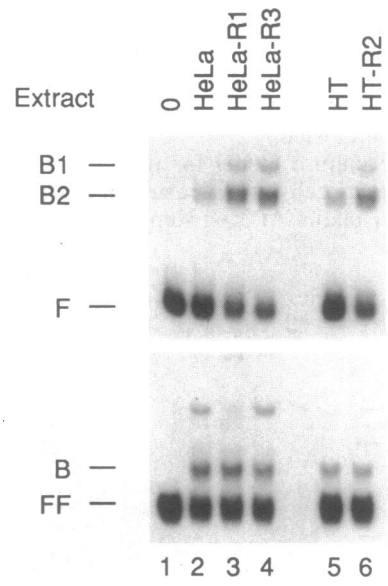

FIG. 3. XPE binding factor is increased in cisplatin-resistant cells. The human tumor cell lines HeLa and HT1080 (HT) were grown in the presence of cisplatin to produce resistant lines HeLa-R1, HeLa-R2, and HT-R2. Equal amounts (1 μg) of nuclear extract were incubated with either UV-f148 or Pt-f148 probe DNA to assay for expression of XPE and CCD binding factors (upper and lower gels, respectively). XPE but not CCD binding factor was increased in the resistant cells. The higher bands seen for the Pt-f148 probe in lanes 2 and 4 were not a consistent finding in repeated experiments.

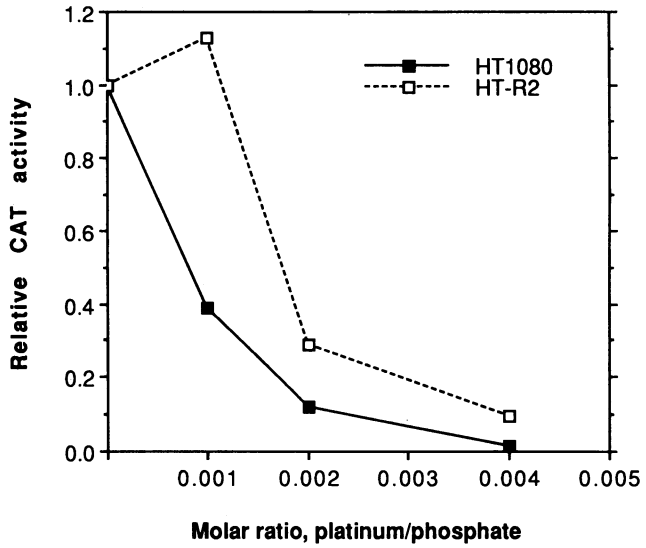

FIG. 4. Increased DNA repair in cisplatin-resistant cells. HT1080 and HT-R2 cells were transfected with pRSVcat DNA crosslinked with cisplatin. Relative CAT activity is shown as a function of the degree of cisplatin crosslinking, expressed as the molar ratio r_f. The value $r_f = 0.001$ corresponds to ≈2.7 cisplatin lesions in the CAT transcription unit. CAT activity was normalized to the activity from undamaged DNA for each cell line. The error due to uncertainties in transfection and the CAT assay was estimated to be about 30% from a second independent transfection experiment and from cotransfection of a second marker plasmid (see text).

We next wanted to see if the increased expression of XPE binding factor in the cisplatin-resistant cells was accompanied by increased DNA repair. Cisplatin-crosslinked plasmid DNA carrying the marker CAT gene, pRSVcat, was transfected into sensitive and resistant cells. Earlier work had shown that such an assay is a sensitive probe for the DNA repair defect in XP cells (13). Therefore, transient expression of CAT activity may be interpreted as a measure of cellular DNA repair capacity. When HT1080 cells and the resistant line HT-R2 were tested, the resistant cells showed increased CAT activity for each level of cisplatin damage tested (Fig. 4). The increase was 2.9-fold for $r_f = 0.001$, 2.4-fold for $r_f = 0.002$, and 6.7-fold for $r_f = 0.004$. (There is significant uncertainty in the last value due to the low levels of CAT activity.) The increase in CAT activity was thus the same order of magnitude as the increase in XPE binding factor expression. These results suggest that the cisplatin-resistant cells had acquired increased levels of DNA repair.

