ABSTRACT

We have evaluated the role played by BRCA1 in mediating the phenotypic response to a range of chemotherapeutic agents commonly used in cancer treatment. Here we provide evidence that BRCA1 functions as a differential mediator of chemotherapy-induced apoptosis. Specifically, we demonstrate that BRCA1 mediates sensitivity to apoptosis induced by antimicrotubule agents but conversely induces resistance to DNA-damaging agents. These data are supported by a variety of experimental models including cells with inducible expression of BRCA1, siRNA-mediated inactivation of endogenous BRCA1, and reconstitution of BRCA1-deficient cells with wild-type BRCA1. Most notably we demonstrate that BRCA1 induces a 10–1000-fold increase in resistance to a range of DNA-damaging agents, in particular those that give rise to double-strand breaks such as etoposide or bleomycin. In contrast, BRCA1 induces a >1000-fold increase in sensitivity to the spindle poisons, paclitaxel and vinorelbine. Fluorescence-activated cell sorter analysis demonstrated that BRCA1 mediates G2/M arrest in response to both antimicrotubule and DNA-damaging agents. However, poly(ADP-ribose) polymerase and caspase-3 cleavage assays indicate that the differential effect mediated by BRCA1 in response to these agents occurs through the inhibition or induction of apoptosis. Therefore, our data suggest that BRCA1 acts as a differential modulator of apoptosis depending on the nature of the cellular insult.

INTRODUCTION

Substantial evidence exists to support a role for BRCA1 in mediating the cellular response to DNA damage and, in particular, double-strand DNA breaks. BRCA1 becomes hyperphosphorylated in response to various DNA-damaging agents including γ-irradiation, an effect that is mediated in part by the ATM (1) and chk2 kinases (2). BRCA1 has been shown to colocalize at sites of DNA damage with RAD51, the human homologue of bacterial RecA, which is involved in homologous recombination repair after ionizing radiation (3). Furthermore, BRCA1 is a component of the RAD50, Mre11, NBS1 complex implicated in homologous recombination and nonhomologous end joining (4). Genetic studies support a role for BRCA1 in the repair of double-strand breaks. Significantly, ES cells from BRCA1 knockout mice exhibit a defect in the repair of double-strand breaks by homologous recombination (5). More recently, it has been reported that BRCA1 resides within a large DNA repair protein complex called BRCA1-associated genome surveillance complex that includes various mismatch repair proteins including MLH1, MSH2, and MSH6, suggesting a role for BRCA1 in mismatch repair (6). Interestingly, BRCA1 has also been implicated in the transcription-coupled repair of oxidative-induced DNA damage (7) and global genomic repair of UV-induced cyclobutane pyrimidine dimers (8). Taken together, these reports suggest that BRCA1 is a component of multiple repair pathways, which remain to be fully investigated.

It has also been suggested that BRCA1 functions as a sensor of DNA damage relaying signals to either the cell cycle checkpoint or cell death machinery. A number of studies have correlated BRCA1 deficiency with defects in cell cycle checkpoint control. Human tumor cells lacking functional BRCA1 demonstrate a high frequency of chromosome aneuploidy, characteristic of a defective G2/M checkpoint (9). In addition it has been demonstrated that BRCA1 is required for both S phase and G2 arrest after ionizing irradiation, an effect that is dependent on differential phosphorylation by ATM (10, 11). Furthermore, genetic instability has been observed in BRCA1-exon 11 isoform-deficient MEFs resulting from a defective G2/M checkpoint and centrosome amplification (12). We have reported previously that inducible expression of BRCA1 can activate both the G2 and mitotic checkpoints after treatment with paclitaxel, an antimicrotubule agent that functions by inhibiting the depolymerization of tubulin, thereby disrupting the mitotic spindle (13). Therefore, it appears that BRCA1 can act in a more general capacity to activate cell cycle checkpoints in response to different types of cellular stress.

