

Enhanced Cisplatin Cytotoxicity by Disturbing the Nucleotide Excision Repair Pathway in Ovarian Cancer Cell Lines¹

Muthu Selvakumaran,² Debra A. Pisarcik, Rudi Bao, Anthony T. Yeung, and Thomas C. Hamilton³

Ovarian Cancer Program, Medical Oncology, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

ABSTRACT

Ovarian cancer is the leading cause of death among women from gynecological malignancies in the United States. Resistance to the chemotherapeutic agent cisplatin is a major limitation for the successful treatment of ovarian cancer. In an effort to overcome the cisplatin resistance problem in ovarian cancer treatment, we have sought to enhance cisplatin cytotoxicity by perturbing the nucleotide excision repair (NER) pathway. The NER pathway is responsible for repairing cisplatin bound to DNA. Expression of one of the NER components, ERCC1, is correlated with cisplatin drug resistance. Hence, we targeted ERCC1 by antisense RNA methodologies, and we show that we could sensitize a relatively sensitive A2780 cell line and also the highly resistant OVCAR10 cell line to cisplatin by expressing antisense ERCC1 RNA in them as measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assays. The A2780 cell lines expressing antisense ERCC1 had 1.9–8.1-fold enhancements in cisplatin sensitivity. The OVCAR10 antisense ERCC1 cell lines had IC₅₀ values ranging from 2.28 μ M to 2.7 μ M cisplatin as compared with 9.52 μ M for control OVCAR10 cells. The OVCAR10 antisense ERCC1 cells also show reduced DNA-damage repair capacity as assessed by host cell reactivation. Furthermore, immunocompromised mice transplanted with the antisense cell lines survived longer than the mice bearing control cells after response to cisplatin treatment. These data suggest that it is possible to substantially enhance the cisplatin cytotoxicity by disturbing the NER pathway in cisplatin-resistant cell lines and to enhance the survival capacity of mice in an ovarian cancer xenograft model.

INTRODUCTION

Ovarian carcinoma is the leading cause of death from gynecological malignancies and is most frequently diagnosed at an advanced stage. Platinum-based chemotherapy is the primary treatment for ovarian cancer and is also used in a wide variety of other malignancies (1). Platinum is used currently either alone or in combination with other chemotherapeutic agents in ovarian cancer. Despite such combinations of drugs yielding complete responses in 60–80% of patients with advanced-stage disease, the majority of ovarian cancer patients eventually relapse and become refractory to additional treatment (2). In most studies, the long-term survival for patients with advanced-stage disease rarely exceeds 30%.

Intuitively, the failure of chemotherapy must be the result of development of drug resistance by tumor cells. Therefore, the occurrence of resistance to platinum is a major problem that undermines efforts to effectively treat ovarian cancer. One approach to overcome this limitation in ovarian cancer treatment is to elucidate the mecha-

nisms responsible for drug resistance and then develop ways to treat resistant disease effectively or prevent its occurrence.

The basis for the therapeutic effectiveness of cisplatin is not fully understood, but its cytotoxic action against tumor cells is thought to be mediated through the formation of cisplatin-DNA adducts, which inhibit DNA replication and/or transcription (3). Cisplatin forms primarily 1,2-intrastrand cross-links between adjacent purines in DNA, and also introduces other adducts including 1,3 cross-links, interstrand cross-links and monoadducts. The main mechanism for removing intrastrand crosslinks is NER.⁴ NER in human cells is comprised of many different highly coordinated steps and is carried out by perhaps >30 distinct proteins that recognize DNA damage, incise the lesion, and resynthesize and ligate the repair patch (4, 5). The human ERCC1 gene product forms heterodimeric complex with XPF (ERCC1-XPF). This complex is involved in 5' side incision of DNA adducts.

Resistance to cisplatin in ovarian cancer may be multifactorial and include decreased drug accumulation, increased inactivation, enhanced repair capacity, and tolerance to DNA damage (6). 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 (7, 8), reactivation of cisplatin-damaged plasmid DNA (9), atomic absorption spectrometry (10, 11), and renaturing agarose gel electrophoresis (12). Our previous studies demonstrated a clear association between increased platinum-DNA adduct removal and increased cisplatin resistance in the A2780/C-series cisplatin-resistance model (7, 8, 11, 12). More recently, we have demonstrated increased NER in cisplatin-resistant ovarian cancer cells using a highly NER-specific single lesion assay (13). This work included evidence that, at least in our model, a rate-limiting component for NER is the amount of ERCC1 expression. Others have shown an association between ERCC1 and clinical response to platinum therapy (14).

