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Egr-1 regulates the transcription of the *BRCA1* gene by etoposide

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The breast cancer susceptibility gene *BRCA1* encodes a nuclear protein, which functions as a tumor suppressor and is involved in gene transcription and DNA repair processes. Many families with inherited breast and ovarian cancers have mutations in the *BRCA1* gene. However, only a few studies have reported on the mechanism underlying the regulation of *BRCA1* expression in humans. In this study, we investigated the transcriptional regulation of *BRCA1* in HeLa cells treated with etoposide. We found that three Egr-1-binding sequences (EBSs) were located at −1031, −1005, and −385 within the enhancer region of the *BRCA1* gene. Forced expression of Egr-1 stimulated the *BRCA1* promoter activity. EMSA data showed that Egr-1 bound directly to the EBS within the *BRCA1* gene. Knockdown of *Egr-1* through the expression of a small hairpin RNA (shRNA) attenuated etoposide-induced *BRCA1* promoter activity. We conclude that Egr-1 targets the *BRCA1* gene in HeLa cells exposed to etoposide. [BMB Reports 2013; 46(2): 92-96]

INTRODUCTION

The *BRCA1* gene is a nuclear phosphoprotein of 1863 amino acids that contains an N-terminal Cys_3-His-Cys_4 zinc finger domain and a C-terminal acidic domain (1). BRCA1 interacts with several proteins, including Rad51 p53, RNA polymerase II holoenzyme, RNA helicase A, CtBP-interacting protein, CBP/p300 and c-Myc; it plays important roles in DNA damage repair, cell cycle check-point control and apoptosis (2-7). Germline mutations in the BRCA1 gene are closely linked to an increased risk for the development of breast cancer, ovarian cancer and other malignancies (1, 8, 9), suggesting a tumor suppressor role for *BRCA* gene.

Several lines of evidence suggest that *BRCA1* is associated with the transcriptional regulation of diverse genes, including the upregulation of $p21^{Waf1/Cip1}$, GADD45, 14-3-3δ, $p27^{Kip1}$, XPC and TNFα, as well as with the downregulation of cyclin B1, estrogen receptor α-responsive genes and insulin growth factor 1 (7). In MCF7 cells, *BRCA1* mRNA expression was increased in response to gamma-irradiation and etoposide, as a response to DNA damage sensing (10). In other studies, *BRCA1* was found to be down-regulated at the mRNA level (11) and specifically cleaved and activated by caspase-3 (12) during UVC-irradiation. Although BRCA1 expression is known to be controlled by DNA-damaging agents in diverse cell types, little information is available regarding the regulation of *BRCA1* gene expression.

In the present study, we investigated whether *BRCA1* expression in HeLa cervix carcinoma cells is regulated at the transcriptional level by etoposide, which is a DNA topoisomerase II inhibitor that induces DNA strand breakage. We show that the transcription factor Egr-1 bound directly to the enhancer region of the *BRCA1* gene and that etoposide-induced BRCA1 promoter activity is mediated through Egr-1 activation. These results identify a functional linkage between the DNA damage response and the immediate-early response gene *Egr-1* in the regulation of DNA repair and/or induction of apoptosis.

RESULTS AND DISCUSSION

To determine whether etoposide induces the expression of BRCA1 in HeLa cells, Western blot analysis was performed on cells that were exposed to 100 μM etoposide for different time periods. The p53 and p21 proteins were used as positive controls for etoposide stimulation (13). Following exposure of the cells to etoposide, the level of BRCA1 protein increased in a time-dependent manner (Fig. 1A). In addition, the BRCA1 protein displayed retarded electrophoretic migration, probably reflecting phosphorylation of the protein after DNA damage (3, 12, 14). During UV-induced apoptosis, BRCA1 is cleaved to a ∼90-kDa C-terminal fragment by caspase-3 and plays an important role in the induction of apoptosis (12). We also observed BRCA1 fragments of ∼90 kDa after etoposide treatment of HeLa cells.

