Cisplatin Induction of ERCC-1 mRNA Expression in A2780/CP70 Human Ovarian Cancer Cells*

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ERCC-1 is a critical gene within the nucleotide excision repair pathway, and cells without a functional ERCC-1 do not perform cisplatin-DNA adduct repair. We therefore investigated the cisplatin effect on ERCC-1 mRNA expression in vitro. In response to a 1-h cisplatin exposure, A2780/CP70 human ovarian cancer cells showed a 6-fold increase in steady-state level of ERCC-1 mRNA. This rise was attributable to increased transcription as measured by nuclear run-on assays and a 60% increase in ERCC-1 mRNA half-life. The increase in ERCC-1 mRNA was preceded by a 4–5-fold rise in mRNA expressions of c-fos and c-jun, a 14-fold increase in c-Jun protein phosphorylation, and an increase in in vitro nuclear extract binding activity to the AP-1-like site of ERCC-1. These data suggest that the induction of ERCC-1 expression in A2780/CP70 cells exposed to cisplatin results from two major factors: (a) an increase in the expression of transactivating factors that bind the AP-1-like site in the 5'-flanking region of ERCC-1 and (b) an increase in the level of c-Jun phosphorylation that enhances its transactivation property.

 cis-Diaminedichloroplatinum (II) (cisplatin)1 is one of the most widely used chemotherapeutic agents for the treatment of human ovarian cancer and other tumors (1–4). However, the efficacy of cisplatin is hampered by intrinsic or acquired resistance of cancer cells to its cytotoxicity. Although the mechanism of cisplatin resistance in vivo is not clearly understood, laboratory studies on tumor tissues and cell lines suggest that resistance to cisplatin is nearly always multifactorial (5–7). These factors include impaired cellular uptake of cisplatin (5, 7), enhanced intracellular detoxification by glutathione and metallothionein systems (5, 8–10), altered patterns of DNA platinatation (11, 12), increased tolerance of platinum-DNA damage (11, 12), and enhanced repair of DNA damage (5–7, 13).

The cellular toxicity of cisplatin occurs primarily through its ability to bind covalently to DNA and prevent DNA replication and transcription (3, 14). Cisplatin reacts with DNA to form intrastrand and/or interstrand cross-links of platinum adducts (3). Cells exposed to cisplatin must either repair or tolerate the DNA damage if they are to survive. A percentage of platinum-DNA lesions formed in vivo are repaired by human cells.

Nucleotide excision repair (NER) appears to be responsible for the repair (15–19) since repair-defective cells are hypersensitive to the drug (20, 21), and enhanced DNA repair has been implicated in the cisplatin-resistance phenotype (7, 13, 22). Furthermore, increased removal of cisplatin-induced interstrand and intrastrand adducts have been reported in laboratory-derived cisplatin-resistant sublines (7, 23). Increased gene-specific repair of cisplatin interstrand cross-links may be associated with resistance in Chinese hamster ovary cells (17) as well as in human ovarian cancer cells (18). Human excision-repair gene ERCC-1 (excision repair cross-complementation group 1) is one of the critical repair genes in NER (15, 16, 20, 23–26). Overexpression of ERCC-1 and other NER genes has been associated with repair of cisplatin-induced DNA damage (15–18) and clinical resistance to cisplatin (25). In contrast, the levels of expression of ERCC-1 in cisplatin hypersensitive, repair-deficient cells are 50–to 30-fold lower than in inherently resistant cells (26).

The 414-bp sequence in the 5'-flanking region of the ERCC-1 gene has been studied in detail by Hoeijmakers and colleagues (27, 28), who have shown that constructs of this region may drive transcription of ERCC-1. Classical promoter elements like CAAT, TATA, and GC boxes are absent from the −1 to −170-bp portion of this region (28), although an AP-1-like site exists further upstream (Fig. 1). We have previously demonstrated that there are elevated levels of ERCC-1 mRNA in ovarian cancer tissues of patients clinically resistant to platinum compounds (25). However, the fundamental molecular basis of transcriptional activation and regulation of ERCC-1 expression is not well elucidated.

