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Advances in Brief

BRCA1 Up-Regulation Is Associated with Repair-mediated Resistance to *cis*-Diamminedichloroplatinum(II)[1]

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Abstract

We sought to identify novel genes associated with *cis*-diamminedichloroplatinum(II) (CDDP) resistance, and by differential display analysis, we found that the human breast and ovarian cancer susceptibility gene *BRCA1* was overexpressed in CDDP-resistant MCF-7 cells. A recent report that BRCA1 and human Rad51 colocalize in S-phase cells suggests a role for BRCA1 in DNA damage repair. We hypothesized that *BRCA1* plays a role in DNA damage repair-mediated CDDP resistance. In CCDP-resistant variants of breast and ovarian carcinoma cell lines, MCF-7 CDDP/R and SKOV-3 CDDP/R, we found increased levels of BRCA1 protein, and we determined that the SKOV-3 CDDP/R cell line is significantly more proficient at DNA damage repair. Antisense inhibition of BRCA1 in this cell line resulted in an increased sensitivity to CDDP, a decreased proficiency of DNA repair, and an enhanced rate of apoptosis. These data support the hypothesis that *BRCA1* is a gene involved in DNA damage repair.

Introduction

The human breast and ovarian tumor suppressor gene *BRCA1* was cloned in 1994 (1), but the function of the BRCA1 protein product remains unknown. In breast and ovarian cancers, *BRCA1* mutations are found in 50–80% of familial cancers but in only a small percentage of sporadic tumors (2). A recent report has shown that BRCA1 and human Rad51 colocalize in S-phase cells and interact physically (3). Rad51 is one of several key components of the double-strand-break DNA repair pathway (4), and the strong association found between Rad51 and BRCA1 supports a role for BRCA1 in DNA damage repair. Cells exposed to CDDP[3] undergo cell cycle arrest and subsequently either repair CDDP-induced DNA damage or undergo programmed cell death (5). We hypothesized that *BRCA1* might play a role in CDDP resistance, and loss of *BRCA1* function might increase CDDP cytotoxicity. In CDDP-resistant breast and ovarian carcinoma cell lines, we found increased levels of BRCA1 protein in association with Pt exposure and acquired CDDP resistance. Antisense suppression of endogenous BRCA1 resulted in a reduced proficiency of repair of damaged DNA, a more CDDP-sensitive phenotype, and an enhanced rate of apoptosis. Our data support the hypothesis that *BRCA1* may be a gene involved in DNA damage repair.

Materials and Methods

Cell Culture. The human ovarian cancer cell line SKOV-3 and the human breast cancer cell line MCF-7 were obtained from American Type Culture Collection. Cells were maintained in DMEM supplemented with 10% (v/v) FCS, 200 mM glutamine, 0.24 unit/ml insulin, 100 units/ml penicillin, and 100 μg/ml streptomycin. The *BRCA1* gene was sequenced in SKOV-3 and SKOV-3 CDDP/R by Myriad Genetic Laboratories, Inc. (Salt Lake City, UT), and no mutations were detected. The MCF-7 cells have been reported by others to be wild type, but the *BRCA1* gene was not sequenced in this cell line as part of these studies.

Cytotoxicity Assays. Cells were plated in 96-well culture plates and treated with varying concentrations of CDDP. Plates were incubated at 37°C for 5 days, after which cell viability was measured using the Alamar blue vital dye indicator assay (6). Dose-response curves were plotted as surviving percentage *versus* drug concentration, in which the surviving fraction was determined by comparing the growth of drug-treated cells to untreated controls (each value is the mean of eight determinations). The IC_{50} was determined from a least squares regression fit to the linear portion of the dose-response curve. Curves were compared statistically by permutation-based nonparametric testing.