DISCUSSION

The inherited disease xeroderma pigmentosum is characterized by the deficient repair of many forms of damaged DNA, including UV-irradiated (16) and cisplatin-crosslinked DNA (13). Somatic cell hybridizations of cells from different patients define multiple genetic complementation groups (17), suggesting that DNA repair in humans involves multiple gene products.

XPE binding factor is a factor that binds to damaged DNA and is deficient in XP complementation group E (7). It bound

to single-stranded, UV-irradiated, or cisplatin-treated DNA. UV radiation under the conditions used here produces primarily cyclobutane pyrimidine dimers (18). However, other lesions are also present at lower levels, including 6-4 photoproducts. Similarly, cisplatin treatment produces intrastrand crosslinks primarily at GpG and less often at ApG sites, but other lesions are seen, including interstrand crosslinks (19). Therefore, the exact nature of the lesions that were recognized by XPE binding factor remains to be determined. Nevertheless, it has the ability to bind multiple forms of damaged DNA. Its affinity for single-stranded DNA suggests that the basis for this versatility may be recognition of a disruption in the DNA duplex rather than the adduct itself. Cyclobutane dimers and cisplatin crosslinks may produce local regions of bending and/or melting of the DNA duplex, structures that may be mimicked by single-stranded DNA.

When human tumor cell lines were selected for resistance to cisplatin, they showed increased expression of XPE binding factor. The effect was specific, since expression of another factor that bound to cisplatin-crosslinked DNA, CCD binding factor, was unchanged. Furthermore, cisplatin-resistant cells were more efficient in the repair of cisplatin damaged DNA, as measured by the transfection of a cisplatin-crosslinked marker plasmid.

Our results support the conclusion that XPE binding factor participates in a versatile DNA repair pathway: (*i*) it is missing in XP group E, a disease characterized by defective DNA repair; (*ii*) it is increased in cells that show increased DNA repair; (*iii*) it has functional activity suggestive of a protein involved in DNA repair—namely, binding to damaged DNA.

We propose that increased expression of XPE binding factor mediates cisplatin drug resistance and that it does so by facilitating more rapid recognition of cisplatin-damaged DNA. Other excision repair proteins are then directed to the site of damage. Definitive proof awaits isolation of the gene and transfection of a high-expression construct into cisplatin-sensitive cells.

Increased expression of XPE binding factor appeared early in the development of resistance to cisplatin, since it was present after the first step of selection (in HeLa-R1 cells). Further selection in cisplatin produced a further increase in resistance to cisplatin (in HeLa-R3 cells) but no further increase in expression of XPE binding factor (Table 1). DNA repair is a multienzyme pathway, so it is not surprising that further increases in cisplatin resistance are not accompanied by unbounded increases in XPE binding factor. At some point another factor in the pathway must become rate limiting. Furthermore, it is likely that as resistance to cisplatin increases, a number of other mechanisms become involved, including those not related to DNA repair such as increased levels of glutathione (20), increased expression of metallothionein (21) or thymidylate synthase (22), and decreased drug accumulation (20).

The tissue culture model for cisplatin resistance has implications for cancer treatment. The increase in XPE binding factor was modest in comparison to the increases that have been observed for *mdr* and *dhfr* expression. However, 3- to 5-fold changes in expression can be clinically important, since the severe toxicity of chemotherapeutic drugs such as cisplatin precludes much increase in the administered dose. Moreover, increased expression may be relevant *in vivo* since it appeared early in the development of drug resistance *in vitro*. Finally, cells that overexpress XPE binding factor may acquire cross-resistance to other DNA active drugs as well, since XPE binding factor is capable of recognizing multiple forms of damaged DNA.

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