Biochemical Pharmacology, Vol. 60, pp. 1305–1313, 2000.

ISSN 0006-2952/00/$–see front matter
PII S0006-2952(00)00441-X

Increased Nucleotide Excision Repair in Cisplatin-Resistant Ovarian Cancer Cells

Role of ERCC1–XPF

Katherine V. Ferry,† Thomas C. Hamilton* and Steven W. Johnson‡§*

*Department of Medical Oncology, Fox Chase Cancer Center, Philadelphia, PA 19111; †Department of Chemistry, Lehigh University, Bethlehem, PA 18017; and ‡Department of Pharmacology, University of Pennsylvania, Philadelphia, PA 19104-6160, U.S.A.

ABSTRACT. Increased platinum–DNA adduct removal has been shown by several DNA repair assays to be associated with cisplatin resistance in the A2780/C-series human ovarian cancer model system. In the present study, we provide further evidence that the resistance phenotype of these cell lines is due, in part, to enhanced nucleotide excision repair (NER). Cisplatin resistance was found to be associated with increased UV resistance. Northern blot analysis revealed that increased expression of *ERCC1* was also associated with cisplatin resistance in this panel. Several other NER genes were found to be constitutively overexpressed in the most resistant cell line, C200, as compared with the parental A2780 cells. A plasmid substrate containing a site-specific cisplatin adduct was used to measure the nucleotide excision activity of cell extracts prepared from cisplatin-sensitive and -resistant cells. Using this *in vitro* assay, extracts prepared from C200 cells exhibited approximately 3-fold more activity than extracts prepared from A2780 cells, similar to the difference in UV sensitivity. Complementation of A2780 extracts with ERCC1–XPF protein resulted in approximately 2-fold increased activity, but had little effect on excision in C200 extracts. Overall, these results support a role for the ERCC1–XPF endonuclease as a determinant of increased NER in this cisplatin resistance model. BIOCHEM PHARMACOL **60**;9:1305–1313, 2000.

KEY WORDS. cisplatin; nucleotide excision repair; ERCC1; XPF; drug resistance; ovarian cancer

Ovarian cancer is the leading cause of death from gynecologic malignancies in the United States. Aggressive treatment with platinum-based chemotherapy results in complete response rates of 70–80% in these patients. Unfortunately, 30–40% of complete responders eventually relapse with disease that is resistant not only to platinum compounds, but also to a wide range of other chemotherapeutic agents [1]. One approach to alleviate this major limitation in ovarian cancer treatment is to elucidate the mechanisms responsible for drug resistance and then develop ways to treat resistant disease effectively or to prevent its occurrence.

Cisplatin [*cis*-diamminedichloroplatinum (II)] is widely used not only in the treatment of ovarian cancer but also for many other solid tumors including those arising in the testes, bladder, head and neck, and lung [2]. The cytotoxicity of this drug is believed to result from the formation of platinum–DNA adducts. Intracellularly, cisplatin becomes aquated to form an electrophilic species that binds preferentially to the N7 atom of guanine and adenine residues [3]. Platinum–DNA adducts consist primarily of monoadducts and intrastrand cross-links (GG ≫ AG > GNG); however, this drug also forms GG-interstrand cross-links and DNA–protein cross-links, although each is thought to comprise a small percentage of the total adducts formed [4, 5].

Studies with human ovarian cancer cell lines have indicated that cisplatin resistance is multifactorial, consisting of mechanisms such as decreased drug accumulation [6–8], increased drug inactivation [9–13], and an enhanced ability to repair [14–19] and tolerate [20] DNA damage. The A2780/C-series of cells was established by treating the human ovarian cancer cell line A2780 with incrementally increasing concentrations of cisplatin. The cell lines of this panel exhibit a wide range of cisplatin sensitivity. All of the cisplatin resistance mechanisms mentioned above have been observed in these cell lines, as well as significant cross-resistance to a wide range of alkylating agents and natural product drugs [9, 13–21]. Therefore, this model is valuable for elucidating the molecular basis for drug resistance mechanisms that ultimately may be relevant to the drug resistance phenotype observed in the clinic.

Evidence for increased repair of platinum–DNA damage in resistant ovarian cancer cells has been demonstrated by a variety of cellular assays including the measurement of unscheduled DNA synthesis, reactivation of cisplatin-damaged plasmid DNA, atomic absorption spectrometry, and renaturing agarose gel electrophoresis. We and others have

§ Corresponding author: Dr. Steven W. Johnson, Center for Cancer Pharmacology, University of Pennsylvania, BRB II/III–Rm. 1252, Philadelphia, PA 19104-6160. Tel. (215) 573-9881; FAX (215) 573-9889; E-mail: Johnson@pharm.med.upenn.edu
Received 21 February 2000; accepted 14 April 2000.

demonstrated a clear association between increased platinum–DNA adduct removal and increased cisplatin resistance in the A2780/C-series [14–18, 22]. For example, in one study, cells were treated to obtain similar initial levels of platinum–DNA adducts, and repair was monitored by comparing total platinum–DNA adduct levels, as measured by atomic absorption spectrometry, of cells harvested immediately after treatment with cells incubated in fresh medium post-treatment. The initial rate of platinum removal from DNA was increased in the resistant derivatives compared with the cisplatin-sensitive A2780 cell line, and, after 12 hr, the cisplatin-resistant cell lines removed 24–40% of platinum from their DNA, whereas the cisplatin-sensitive A2780 cells removed only 14% [18]. In a follow-up study, under drug treatment conditions that yielded similar initial levels of sequence-specific cisplatin-interstrand cross-links, the cisplatin-resistant derivatives removed up to 2.5 times more interstrand cross-links at 12-hr post-treatment than the A2780 parental cell line (82–86 vs 30–36%), as measured by renaturing agarose gel electrophoresis [22].

The repair of platinum–DNA lesions is believed to occur primarily by the process of NER.* Mammalian NER removes DNA damage as part of an oligonucleotide 24–32 residues long. DNA damage is thought to be recognized by the zinc-finger protein XPA in association with the heterotrimeric replication protein RPA. The XPA–RPA complex then is believed to recruit the basal transcription factor TFIIH, a multisubunit protein that also plays a role in transcription, to the site of damage. Two subunits of TFIIH, XPB and XPD, have helicase activities that are believed to function in opening up the DNA around the adduct, thus allowing structure-specific nucleases to incise the DNA. The ERCC1–XPF heterodimer is responsible for cutting the strand on the 5′ side of the damage, and the XPG protein incises on the 3′ side [23]. Principal sites of cleavage flanking a 1,3-intrastrand d(GpTpG)-cisplatin cross-link in a closed circular duplex DNA substrate were identified as the 9th phosphodiester bond 3′ to the lesion and the 16th phosphodiester bond 5′ to the lesion [24]. DNA polymerase δ or ϵ and accessory proteins, replication factor C (RFC), and proliferating-cell nuclear antigen (PCNA), fill in the gap, and the DNA is joined by the action of DNA ligase. In addition to the proteins described above, the incision stage of the reaction requires XPC, a protein that may be involved in DNA damage recognition and/or stabilizing an incision reaction intermediate [23]. Knowledge is currently limited as to which of these NER proteins may be critical or rate-limiting in the enhanced DNA repair phenotype that is observed in the A2780/C-series and other cisplatin-resistant cell lines.