The goal of the work described presently was to examine whether perturbing NER at this ERCC1-XPF incision step would substantially alter platinum sensitivity. To test this probability we used the antisense RNA approach to perturb NER. Transfectants were examined for altered DNA repair capacity, and their sensitivity to cisplatin *in vitro* and *in vivo*.

MATERIALS AND METHODS

Chemicals and Reagents. Cisplatin was obtained from Bristol Myers Squibb Co. (Princeton, NJ). Regular iletin II pork insulin was obtained from Eli Lilly. Glutamine-free concentrated RPMI 1640, penicillin/streptomycin, glutamine, and Geneticin (G-418 Sulfate) were obtained from Life Technologies, Inc. (Grand Island, NY). Fetal bovine serum was obtained from Atlanta Biologicals (Norcross, GA). All of the other chemicals and reagents were obtained from Sigma.

Cell Lines. A2780 is an ovarian cancer cell line derived from an untreated patient (15). Cisplatin-resistance cell lines CP70, C30, and C200 were produced by intermittent, incremental exposure of the sensitive parental A2780

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² Present address: University of Pennsylvania, Philadelphia, PA 19104.

³ To whom requests for reprints should be addressed, at Fox Chase Cancer Center, 7701 Burholme Avenue, Philadelphia, PA 19111. Phone: (215) 728-3679; E-mail: TC_Hamilton@fccc.edu.

⁴ The abbreviations used are: NER, nucleotide excision repair; ERCC1, excision repair cross-complementing group 1; XPF, xeroderma pigmentation group F; UTR, untranslated region; CMV, cytomegalovirus; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT-PCR, reverse transcription-PCR; SCID, severe combined immune deficiency; β -gal, β -galactosidase.

cell line to various concentrations of cisplatin. OVCAR-4 and OVCAR-10 cell lines were developed from patients who were clinically refractory to platinum-based chemotherapy (16, 17). All of the cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 0.28 units/ml insulin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 100 units/ml penicillin, and 0.3 mg/ml glutamine. Cells were grown at 37°C in a humidified atmosphere of 5% CO₂ in air.

Construction of Antisense ERCC1 Expression Vectors. The full-length ERCC1 cDNA containing plasmid was a kind gift from Dr. Jan H. J. Hoeijmakers (Erasmus University Rotterdam, Rotterdam, The Netherlands). This cDNA appeared to have used an alternate promoter to transcribe ERCC1 transcript, resulting in splicing variant of 104 bp 5'-UTR that was different from the one published previously (18). The coding sequences were similar except there was a mutation at position 246 where N has been mutated to T. This mutation was corrected by using site-directed mutagenesis (Promega). This full-length ERCC1 cDNA was excised with *NotI* enzyme and subcloned into expression vector pcDNA3.1 (-; Invitrogen) in an antisense orientation.

Establishing Stable Cell Lines Expressing Antisense ERCC1. The Scal linearized pcDNA3.1-antisense ERCC1 plasmid was stably transfected into A2780 and OVCAR10 cell lines by electroporation. Electroporation and selection for G418-resistant clones was carried out as described earlier (19). The G418-resistant clones were expanded and ultimately used for ERCC1 RNA expression analysis by Northern and RT-PCR, and for protein expression using Western blot analysis.

Western and Northern Blot Analysis. Protein extraction and Western blot analysis were essentially carried out as described earlier (20). The ERCC1 protein was detected using primary monoclonal antibodies to ERCC1 (Ab-1; Neomarkers, Lab Vision Inc., Fremont, CA). RNA extraction and Northern blot analysis were carried out as described earlier (19). The single-stranded sense ERCC1 riboprobe was made according to the manufacturer's protocol (Promega). The ³²P-labeled sense ERCC1 riboprobe was used in Northern hybridization to identify antisense ERCC1 transcripts.