As the cleavage of BRCA1 is an irreversible reaction, we hypothesized that BRCA1 expression is upregulated by etoposide treatment. To test this theory, we examined whether the *BRCA1* gene is activated by etoposide. Quantitative Real-Time

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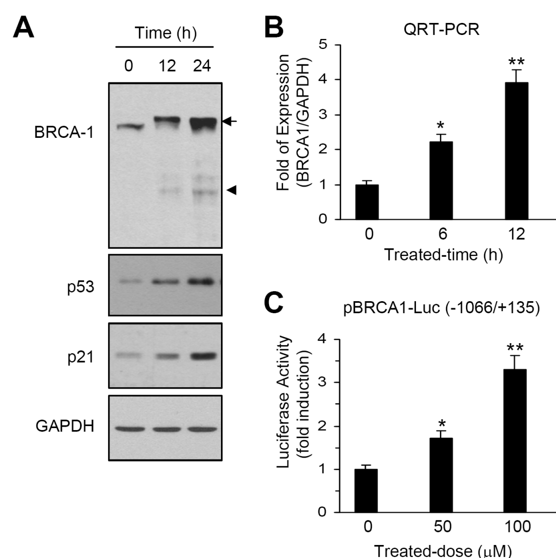

Fig. 1. Effect of etoposide on the induction of BRCA1 expression. (A) HeLa cells were treated with 100 μM etoposide for different time periods. Whole cell extracts were prepared and subjected to Western blotting with antibodies directed against BRCA1, p53 and p21. The ~220-kDa full length and ~90-kDa fragment of BRCA1 are indicated by an arrow and an arrowhead, respectively. The same blot was reprobed with anti-GAPDH antibody as an internal control. The blots shown are representative of the results obtained from three independent experiments. (B) Total RNA was isolated and the levels of *BRCA1* mRNA were measured by QRT-PCR. Relative levels are normalized to the level of *gapdh* mRNA. The data shown represent the mean ± SD of three independent experiments. *P < 0.05; **P < 0.01, compared with the untreated control cells. (C) HeLa cells grown in 12-well plates were transfected with 0.5 μg of the *BRCA1* promoter reporter plasmid, p*BRCA1*-Luc(−1066/+135), along with 50 ng of the pRL-null vector. After 24 h, the cells were either untreated or treated with 50 μM or 100 μM etoposide for 8 h. The firefly luciferase activity was normalized to the *Renilla* activity. The data shown represent the mean ± SD of three independent experiments performed in triplicate. *P < 0.05; **P < 0.01, compared with the untreated control cells.

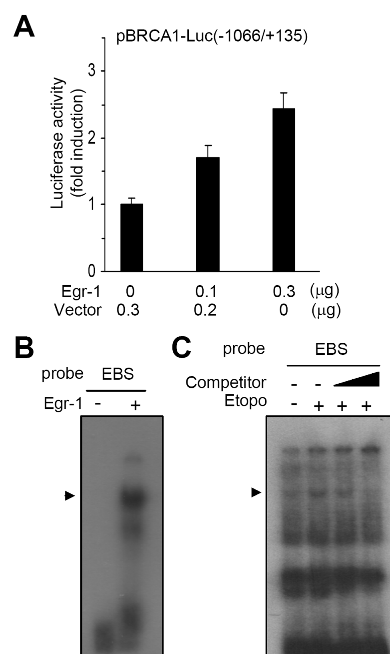

Fig. 2. Egr-1 transactivates the *BRCA1* promoter through direct binding to the EBS. (A) HeLa cells were co-transfected with 0.5 μg wild-type (WT) p*BRCA1*-Luc(−1066/+135) and different concentrations of the empty vector (pcDNA3.1zeo) or Egr-1 expression plasmid (pcDNA3.1zeo/Egr1), as indicated. After 48 h, the cells were collected and analyzed for luciferase activity. The firefly luciferase activity was normalized to the *Renilla* activity. The data shown represent the mean ± SD of three independent experiments performed in triplicate. (B and C) Purified recombinant Egr-1 protein (B) and nuclear extracts from HeLa cells treated with 100 μM etoposide for 1 h (C) were incubated with ^{32}P-labeled oligodeoxynucleotide probes that contain EBS. For competition, unlabeled oligodeoxynucleotides (Competitor) were added at 10-fold or 100-fold excess. Arrowheads indicate DNA-Egr-1 complexes.