In the present study, the DNA repair profile of members of the A2780/C-series of cells was examined in several ways: (a) cytotoxicity assays were performed to determine sensitivity to cisplatin and UV irradiation; (b) the constitutive expression of several NER genes was examined; and (c) an *in vitro* DNA excision assay was performed to analyze the activity of cellular extracts and to monitor the impact of the addition of purified protein on excision. Furthermore, we present evidence that a molecular determinant of increased nucleotide excision repair in the cisplatin-resistant C200 cell line may be due, in part, to increased activity of the ERCC1–XPF heterodimeric endonuclease.

* *Abbreviations*: NER, nucleotide excision repair; DTT, dithiothreitol; and MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide.

MATERIALS AND METHODS

Chemicals and Reagents

Cisplatin was obtained from Bristol Myers Squibb. Cell culture reagents were obtained from Life Technologies, Inc., except for fetal bovine serum, which was obtained from Atlanta Biologicals. All other chemicals were obtained from the Sigma Chemical Co. unless otherwise indicated. The M13mp18GTGx construct and ERCC1–XPF protein were provided by Dr. Richard Wood of the Imperial Cancer Research Fund.

Cell Culture

A2780 is a human ovarian cancer cell line derived from a patient prior to chemotherapy. The A2780/C-series of cisplatin-resistant cell lines was developed by nearly continuous, incremental exposure of the A2780 cells to cisplatin (8–200 μM). GMO8437A and GMO4312B, SV40-immortalized fibroblasts from patients with the NER-deficient syndrome xeroderma pigmentosum of complementation groups XPF and XPA, respectively, were obtained from the NIGMS Human Genetic Mutant Cell Repository. HeLa is a human cervical cancer cell line. All cell lines were maintained at 37°, 5% CO_2 in RPMI 1640 medium containing 10% (v/v) fetal bovine serum, 100 μg/mL of streptomycin, 100 U/mL of penicillin, 0.3 mg/mL of glutamine, and 0.3 U/mL of insulin (porcine, Novo Nordisk).

Cisplatin Cytotoxicity Assay

Cisplatin cytotoxicity was determined using the MTT assay [25]. Cells were plated in 150 μL of medium/well in 96-well plates. Following overnight incubation, cells were exposed to various concentrations of cisplatin. Following a 72-hr incubation, 40 μL of a 5 mg/mL solution of MTT was added per well. After 2 hr at 37°, the cells were lysed by adding 100 μL of 20% (w/v) SDS and 50% (v/v) *N*,*N*-dimethylformamide (pH 4.7) and incubated overnight at 37°. The absorbance at 570 nm was determined using a microplate reader (Bio-Rad model 3550). The reported values are the averages of duplicate determinations.

UV Sensitivity Assay

An MTT assay was also used to determine the relative sensitivity of the cell lines to UV light. Cells were plated in 2 mL of medium in 35 × 10 mm dishes. Following overnight incubation, cells were exposed to UV light at 254 nm at approximately 0.2 J/m^2/sec for time points ranging from 10 to 240 sec in medium. Following a 72-hr incubation, 500 μL of a 5 mg/mL solution of MTT was added per dish. After 2 hr at 37°, the cells were lysed by adding 1.3 mL of 20% (w/v) SDS and 50% (v/v) *N*,*N*-dimethylformamide (pH 4.7) and incubated overnight at 37°. The absorbance at 570 nm was measured for each dish. The reported values are the averages of duplicate measurements.

Northern Blot Analysis

For RNA isolation, cells were harvested at approximately 80% confluence. Total cellular RNA was isolated by the guanidine isothiocyanate–phenol–chloroform extraction method. Approximately 15 μg of each RNA sample was denatured, electrophoresed through agarose/formaldehyde gels, and blotted onto nylon membrane filters (MagnaGraph, Micron Separations, Inc.) using standard techniques. Gels were stained with ethidium bromide and photographed by UV illumination to check for even loading prior to membrane transfer. Northern blots were probed by standard methods. Primers were designed to amplify portions of the coding regions of the various DNA repair genes. Polymerase chain reaction (PCR) products were cloned using plasmid vectors (TA Cloning Kit, Invitrogen), and insert sequences were verified by DNA sequence analysis. Fragments were isolated by restriction digestion, agarose gel electrophoresis, and glass powder purification. The fragments, which ranged in size from 259 bp to 1.2 kb, were labeled by the method of random priming (Prime-It II kit, Stratagene). Quantitation was done by phosphorimaging using the Fuji Macbas 2000 imaging system from Fuji Medical Systems.

Preparation of Whole Cell Extracts

Extracts were prepared essentially by the method of Manley *et al.* [26] with modifications noted below. Briefly, cells were plated in four 245 × 245 × 20 cm culture dishes and harvested at 48–72 hr (80–90% confluency). Cells were either untreated or treated for 16 hr at an IC_{50} concentration of drug (IC_{50} values based on previous determinations: A2780, 0.2 μM; C200, 225 μM) prior to harvesting. After rinsing the plates with ice-cold PBS, the cells were scraped into 5 mL of hypotonic lysis buffer containing protease inhibitors [10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, and 0.5 μg/mL each of leupeptin, pepstatin, and chymostatin]. After 20 min on ice, the cells were lysed by 30 strokes with a Dounce homogenizer. Then an equal volume of 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 2 mM DTT, 25% sucrose and 50% glycerol (Fluka), and 1.25 mL neutralized, saturated ammonium sulfate was added with gentle mixing, and stirring was continued for 50 min on ice. The mixture then was centrifuged at 125,000 *g* for 3 hr at 2°. Proteins were precipitated from the resulting supernatant by adding 0.33 g/mL of ammonium sulfate and neutralized with 10 μL of 1 N NaOH per g ammonium sulfate added. The precipitate was collected by centrifugation and dialyzed for 16 hr against 1 L of 25 mM HEPES–KOH (pH 7.9), 0.1 mM KCl, 12 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, and 17% glycerol. Insoluble material was removed by centrifugation, and the extract was frozen in small aliquots and stored at −80°. Protein quantitation was done by a modified Lowry procedure that utilizes bicinchoninic acid and $CuSO_4$.