In addition to cell cycle arrest, BRCA1 has also been implicated in the regulation of apoptosis. It was initially demonstrated that exogenous expression of BRCA1 induced apoptosis, an effect that was dependent on JNK4/SAPK activation (14). It was reported subsequently that BRCA1 modulates stress-induced apoptotic signaling through a pathway that sequentially involves the H-ras oncoprotein, mitogen-activated protein kinase kinase kinase 4, JNK, Fas, and Fas ligand, and the activation of caspase-9 (15, 16). We have reported recently that BRCA1 dramatically sensitizes breast cancer cell lines to IFN-γ-mediated apoptosis, indicating that BRCA1 may regulate apoptosis in response to diverse stress signals (17). In contrast, BRCA1-deficient cells exhibit a radiosensitive phenotype after exposure to a range of DNA-damaging agents including ionizing radiation (2) and the DNA cross-linking agents, cisplatin and mitomycin C (18, 19). This radiosensitive phenotype, which can be reversed after exogenous expression of wild-type BRCA1, has been associated with a failure in BRCA1-dependent DNA damage repair pathways. Consistent with this, antisense inhibition of BRCA1 has been reported to decrease repair proficiency and enhance apoptosis in response to cisplatin (20). Therefore, it appears that BRCA1 in general confers an antiapoptotic-resistant phenotype after DNA damage.

In the current study we provide new evidence to clarify the role played by BRCA1 as a mediator of apoptosis and show that BRCA1 functions as a molecular determinant of response to a range of cytotoxic chemotherapeutic agents. Specifically, we demonstrate that BRCA1 abrogates the apoptotic phenotype induced by a range of DNA-damaging agents including cisplatin, etoposide, and bleomycin, whereas inducing dramatic sensitivity to a range of antimicrotubule agents including paclitaxel and vinorelbine. Furthermore, we also provide mechanistic evidence to indicate that BRCA1 regulates the G2/M checkpoint in response to both spindle poisons and DNA-damaging agents. Therefore, these data indicate that BRCA1 functions as a differential regulator of chemotherapy-induced apoptosis.
and suggest that BRCA1 is worthy of additional investigation as a potential predictive marker of response to these agents.

**MATERIALS AND METHODS**

**Generation of Cell Lines.** MBR62-bcl2 cells were generated as described previously (13). HCC-empty vector and HCC-BRCA1 cells were generated by the stable transfection of the HCC1937 breast cancer cell line with the Rc/CMV (Invitrogen) or Rc-CMV-BRCA1 constructs under selection with geneticin (G418; Sigma). The Rc/CMV construct that expresses the neomycin resistance gene was obtained from Invitrogen, and the Rc-CMV-BRCA1 construct was generated as described previously (17). These constructs were transfected into the HCC1937 cell line using Genejuice methodology (Invitrogen) according to the manufacturer’s instructions. Transfected cells were selected in 0.2 mg/ml G418, assessed by RT-PCR for expression of BRCA1, and the resultant HCC-EV1, HCC-EV2, and HCCEV3, and HCC-BR116, HCC-BR18, and HCC-mixed clones (derived from a mixed population of transfected cells) were selected. Exogenous BRCA1 was detected by vector insert RT-PCR using a forward primer to BRCA1 and a reverse primer to the Rc/CMV-R.

**BRCA1-F, 5'-AGGACGCTTTGACATTACACCC-3'**

**Re-CMV-R 5'-AAGACTGAAGCCACGTCGAGG-3'**

**Cell Culture.** The MBR62-bcl2 cell line was maintained as described previously (13). The HCC1937 and T47D breast cancer cell lines were maintained in RPMI 1640 supplemented with 20% FCS, 1 mM sodium pyruvate, and 100 μg/ml penicillin-streptomycin (all from Life Technologies, Inc.). The HCC-EV1, EV2, EV3, BR116, BR18, and BR-mixed cell lines were grown in HCC1937 medium supplemented with 0.2 mg/ml G418.