PCR (QRT-PCR) analysis revealed an approximately 4-fold increase in the level of *BRCA1* mRNA after 6 h of treatment with 100 μM etoposide (Fig. 1B). To determine whether etoposide stimulates BRCA1 expression at the transcriptional level, we isolated the 5'-end regulatory region of the human *BRCA1* gene, located within 1,066 bp upstream of the transcriptional start site (+1), and subcloned this region into the pGL3-Luc luciferase reporter vector in order to yield pBRCA1-Luc(−1066/+135). This construct was transfected into HeLa cells, and the luciferase activity was measured. Treatment with etoposide resulted in a dose-dependent increase in luciferase reporter activity (Fig. 1C). Approximately a 3.3-fold increase in reporter activity was observed after treatment with 100 μM etoposide (P < 0.01, compared to the mock-treated control). Thus, *BRCA1* mRNA expression in HeLa cells is upregulated following etoposide treatment.

We found that three Egr-1-binding sequences (EBSs) were located at −1031, −1005 and −385 within the enhancer region of the *BRCA1* gene. *Egr-1* is an immediate-early response gene that is induced by multiple stimuli, including stress, injury, mitogens and DNA damaging agents (15-17). Egr-1 target genes, which include p21, p53, PTEN, TGFβ1, fibronectin and Gadd45, are involved in the regulation of cellular growth, DNA repair and apoptosis (16, 18-21). To evaluate whether Egr-1 transactivates the *BRCA1* promoter, HeLa cells were transfected with the promoter reporter pBRCA1-Luc(−1066/+135) together with increasing concentrations of the Egr-1 expression plasmid. Forced expression of Egr-1 resulted in the stimulation of pBRCA1-Luc(−1066/+135) reporter activity in a plasmid concentration-dependent manner (Fig. 2A). To investigate whether Egr-1 binds directly to the putative Egr-1-binding sequences (EBS) within the *BRCA1* gene, EMSA was performed using the purified recombinant Egr-1 protein. Recombi-

nant Egr-1 bound to the ^{32}P-labeled oligodeoxynucleotides that contained the consensus EBS (Fig. 2B). To determine whether Egr-1 binds to the EBS in response to etoposide treatment, nuclear extracts of HeLa cells were prepared and incubated with the radiolabeled Egr-1-binding probe. Treatment with etoposide increased the level of DNA-protein complex (Fig. 2C). The specificity of Egr-1 binding was confirmed by the loss of the DNA-protein complex when an excess of unlabelled Egr-1-binding oligodeoxynucleotides was added to the reaction. These data demonstrate that Egr-1 binds directly to the EBS site within the *BRCA1* promoter region.

To investigate whether Egr-1 expression is regulated by etoposide, Egr-1 expression was analyzed by Northern and Western blot analyses. The amounts of *Egr-1* mRNA (Fig. 3A) and Egr-1 protein (Fig. 3B) were increased by etoposide treatment in a time-dependent manner. The level of *Egr-1* mRNA peaked 15 min after etoposide treatment, while the level of Egr-1 protein peaked 1 h after etoposide treatment and gradually decreased thereafter. To confirm that Egr-1 is required for etoposide-induced *BRCA1* promoter activity, we used the RNA interference approach. Egr-1 knockdown was verified after etoposide treatment of serum-starved cells. The ability of etoposide to activate the *BRCA1* promoter was substantially attenuated by the introduction of *Egr-1* siRNA (Fig. 3C). Western blot analysis demonstrated that both basal and etoposide-induced BRCA1 protein levels were substantially reduced in HeLa cells expressing *Egr-1* siRNA (Fig. 3D). These results demonstrate that Egr-1 expression is necessary for the etoposide-induced upregulation of BRCA1.

In the present study, we demonstrate that BRCA1 expression is upregulated *via* Egr-1 following etoposide treatment. Egr-1 binds directly to the EBSs within the *BRCA1* promoter region. The essential role of Egr-1 in the activation of *BRCA1* transcription is supported by our findings that: (i) Egr-1 binds directly to the EBSs within the *BRCA1* enhancer region; (ii) forced expression of Egr-1 transactivates the *BRCA1* promoter; and (iii) the expression of Egr-1 siRNA abrogates etoposide-induced activation of the *BRCA1* promoter. These results imply that Egr-1 plays a critical role in DNA damage-induced cellular responses.