Single Lesion Excision Assay

The *in vitro* excision experiments were done essentially by methods described by Moggs *et al.* [24]. Briefly, the 24-mer 5′-TCTTCTTCTGTGCACTCTTCTTCT-3′ was reacted with cisplatin and gel purified to isolate the major product of this reaction, the oligonucleotide containing a 1,3-intrastrand d(GpTpG)-cisplatin cross-link. This particular platinum lesion was selected since it was shown previously to be repaired more efficiently than the d(GpG)Pt intrastrand cross-link and would result in higher signal to noise ratios in the excision assay [27]. The oligonucleotide or a purified undamaged oligonucleotide was used to prime the plus strand of a modified M13mp18 molecule (M13mp18GTGx), and the second strand was synthesized by incubation in a reaction mixture containing 10 mM Tris–HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 600 μM each of dATP, dCTP, dGTP, and TTP, 75 U T4 DNA polymerase gp43 subunit (Promega), and 1600 cohesive end units T4 DNA ligase (NEB). Closed circular DNA then was isolated by CsCl/EtBr density gradient centrifugation. Reaction mixtures (50 μL) contained 250 ng of the modified M13mp18 molecule containing a site-specific 1,3-intrastrand d(GpTpG)-cisplatin cross-link (Pt-GTG) or an undamaged control substrate (Con-GTG) and 125 μg of whole cell extract protein. For complementation experiments, 5 μL of a 20 ng/μL preparation of purified ERCC1–XPF protein was added per reaction. The substrate and protein were incubated in a buffer containing 45 mM HEPES–KOH (pH 7.8), 70 mM KCl, 7.5 mM $MgCl_2$, 0.9 mM DTT, 0.4 mM EDTA, 2 mM ATP, 20 μM each of dATP, dCTP, dGTP, and TTP, 40 mM phosphocreatine, 2.5 μg creatine phosphokinase, 3.4% (v/v) glycerol, and 18 μg BSA for 30 min at 30°. Purified DNA was electrophoresed through a 12% denaturing polyacrylamide gel, and the DNA was transferred onto a nylon membrane (Hybond N+, Amersham) by capillary transfer. Fixed membranes were incubated in 40 mL of buffer containing 7% (w/v) SDS, 10% (w/v) polyethylene glycol 8000, 250 mM NaCl, 130 mM potassium phosphate buffer (pH 7.0), and 100 pmol of ^{32}P-labeled oligonucleotide with the sequence

TABLE 1. Sensitivity of the A2780/C-series and control cell lines to cisplatin and UV irradiation

Cell line	Cisplatin IC_{50}* (μM)	UV ID_{50}† (J)
A2780	0.28	0.018
CP70	7.0	0.028
C30	60	0.035
C200	265	0.045

Measurements were determined using an MTT assay. Each value shown is the result of duplicate measurements.

*Concentration of cisplatin that inhibited growth by 50%.

†Dose of UV light (254 nm at 0.2 J/m^2/sec) that inhibited growth by 50%.

5′-GAAGAGTGCACAGAAGAA-GAGGCCTGG-3′. After washing in 1X SSC [3 M sodium chloride + 0.3 M sodium citrate (pH 7.0)] with 0.1% (w/v) SDS, the membranes were exposed to x-ray film (X-OMAT AR, Kodak). Quantitation was done by densitometry using the AMBIS optical imaging system (AMBIS).

RESULTS

The cisplatin-resistant cell lines CP70, C30, and C200 were derived from the human ovarian cancer cell line A2780 by nearly continuous, incremental exposure to cisplatin. Cisplatin sensitivities were determined using the MTT assay (Table 1). The results were similar to those reported previously [13, 22] in which cisplatin sensitivity was significantly lower in the resistant sublines (CP70, C30, C200) with IC_{50} values ranging from 7 to 265 μM as compared with 0.28 μM in A2780, yielding relative resistance values of 25- to 946-fold. These values are also very similar to those reported by Perez *et al.* [28] using a clonogenic assay.

Figure 1 shows the UV sensitivity of the A2780/C-series as well as the HeLa, GMO8437A (XPF), and GMO4312B (XPA) cell lines as measured by an MTT assay. Decreased UV sensitivity was found to be associated with decreased cisplatin sensitivity in the A2780/C-series. The exposure time to UV light with a wavelength of 254 nm at approximately 0.2 J/m^2/sec necessary to eliminate one-half of the A2780 cells was approximately 94 sec, whereas for CP70 it was 145 sec (1.5-fold increase), for C30 it was 180 sec (1.9-fold increase), and for C200 it was 235 sec (2.5-fold-increase) (Table 1). HeLa cells were about as sensitive as A2780 in this assay, and the NER-deficient cell lines exhibited hypersensitivity to UV irradiation.

The relative constitutive mRNA levels of several nucleotide excision repair genes were evaluated by northern blot analysis (Fig. 2). Total cellular RNA was isolated from subconfluent cultures of A2780, CP70, C30, and C200 cells, and northern blots were prepared. The constitutive expression levels of many of the genes examined were quite low; however, they were expressed at a significantly higher level in the C200 cell line as compared with the A2780 cells. The largest increase in steady-state mRNA expression in C200 compared with A2780 was observed for *ERCC1*

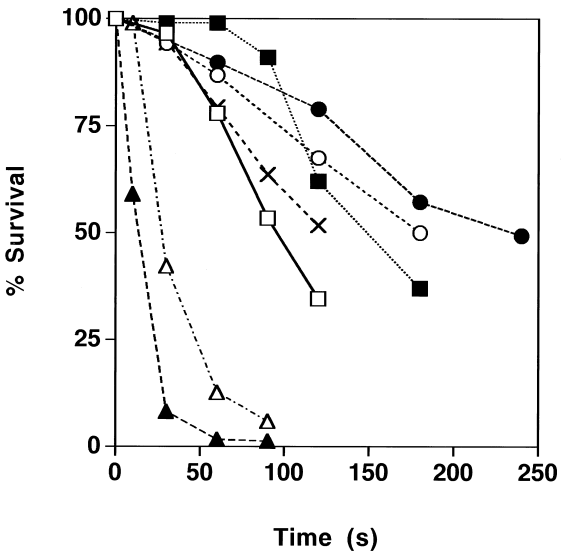

FIG. 1. Relationship between cell survival and time of exposure to UV light for the A2780/C-series and control cell lines as measured by an MTT assay. Cells (approx. 5 × 10^4) were treated with UV light at 254 nm of approximately 0.2 J/m^2/sec. Measurements were obtained using an MTT assay, and data points shown are the average of two independent measurements. The cell lines analyzed were: A2780 (□), CP70 (■), C30 (○), C200 (●), HeLa (X), XPA (▲), and XPF (△).