**Flow Cytometry Analysis.** DNA content was evaluated after propidium iodide staining of cells as described previously (13). In all of the cases the FACS analysis was carried out using an EPICS ELITE flow cytometer (Coulter).

**Drug Sensitivity Assays.** For assays in MBR62-bcl2 cells were seeded in 24-well plates, and induced to express BRCA1 by withdrawal of tetracycline from medium and at the same time were treated or untreated with 10 μM paclitaxel (Bristol Myers Squibb). For drug sensitivity assays in HCC-EV and HCCBR cells were seeded onto 24-well tissue culture plates at a density of 100,000 cells/well. After 24 h, cells were incubated in HCC1937 medium supplemented with the described concentration ranges of paclitaxel (Bristol Myers Squibb), vinorelbine (Novartis), cisplatin (Faulding Pharmaceuticals), etoposide “etopo-phos” (Bristol Myers Squibb), and 5-fluorouracil (David et al.).

**IC50–IC90 values were calculated for each drug from the respective sigmoidal dose response curves using Prism software.**

**Antibodies and Western Blot Analysis.** MBR62-bcl2, HCC-EV, and HCC-BR116 cells were treated in the presence of 10 nM paclitaxel, 1 μM vinorelbine, 1 μM cisplatin, 1 μM etoposide, and 10 μM 5-fluorouracil. Protein lysates were extracted in radioimmunoprecipitation assay buffer [50 μM HEPES (pH 7.0), 150 mM NaCl, 0.1% Triton X-100, 0.1% sodium deoxycholate, and 0.1% SDS] separated on a 12% SDS polyacrylamide gel and transferred to polyvinylidene difluoride membrane followed by immunoblotting. BRCA1 Westerns were carried out using the rabbit polyclonal, BRCA1 NH2-terminal antibody, D-20 (Santa Cruz Biotechnology). For PARP assays, the monoclonal antibody #556494 (BD Bioscience) was used, which specifically recognizes the full-length M, 116,000 PARP protein and its M, 85,000 and 25,000 cleaved products. Cleaved caspase-3 was detected using the rabbit polyclonal cleaved caspase-3 (Asp 175) antibody #9661 (Cell Signaling) that specifically recognizes the cleaved M, 17,000 caspase-3 products. β-Tubulin was detected using the monoclonal antibody T-4026 (Sigma).

**siRNA.** T47D cells were transfected with a BRCA1-specific siRNA oligonucleotide or a control oligonucleotide and treated with a range of paclitaxel concentrations. After 72 h of continuous drug exposure, cells were detached using trypsin, and counted using a Z2 particle and size analyzer (Coulter). The IC50 values were calculated from the respective sigmoidal dose response curves: BRCA1 siRNA oligonucleotide 5'-aacc ctt tet cca caa aat ggt-3' and control siRNA oligonucleotide 5'-aaa acc cgu caa ggc ugu uac-3'.

**RESULTS**

**BRCA1 Enhances Paclitaxel-induced Apoptosis.** To study the functional properties of BRCA1 we generated inducible tetracycline-regulated expression in a MDA435 breast cancer-derived cell line, termed MBR62-bcl2 (13, 14). BRCA1 expression levels were shown to increase >4-fold above endogenous levels in this cell line after tetracycline withdrawal (Fig. 1A). This is well within the physiological levels observed for this protein in mouse models where BRCA1 expression has been shown to increase 10-fold during pregnancy, a level that is maintained through postlactational involution (21). We demonstrated previously that induction of BRCA1 in the presence of paclitaxel resulted in acute arrest at the G2/M phase of the cell cycle, an effect that correlated with BRCA1-mediated induction of GADD45 (13). To extend these studies additionally we evaluated the effect of inducible BRCA1 expression on the sensitivity of MBR62-bcl2 cells to paclitaxel. We generated IC50 curves after treatment of the MBR62-bcl2 cells with a range of different paclitaxel concentrations in the presence and absence of inducible BRCA1 expression. Inducible expression of BRCA1 increased paclitaxel sensitivity 100-fold in the MBR62-bcl2 cells with an observed decrease in IC50 from 7.7 μM to 96.4 μM (Fig. 1B). To determine whether BRCA1-induced sensitivity to paclitaxel might reflect activation of an apoptotic pathway in these cells we carried out PARP cleavage assays. The presence of the