RT-PCR. Total RNA was obtained using the Trizol (Invitrogen) reagent. The SuperScript one-step RT-PCR with the Platinum Taq system (Invitrogen) was performed to analyze RNA molecules. Reverse transcription was performed at 50°C for 30 min, and PCR was performed with specific primers in volumes of 50 μl according to the protocol provided by the manufacture (Invitrogen). The 5'-UTR in endogenous ERCC1 transcript is different from the exogenous antisense ERCC1 transcript that allowed us to design primers for sense and antisense ERCC1 in the RT-PCR reaction. The endogenous ERCC1 RNA molecules were amplified using forward primer, 5'-CTTTC-CCTTGAGGCTCCAAGAC-3', and reverse primer, 5'-AGATCTTCTT-GATGCGGCG-3'. The antisense ERCC1 RNA molecules were amplified with primers of 5'-ACAAAACCTCGAGAGCTCCA-3' and 5'-CAACAGATG-GCTGGCAACTA-3'. Glyceraldehyde-3-phosphate dehydrogenase was used as an internal control in RT-PCR reaction with primers 5'-CCACATCGCT-CAGACACCAT-3' and 5'-CCTCCG ACGCCTGCTTACCAC-3'. The amplification reaction was involved denaturation at 95°C for 15 s, annealing at 55°C for 30 s, and 72°C for 1 min and 30 s using a thermal cycler (PTC-1000; MJ Research). The resultant RT-PCR products were analyzed on ethidium bromide-stained 1% agarose gel.

Cytotoxicity Assays: MTT Assay. Cisplatin cytotoxicity was determined by the tetrazolium (MTT) assay (21). Cells (1000–8000) were plated in 150 μl of medium/well in 96-well plates (Costar, Corning Inc., Rochester, NY). After incubation overnight, cisplatin was added in various concentrations. After 72 h, 40 μl of 5 mg/ml MTT were added per well. After a 2-h incubation, the cells were lysed with 100 μl /well of lysis buffer [20% (w/v) SDS-50% *N,N*-dimethylformamide (pH 4.7)], and incubation overnight. The absorbance at 570 nm was measured using wells without cells as blanks. IC₅₀ was used as the measure of relative cytotoxicity. The fold enhancement in cisplatin sensitivity was considered to be the ratio of the IC₅₀ of the parental cell line to antisense-ERCC1 transfected cell lines or clones.

Host Cell Reactivation. The PGL3-control vector was obtained from Promega and was used for reactivation studies of cisplatin-damaged reporter plasmids. The luciferase gene in PGL3 control vector is driven by SV40 promoter and enhancer. The pGL3 control plasmid was incubated with various concentrations of cisplatin (50–250 nm) for 4 h at 37°C. The cisplatin-damaged plasmids were precipitated with sodium acetate and ethanol, and washed three times with 80% ethanol to eliminate the unbound cisplatin. The level of plasmid platinumation was confirmed by atomic absorption spectrometry.

It was approximately 2–4 cisplatin damage occurred at 10 nm cisplatin concentration. Cells growing at 50–60% confluence were used for transient transfection assays as described earlier (19). A total of 5 μg of plasmid (4.5 μg of platinated PGL3-control and 0.5 μg of pCMV- β) were used for transfection with LT-1 reagent (Mirus). Cells were lysed, after 48 h of incubation, with passive lysis buffer (Promega). The luciferase activity was measured with the luciferase assay system from Promega. The transfection efficiency was normalized with β -gal activity. The β -gal was assayed with an *O*-nitrophenyl- β -D-galactopyranoside substrate based assay system (Promega). The level of luciferase activity associated with a zero level of cisplatin molecules per plasmid was assigned a value of 100% luciferase activity, and the luciferase activity associated with damaged plasmid was assessed relative to this control value.

In Vivo Sensitivity of OVCAR10 Transfectants to Cisplatin. *In vivo* animal studies were carried out in immunocompromised C.B.17/ICR SCID mice. They were bred in the laboratory animal facility at Fox Chase Cancer Center and kept under specific pathogen-free conditions in plastic cages equipped with air filters. The animals received commercial food and water *ad libitum*. All of the SCID mice used in this study were female, 10–12 weeks of age, with body weights between 18 and 22 g. The animal care and use were in accord with the institutional guidelines.