The AP-1 (activator protein 1) family is a group of transcription factors responsible for the activation of a wide variety of genes in different cell types and tissues (29–31). The AP-1 transcription factor consists of either heterodimers formed between Jun and Fos family members of proto-oncoproteins or homodimers of Jun proteins (29–31). AP-1-binding sites (5'-TGGTCA-3') are frequently found in promoters or enhancers of genes that are inducible by a wide range of extracellular signals. Evidence showed that cisplatin induced expression of proto-oncogenes c-fos/c-jun (32, 33) and activated c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) (34, 35) in ovarian cancer cells and other tumor cells. JNK/SAPK is a subfamily of MAP kinases in the Ras pathway which

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1 The abbreviations used are: cisplatin, cis-diaminedichloroplatinum (II); NER, nucleotide excision repair; ERCC-1, excision repair cross-complementation group 1; AP-1, activator protein 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; JNK/SAPK, c-Jun NH2-terminal kinase/stress-activated protein kinase; MTT, microculture tetrazolium; EMSA, electrophoretic mobility shift assay; CREB, cAMP response element-binding protein; bp, base pair(s).
is responsible for the phosphorylation of Jun protein. Phosphorylation of the c-Jun at serine residues 63 and 73 in its NH2-terminal domain greatly enhances the transcriptional activity of the AP-1-binding sites (36–38) and AP-1-regulated genes (39–41). Therefore, it is possible that the effect of cisplatin on ERCC-1 could be through AP-1 induction or c-Jun phosphorylation. Because methodological problems would limit investigations of mechanism in ovarian cancer tissues taken from patients, we have now conducted studies in the human ovarian cancer cell line, A2780/CP70, to investigate these possibilities.

EXPERIMENTAL PROCEDURES

Cell Line and Cell Culture Conditions—The human ovarian cancer cell line A2780/CP70 has been described previously (42) and were used in all experiments. Cells were cultured in monolayer using RPMI 1640 media supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 0.2 units/ml human insulin, 150 units/ml penicillin, 50 μg/ml streptomycin (Life Technologies, Inc., Gaithersburg, MD). Cells were grown in logarithmic growth at 37 °C in a humidified atmosphere consisting of 5% CO2, 95% air. Cells were routinely tested for mycoplasmal infection using a commercial assay system (MycoTect; Life Technologies, Inc.), and the results reproduced in two or more separate experiments. Equal viability was determined in triplicate by trypan blue dye exclusion. Before starting the experiments, the cells were

Preparation of cDNA Probes—A 1.05-kilobase cDNA probe for human ERCC-1 was obtained from Dr. Aziz Sancar (University of North Carolina, Chapel Hill, NC). A second cDNA for human GAPDH was obtained from Dr. Mitchell Olman (University of California, San Diego, CA). The c-fos and c-jun probes were obtained commercially from Oncogene Research Products (Cambridge, MA). cDNA inserts were excised using appropriate restriction enzymes, isolated by electrophoresis through 1% agarose onto DEAE-membrane (NA-45, Schleicher & Schuell Inc.), and purified by using the GeneClean II Kit (BIO 101 Inc., La Jolla, CA). cDNA was labeled with 32P using a commercial random primer kit (Life Technologies, Inc.) according to the manufacturer’s instructions.

Measurement of Transcription Rate—ERCC-1 transcription rate was measured using a modification of previously described nuclear run-on analysis (46). A2780/CP70 cells grown to ~90% confluence were lysed in 10 ml of lysis buffer, containing 10 μM Tris (pH 8.0), 2.5 mM MgCl2, 0.25% Triton X-100, 0.3 mM succrose, and 1 mM dithiothreitol, and nuclei were collected by centrifugation for 5 min at 500 × g. Isolated nuclei were incubated with 250 μCi of [α-32P]UTP (NEN Life Science Products, Wilmington, DE) for 30 min at 37 °C. cDNA for ERCC-1 and GAPDH, or vector DNA (the plasmid without ERCC-1 cDNA insert) (5 μg) in a run-on assay was heat denatured and transferred to supported nitrocellulose (Life Technologies, Inc.) by vacuum filtration using a 24-well manifold (Hybri-Dot; Life Technologies, Inc.). The membrane was rinsed with 6 × sodium chloride-sodium citrate (SSC), air dried, and baked at 80 °C for 2 h in a vacuum oven. Membranes were prehybridized for 1 h at 42 °C in 50% formamide, 6 × SSC, 5 × Denhardt’s solution, 0.5% sodium dodecyl sulfate, and 100 μg/ml denatured salmon testes DNA and then hybridized at 42 °C for 3 days in prehybridization buffer containing run-on reaction mixtures adjusted to equalize radioactivity added in all reactions. Membranes were extensively washed with increasing stringency and then treated with 1 μg/ml ribonuclease for 30 min at 37 °C. Membranes were air dried, exposed to XAR-5 film, and quantitation of the results was achieved by densitometric scanning normalized to the signal for GAPDH.