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**A**

**B**

**C**

![Image](https://cancerres.aacrjournals.org)

**Fig. 1.** Inducible expression of BRCA1 sensitizes MBR62-bcl2 cells to paclitaxel-induced apoptosis. A. IP-Western demonstrating inducible expression of BRCA1 in the MBR62-bcl2 cells at 12 and 24 h after withdrawal of tetracycline (+tet, BRCA1 off; -tet, BRCA1 on). B. Dose response curves detailing the effect of inducible expression of BRCA1 on the cytotoxicity of paclitaxel in the MBR62-bcl2 cells. The IC50 values were calculated from the sigmoidal dose response curves. C. PARP cleavage assay demonstrating that BRCA1 sensitizes MBR62-bcl2 cells to paclitaxel-induced apoptosis. MBR62-bcl2 cells were induced (+tet) or uninduced (-tet) to express BRCA1 for 24 or 48 h in the presence or absence of 10 μM paclitaxel. The presence of the cleaved M, 85,000 PARP protein is indicative of apoptosis. The identical blot was reprobed with β-tubulin as a loading control.

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cleaved PARP Mr 85,000 product, which is an early hallmark of apoptosis, was initially observed as early as 24 h after inducible expression of BRCA1 and was dramatically increased 48 h after BRCA1 induction in the presence of paclitaxel, suggesting that BRCA1 can sensitize these cells to paclitaxel-induced apoptosis (Fig. 1C). Paclitaxel treatment alone resulted in moderate PARP cleavage 48 h after treatment as expected (Figs. 1C). Similarly, induction of BRCA1 in the absence of paclitaxel treatment also resulted in moderate PARP cleavage consistent with our previous report that exogenous expression of BRCA1 alone can induce apoptosis in these cells (14).

**BRCA1 Is Required for Paclitaxel-mediated Apoptosis.** We used the HCC1937 breast cancer cells that express a single copy of a COOH-terminal truncated BRCA1 protein, which is transcriptionally inactive, as a model to investigate the requirement for BRCA1 in paclitaxel-mediated apoptosis (9). We generated the HCC1937-derived cell lines, termed HCC-BR18, HCC-BR116, and HCCBR-mixed (derived from a mixed transfected population) with constitutive expression of exogenous wild-type BRCA1 and the control cell lines HCC-EV1, HCC-EV2, and HCC-EV3 reconstituted with empty Rc/CMV vector. To confirm expression of exogenous BRCA1 we carried out vector-insert RT-PCR from cDNA derived from the various cell lines. RT-PCR analysis confirmed expression of exogenous BRCA1 expression in both the HCC-BR18 and HCC-BR116 clones (Fig. 2A). To confirm exogenous BRCA1 expression at the protein level we carried out Western blot analysis to detect both endogenous mutant and exogenous wild-type BRCA1. Western blot analysis confirmed increased expression of wild-type BRCA1 in the HCC-BR116 cells relative to the mutant BRCA1 in vector only transfected controls (Fig. 2B).