The two antisense ERCC1-transfected cell lines, OVCAR10^{AS-ERCC1-8} and OVCAR10^{AS-ERCC1-9}, and control plasmid transfected OVCAR10 were used for *in vivo* cisplatin sensitivity study. Cells were grown as a monolayer, and were trypsinized, washed, and resuspended in PBS. A total of 10 \times 10⁶ cells were injected i.p. into each SCID mouse (*n* = 6). One week after the implantation of cells, mice were treated i.p. twice in 2 weeks with cisplatin as 3 mg/kg body weight. The untreated mice received physiological saline. The side effects including body weight changes were observed closely. Survival was used as an end point for the efficacy of this therapy.

Statistical Analysis. The Wilcoxon test for paired samples was used to evaluate differences between animal groups.

RESULTS

ERCC1 Protein Expression in Cisplatin-resistant Human Cell Lines. ERCC1 plays an essential role in NER (5). Our previous findings showed that the DNA repair capacity of cisplatin-resistant cells was higher than that of relatively cisplatin-sensitive cells (13). We have also shown previously that the steady-state mRNA levels of some of the NER components were higher in cisplatin-resistant ovarian cancer cells. The mRNA amount that most closely correlated with cisplatin resistance was that of ERCC1 (13). Hence, we wished to determine whether ERCC1 protein followed this trend. ERCC1 protein expression was analyzed by immunoblotting using ERCC1-specific antibodies by Western blotting of extracts from different ovarian cancer cell lines. As is shown in Fig 1, ERCC1 protein expression level was noticeably higher in cisplatin-resistant cell lines C200, OVCAR4, and OVCAR10.

Generation of A2780 Ovarian Cancer Cell Lines Expressing the ERCC1 Gene in the Antisense Orientation. Our present study was undertaken to analyze whether it is possible to enhance the cisplatin sensitivity by altering NER pathway. As a proof of principle, we

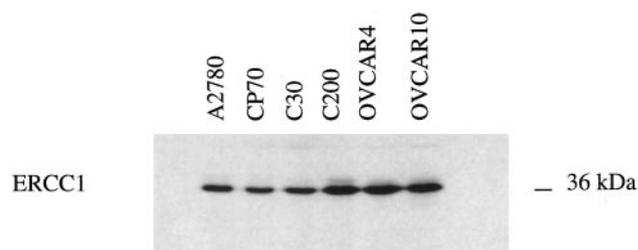


Fig. 1. Expression of ERCC1 protein by Western blot analysis. ERCC1 protein was detected in ovarian cancer cell lines A2780, CP20, C30, C200, OVCAR4, and OVCAR10 by Western blot with antihuman ERCC1 monoclonal antibodies.