Northern Blot Analysis. Total RNA was prepared by lysing cell monolayers in guanidine isothiocyanate and centrifuging over a cesium chloride cushion. Twenty μg of RNA were electrophoresed on denaturing formaldehyde gels and transferred to Nylon membrane (Magna Graph. Micron Separation, Inc., Westborough, MA). The probe used for the Northern blot was a 1.1-kb fragment of BRCA1 exon 11 cDNA obtained by PCR amplification using primers 5′-CACTCTAGCGAAGCAAAAC-3′ (forward) and 5′-CCCCTAATCTAAGCATAGCAT-3′ (reverse) and a β-actin cDNA fragment from American Type Culture Collection. All probes were labeled with [α-^{32}P]dCTP using a random-primed labeling kit (Amersham Life Science, Arlington Heights, IL).

Western Blot Analysis. Whole-cell protein extracts were separated by SDS-PAGE using standard procedures, and BRCA1 was detected with commercially available antibodies (D-20; Santa Cruz Biotechnology, Santa Cruz, CA). Antibody reaction was revealed using enhanced chemiluminescence reagents (DuPont New England Nuclear, Boston, MA).

Assay for Reactivation of Damaged Plasmid DNA. The degree of luciferase activity was used as a measure of the proficiency of DNA repair after transfection with a luciferase expression vector, pRL-CMV (Promega, Madison, WI), treated with different amounts of CDDP to produce stoichiometric numbers of Pt-DNA adducts (16 and 32). The untreated plasmid and the CDDP-treated plasmid were transfected into cell lines, and 48 h after transfection, cells were harvested, and extracts were assayed for luciferase activity using the luciferase assay kit (Promega). The control unplatinated plasmid luciferase activity was defined as 100%, and the transfection efficiency of unplatinated plasmid was equivalent in all cell lines (six replicates of all conditions were assayed). A resampling scheme (bootstrap) was used to estimate the confidence intervals for the proportions as well as test the differences for significance.

Construction of Antisense and Sense Vectors. A BRCA1 antisense expression vector was constructed by cloning an *Eco*RI/*Bam*HI fragment of *BRCA1* cDNA (bases 1026–4058) in the antisense direction into the multiple cloning site of the CMV-driven Neo-resistant mammalian expression vector pcDNA3 (Invitrogen, Inc., San Diego, CA). A sense control construct was similarly made by cloning the *BRCA1* exon 11 cDNA segment including bases 1026–3996 into pcDNA3 in the sense direction. These vectors were transfected into the SKOV-3 CDDP/R cell line using the *N*-[1-(2,3-dioleoyloxyl) propyl]-*N,N,N*-trimethylammoniummethyl sulfate reagent per the manufacturer's instructions (Boehringer Mannheim Corp., Indianapolis, IN). G418-

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[3] The abbreviations used are: CDDP, *cis*-diamminedichloroplatinum(II); Pt, platinum.

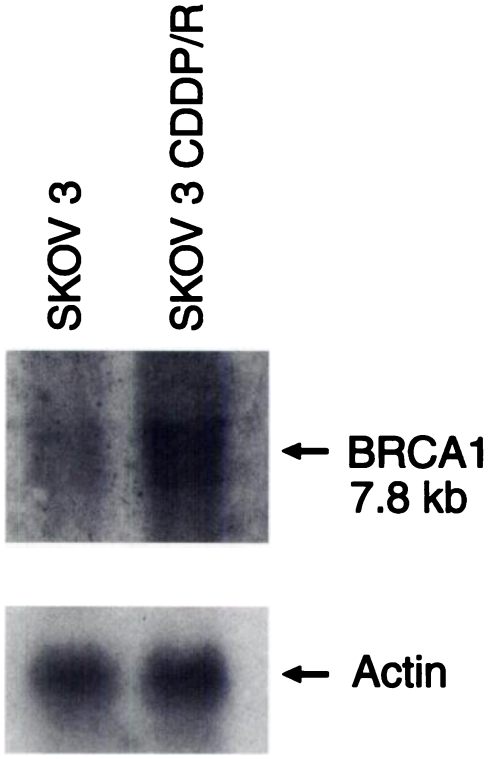

Fig. 1. Northern blot analysis of total RNA extracted from SKOV-3 and SKOV-3 CDDP/R cells probed with ^{32}P-labeled BRCA1 exon 11 cDNA shows increased mRNA levels in the CDDP-resistant cell line ($P < 0.01$). The membrane was reprobed with ^{32}P-labeled β-actin probe to demonstrate equal loading. The density of bands was quantified using Adobe Photoshop software and normalized to the density of the actin bands, and statistical significance was determined by paired *t* test.

resistant colonies were selected and screened by Western blot for suppression of endogenous BRCA1 protein.