Measurement of mRNA Stability—A standard technique for measuring stability of labile transcripts was used (47). Cells were incubated with fresh drug-free medium for 36 h following treatment with 40 μM cisplatin for 1 h or left without treatment as control, after which α-amanitin (5 μg/ml) or actinomycin D (5 μg/ml) was added to the treatment conditions. Total RNA was isolated at the time of α-amanitin or actinomycin D addition or at different times thereafter. Northern blot analysis was performed to determine mRNA levels.

Whole Cell Extract Preparation and Western Immunoblot Analysis—To prepare whole cell lysates, 2 × 107 cells were washed 3 times in ice-cold phosphate-buffered saline, and resuspended in 500 μl of buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.25% sodium deoxycholate, 1% Nonidet P-40, 1 mM EDTA, 1 μM leupeptin, 1 μM pepstatin, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM orthovanadate, 1 mM sodium fluoride at 4 °C. Lysates were sheared through a 21-gauge needle and clarified at 4 °C by microcentrifugation. Protein content in the supernatants was determined by means of the BCA protein assay (Pierce, Rockford, IL) using bovine serum albumin as the standard.

The cell extract proteins (25 μg) were loaded on a 12% Tris glycine gel (Novex, San Diego, CA), electrophoresed, and transblotted to a Protran (pure nitrocellulose) membrane (Schleicher & Schuell) by the procedure described by Towbin et al. (48). The blot was then rinsed with TBS, and incubated with a blocking buffer (1 × TBS, 0.1% Tween 20 with 5% (w/v) nonfat dry milk) overnight at 4 °C. After rinsing with TBS/Tween (0.1% Tween) three times, the blot was incubated with phosphorylated Jun (Ser73) antibodies (1:1,000 in TBS/Tween with milk) overnight at 4 °C on a shaker. The blot was washed three times with TBS/Tween, and incubated with a 1:2,000 dilution of secondary antibody coupled with horseradish peroxidase for 2 h at room temperature on a shaker. The blot was washed three times with TBS/Tween and then incubated with a 1:2,000 dilution of secondary antibody coupled with horseradish peroxidase for 2 h at room temperature on a shaker. The blot was washed three times with TBS/Tween and then incubated with ECL Western blotting detection reagents (Amersham) for 1 min at room temperature. The results were visualized by autoradiography and quantitated by densitometric scanning.

Preparation of Nuclear Extracts—Nuclear extracts were prepared from resting or cisplatin-treated A2780/CP70 cells by a modification of the procedure described by Dignam et al. (49). Cells were harvested by scraping and washed once with ice-cold phosphate-buffered saline. The cells were then resuspended in 1.5 volumes of lysis buffer (70 mM KCl, 1.5 mM MgCl2, 0.5 mM sodium orthovanadate, 0.4 mM sodium...
fluoride, 0.5 mM phenylmethylsulfonyl fluoride, 1.0 mM dithiothreitol, 25 mM HEPES, pH 7.5). The mixture was incubated on ice for 20 min and then extracted by adding 1.6 volumes of extraction buffer (0.5 mM EDTA, 20% glycerol, 1.66 mM KCl, 0.4 mM sodium fluoride, 0.4 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1.0 mM 1,1,1-trichloro-2,2-bis(p-chlorophenyl)ethane, 25 mM HEPES, pH 7.5) with constant shaking at 4°C for 4 h. Samples were centrifuged at 55,000 × g for 1 h at 4°C, and the supernatant was dialyzed at 4°C for 4 h in a buffer containing 20 mM HEPES (pH 7.5), 50 mM KCl, 0.1 mM EDTA, 10% glycerol, 0.4 mM sodium fluoride, 0.4 mM sodium orthovanadate, 0.1 mM phenylmethylsulfonyl fluoride, 1.0 mM dithiothreitol. Samples were stored at −80°C. Protein content was determined by the BCA protein assay (Pierce).