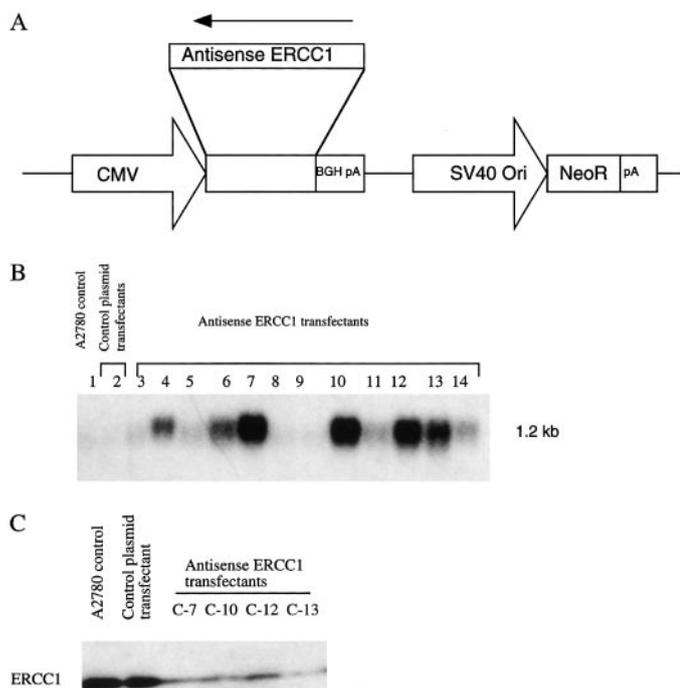


Fig. 2. A, structure of antisense-ERCC1 expression vector. The antisense-ERCC1 expression construct was made by cloning a full-length human ERCC1 cDNA in an antisense orientation into expression vector, pcDNA3.1 (-). B, Northern analysis for antisense ERCC1 transcripts in A2780 cells. Antisense ERCC1 transcripts in antisense-ERCC1 and empty vector-transfected A2780 cells were detected in Northern hybridization using ^{32}P -labeled sense ERCC1 riboprobe. C, Western blot analysis for ERCC1 protein. A2780 antisense ERCC1 RNA-expressing cell lines C-7, C-10, C-12, and C-13, A2780 empty vector-transfected cell line, and control A2780 cells were analyzed for ERCC1 protein expression in Western blot with an anti-ERCC1 monoclonal antibodies.

studied the effect of antisense ERCC1 RNA expression in the relatively cisplatin-sensitive A2780 cell line. We constructed plasmid expressing ERCC1 in the antisense orientation under the control of CMV-promoter (pcDNA3.1-antisense ERCC1) as described in "Materials and Methods" (Fig. 2A). The antisense ERCC1 construct was stably transfected into A2780. The resultant neomycin-sulfate-resistant clones were analyzed for the expression of antisense ERCC1 RNA by Northern analysis. In Northern analysis, antisense RNA transcripts were detected using sense ERCC1 RNA-based ^{32}P -labeled riboprobe (Fig. 2B). The stable cell lines were established based on the expression of the antisense ERCC1 transcripts (Fig. 2B) and were analyzed for ERCC1 protein expression levels. The cell lines expressing antisense ERCC1 RNA showed reduced amounts of ERCC1 protein as compared with A2780 control cells (Fig. 2C).

In Vitro Sensitivity of A2780-Antisense-ERCC1 Transfectants to Cisplatin. To determine the impact of ERCC1 down-regulation on cell survival in response to cisplatin, MTT assays were used for antisense and control cells. In MTT assays, antisense ERCC1 transfectants had IC_{50} values of 0.093–0.022 μM as compared with 0.18 μM for A2780 control. The empty vector transfectants of A2780 (pcDNA3.1– alone, without any insert) had similar IC_{50} values to the A2780 control cells. The antisense cell lines had 1.9–8.1-fold enhancement (Tables 1 and 2) in cisplatin sensitivity when compared with control.

Generation of OVCAR10 Cell Lines Expressing Antisense ERCC1. Because the antisense ERCC1 showed enhancement in cisplatin sensitivity in relatively sensitive A2780 cells, we wished to test the effect on one of the highly resistant OVCAR10 cells. The OVCAR10 cells were stably transfected with antisense ERCC1 plasmid, and G418-resistant clones were analyzed by RT-PCR. In RT-

PCR, the antisense transcripts were detected with the strand-specific primers for pcDNA3.1(-) and the 5'-UTR of the antisense-ERCC1. As it can be seen in Fig. 3A, the antisense PCR products were only present in the samples from antisense plasmid-transfected cells and not in the control cells. To determine the level of endogenous sense ERCC1 RNA, the selected positive clones were subjected to RT-PCR using strand-specific primers for the endogenous ERCC1. As shown in Fig. 3B, the antisense clones AS-8 and AS-9, and pooled transfectants had considerably lower levels of ERCC1 as compared with control cells, indicating that the antisense ERCC1 down-regulates ERCC1 at the RNA level. The ERCC1 protein levels were also reduced in antisense cell lines (Fig. 3C) as compared with control OVCAR10 cells.

The DNA Repair Capacity by Host Cell Reactivation. To determine the impact of reduced ERCC1 on DNA repair capacity, the host cell reactivation method was used with the luciferase gene as a reporter. The SV40-Luc plasmid was treated with various concentrations of cisplatin and used for host cell reactivation assay. To determine the capacity of the cells to restore luciferase activity, the cisplatin-damaged reporter plasmids were transfected into OVCAR10 control and two representative clones of the antisense ERCC1 transfectants. As it is shown in Fig. 4, the antisense cell lines had ~2-fold lower capacity to restore luciferase activity as compared with control OVCAR10 cells.