Assessment of Apoptosis. Apoptosis was measured as described previously (7) by quantitative fluorescence microscopy of nuclear changes induced by apoptosis determined by bis-benzimide trihydrochloride (Hoechst 33258) staining of nuclear chromatin. Four hundred cells were counted and scored for the incidence of apoptotic chromatin condensation (done in triplicate). Statistical analysis was by Wilcoxon test.

Results and Discussion

We examined the differences in gene expression associated with CDDP resistance through differential display studies on the human breast cancer cell line MCF-7 and an acquired CDDP-resistant subline, MCF-7 CDDP/R. A number of differentially expressed sequences were identified in this analysis, and one of the sequences found to be increased in the resistant cell line was homologous with published 3′ sequences of *BRCA1*.[4] We investigated the function of BRCA1 in CDDP resistance using ovarian carcinoma cell line SKOV-3 and breast carcinoma cell line MCF-7. Variants with acquired resistance to CDDP of the SKOV-3 and MCF-7 cell lines were developed in our laboratory by stepwise exposure to increasing concentrations of CDDP (SKOV-3 CDDP/R and MCF-7 CDDP/R). The IC_{50}s, as measured by the Alamar blue vital dye indicator assay, are 6-fold and 3.4-fold higher, respectively, for the CDDP-resistant sublines (Table 1). The doubling times of the resistant cell lines differ by less than 10% from those of the parental lines, and the cell cycle distribution of the parental and resistant cell lines is similar.

It was necessary to confirm that the increase in *BRCA1* sequences identified by differential display and the increased levels of *BRCA1*