MATERIALS AND METHODS

Cell culturing and reagents

The human cervix carcinoma cell line HeLa was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) that was supplemented with 10% fe-

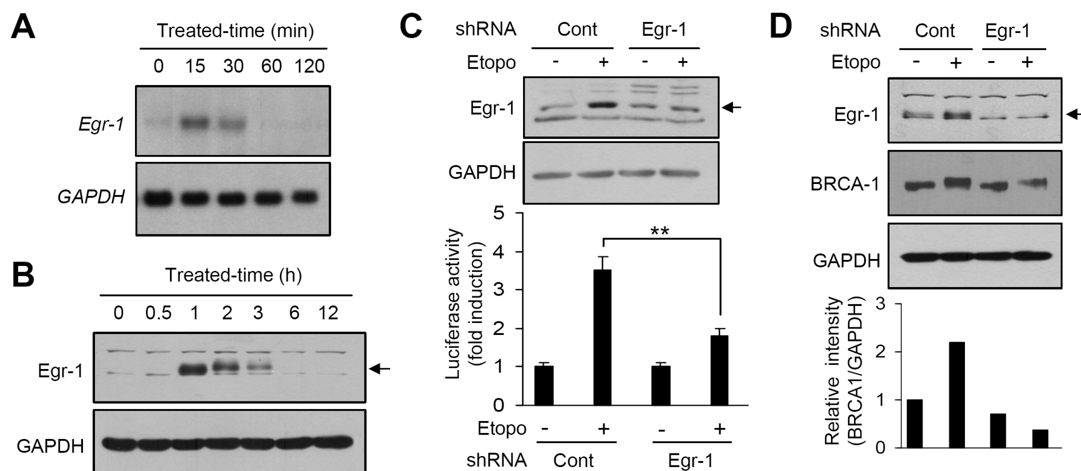

Fig. 3. Role of Egr-1 in etoposide-induced BRCA1 expression. (A) Serum-starved HeLa cells were treated with 100 μM etoposide for different time periods. Total RNA was isolated and Egr-1 mRNA expression was assessed by Northern blotting with ^{32}P-labeled Egr-1 cDNA. The same blot was re-probed with ^{32}P-labeled GAPDH cDNA as an internal control. (B) Serum-starved HeLa cells were treated with 100 μM etoposide for different time periods. Total cell lysates were prepared and subjected to Western blot analysis with rabbit anti-Egr-1 antibody. The same blot was reprobed with anti-GAPDH antibody as an internal control. (C) HeLa cells were transiently co-transfected with 0.5 μg pBRCA1-Luc(−1066/+135) and an shRNA plasmid, pSilencer/scrambled (control siRNA; *Cont*) or pSilencer/siEgr1 (*Egr-1*), along with 50 ng of the pRL-null vector plasmid. After 24 h, the cells were left untreated or treated with 100 μM etoposide for 8 h, and the luciferase activity was measured. Egr-1 is indicated by an arrow. The knockdown of Egr-1 expression was verified by Western blot analysis (*upper panel*). Luciferase activity is shown as the mean ± SD of three independent experiments performed in triplicate (bottom graph). **P < 0.01. (D) HeLa cells were transiently transfected with 0.5 μg shRNA plasmid, pSilencer/scrambled (control siRNA; *Cont*) or pSilencer/siEgr1 (*Egr-1*). After 24 h, the cells were left untreated or treated with 100 μM etoposide for 3 h. Whole cell extracts were prepared and subjected to Western blotting with antibodies directed against Egr-1 and BRCA1. Egr-1 is indicated by an arrow. The same blot was reprobed with anti-GAPDH antibody as an internal control. The relative band intensities were measured by quantitative scanning densitometer (*bottom graph*).

tal bovine serum (FBS; Hyclone, Logan, UT, USA). Etoposide was purchased from Calbiochem (San Diego, CA, USA). Antibodies directed against Egr-1 and GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The firefly and *Renilla* Dual-Glo™ Luciferase Assay System was purchased from Promega (Madison, WI, USA).

Northern blot analysis
Total RNA (10 μg) from each sample was separated by electrophoresis on a formaldehyde/agarose gel and transferred to a Hybond N^+ nylon membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Northern blotting was performed using a [α-^{32}P]dCTP-labeled cDNA probe, followed by hybridization with a glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) cDNA probe, as described previously (22).