(approximately 3-fold), followed by *XPA*, *XPB*, *XPC*, and *XPG* (approximately 2-fold), whereas *PCNA* and *XPF* were elevated slightly (approximately 1.4-fold) (Table 2). Constitutive mRNA levels for *RPA* and *XPD* were similar in these cell lines. Several of the genes, including *XPB*, *XPC*, *XPF*, *XPG*, and *PCNA*, were expressed at a lower level in the cell lines of intermediate resistance levels (CP70 and C30) relative to A2780. Although most of the genes examined were overexpressed in the C200 cell line as compared with the A2780 cells, the only transcript in which the steady-state level was increased significantly relative to the parental cells in all of the resistant sublines was *ERCC1*. Furthermore, increased expression of this gene was associated with increased cisplatin resistance in the C-series ($r = 0.99$).

Figure 3 shows the results of a single lesion excision assay that utilizes a closed circular DNA molecule containing a site-specific 1,3-intrastrand d(GpTpG) cisplatin adduct incubated with whole cell extracts from the A2780 and C200 cell lines. Two independent whole cell extracts, prepared from cells that were untreated (A2780U, C200U) or treated (A2780T, C200T) with an IC_{50} concentration of cisplatin, were analyzed. Reaction products were separated on a 12% denaturing polyacrylamide gel, and the DNA was transferred onto a nylon membrane and probed with a ^{32}P-labeled oligonucleotide complementary to the region of the substrate that is removed during the dual incision process of NER. The autoradiograph shown in Fig. 3 revealed that the C200 extracts from untreated cells possessed a significantly greater ability (2.8-fold, $P = 4.75 \times 10^{-4}$) to complete the NER dual incision event as com-

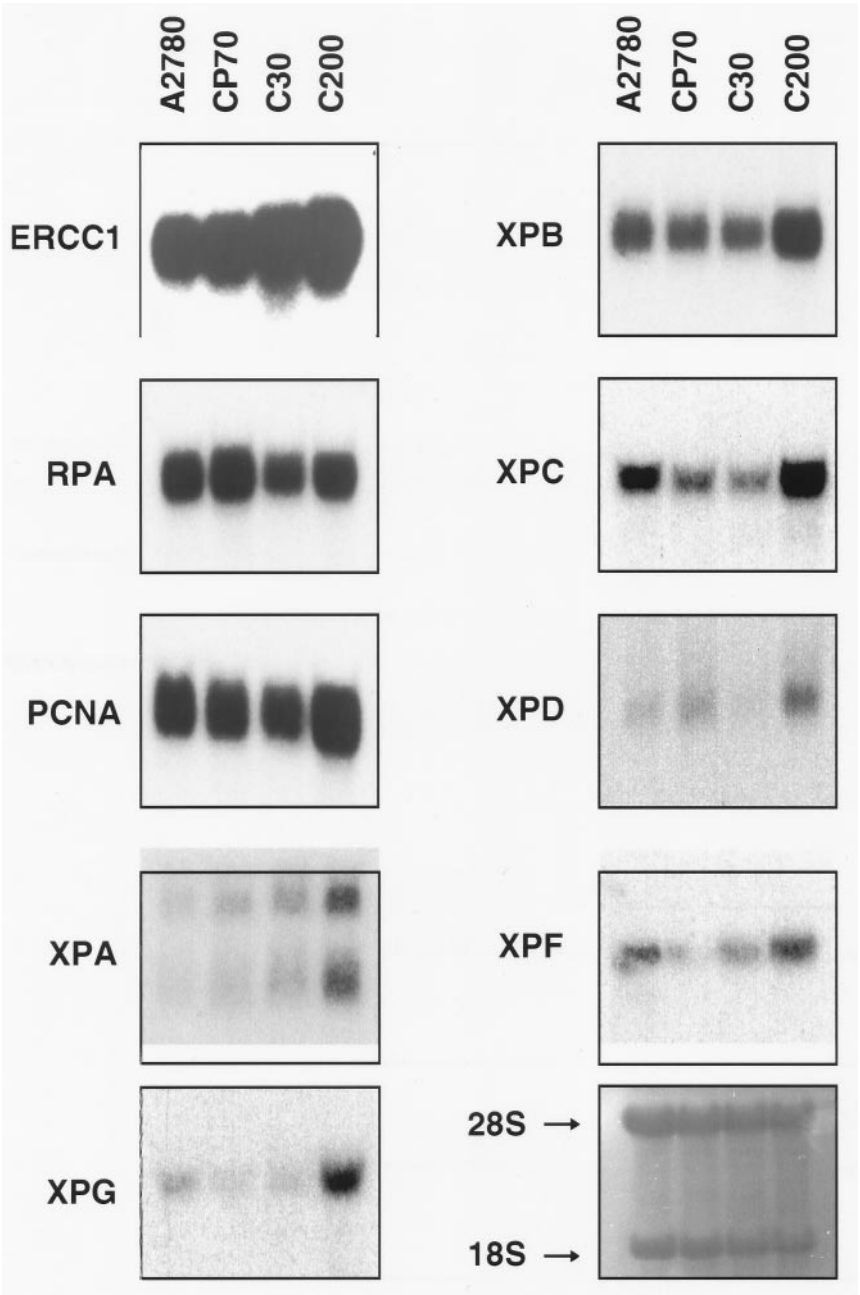

FIG. 2. Northern blot analysis of the A2780 cell line and its resistant derivatives CP70, C30, and C200 showing the constitutive expression of various NER genes. RNA was isolated from subconfluent cells by the guanidine isothiocyanate–phenol–chloroform method, and northern blots were prepared and probed by standard methods. The last panel is an ethidium bromide stained gel showing the even loading of 28S and 18S ribosomal RNA in each lane.

TABLE 2. Expression of NER genes in CP70, C30, and C200 relative to A2780

Cell line	*ERCC1*	*PCNA*	*RPA*	*XPA*	*XPB*	*XPC*	*XPD*	*XPF*	*XPG*
A2780	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
CP70	1.37 ± 0.14	0.76 ± 0.10	0.97 ± 0.51	1.15 ± 0.33	0.82 ± 0.15	0.59 ± 0.13	1.07 ± 0.03	0.53 ± 0.09	0.63 ± 0.07
C30	1.57 ± 0.19	0.73 ± 0.02	0.82 ± 0.06	1.33 ± 0.35	0.94 ± 0.14	0.74 ± 0.11	0.71 ± 0.04	0.64 ± 0.08	0.63 ± 0.08
C200	2.95 ± 0.47	1.44 ± 0.39	0.91 ± 0.14	2.01 ± 0.32	2.06 ± 0.29	1.82 ± 0.08	1.29 ± 0.09	1.41 ± 0.13	1.70 ± 0.10

Quantitation of northern blots was done by phosphoimaging using the Fuji Macbas 2000 imaging system from Fuji Medical Systems. Values given are relative to expression in the A2780 parental cell line and are the result of two independent measurements with the range indicated.