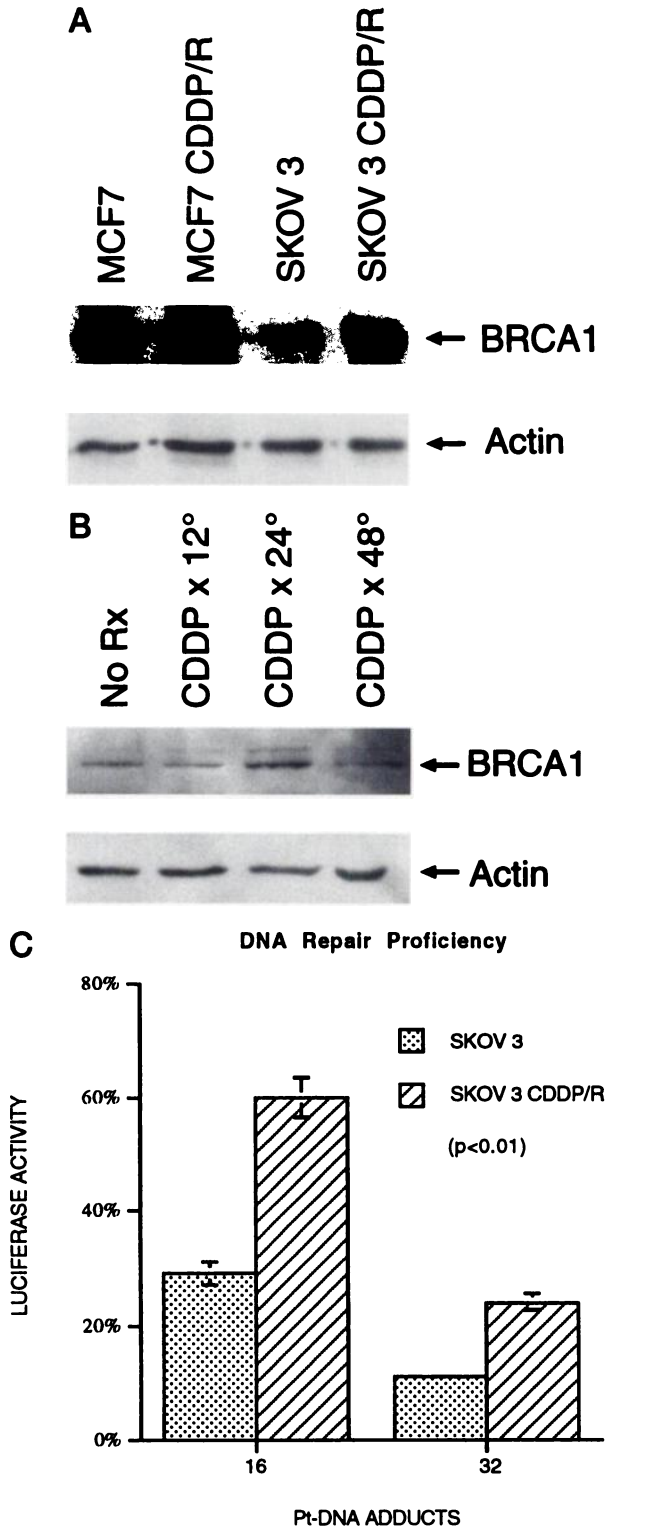

Fig. 2. *A*, CDDP resistance is associated with increased expression of BRCA1 protein as seen by Western blot analysis of BRCA1 protein in SKOV-3 CDDP/R and MCF-7 CDDP/R resistant cell lines as compared to the parental cell lines SKOV-3 and MCF-7 ($P < 0.01$). The density of bands was quantified using Adobe Photoshop software and normalized to the density of the actin bands, and statistical significance was determined by paired *t* test. *B*, acute exposure to CDDP results in a transient increase in BRCA1 protein levels as seen by Western immunoblot of whole-cell extracts from SKOV-3 cells. After exposure to cytotoxic concentrations of CDDP, BRCA1 levels peak at 24 h ($P < 0.01$). The density of bands was quantified using Adobe Photoshop software and normalized to the density of the actin bands, and statistical significance was determined by paired *t* test. *C*, CDDP resistance is associated with an increased proficiency of DNA repair as measured by reactivation of a platinated luciferase-expressing plasmid, pRL-CMV. The CDDP-resistant cell line SKOV-3 CDDP/R has an increased capacity for repair of plasmid DNA with 16 and 32 Pt-DNA adducts/plasmid as compared to the parental SKOV-3 ($P < 0.01$).

[4] Unpublished data.

Table 1 *Cytotoxicity of CDDP in the cell lines studied*

IC_{50} of CDDP in the parental and CDDP-resistant variants of the SKOV 3 and MCF 7 cell lines.

	SKOV-3	SKOV-3 CDDP/R	MCF-7	MCF-7 CDDP/R
IC_{50}	5 μM	30 μM	5 μM	17 μM

mRNA as seen on Northern blot analysis (Fig. 1) were associated with increased protein production. Western immunoblot analysis of whole-cell protein extract using BRCA1 antibody (D-20; Santa Cruz Biotechnology) showed increased levels of full-size BRCA1 protein (Fig. 2*A*) in resistant cell lines SKOV-3 CDDP/R and MCF-7 CDDP/R as compared to parental cell lines SKOV-3 and MCF-7. Thus, it seems that the *BRCA1* gene is transcribed and translated to a significantly greater degree in tumor cells with acquired CDDP resistance.

Because chronic exposure to CDDP increased BRCA1 expression, the effect of acute CDDP treatment on BRCA1 protein levels in the SKOV-3 cells was also examined. Cells were treated with cytotoxic concentrations of CDDP (20 μM), and Western blot analysis was performed on whole-cell extracts obtained at multiple time points after CDDP treatment. In the SKOV-3 cells, BRCA1 protein levels peak after 24 h of treatment and decline by 48 h (Fig. 2*B*); in the SKOV-3 CDDP/R cells, no further increase above the already elevated basal level of BRCA1 was noted (data not shown). In the SKOV-3 cells, CDDP treatment causes an arrest in G_2 that peaks at 48 h from exposure (8). BRCA1 expression has been determined to occur maximally in late G_1 before the phase of increased DNA synthesis in S phase (9). The increased BRCA1 protein level correlates temporally with a period of increased DNA repair that occurs after CDDP-induced DNA damage (10).

