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

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

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 μM to 2.7 μM cisplatin as compared with 9.52 μM 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 mechanisms 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 (3). NER in human cells is comprised of more than 30 different highly coordinated steps and is carried out by perhaps >30 different 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 (7, 8), including the measurement of unscheduled DNA synthesis (7, 8), reactivation of cisplatin-damaged plasmid DNA (9), and renaturing agarose gel electrophoresis (10, 11). 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 Gentamicin (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 a untreated patient (15). Cisplatin-resistant cell lines C70, C30, and C200 were produced by intermittent, incremental exposure of the sensitive parental A2780...
increased platinum sensitivity in cisplatin resistance

1. INTRODUCTION

Antisense ERCC1 Expression Vectors. The full-length ERCC1 cDNA contained 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 full linearized pcDNA3.1-antisense ERCC1 plasmid was stably transfected into A2780 and OVCAR10 cell lines by electroporation. Electroporation and selection for G418-resistant clones were 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 manufacturer (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'-CTTGCCTTGGAGCTCCAAGAC-3', and reverse primer, 5'-AGTCTTAGCCCTTATGTCGCGC-3'. The antisense ERCC1 RNA molecules were amplified with primers of 5'-ACAAATCCTTGGAGCTCCAAGAC-3' and 5'-CACAAGATGCCTGTCACACA-3'. The antisense ERCC1 RNA molecules were used as an internal control in RT-PCR reaction with primers 5'-CCATCGCTCAGACATCAT-3' and 5'-CTCTCATGACGCTGATCCACAC-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 in well/well plates (Costar, Coning Inc., Rochester, NY). After incubation overnight, cisplatin was added in various concentrations. After 72 h, the absorbance at 570 nm was measured using cells 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 PGL-3 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 platination was confirmed by atomic absorption spectrometry.

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.
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 32P-labeled riboprobe. The stable cell lines were established based on the expression of the antisense ERCC1 transcripts (Fig. 2C) 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 IC50 values of 2.28–4.17 μM as compared with 9.52 μM for A2780 control. The empty vector transfectants of A2780 (pcDNA3.1—alone, without any insert) had similar IC50 values to the A2780 control cells. 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

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cisplatin (μM)</th>
<th>Fold Increase</th>
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<tbody>
<tr>
<td>A2780</td>
<td>0.18</td>
<td>1</td>
</tr>
<tr>
<td>Antisense ERCC1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Transfectants</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C-7</td>
<td>0.022</td>
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</tr>
<tr>
<td>C-10</td>
<td>0.0247</td>
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</tr>
<tr>
<td>C-12</td>
<td>0.031</td>
<td>5.8</td>
</tr>
<tr>
<td>C-13</td>
<td>0.093</td>
<td>1.9</td>
</tr>
</tbody>
</table>

*Cytoxicity is expressed as IC50 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.

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

<table>
<thead>
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<th>Cell line</th>
<th>Cisplatin (μM)</th>
<th>Fold Increase</th>
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</thead>
<tbody>
<tr>
<td>OVCAR10</td>
<td>9.52</td>
<td>1</td>
</tr>
<tr>
<td>Antisense ERCC1</td>
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<td>Transfectants</td>
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<td>As-Pooled</td>
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<td>3.9</td>
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</table>

*Cytoxicity is expressed as IC50 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.

In Vivo Sensitive OFVCA10-Antisense-ERCC1 Transfectants to Cisplatin. 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 is shown in Fig. 4, the antisense cell lines had ~2-fold lower capacity to restore luciferase activity 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. The SV40-Luc plasmid was treated with various concentrations of cisplatin and used for host cell reactivation assay. The SV40-Luc plasmid was treated with various concentrations of cisplatin and used for host cell reactivation assay.
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 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 ~3 μM 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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REFERENCES


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