To investigate the ability of BRCA1 to sensitize these cells to paclitaxel-mediated apoptosis we carried out PARP and caspase-3 cleavage assays. PARP assays demonstrated that the empty vector-transfected HCC-EV1 and EV2 cells were resistant to paclitaxel-mediated apoptosis after treatment with 10 nm paclitaxel (Fig. 2C). In contrast, the BRCA1 reconstituted HCC-BR18 and HCC-BR116 cells displayed the presence of the Mr 85,000 cleaved PARP product, indicative of apoptosis after treatment with identical concentrations of paclitaxel (Fig. 2C). Identical data were obtained after caspase-3 cleavage assays. Reconstitution of BRCA1 in the HCC-BR18 and HCC-BR116 cells resulted in a dramatic increase in the Mr 17,000 cleaved caspase-3 products after treatment with 10 nm paclitaxel (Fig. 2D). In contrast, the HCC-EV1 and HCC-EV2 cells failed to display the presence of cleaved caspase-3 suggesting that these cells are resistant to paclitaxel-mediated apoptosis (Fig. 2D). We extended these studies to look at paclitaxel sensitivity in the T47D breast cancer cell line after inhibition of endogenous BRCA1 using a siRNA approach. T47D cells transfected with a BRCA1-specific siRNA oligonucleotide displayed a >50-fold increase in resistance to paclitaxel compared with cells transfected with a control siRNA oligonucleotide with the IC₅₀ values calculated as >0.1 mM and 2.2 µM, respectively (Fig. 2E). Inhibition of endogenous BRCA1 after transfection with the BRCA1-

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**Fig. 2. BRCA1 is required for paclitaxel-induced apoptosis.** A, vector insert RT-PCR demonstrating the presence of exogenous BRCA1 in the HCC-BR116 (Lane 3) and HCC-BR18 (Lane 4) compared with EV-1 (Lane 1) and EV-2 (Lane 2). A glyceraldehyde-3-phosphate dehydrogenase RT-PCR was included as a control to confirm equal amounts of template. M = 123 bp marker. B, Western blot analysis confirming increased expression of BRCA1 in the HCC-BR116 cells (Lane 2) compared with the control vector transfected HCC-EV1 cells (Lane 1). A β-tubulbin Western was carried out on the identical protein lysate as a control for loading. C, PARP cleavage assay demonstrating the requirement for functional BRCA1 in paclitaxel-mediated apoptosis. HCC-EV1 (Lane 3), HCC-EV2 (Lane 5), HCC-BR116 (Lanes 4), and HCC-BR18 (Lane 6) were treated with paclitaxel for 24 h. The presence of the cleaved Mr 85,000 PARP protein is indicative of apoptosis and is only observed in the BRCA1 reconstituted HCC-BR116 and HCC-BR18 cells after paclitaxel treatment (Lanes 4 and 6). Untreated HCC-EV1 (Lane 1) and HCC-BR116 cells (Lane 2) are included as a control. The identical blot was reprobed with β-tubulbin as a loading control. D, caspase-3 cleavage assay demonstrating the requirement for functional BRCA1 in paclitaxel-mediated apoptosis. HCC-EV1 (Lane 3), HCC-EV2 (Lane 5), HCC-BR116 (Lanes 4), and HCC-BR18 (Lane 6) were treated with paclitaxel for 24 h. The presence of the cleaved Mr 17,000 caspase-3 fragments is indicative of apoptosis and is only observed in the BRCA1 reconstituted HCC-BR116 and HCC-BR18 cells after paclitaxel treatment. Untreated HCC-EV1 (Lane 1) and HCC-BR116 cells (Lane 2) are included as a control. The identical blot was reprobed with β-tubulbin as a loading control. E, dose-response curve demonstrating a >50-fold increase in resistance of T47D cells to paclitaxel after transfection with a BRCA1-specific siRNA oligonucleotide compared with a control siRNA oligonucleotide. IC₅₀ values for paclitaxel were calculated from the respective sigmoidal dose response curves. F, IP-Western demonstrating a marked reduction in endogenous BRCA1 protein levels in T47D cells transfected with a BRCA1 specific siRNA oligonucleotide (Lane 2) compared with a control siRNA oligonucleotide (Lane 1). A β-tubulbin Western was carried out using the identical protein lysate as a control for the specificity of the BRCA1 siRNA.
specific siRNA oligonucleotide was confirmed by immunoprecipitation-Western blot analysis relative to T47D cells transfected with the control siRNA oligonucleotide (Fig. 2F).