Preparation of Oligonucleotide Probes—The oligonucleotide sequence used in the following electrophoretic mobility shift assay was based on sequence analysis of the 5′-flanking region of ERCC-1 gene as described previously (28). Two duplex 21-mer oligonucleotides which encompassed a ERCC-1 AP-1-like site (5′-TACCTGCTGTCACCGC-3′), within −355 to −375 from the transcriptional start site at +1 in the ERCC-1 promoter region) (see Fig. 1) and an altered ERCC-1 AP-1-like site (5′-TACCTGCTGACCGC-3′, −355 to −375) were synthesized by Lofstrand Labs Limited (Gaithersburg, MD) and purified by reverse-phase cartridge chromatography. The altered ERCC-1 AP-1-like site contains a consensus AP-1 site produced by a 1-bp substitution with changes in the steady-state levels of GAPDH mRNA in A2780/CP70 cell nuclei. As seen in Fig. 3, the transcription rate for a given probe was increased by about 6-fold over the corresponding transcription factor and do not interfere with nuclear factor binding.

RESULTS

Up-regulation of ERCC-1 Gene Expression by Cisplatin and Modulation by Cycloheximide and α-Amanitin—The A2780/CP70 cell line was treated with 40 μM cisplatin for 1 h, and the expression of ERCC-1 mRNA was measured at various time points following drug exposure. Cisplatin caused time and dose-dependent increases in ERCC-1 mRNA levels (51). Northern analysis showed that ERCC-1 mRNA accumulation was increased by more than 2-fold as early as 6 h after incubation with 40 μM cisplatin and eventually attained a peak level of 5.5-fold increase at 24–48 h after cisplatin administration. Dose-response experiments showed that the effect of cisplatin was maximal at 40–80 μM with about a 6-fold increase in the ERCC-1 mRNA level. The ERCC-1 increase was not associated with changes in the steady-state levels of GAPDH mRNA in A2780/CP70 cells.

We next examined the importance of protein synthesis in the observed increases in ERCC-1 mRNA. The effect of cycloheximide (a protein synthesis inhibitor) was investigated. When the cells were cultured in the presence of cycloheximide (10 μM) after a 1-h exposure to cisplatin, the expected increase in ERCC-1 mRNA did not occur (Fig. 2, lanes 2 and 5). Cycloheximide alone had little effect on baseline ERCC-1 mRNA levels (Fig. 2, lanes 1 and 6), suggesting that on-going protein synthesis is required for cisplatin-dependent ERCC-1 mRNA induction. To determine whether the cisplatin effect on ERCC-1 expression was at the transcriptional level, cisplatin-induced cells were cultured with the transcriptional inhibitor α-amanitin at a concentration of 5 μg/ml. Again the expected increase in ERCC-1 was not seen (Fig. 2, lane 3). The same results were observed when another transcriptional inhibitor (actinomycin D) was used at a concentration of 5 μg/ml (data not shown). Moreover, α-amanitin repressed the basal expression of ERCC-1 mRNA (Fig. 2, lane 4). These data suggest that ERCC-1 mRNA induction by cisplatin is transcriptionally regulated. Since α-amanitin has been shown to be a specific inhibitor for RNA polymerase II (52), this suggests that these genes are normally transcribed by RNA polymerase II.

Transcriptional Regulation of the ERCC-1 Gene by Cisplatin—To determine more directly whether cisplatin can induce the transcription of the ERCC-1 gene, in vitro transcription elongation (nuclear run-on) assays were performed using purified A2780/CP70 cell nuclei. As seen in Fig. 3, the transcription rate more than doubled by 2 h after incubating A2780/CP70 cells with 40 μM cisplatin. Transcription activity increased to about 4 times control (untreated cells) by 4 h and decreased gradually afterward, but still remained at a relatively higher level for up to 12 h of incubation after cisplatin. No signal was detectable when the “run-on” analysis was carried out using the plasmid vector, which is serving as a specific binding control for ERCC-1 cDNA in this experiment (Fig. 3). Transcription of the ERCC-1 gene in these assays was blocked by 5 μg/ml α-amanitin (data not shown).