Western blot analysis
Cells were lysed in 20 mM HEPES (pH 7.2) that contained 1% Triton X-100, 10% glycerol, 150 mM NaCl, 10 μg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF). The protein samples (15 μg each) were separated by 10% SDS-PAGE, and transferred onto nitrocellulose filters. The blots were incubated with appropriate antibodies and were developed using an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). The relative band intensities were measured by a quantitative scanning densitometer and image analysis software, Bio-1D version 97.04.

Quantitative Real-Time PCR (QRT-PCR)
Total RNA was extracted using the Trizol RNA extraction kit (Invitrogen, Carlsbad, CA, USA) from cells that were stimulated with etoposide (100 μM) for various periods of time. The first-strand cDNA was synthesized from 500 ng of total RNA using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA). QRT-PCR was performed with the iCycler iQ Real-Time PCR Detection System (Bio-Rad), using the TaqMan-iQ™ Supermix Kit (Bio-Rad), according to the manufacturer's recommendation. The TaqMan™ fluorogenic probes and PCR primers for *BRCA1* and *gapdh* were designed by Metabion International AG (Martinsried, Germany). The primer sequences (forward and reverse, respectively) were as follows: for *BRCA1*, 5'-TGGGAGCCAGCCTTCTAACAG-3 and 5'-GTT AATACTGCTTTTTCTGATGTGCTTTG-3'; and for *gapdh*, 5'-T CGACAGTCAGCCGCATCTTC-3' and 5'-CGCCCAATACGACC AAATCCG-3'. The TaqMan™ fluorogenic probes used were: 5'-FAM-TGACTCTTCTGCCCTTGAGGACCTGCGA-BHQ-1-3' (for *BRCA1*); and 5'-Yakima Yellow™-CGTCGCCAGCCGAGCC ACATCGC-BHQ-1-3' (for *gapdh*). The threshold cycle, C_t, which correlates inversely with the target mRNA levels, was calculated as the cycle number at which the reporter fluorescent emission increased above a threshold level. The relative changes in *BRCA1* mRNA levels were normalized for *gapdh* mRNA in the same samples.

Construction of human *BRCA1* promoter-reporter construct
A *BRCA1* promoter fragment spanning nucleotides (nt) −1066 to +135 was PCR-amplified from human genomic DNA (Promega) using the primers 5'-CACTTGCCCTCAAAACGACC-3' (forward) and 5'-GTTATCTGAGAAACCCCACA-3' (reverse). The amplicons were ligated into the pGL4-basic vector, yielding pBRCA1-Luc(−1066/+135). The resultant construct was verified by DNA sequencing and by restriction enzyme digestion.

Promoter reporter assay
HeLa cells were seeded into 12-well plates and transfected with 0.5 μg of the *BRCA1* promoter construct using Lipofectamine™ 2000 (Invitrogen), according to the manufacturer's instructions. To monitor transfection efficiency, 50 ng of the pRL-null plasmid that encodes *Renilla* luciferase were included in all samples. Where indicated, the empty vector or Egr-1 expression plasmid (pcDNA3.1zeo/Egr1) was included. At 24 h post-transfection, the levels of firefly and *Renilla* luciferase activity were measured, as described previously (23).

EMSA
Synthetic oligodeoxynucleotides (4 pmol) corresponding to EBS (−385/−360; 5'-AAGTACAAGCGCGCACAGGTCTCC-3') within the *BRCA1* enhancer were radioactively labelled by incubation with 10 U of T4 polynucleotide kinase, 5 μl of T4 polynucleotide kinase buffer and 20 μCi of [γ-^{32}P]dATP (Amersham Biosciences) for 30 min at 37°C, followed by inactivation at 65°C for 10 min. For EMSA, 10 μg of nuclear extract were mixed with the binding buffer (50 mM Tris-HCl [pH 7.5], 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 2.5 mM EDTA, 250 mM NaCl, 20% glycerol) together with 1 μg of poly(dI-dC) (Amersham Biosciences) as a non-specific competitor. The DNA-protein complexes were electrophoresed in non-denaturing 6% polyacrylamide gels and visualised by autoradiography.

Expression of *Egr-1* siRNA
The generation of a small hairpin RNA (shRNA) plasmid that targets human *Egr-1* mRNA (pSilencer/siEgr1) and its expression were described elsewhere (20).

Statistical analysis
All data are expressed as the means ± SD of at least three independent experiments. The Student's *t*-test and ANOVA were used to identify statistically significant differences. P values <0.05 were considered to be statistically significant.

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