**BRCA1 Functions as a Differential Modulator of Sensitivity to Chemotherapeutic Agents.** The ability of BRCA1 to mediate sensitivity to paclitaxel-induced apoptosis prompted us to examine the role played by BRCA1 in mediating sensitivity or resistance to a range of chemotherapeutic agents commonly used in cancer treatment. To do this we generated dose response curves for the HCC-BR116 and HCC-EV1 cells after treatment with the antimicrotubule agents paclitaxel and vinorelbine, which disrupt the mitotic spindle, the radiomimetic bleomycin, which gives rise to double-strand breaks, the topoisomerase II poison etoposide, the inter and intra-strand DNA cross-linking agent cisplatin, and the antimetabolite, 5-fluorouracil. The HCC-EV1 and HCC-BR116 cells were plated at equal densities and exposed to a range of concentrations of each drug for 72 h after which time cell counts were carried out. Reconstitution of BRCA1 in the HCC1937 cells resulted in a 1000-fold increase in sensitivity of the HCC-BR116 cells to paclitaxel, with the IC50 value decreasing from 6.2 μM in the HCC-EV1 cells compared with 7.7 nM in the HCC-BR116 cells (Fig. 3A). Similar to that observed for paclitaxel, the HCC-BR116 cells displayed a dramatic increase in sensitivity to vinorelbine over many concentrations compared with HCC-EV1 cells. Specifically, the HCC-BR116 cells displayed an IC30 value of 1.9 nM compared with an IC30 value of 17.0 μM in the HCC-EV1 cells, representing a 10,000-fold increase in sensitivity after reconstitution with BRCA1 (Fig. 3B).

We observed the opposite effects when these cell lines are exposed to DNA damaging agents. The IC50 value for the HCC-EV1 cells after bleomycin treatment was calculated at 0.5 mM. However, we failed to obtain an IC50 value for the HCC-BR116 cells obtaining a value of >1.0 mM, suggesting that BRCA1 induces extreme resistance to bleomycin in these cells (Fig. 3C). Similarly, treatment with the topoisomerase II poison etoposide resulted in an IC50 value of 0.9 μM in the HCC-EV1 cells compared with an IC50 value of >0.1 mM in the HCC-BR116 cells representing at least a 1000-fold increase in resistance (Fig. 3D). BRCA1 also induced 20-fold resistance to cisplatin with an IC50 value of 0.2 μM observed in the HCC-EV1 cells compared with an IC50 value of 4.1 μM in the HCC-BR116 cells (Fig. 3E). Interestingly, BRCA1 failed to modulate resistance or sensitivity to the antimetabolite 5-fluorouracil with equivalent IC50 values obtained for both cell lines (Fig. 3F), probably reflecting the distinct mode of action of this agent.