Effect of Cisplatin on the Turnover of ERCC-1 mRNA—It is possible that the increase in the mRNA level reflects a decreased rate of mRNA degradation. This possibility is supported by the experiment shown in Fig. 4, in which the half-life of ERCC-1 mRNA, as determined by α-amanitin chase (5 μg/ml), is seen to be prolonged by cisplatin. Panel A shows ERCC-1 mRNA levels after cisplatin treatment; panel B shows ERCC-1 mRNA levels under control conditions; and panel C shows a
Numerical analysis of the rate of ERCC-1 decay in panels A and B. The $t_{1/2}$ of ERCC-1 mRNA is 23 ± 1 h in the presence of cisplatin, as compared with a $t_{1/2}$ of 15 ± 1 h in the absence of cisplatin. This difference of 8 h represents a 62% increase in mRNA half-life. The cisplatin-stimulated increase in the half-life of ERCC-1 mRNA was observed in similar experiments where actinomycin D (5 μg/ml) was used as the transcriptional inhibitor (data not shown). Thus, it appears that both increased ERCC-1 gene transcription and increased ERCC-1 mRNA stability underlie the increased steady-state ERCC-1 mRNA levels in cisplatin-treated A2780/CP70 cells.

Induction of Nuclear Factor AP-1 Binding Activity by Cisplatin Exposure—As a step toward determining how the ERCC-1 gene is regulated, we examined the effect of cisplatin on the expression of AP-1 in A2780/CP70 cells. As shown in Fig. 5, cisplatin exposure induced transient expression of c-fos and c-jun mRNA in a time-dependent manner with peak levels of 5-fold increase at 1–2 h in this system.

We next determined whether cisplatin exposure induced an increase in c-Jun protein content or its level of protein phosphorylation in these cells, by Western blot (immunoblot) (Fig. 6). Consistent with the Northern (RNA) analysis (Fig. 5), expression of c-Jun protein was evident at 1 h and peaked at 3 to 5 h with an approximate 3.5-fold increase after the cisplatin exposure (Fig. 6). Similarly, levels of protein phosphorylation of c-Jun at serine residues 63 and 73 increased also under the same conditions (Fig. 6). However, the change was much more pronounced. Cisplatin caused a 3.5-fold increase in c-Jun protein level, but a 14-fold increase in c-Jun phosphorylation (Fig. 6, panels B and C).

On the basis of these findings, it was of interest to examine whether cisplatin treatment may result in altered amounts of transcription factors capable of binding the AP-1 site. We analyzed the 5’-flanking region of the ERCC-1 gene (28) and found that it contained one region with high homology to AP-1-binding sites. An AP-1-like site located between −361 and −367 bp in the ERCC-1 promoter consisted of TGTGTCA, which is shown schematically in Fig. 1. No consensus sequence for AP-2, CREB, or NFκB was found in this region. We initially evaluated the AP-1 element and its transcription factor. We constructed two oligonucleotides of 21 bp (−355 to −375), as described above, encompassing this potential binding region and performed EMSA with these oligonucleotides. Induction of AP-1 nuclear factor binding to ERCC-1 AP-1-like site (TGTGTCA) was found as an early event immediately after cisplatin exposure, and binding activity peaked at 3 h and decreased to the baseline level at about 8 h (Fig. 7). The same results were observed with a ERCC-1 AP-1-like site was altered to contain a consensus AP-1 site (TGAGTCA) and an oligonucleotide which contained the consensus AP-1 sequence (5’-CGCTTGA-GAGTCAGCCCGGA-3’) of the collagenase promoter (Promega) were utilized in this experiment (data not shown).
consensus sequence for AP-1 as a competitor in the EMSA. This oligonucleotide at 50-fold excess abolished binding to the experimental 32P-labeled AP-1-like site (Fig. 7). Same results were observed when a 21-bp oligonucleotide containing the ERCC-1 AP-1-like site was used as a competitor at identical concentrations (Fig. 7). In contrast, TFIID (Fig. 7) or other oligonucleotides used included AP-2, CREB, and NFκB did not (data not shown).