In Vitro Sensitivity of OVCAR10-Antisense-ERCC1 Transfectants to Cisplatin. To determine the impact of ERCC1 down-regulation on cell survival in response to cisplatin, MTT assays were used for antisense and control cells. In MTT assays, antisense ERCC1 transfectants had IC_{50} values of 2.28–2.70 μM as compared with 9.52 μM for control OVCAR10. The empty vector transfectants of OVCAR10 (pcDNA3.1– alone, without any insert) had similar IC_{50} values to the OVCAR10 control. Hence, the antisense cell lines had 3.5–4.38-fold enhancement (Table 2) in cisplatin sensitivity when compared with control cells.

In Vivo Sensitivity of Antisense-ERCC1 Transfectants to Cisplatin. To evaluate the *in vivo* efficacy of cisplatin on antisense-ERCC1 transfectants, the two antisense-ERCC1 clones and control cells were transplanted i.p. into SCID mice to establish i.p. disease.

Table 1 Effects of antisense ERCC1 on cisplatin sensitivity in A2780 cells

Cell line	Cisplatin (μM) ^a	Fold Increase
A2780	0.18	1
Antisense ERCC1 Transfectants		
C-7	0.022	8.1
C-10	0.0247	7.5
C-12	0.031	5.8
C-13	0.093	1.9

^a Cytotoxicity is expressed as IC_{50} values for each cell line which is the concentration of drug that caused a 50% reduction of absorbance at 570 nm relative to untreated cells using the MTT assay. These experiments were done thrice with similar results.

Table 2 Effects of antisense ERCC1 on cisplatin sensitivity in OVCAR10 cells

Cell line	Cisplatin (μM) ^a	Fold Increase
OVCAR10	9.52	1
Antisense ERCC1 Transfectants		
As-8	2.28	4.17
As-9	2.7	3.5
As-Pooled	2.42	3.9

^a Cytotoxicity is expressed as IC_{50} values for each cell line which is the concentration of drug that caused a 50% reduction of absorbance at 570 nm relative to untreated cells using the MTT assay. These experiments were done thrice with similar results.

One week after tumor implantation, mice were treated i.p. with cisplatin at a dose of 3 mg/kg twice during 2 weeks. At this dose level, the mice tolerated the treatment without loss of body weight or other noticeable side effects. Survival was used as an end point for the efficacy of the therapy, and mice were followed for 300 days. These genetically modified cancer cell lines (transfectants) retained the biological properties of their parental cell line, such as formation of

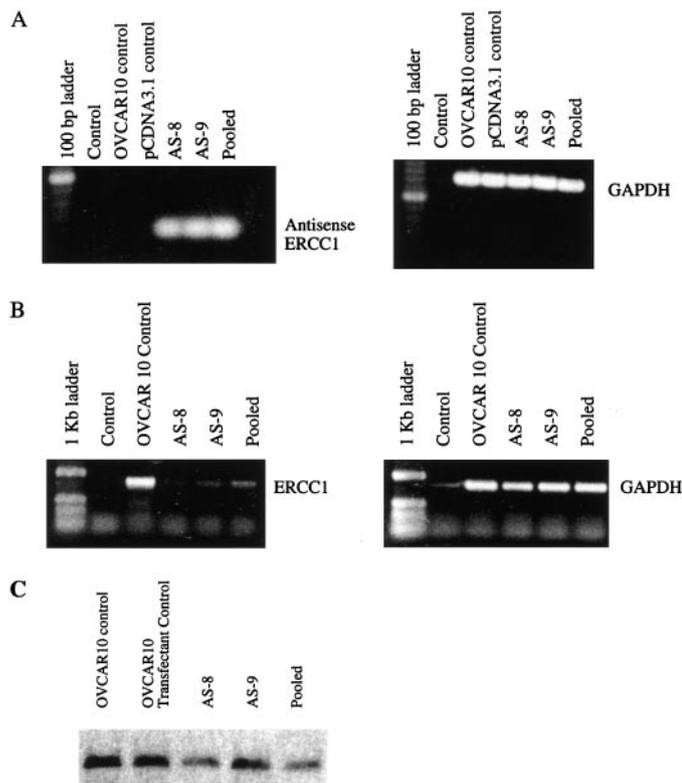


Fig. 3. *A* and *B*, expression of the ERCC1 antisense and sense transcripts in OVCAR10 cells. RT-PCR analysis was performed in OVCAR10 antisense ERCC1-transfected cells AS-8, AS-9, and pooled transfectants using antisense and sense ERCC1 strand-specific primers. The OVCAR10 cells and empty vector-transfected OVCAR10 cells were used as control in RT-PCR. *C*, expression of ERCC1 protein. The expression of ERCC1 protein in OVCAR10 antisense-ERCC1 cell lines AS-8, AS-9, and pooled transfectants, control empty vector-transfected OVCAR10, and OVCAR10 control cells was evaluated by Western blot analysis.