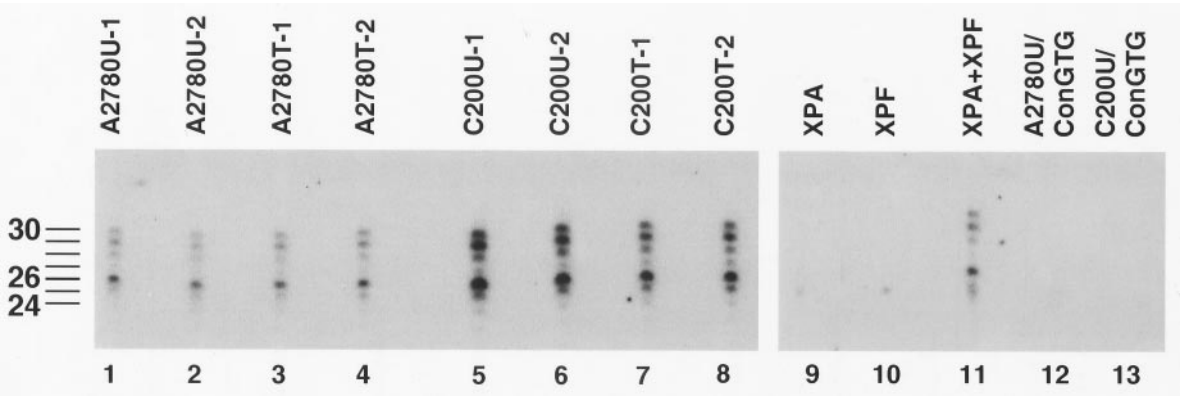

FIG. 3. Single lesion excision assay of A2780 and C200 both untreated and treated at an IC_{50} concentration of cisplatin along with control cell lines. Shown is the autoradiograph of the Southern blot, in which the reaction products formed during the dual incision reaction were transferred onto a nylon membrane and probed with a ^{32}P-labeled complementary probe. The sizes (bases) of the excised oligonucleotides are indicated and were determined by comparison with the mobility of the platinated 24-mer used in the synthesis of the substrate. Two independent extracts of A2780, C200, and of both cell lines treated at their respective IC_{50} concentrations of drug were prepared, and the activity of 125 μg of each was examined as indicated in lanes 1–8. Results from reactions using XPA extract (125 μg), XPF (125 μg), and XPA + XPF (62.5 μg of each) are shown at a longer exposure in lanes 9–11, respectively. Results from reactions in which 125 μg of an A2780U or C200U was incubated with a substrate devoid of the Pt-GTG adduct are shown in lanes 12 and 13, also at a longer exposure time.

pared with A2780 extracts from untreated cells (Table 3). Linear reaction conditions used for this comparison were determined in a separate experiment (data not shown). Drug treatment resulted in slightly increased activity of the A2780T extracts (1.2-fold), whereas treatment with an equitoxic dose in C200 cells resulted in decreased extract activity (1.5-fold). Similarly, *ERCC1* expression increased in cisplatin-treated A2780 cells, whereas *ERCC1* expression decreased in cisplatin-treated C200 cells (data not shown). Dual incision of the Pt-(GTG) substrate results in products that range in size from 25 to 30 nucleotides with the most predominant being 26 nucleotides in length [24]. The approximate size of these bands was verified by comparison with the migration of an end-labeled, platinated 24-mer used in the construction of the single lesion substrate (data not shown). Control samples are shown in lanes 9–13 (shown at a longer exposure time). Extracts prepared from XPA and XPF cells showed no indication of dual incision activity, as expected. Dual incision activity was reconstituted by mixing these extracts, while keeping the total protein constant. Lanes 12 and 13 represent an A2780 (untreated) and a C200 (untreated) extract incubated with Con-GTG, a substrate devoid of the Pt-GTG lesion.

Figure 4 shows a representative result of a single lesion assay in which the effect of the addition of purified ERCC1–XPF protein to both A2780U and C200U extracts was examined. In this experiment, the addition of ERCC1–XPF protein to an A2780 extract resulted in an increase in excision activity (2.2-fold), whereas addition of this het-

TABLE 3. Results of the single lesion excision assay of A2780 and C200 untreated and treated with an IC_{50} concentration of cisplatin

Cell extract	Cisplatin (μM)	Average relative intensity
A2780U	0	9.65 ± 0.65
A2780T	0.2	12.1 ± 0.20
C200U	0	27.4 ± 1.50
C200T	225	17.8 ± 0.60

Average relative intensity was derived by densitometric scanning of the autoradiograph using the AMBIS system (AMBIS). The average values obtained from the assay of two independent extracts are shown, and the range is indicated.

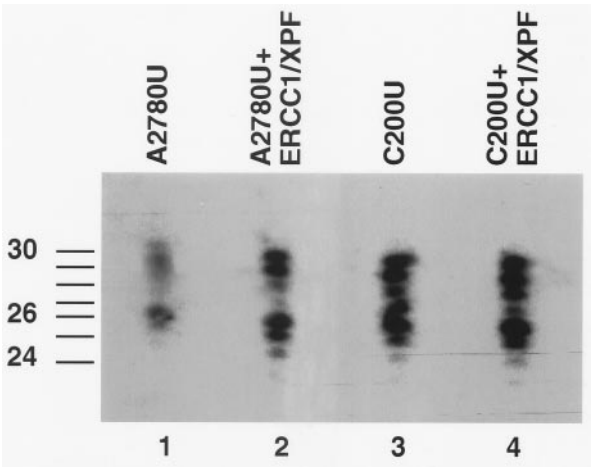

FIG. 4. Complementation of A2780 and C200 extracts with purified ERCC1–XPF protein as measured by a single lesion excision assay. Shown is the autoradiograph of the Southern blot, in which the reaction products formed during the dual incision reaction were transferred onto a nylon membrane and probed with a ^{32}P-labeled complementary probe. The sizes (*bases*) of the excised oligonucleotides are indicated and were determined by comparison with the mobility of the platinated 24-mer used in the synthesis of the substrate. Lanes 1 and 3 result from the reaction of the single lesion substrate with 125 μg of A2780 and C200 extract protein, respectively. Lanes 2 and 4 result from the same reactions with the addition of 100 ng of purified ERCC1–XPF protein.

erodimeric protein had little effect on the activity of a C200 extract (1.2-fold). A second experiment examining an independently prepared A2780 extract showed a reproducible (extract and extract plus protein examined in triplicate) but more modest complementation of 1.5-fold, whereas the addition of the purified protein had no impact on an independently prepared C200 extract. The resulting average complementation factor of ERCC1–XPF for A2780 was 1.86 ± 0.36-fold and for C200 was 1.13 ± 0.08-fold.