**BRCA1 Is a Differential Modulator of Chemotherapy-induced Apoptosis.** To investigate whether the observed chemosensitivity or resistance induced by BRCA1 was because of activation or inhibition...
of apoptosis we carried out PARP assays 24 h after treatment with paclitaxel (10 nM), vinorelbine (1 nM), cisplatin (1 μM), etoposide (1 μM), bleomycin (0.1 μM), and 5-fluorouracil (10 μM). PARP assays demonstrated cleavage of the M₈ 116,000 PARP product into its M₆ 85,000 cleaved fragment when HCC-BR116 cells were treated with either paclitaxel or vinorelbine (Fig. 4, A and B). In agreement with the drug sensitivity assays the HCC-EV1 cells were completely resistant to paclitaxel and vinorelbine-induced apoptosis at these concentrations (Fig. 4, A and B). We observed the exact opposite after treatment of the HCC-BR116 cells with the DNA damaging agents bleomycin, etoposide, and cisplatin. HCC-BR116 cells were resistant to apoptosis induced by these agents, whereas the HCC-EV1 cells displayed the characteristic M₈ 85,000 PARP cleavage product indicative of apoptosis (Fig. 4A). We repeated the drug sensitivity experiments with two additional clones, termed HCC-BR118 and HCCBR-mixed, using paclitaxel and etoposide as representative agents. In keeping with our previous observations, reconstitution of BRCA1 in the HCC-BR18 and HCCBR-mixed cells resulted in an ~5,000-fold and 10,000 fold increase in sensitivity to paclitaxel, respectively, compared with the HCC-EV2 and HCC-EV3 cells (Fig. 5, A and C). Similarly, the HCC-BR18 cells were observed to be 100-fold more resistant and the HCCBR-mixed cells ~1,000 fold more resistant to etoposide compared with the HCC-EV2 and HCC-EV3 cells, respectively (Fig. 5, A and C). Finally, to illustrate that this difference reflected a corresponding increase or decrease in apoptosis we carried out PARP cleavage assays in these cells. The HCC-BR18 and HCCBR-mixed cells displayed the characteristic M₈ 85,000 PARP cleavage product after treatment with paclitaxel but not with etoposide, whereas the reverse was true for the HCC-EV2 and HCC-EV3 cells (Fig. 5, B and D).

**BRCA1 Induces G₂/M Arrest in Response to Antimicrotubule and DNA Damaging Agents.** To determine whether the differential effects on apoptosis mediated by BRCA1 in response to DNA-damaging agents and spindle poisons reflected differential modulation of cell cycle checkpoints we carried out cell cycle analysis. The HCC-BR116 and HCC-EV1 cells were treated with IC₅₀ concentrations of paclitaxel (10 nM) or etoposide (1 μM) for 12 h, followed by FACS analysis to determine DNA content. Reconstitution of BRCA1 in the absence of drug treatment had no effect on cell cycle profile, compared with vector-transfected controls (Fig. 6A). However, a significant increase in the G₂/M population was observed in the HCC-BR116 (50%) cells compared with HCC-EV1 cells (21%) after treatment with paclitaxel (Fig. 6B). Similar results were obtained with vinorelbine (data not shown), suggesting that BRCA1 can regulate the G₂/M checkpoint after treatment with agents that disrupt the mitotic spindle. In agreement with previous studies using ionizing radiation (10, 22), treatment of the HCC-BR116 cells with etoposide also resulted in a significant increase in the G₂/M content in HCC-BR116 cells (50%) compared with HCC-EV1 cells (31%; Fig. 6C). Similar results were obtained for bleomycin (data not shown), suggesting that BRCA1 regulates the G₂/M checkpoint in response to agents that induce double-strand breaks in DNA.