In studies of mouse leukemia L1210 cells resistant to CDDP, both repair of genomic intrastrand cross-links and platinated plasmid DNA were increased in the CDDP-resistant L1210 cells (11, 12). The capacity for repair of DNA damage in SKOV-3 cells was assayed by using a plasmid reactivation assay. The luciferase activity in extracts from SKOV-3 CDDP/R transfected with plasmid having 16 Pt-DNA adducts was 60.4% of control, whereas in the SKOV-3 parental cell line, it was only 29.1% of control, and with 32 Pt-DNA adducts, the reactivation rate of the luciferase vector was 24.5% in the CDDP-resistant cell line as compared to 11.7% ($P < 0.01$; Fig 2*C*). The implication is that the SKOV-3 cell line with acquired CDDP resistance is significantly more proficient at repairing an equal number of Pt-DNA adducts than the parental cell line.

To assess the effect of BRCA1 protein expression on CDDP cytotoxicity, an expression vector containing a segment of *BRCA1* exon 11 cDNA cloned in both the sense and antisense directions was stably transfected into the SKOV-3 CDDP/R cell line. Colonies were selected, and a decrease in endogenous BRCA1 protein was confirmed in the antisense transfectants by Western blot analysis (Fig. 3*A*). The two cell lines thus established were SKOV-3 CDDP/R^{AS} and SKOV-3 CDDP/R^{sense}.

The proficiency of DNA repair was compared in these transfected cell lines by using the plasmid reactivation assay. When the *BRCA1* antisense-expressing cell line SKOV-3 CDDP/R^{AS} was transfected with the platinated luciferase expression vector having 16 and 32 Pt-DNA adducts, it was found to be markedly less proficient at repairing damaged plasmid DNA (Fig. 3*B*). In the SKOV-3 CDDP/R^{AS}, luciferase activity was 57% of control as compared to 75% in the SKOV-3 CDDP/R^{sense}. This represents a significant ($P < 0.01$) decrease in the proficiency of DNA damage repair when BRCA1 protein is suppressed in the SKOV-3 CDDP/R cells.

CDDP-induced DNA damage is cytotoxic and leads to the induction of programmed cell death (5). Cytotoxicity assays using these cell lines