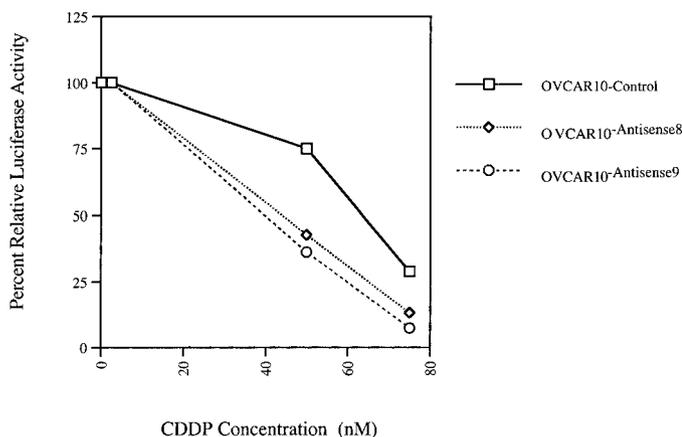


Fig. 4. Host cell reactivation assay. The DNA repair capacity of OVCAR10 control and antisense-ERCC1-transfected cell lines AS-8 and AS-9 was evaluated by host cell reactivation assay. Cells were transiently transfected with cisplatin-damaged SV40-Leu plasmid for the evaluation of DNA repair capacity. These experiments were done twice with similar results.

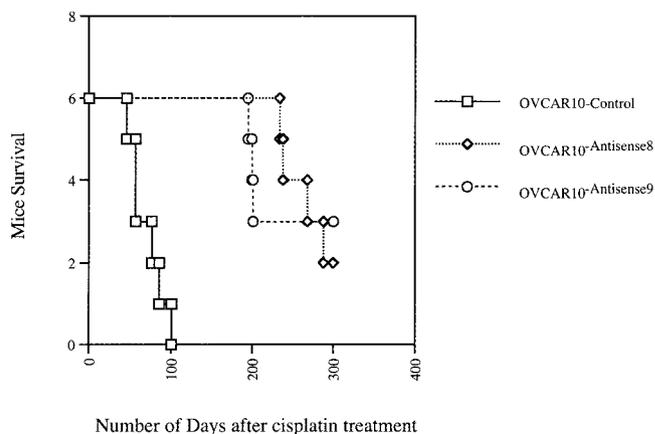


Fig. 5. *In vivo* sensitivity of OVCAR10 transfectants to cisplatin. The antisense ERCC1-transfected cell lines OVCAR10^{Antisense-8} and OVCAR10^{Antisense-9}, and control plasmid-transfected OVCAR10 cells were injected i.p. at a concentration of 10⁷ cells/mouse in SCID mice (*n* = 6). One week after implantation of cells, mice were treated i.p. twice in 2 weeks with cisplatin as 3 mg/kg body weight.

ascites and peritoneal nodules, unchecked growth, and metastasis, which finally led to host death. With cisplatin treatment, animals bearing OVCAR10 control and empty vector control cells developed progressive ascites and peritoneal tumors, and finally died from these complications within 100 days after cisplatin treatments. Animals bearing OVCAR10 control and empty vector control cells, and OVCAR10 antisense transfectants died without cisplatin treatments within 70 days of tumor implantation. On the contrary, after cisplatin treatment animals bearing the antisense ERCC1 transfectants survived longer than control mice (*P* = 0.002). Among the 12 mice treated with cisplatin from two groups of antisense ERCC1 transfectants, 3 (50%) of the mice from one group and 2 from another group survived for 300 days (Fig. 5). Among the 5 mice surviving, 1 had tumor and others appeared to be tumor-free survivors.

DISCUSSION

The emergence of cisplatin resistance is a major stumbling block to the successful treatment of ovarian cancer. To effectively treat or overcome the problem, one has to understand the various mechanisms behind the resistance to cisplatin. Study of various platinum resistance models has shown 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 (7-12, 22, 23).