DISCUSSION

The A2780 human ovarian cancer cell line and its cisplatin-resistant derivatives CP70, C30, and C200 exhibit a wide range of cisplatin resistance. The resistant sublines in this model system contain several putative cisplatin resistance mechanisms including: (a) decreased platinum accumulation; (b) increased drug inactivation; (c) enhanced platinum–DNA adduct repair capacity; and (d) an increased ability to tolerate platinum–DNA damage [9, 13–21]. We would like to emphasize that this is an exaggerated model of resistance, developed by repeatedly exposing cells to high concentrations of cisplatin. It is valuable for identifying resistance mechanisms and determining their underlying molecular basis, but it may not necessarily reflect the clinical drug resistance phenotype. We do believe, however, that this model offers a unique opportunity to gain insight into the molecular basis for the enhanced platinum–DNA adduct repair phenotype, since differences in repair activity are easily measured and clearly evident in this panel of cell lines. Therefore, if it turns out that increased platinum–DNA adduct repair is shown to be a clinically relevant resistance mechanism, then our results using this model may provide insight into the basis for the phenotype and may be useful for designing and testing strategies to circumvent resistance caused by this mechanism.

To focus on the DNA repair component of cytotoxicity, the UV sensitivity of this panel of cell lines was measured. Cells irradiated with UV light at 254 nm incur DNA damage mainly in the form of pyrimidine dimers, circumventing factors such as accumulation and inactivation, which contribute to the cytotoxicity of cisplatin and other chemical DNA-damaging agents. Pyrimidine dimers, like platinum–DNA adducts, are repaired by the nucleotide excision repair pathway, and thus UV sensitivity is an indication of the capacity of a cell to perform this type of repair or, perhaps, to tolerate these types of lesions [29]. A clear association between increased cisplatin resistance and decreased UV sensitivity was observed in this model system, providing additional evidence that the cellular measurements of increased removal of platinum–DNA adducts in the resistant members of the panel is the result of enhanced NER activity. In addition, the decreased UV sensitivity of C200 cells relative to A2780 cells (2.5-fold) was nearly identical to the difference in platinum–DNA excision activity between the two cell lines (2.8-fold).

To gain insight into the molecular basis for enhanced repair in our model system, the constitutive expression of several NER genes was determined. Most of the transcripts examined were increased in abundance in C200 cells as compared with A2780 cells, including *ERCC1*, *XPA*, *XPB*, *XPC*, *XPG*, and *PCNA*. However, *ERCC1* was the only transcript examined that consistently showed a significant increase in all of the resistant cell lines and that was present at steady-state levels that correlated with the level of cisplatin resistance ($r = 0.99$). An additional observation was that *ERCC1* expression was strongly associated with UV resistance ($r = 0.93$).

To investigate further the molecular determinant(s) of increased NER in our model system, a cell-free excision assay was performed. Initial attempts at measuring repair using an assay that relies on the measurement of DNA damage-dependent repair synthesis were unsuccessful due to the significant contribution of the mismatch repair system to nucleotide incorporation. Therefore, to avoid the contribution of the mismatch repair system to the overall signal, we chose to use an assay that measured platinum–DNA adduct excision. Using this single lesion excision assay, we found that C200 cell extracts had a significantly greater capacity than A2780 extracts to excise an oligonucleotide containing the platinum–DNA adduct. Since ERCC1 was the strongest candidate for the molecular determinant of increased NER activity in C200 cells based on northern analysis, complementation experiments using purified ERCC1–XPF heterodimeric protein were performed. ERCC1 forms a tight complex with its heterodimeric partner XPF and, therefore, copurifies with it [30]. Furthermore, within the cell, ERCC1 protein is very unstable in its absence [31]. The addition of ERCC1–XPF to A2780 extracts resulted in an average increase in excision activity of approximately 2-fold, whereas the addition of this protein had little effect on the excision of C200 extracts. The lack of an association between the constitutive mRNA levels of XPF throughout the entire panel of cell lines and both cytotoxicity and repair measurements, as seen for ERCC1 mRNA, may indicate that, in this model system translation of ERCC1 mRNA may be rate-limiting in the synthesis of the heterodimeric endonuclease. We conclude from these experiments that increased activity of the ERCC1–XPF endonuclease is likely to be one factor involved in the increased NER activity in the cisplatin-resistant cell line C200. Increased abundance of other NER components or as yet undiscovered proteins may be involved in the full increase in excision activity that we observed in C200 extracts.

Reconstitution of the specific dual incisions characteristic of human nucleotide excision repair has been accomplished by the mixture of six repair proteins: XPA, XPC, XPG, TFIIH, RPA, and ERCC1–XPF [32]. ERCC1–XPF is a 5′ junction-specific endonuclease, believed to form the 5′-incision during NER [30, 33]. It should be mentioned that in addition to having a role in NER, this protein complex is also believed to be involved in a separate, poorly

understood recombination-dependent pathway for removing interstrand cross-links known as recombinational repair [34]. The ERCC1–XPF protein is believed to directly interact with both XPA and RPA [35]. These interactions suggest that ERCC1–XPF may play a role in both the damage-recognition and incision steps of nucleotide excision repair. This article presents data to support a role for the ERCC1–XPF endonuclease in the increased repair observed in our cellular model of cisplatin resistance. A number of studies by Reed and colleagues have also implicated ERCC1 as an important contributor to enhanced NER in cisplatin resistance. First of all, this group presented clinical data to support this protein as having a role in resistance to cisplatin. They showed that ERCC1 mRNA levels in tumors from previously untreated ovarian cancer patients correlate well with subsequent response to platinum-based therapy [36]. They also recently presented evidence showing that cisplatin induces *ERCC1* expression in A2780/CP70 cells following cisplatin treatment and that this induction is associated with AP-1 activity [37]. These results complement those of our previous study in which we demonstrated that increased AP-1 activity is associated with cisplatin resistance in the A2780/C-series [38]. Our study, the clinical correlates, and evidence implicating ERCC1–XPF in the recognition and excision of adducts formed by cisplatin and other DNA-damaging agents suggest that the ERCC1–XPF endonuclease has a rate-limiting or regulatory role in the NER process.