To characterize further the proteins binding to the AP-1-like site in cisplatin-treated cells, we used supershift assays with antibodies specific to Fos, Jun, and AP-2. The results (Fig. 8) show that antibodies to Fos or Jun, respectively, cause an altered gel migration pattern for the AP-1 DNA complex (the two far right lanes). Antibody to AP-2 protein does not cause such a supershift. Data presented in Fig. 8 confirm the data above, suggesting that AP-1 is the relevant DNA binding factor which is composed of Fos/Jun heterodimers. Collectively, these results suggest that both increased AP-1 levels and increased levels of Jun phosphorylation contribute to the increased transactivation of ERCC-1 expression in cisplatin-treated A2780/CP70 human ovarian carcinoma cells.

**DISCUSSION**

ERCC-1 is a single-stranded DNA endonuclease (53) which forms a tight heterodimer with xeroderma pigmentosum complementation group F (54, 55). Its role in NER is to incise DNA on the 5' side of a lesion such as platinum-DNA adduct (53–55). Therefore, overexpression of ERCC-1 and other NER enzymes during ovarian cancer chemotherapy with cisplatin appears to be implicated in the formation of cellular and clinical drug resistance (25). By contrast, repair of cisplatin-DNA adduct does not occur in the absence of a functional ERCC-1 (15). Thus, understanding the mechanism of regulation and control of ERCC-1 expression in ovarian tumors is of pathophysiological importance. In the present study, we show that cisplatin induces ERCC-1 up-regulation in the A2780/CP70 human ovarian carcinoma cell line and that this induction by cisplatin is regulated at both the transcriptional and post-transcriptional levels. Our results also indicate that the binding activity for transcription factor AP-1 is induced in response to cisplatin in our system.

Protein kinases regulate signaling pathways for a broad spectrum of cellular processes, including differentiation, oncogenesis, and damage response (56). There is considerable evidence that one or more protein kinases are involved in the
A2780/CP70 cell nuclear extracts to experimental ERCC-1 cisplatin-treated and untreated A2780/CP70 cells. Cells were incubated with 80 μM cisplatin for 1 h. Fresh medium was then replaced, and nuclear extract was prepared from cells harvested at different times after drug removal. EMSAs were performed by using a 32P-labeled synthetic double-stranded oligonucleotide containing the ERCC-1 promoter sequence of AP-1-like binding site as probe. The protein-DNA complexes formed are indicated (arrow). The unbound probe in the gel is indicated at the bottom. Cisplatin-inducible DNA binding activity at 1 to 3 h following the drug exposure was abolished by competition with a 50-fold molar excess of unlabeled natural AP-1 oligonucleotide (lanes labeled AP-1 competitor) and by an unlabeled oligonucleotide containing the ERCC-1 promoter sequence of AP-1-like binding site (lanes labeled AP-1-like competitor), but not by an identical concentration of TFII D oligonucleotide (lanes labeled TFII D competitor).

FIG. 7. EMSA of AP-1 binding activity in nuclear extracts from cisplatin-treated and untreated A2780/CP70 cells. Cells were incubated with 80 μM cisplatin for 1 h. Fresh medium was then replaced, and nuclear extract was prepared from cells harvested at different times after drug removal. EMSAs were performed by using a 32P-labeled synthetic double-stranded oligonucleotide containing the ERCC-1 promoter sequence of AP-1-like binding site as probe. The protein-DNA complexes formed are indicated (arrow). The unbound probe in the gel is indicated at the bottom. Cisplatin-inducible DNA binding activity at 1 to 3 h following the drug exposure was abolished by competition with a 50-fold molar excess of unlabeled natural AP-1 oligonucleotide (lanes labeled AP-1 competitor) and by an unlabeled oligonucleotide containing the ERCC-1 promoter sequence of AP-1-like binding site (lanes labeled AP-1-like competitor), but not by an identical concentration of TFII D oligonucleotide (lanes labeled TFII D competitor).

FIG. 8. Supershift assay of binding activity in cisplatin-treated A2780/CP70 cell nuclear extracts to experimental ERCC-1 AP-1-like binding site. The use of anti-Fos or anti-Jun before EMSA is seen to result in a super-retardation in band mobility consistent with the formation of protein-antibody (Ab)-DNA complexes (arrow with AP-1-Ab/DNA complex), whereas a supershift in band mobility is not seen when anti-AP-2 antibody was used before EMSA (lanes labeled anti-AP-2).

activation of gene expression in response to DNA damage, and JNK appears to be one such kinase.