Recent evidence from our laboratory indicated that the ability of cells to repair DNA damage may be a critical determinant of cisplatin sensitivity and resistance in our cisplatin resistance model (13). In our model system, NER activity was higher in resistant cells as determined by a single lesion assay, and the amount of ERCC1 RNA expression was also elevated in cells resistant to platinum. Moreover, complementation of ERCC1 and XPF proteins in the cell-free extract of cisplatin-sensitive A2780 cells resulted in increased repair activity (13), indicating the rate-limiting effect of ERCC1 in the NER pathway. Others have also shown an association between ERCC1 and clinical response to platinum therapy (14). In human ovarian cancer patients, high tumor tissue levels of ERCC1 mRNA expression were associated with clinical resistance to platinum, whereas low mRNA levels were associated with clinical sensitivity (14, 24).

On the basis of the above described work indicating that ERCC1 activity may be critical to NER-mediated platinum resistance, we have

studied whether we could increase cisplatin sensitivity by down-regulating ERCC1 functions. To accomplish this goal, we decreased expression of ERCC1 protein by antisense RNA expression in A2780 ovarian cancer cell lines. These approaches enhanced cisplatin sensitivity *in vitro*. Antisense-ERCC1 clones from A2780 and OVCAR10 cell lines had different levels of sensitivity to cisplatin despite having low levels of ERCC1 protein expression. It may be attributed to the difference between ERCC1 expression level and NER activity. Our future experiments with NER-specific single lesion assays will overcome these problems.

On the basis of the results that antisense ERCC1 enhanced the cisplatin sensitivity in a relatively sensitive A2780, we wanted to study this effect in the highly cisplatin-resistant OVCAR10 cell line. This cell line was developed from a patient after extensive platinum therapy and relapse. To accomplish this goal we decreased expression of ERCC1 protein by antisense ERCC1 RNA in OVCAR10 cells. This manipulation enhanced cisplatin sensitivity *in vitro* and *in vivo*. The highly resistant OVCAR10 cell line with an IC₅₀ value of 10 μM *in vitro* had only modest sensitivity to cisplatin *in vivo*. However, cisplatin at a physiological range of $\sim 3 \mu\text{M}$ was quite effective when the cells constitutively expressed antisense ERCC1. These results suggest that cisplatin resistance reversal may be feasible in ovarian cancer patients when they become refractory to cisplatin treatment using methods that perturb ERCC1 levels/function.

The cell lines expressing antisense ERCC1 showed decreased DNA repair capacity. Our results are supported by those of others that indicate cells defective in NER exhibit high sensitivity to cisplatin when compared with NER-proficient cells (25–27). Elevated DNA repair capacity has also been shown in bladder carcinoma (28), lung cancer (29), breast cancer, cervical cancer (30, 31), and glioma (32), and the levels of ERCC1 expression in gastric cancer patients were correlated with cisplatin sensitivity (33). A truncated form of xeroderma pigmentosum complementation group A (XPA) protein expression has been shown to increase the cisplatin sensitivity in lung cancer cell line (34). Cisplatin plus gemcitabine chemotherapy has been shown to increase survival of non-small cell lung cancer patients (35), and the prolonged survival was correlated with low-level ERCC1 expression. Gemcitabine has also increased cisplatin sensitivity in colon tumor cells when ERCC1 expression was down-regulated (36). These studies suggest a critical role for efficient DNA repair in sensitivity to tumor cells to platinum drugs and other DNA-damaging agents, and suggest that manipulation of ERCC1 in the context of platinum treatment may have relevance to other malignancies in addition to ovarian cancer.

In summary, this study suggests that by targeting ERCC1 using antisense ERCC1 RNA, it is possible to alter the DNA repair capacity of cisplatin-resistant ovarian cancer cells and increase their sensitivity to cisplatin. We hypothesize that the strategy of inhibition of the damage recognition and incision step of NER may be more effective or may be used in combination with inhibition of repair synthesis, *e.g.*, gemcitabine, to make platinum drugs or classical alkylating agents more effective.

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Enhanced Cisplatin Cytotoxicity by Disturbing the Nucleotide Excision Repair Pathway in Ovarian Cancer Cell Lines

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