The capacity to efficiently repair DNA damage clearly plays a role in determining the sensitivity of a tumor cell to platinum drugs and other DNA-damaging agents. There is recent evidence suggesting that cell lines derived from tumors that are unusually sensitive to cisplatin, such as testicular non-seminomatous germ cell tumors, are deficient in their ability to repair platinum–DNA adducts as compared with tumor cells derived from intrinsically resistant bladder carcinoma [39]. Similarly, the relative unresponsiveness of non-small cell lung cancer as compared with small cell lung cancer has been associated with an elevated DNA repair capacity [40]. Increased repair of platinum–DNA lesions in cisplatin-resistant cells as compared with their sensitive counterparts has been shown for several human cancers including ovarian [18, 19], breast [41], and glioma [42]. Evidence for increased repair of cisplatin interstrand cross-links in specific gene and non-gene regions in cisplatin-resistant cell lines has also been demonstrated [22, 43]. Due to the complex nature of the NER process, the molecular basis for the increased platinum–DNA adduct repair capacity of cisplatin-resistant cell lines has been elusive. The recent development of highly specific assays for NER has made it possible to both confirm the increase in NER capacity in these and other drug-resistant cell lines and to examine the molecular determinants of this resistance mechanism, which was done in this study. Importantly, this and other studies point to ERCC1–XPF as having a role in determining the overall NER capacity of cell lines resistant to cisplatin and other DNA-damaging agents and suggest that this protein may be an important regulatory component of the NER machinery.

The authors would like to thank Drs. Richard Wood and Jonathan Moggs for excellent technical guidance. This work was supported, in part, by Grant CA51228 (to T. C. H) and a UICC ICRETT grant (to K. V. F.).

References

1. McGuire WP and Ozols RF, Chemotherapy of advanced ovarian cancer. *Semin Oncol* **25:** 340–348, 1998.
2. O'Dwyer PJ, Hamilton TC and Johnson SW, Cisplatin and its analogues. In: *Principles and Practice of Oncology* (Ed. DeVita VJ), pp. 418–432. J.B. Lippincott, Philadelphia, 1997.
3. Eastman A, The formation, isolation and characterization of DNA adducts produced by anticancer platinum complexes. *Pharmacol Ther* **34:** 155–166, 1987.
4. Plooy AC, Fichtinger-Schepman AM, Schutte HH, van Dijk M and Lohman PH, The quantitative detection of various Pt-DNA-adducts in Chinese hamster ovary cells treated with cisplatin: Application of immunochemical techniques. *Carcinogenesis* **6:** 561–566, 1985.
5. Fichtinger-Schepman AM, van der Veer JL, den Hartog JH, Lohman PH and Reedijk J, Adducts of the antitumor drug *cis*-diamminedichloroplatinum(II) with DNA: Formation, identification, and quantitation. *Biochemistry* **24:** 707–713, 1985.
6. Andrews PA, Velury S, Mann SC and Howell SB, *cis*-Diamminedichloroplatinum(II) accumulation in sensitive and resistant human ovarian carcinoma cells. *Cancer Res* **48:** 68–73, 1988.
7. Kuppen PJ, Schuitemaker H, van't Veer LJ, de Bruijn EA, van Oosterom AT and Schrier PI, *cis*-Diamminedichloroplatinum(II)-resistant sublines derived from two human ovarian tumor cells. *Cancer Res* **48:** 3355–3359, 1988.
8. Kelland LR, Mistry P, Abel G, Loh SY, O'Neill CF, Murrer BA and Harrap KR, Mechanism-related circumvention of acquired *cis*-diamminedichloroplatinum(II) resistance using two pairs of human ovarian carcinoma cell lines by ammine/amine platinum(IV) dicarboxylates. *Cancer Res* **52:** 3857–3864, 1992.
9. Schilder RJ, Hall L, Monks A, Handel LM, Fornace AJ Jr, Ozols RF, Fojo AT and Hamilton TC, Metallothionein gene expression and resistance to cisplatin in human ovarian cancer. *Int J Cancer* **45:** 416–422, 1990.
10. Behrens BC, Hamilton TC, Masuda H, Grotzinger KR, Whang-Peng J, Louie KG, Knutsen T, McKoy WM, Young RC and Ozols RF, Characterization of a *cis*-diamminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. *Cancer Res* **47:** 414–418, 1987.
11. Mistry P, Kelland LR, Abel G, Sidhar S and Harrap KR, The relationships between glutathione, glutathione-S-transferase and cytotoxicity of platinum drugs and melphalan in eight human ovarian carcinoma cell lines. *Br J Cancer* **64:** 215–220, 1991.
12. Hamilton TC, Winker MA, Louie KG, Batist G, Behrens BC, Tsuruo T, Grotzinger KR, McKoy WM, Young RC and Ozols RF, Augmentation of adriamycin, melphalan, and cisplatin cytotoxicity in drug-resistant and -sensitive human ovarian cancer cell lines by buthionine sulfoximine mediated glutathione depletion. *Biochem Pharmacol* **34:** 2583–2586, 1985.
13. Godwin AK, Meister A, O'Dwyer PJ, Huang CS, Hamilton TC and Anderson ME, High resistance to cisplatin in human ovarian cancer cell lines is associated with marked increase of