JNK is part of a protein kinase cascade in the Ras pathway that eventually leads to increased phosphorylation of the transcription factor c-Jun. Recent work has shown that cellular damage induced by DNA damaging agents, including UV-C radiation (39), ionizing radiation (57), alkylating agents (39, 40, 58), and cisplatin (34, 35), results in activation of the JNK/SAPK pathway involving the transcription factor AP-1. This signal transduction pathway has been reported to protect against cisplatin-induced DNA damage and that this response is required for DNA repair and survival following cisplatin treatment (34). Furthermore, inhibition of this pathway in cells modified by overexpression of a dominant negative mutant of c-Jun, blocks DNA repair, and leads to decrease in viability following treatment with cisplatin (34). These reports suggest that the Ras/JNK pathway may mediate a physiological response to DNA damage such as induction of one or more DNA repair enzymes. However, numerous protein constituents of a complex enzyme system are likely involved in NER, and which of these constituents might be the downstream targets of Ras/JNK cascade-dependent events and mediate protection against cisplatin-induced damage are currently not known.

In this study, we found that cisplatin treatment of A2780/CP70 cells led to an increase in ERCC-1 mRNA expression and in nuclear AP-1 binding activity in a time- and dose-dependent manner. In vitro run-on assays in nuclei isolated from control and cisplatin-stimulated A2780/CP70 cells revealed that the appearance of ERCC-1 mRNA occurred in large part because of an increase in the level of de novo transcription. The induction of AP-1 binding activity was selective, insofar as basal DNA binding activities for the inducible transcription factors AP-2, CREB, and NFκB were not affected by cisplatin at the doses used and the time points examined in this study. Although our analysis of trans-acting DNA-binding proteins is limited, these findings suggest that cisplatin has a differential effect on the inducible transcription factor activity, and AP-1 is involved, at least partially, in the transactivation of the ERCC-1 gene expression in our system. Evidence for the presence of Jun and Fos proteins in cisplatin-induced AP-1 binding activity was demonstrated by highly specific anti-c-Jun and anti-c-Fos antibodies in supershift analysis of the DNA binding activity in nuclear extracts from A2780/CP70 cells exposed to cisplatin. The ability of Jun/AP-1 to activate transcription is modulated through phosphorylation of the NH2-terminal transactivating domain by the JNK. We suspected, therefore, that JNK activity might be affected by cisplatin. In support for this hypothesis, phosphorylation of the JNK substrate c-Jun at serine residues 63 and 73 was rapidly increased following cisplatin exposure. Thus, Jun protein activity is both transcriptionally and post-translationally activated in cisplatin-treated cells.

Overexpression of Ras proteins has been associated with resistance to chemotherapeutic agents and radiation (59–61). Sklar (59) initially reported that NIH3T3 cells transfected by either the normal or mutant c-Ha-ras oncogene were significantly more resistant to cisplatin than control cells. This study has been confirmed in cisplatin-resistant human cells in vitro and from patients (60, 62, 63). Moreover, protein kinase inhibitors are able to enhance the cytotoxicity of cisplatin. Although the detailed mechanisms responsible for these resistance phenomena are not entirely clear, evidence is accumulating that Ras transactivates a set of genes coding for enzymes or proteins involved in DNA repair and drug detoxification which affect the sensitivity of cells to cisplatin. These proteins may include AP-1, the products of c-jun and c-fos genes (64), and possibly some proteins in NER. This is supported by the evidence that overexpression of wild-type c-Jun is associated with cisplatin resistance (34), whereas dominant negative Jun expressing cells were inhibited in repair of cisplatin adducts and are sensitized to the cytotoxic effects of cisplatin (34). In addition, increased levels of xeroderma pigmentosum complementation group A and ERCC-1 have been correlated to cis-platinum

*Q. Li and E. Reed, unpublished observations.*
resistance (25). Our data in this work suggest that AP-1 may directly modulate ERCC-1 gene expression through some specific targets of its cis-regulatory elements. That would place this critical NER protein under the influence of the Ras/JNK pathway of signal transduction. This potentially opens the door for molecular based strategies to control or modulate DNA repair activity through direct influence on this specific signal pathway, or through direct influence on AP-1.

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