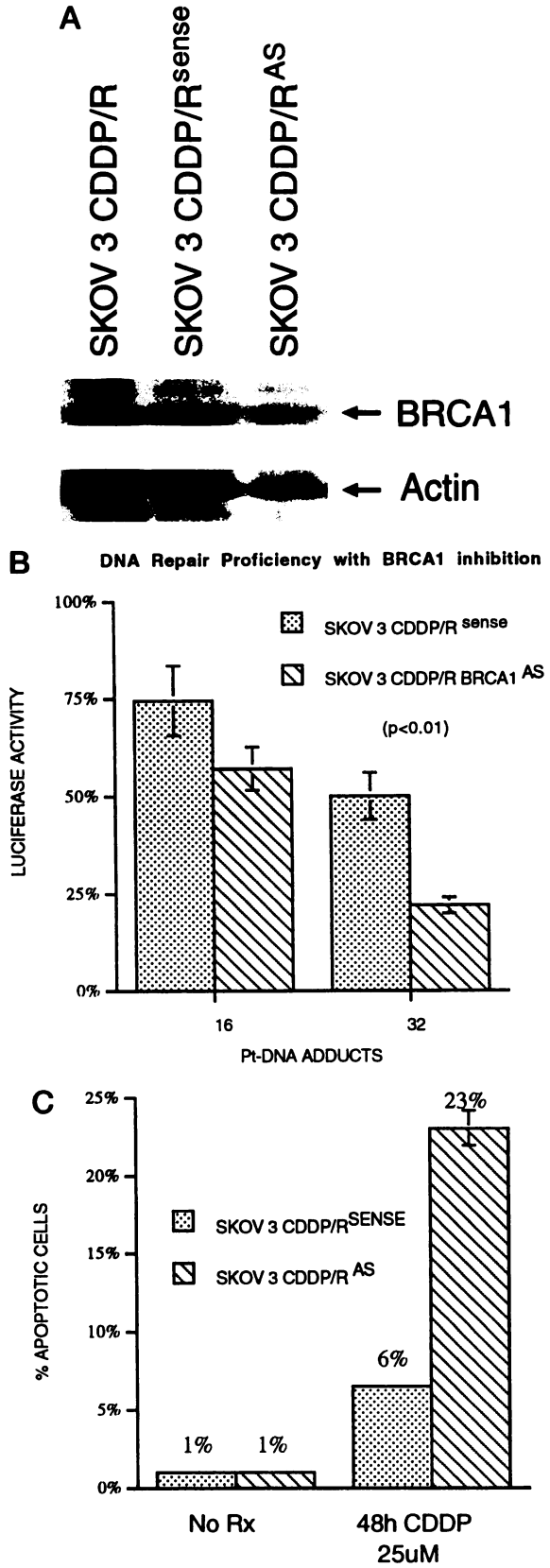

Fig. 3. Suppression of BRCA1 by cloning an antisense construct into the SKOV-3 CDDP/R cell line results in: *A*, decreased protein expression as measured by Western immunoblot analysis of the SKOV-3 CDDP/R^{AS} cell line; *B*, decreased proficiency of DNA repair in the SKOV-3 CDDP/R^{AS} cells in comparison to the control SKOV-3 CDDP/R^{sense} cells as measured by reactivation of a DNA-damaged luciferase-expressing plasmid ($P < 0.01$); and *C*, an increased rate of CDDP-induced apoptosis as measured by quantitative fluorescence microscopy in the SKOV-3 CDDP/R^{AS} cells compared to the SKOV-3 CDDP/R^{sense} cells ($P = 0.02$).

found a statistically significant ($P < 0.01$) increase in sensitivity to CDDP in the SKOV-3 CDDP/R^{AS} cell line, in which BRCA1 protein expression is partially inhibited by antisense sequences. As determined by dose-response curves, the IC_{50} of the antisense-expressing cell line SKOV-3 CDDP/R^{AS} was decreased to 17.5 μM as compared to 21 μM in the control cell line SKOV-3 CDDP/R^{sense} (data not shown). The rate of CDDP-induced apoptosis was compared between the antisense-expressing cell line and the sense-expressing control cell line. Using quantitative fluorescence microscopy, we found an increased rate of apoptosis in the SKOV-3 CDDP/R^{AS} cell line (Fig. 3*C*). In SKOV-3 CDDP/R^{sense} cells, 48 h of exposure to 25 μM CDDP resulted in 6.3% apoptotic cells, whereas in SKOV-3 CDDP/R^{AS} cells, the number of apoptotic cells after 48 h of CDDP was 23%. Thus, it seems that suppression of BRCA1 protein decreases the ability of SKOV-3 cells to repair damaged DNA, and this results in an enhanced sensitivity to the cytotoxic effects of CDDP and a higher rate of apoptotic cell death.

This is the first identification of *BRCA1* as a gene up-regulated in association with acute exposure to CDDP and acquired CDDP resistance. The previously described association between BRCA1 and Rad51 implicates BRCA1 in DNA integrity and damage repair (3). These data support the hypothesis that BRCA1 protein is involved in the repair of CDDP-induced DNA damage. Increased expression of DNA repair genes is known to contribute to the development of clinically significant CDDP resistance in ovarian cancers (13). Suppression of BRCA1 function in SKOV-3 CDDP/R cells reduces the proficiency of DNA damage repair, increases the cytotoxicity of CDDP, and promotes CDDP-induced apoptosis. These observations are consistent with the recent report that ovarian cancer patients with *BRCA1* mutations have a more favorable clinical course with a median actuarial survival of 77 months as compared to 29 months in matched controls (14). *BRCA1* is probably only one of several genes that play a role in the process of CDDP resistance and DNA damage repair, and additional studies are needed to delineate how significant the role of BRCA1 is in CDDP resistance and ovarian cancer.

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