glutathione synthesis. *Proc Natl Acad Sci USA* **89:** 3070–3074, 1992.
14. Masuda H, Ozols RF, Lai G-M, Fojo A, Rothenberg M and Hamilton TC, Increased DNA repair as a mechanism of acquired resistance to *cis*-diamminedichloroplatinum(II) in human ovarian cancer cell lines. *Cancer Res* **48:** 5713–5716, 1988.
15. Lai G-M, Ozols RF, Smyth JF, Young RC and Hamilton TC, Enhanced DNA repair and resistance to cisplatin in human ovarian cancer. *Biochem Pharmacol* **37:** 4597–4600, 1988.
16. Masuda H, Tanaka T, Matsuda H and Kusaba I, Increased removal of DNA-bound platinum in a human ovarian cancer cell line resistant to *cis*-diamminedichloroplatinum(II). *Cancer Res* **50:** 1863–1866, 1990.
17. Parker RJ, Eastman A, Bostick-Bruton F and Reed E, Acquired cisplatin resistance in human ovarian cancer cells is associated with enhanced repair of cisplatin-DNA lesions and reduced drug accumulation. *J Clin Invest* **87:** 772–777, 1991.
18. Johnson SW, Perez RP, Godwin AK, Yeung AT, Handel LM, Ozols RF and Hamilton TC, Role of platinum-DNA adduct formation and removal in cisplatin resistance in human ovarian cancer cell lines. *Biochem Pharmacol* **47:** 689–697, 1994.
19. Zhen W, Link CJ Jr, O'Connor PM, Reed E, Parker R, Howell SB and Bohr VA, Increased gene-specific repair of cisplatin interstrand cross-links in cisplatin-resistant human ovarian cancer cell lines. *Mol Cell Biol* **12:** 3689–3698, 1992.
20. Johnson SW, Laub PB, Beesley JS, Ozols RF and Hamilton TC, Increased platinum-DNA damage tolerance is associated with cisplatin resistance and cross-resistance to various chemotherapeutic agents in unrelated human ovarian cancer cell lines. *Cancer Res* **57:** 850–856, 1997.
21. Hamaguchi K, Godwin AK, Yakushiji M, O'Dwyer PJ, Ozols RF and Hamilton TC, Cross-resistance to diverse drugs is associated with primary cisplatin resistance in ovarian cancer cell lines. *Cancer Res* **53:** 5225–5232, 1993.
22. Johnson SW, Swiggard PA, Handel LM, Brennan JM, Godwin AK, Ozols RF and Hamilton TC, Relationship between platinum-DNA adduct formation and removal and cisplatin cytotoxicity in cisplatin-sensitive and -resistant human ovarian cancer cells. *Cancer Res* **54:** 5911–5916, 1994.
23. Wood RD, Nucleotide excision repair in mammalian cells. *J Biol Chem* **272:** 23465–23468, 1997.
24. Moggs JG, Yarema KJ, Essigmann JM and Wood RD, Analysis of incision sites produced by human cell extracts and purified proteins during nucleotide excision repair of a 1,3-intrastrand d(GpTpG)-cisplatin adduct. *J Biol Chem* **271:** 7177–7186, 1996.
25. Hansen MB, Nielsen SE and Berg K, Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *J Immunol Methods* **119:** 203–210, 1989.
26. Manley JL, Fire A, Cano A, Sharp PA and Gefter ML, DNA-dependent transcription of adenovirus genes in a soluble whole-cell extract. *Proc Natl Acad Sci USA* **77:** 3855–3859, 1980.
27. Zamble DB, Mu D, Reardon JT, Sancar A and Lippard SJ, Repair of cisplatin-DNA adducts by the mammalian excision nuclease. *Biochemistry* **35:** 10004–10013, 1996.
28. Perez RP, Godwin AK, Handel LM and Hamilton TC, A comparison of clonogenic, microtetrazolium and sulforhodamine B assays for determination of cisplatin cytotoxicity in human ovarian carcinoma cell lines. *Eur J Cancer* **29A:** 395–399, 1993.
29. Aboussekhra A and Wood RD, Repair of UV-damaged DNA by mammalian cells and *Saccharomyces cerevisiae*. *Curr Opin Genet Dev* **4:** 212–220, 1994.
30. Park C-H, Bessho T, Matsunaga T and Sancar A, Purification and characterization of the XPF-ERCC1 complex of human DNA repair excision nuclease. *J Biol Chem* **270:** 22657–22660, 1995.
31. Sijbers AM, van der Spek PJ, Odijk H, van den Berg J, van Duin M, Westerveld A, Jaspers NG, Bootsma D and Hoeijmakers JH, Mutational analysis of the human nucleotide excision repair gene *ERCC1*. *Nucleic Acids Res* **24:** 3370–3380, 1996.
32. Bessho T, Sancar A, Thompson LH and Thelen MP, Reconstitution of human excision nuclease with recombinant XPF-ERCC1 complex. *J Biol Chem* **272:** 3833–3837, 1997.
33. Evans E, Moggs JG, Hwang JR, Egly JM and Wood RD, Mechanism of open complex and dual incision formation by human nucleotide excision repair factors. *EMBO J* **16:** 6559–6573, 1997.
34. Andersson BS, Sadeghi T, Siciliano MJ, Legerski R and Murray D, Nucleotide excision repair genes as determinants of cellular sensitivity to cyclophosphamide analogs. *Cancer Chemother Pharmacol* **38:** 406–416, 1996.
35. Saijo M, Kuraoka I, Masutani C, Hanaoka F and Tanaka K, Sequential binding of DNA repair proteins RPA and ERCC1 to XPA *in vitro*. *Nucleic Acids Res* **24:** 4719–4724, 1996.
36. Dabholkar M, Vionnet J, Bostick-Bruton F, Yu JJ and Reed E, Messenger RNA levels of XPAC and ERCC1 in ovarian cancer tissue correlate with response to platinum-based chemotherapy. *J Clin Invest* **94:** 703–708, 1994.
37. Li Q, Tsang B, Bostick-Bruton F and Reed E, Modulation of excision repair cross complementation group 1 (ERCC-1) mRNA expression by pharmacological agents in human ovarian carcinoma cells. *Biochem Pharmacol* **57:** 347–353, 1999.
38. Yao K-S, Godwin AK, Johnson SW, Ozols RF, O'Dwyer PJ and Hamilton TC, Evidence for altered regulation of γ-glutamylcysteine synthetase gene expression among cisplatin-sensitive and cisplatin-resistant human ovarian cancer cell lines. *Cancer Res* **55:** 4367–4374, 1995.
39. Koberle B, Grimaldi KA, Sunters A, Hartley JA, Kelland LR and Masters JR, DNA repair capacity and cisplatin sensitivity of human testis tumour cells. *Int J Cancer* **70:** 551–555, 1997.
40. Zeng-Rong N, Paterson J, Alpert L, Tsao M-S, Viallet J and Alaoui-Jamali MA, Elevated DNA repair capacity is associated with intrinsic resistance of lung cancer to chemotherapy. *Cancer Res* **55:** 4760–4764, 1995.
41. Yen L, Woo A, Christopoulopoulos G, Batist G, Panasci L, Roy R, Mitra S and Alaoui-Jamali MA, Enhanced host cell reactivation capacity and expression of DNA repair genes in human breast cancer cells resistant to bi-functional alkylating agents. *Mutat Res* **337:** 179–189, 1995.
42. Ali-Osman F, Berger MS, Rairkar A, and Stein DE, Enhanced repair of a cisplatin-damaged reporter chloramphenicol-O-acetyltransferase gene and altered activities of DNA polymerases α and β, and DNA ligase in cells of a human malignant glioma following *in vivo* cisplatin therapy. *J Cell Biochem* **54:** 11–19, 1994.
43. Petersen LN, Mamenta EL, Stevnsner T, Chaney SG and Bohr VA, Increased gene specific repair of cisplatin induced interstrand crosslinks in cisplatin resistant cell lines, and studies on carrier ligand specificity. *Carcinogenesis* **17:** 2597–2602, 1996.