**DISCUSSION**

In the current study we provide evidence to suggest that BRCA1 acts as a molecular determinant of response to a range of chemotherapeutic agents commonly used in cancer treatment. We demonstrate that BRCA1 mediates G₂/M arrest in response to agents that disrupt the mitotic spindle including paclitaxel and to DNA-damaging agents such as etoposide. In contrast, BRCA1 dramatically sensitizes breast cancer cell lines to apoptosis induced by the antimicrotubule agents paclitaxel and vinorelbine, yet induced resistance to a range of DNA-damaging agents, most notably those that induce double-strand breaks in DNA including bleomycin and etoposide. The observation that BRCA1 mediates G₂/M arrest in response to both antimicrotubule agents and DNA-damaging agents is intriguing considering the differential effects on apoptosis. Standard FACS analysis does not distinguish between the G₂ and mitotic populations of cells. Therefore, it is possible that the differential effect on apoptosis may reflect different subpopulations of cells in the G₂/M and mitotic phases in response to the different agents. In support of this argument we have reported previously that the inducible expression of BRCA1 can activate the mitotic checkpoints in response to paclitaxel (13). In addition a dominant-negative BRCA1 construct encoding the COOH terminus was shown to abrogate G₂/M arrest in response to the spindle poison colchicine (23). Moreover, BRCA1 has been shown to localize to centrosomes via a physical interaction with γ-tubulin, suggesting that BRCA1 plays a direct role in the accurate segregation of duplicated chromosomes during mitosis (24). It has also been reported that BRCA1 is required for the decatenation and, therefore, accurate separation of sister chromatids at anaphase lending additional support for a role in mitotic checkpoint regulation (25). Genetic studies support these observations revealing aneuploidy characteristic of a defect in the mitotic checkpoint in BRCA1 exon 11 isoform-deficient cells (12). Our observation that BRCA1 can specifically sensitize breast cancer cell lines to apoptosis induced by antimicrotubule agents is intriguing in the context of these other studies. Antimicrotubule agents function by promoting the stabilization or destabilization of tubulin, which, in turn, prevents sister chromatid separation and arrest at the metaphase to anaphase transition (26). The mechanisms through which spindle poisons such as paclitaxel induce apoptosis are still a matter of speculation; however, it has been suggested that different pathways are activated depending on the concentrations used. Higher concentrations of paclitaxel in the region of 0.2–3.0 μM give rise to microtubule damage and activation of JNK-dependent apoptosis (26). In contrast, paclitaxel concentrations of 10 μM to 0.1 mM result in
suppression of microtubule dynamics and mitotic arrest in the absence of microtubule damage (26).

In this study we demonstrate that BRCA1 sensitizes HCC1937 cells to apoptosis at concentrations ranging from 1 nM to 10 μM suggesting that BRCA1 may specifically play a role in mediating apoptosis at lower concentrations. Indeed, cellular proliferation assays clearly display a biphasic decrease in cell viability with BRCA1 mediating sensitivity at paclitaxel concentrations as high as 0.1 μM followed by a BRCA1-independent paclitaxel sensitivity at concentrations higher than 10 μM (Fig. 3A; Fig. 5, A and C). Furthermore, we demonstrate that inhibition of endogenous BRCA1 results in a dramatic increase in resistance of breast cancer cells to paclitaxel-induced apoptosis (Fig. 2, E and F) suggesting that BRCA1 expression levels are critical for mediating apoptosis in response to this class of chemotherapeutic agent. Irrespective of the mechanistic basis of antimicrotubule-induced apoptosis it appears that the initial step involves the activation of the spindle checkpoint (27). A number of potential scenarios can then occur that ultimately result in the induction of apoptosis, including the generation of multinucleated cells and the onset of mitotic catastrophe or entrance of cells into a tetraploid G2 state that also results in apoptosis. The observation that BRCA1 enhances antimicrotubule-induced G2/M arrest and apoptosis suggests that BRCA1 may play a role in the regulation of the spindle checkpoint, which, in turn, results in the induction of apoptosis through a number of potential pathways. The mechanistic basis of BRCA1-induced apoptosis in response to paclitaxel and vinorelbine remains to be defined; however, it is reasonable to speculate that BRCA1 may regulate key pro-apoptotic target genes after disruption of the mitotic spindle. Additional elucidation of this pathway may therefore provide critical new information on the mechanism of paclitaxel sensitivity and resistance in breast tumors.

The observation that BRCA1 sensitizes HCC1937 cells to antimicrotubule-induced apoptosis is in direct contrast to that observed after exposure to DNA-damaging agents where BRCA1 clearly mediates resistance. We observed a dramatic increase in resistance to apoptosis in HCC1937 cells reconstituted with wild-type BRCA1 after treatment with the radiomimetic bleomycin and the topoisomerase II inhibitor etoposide, both of which introduce double-strand breaks in DNA, although through different mechanisms. We also demonstrate that BRCA1 can induce resistance to cisplatin-mediated apoptosis suggesting that BRCA1 may also be involved in the repair of inter and intrastrand cross-links. These data are in agreement with previous reports, which demonstrate that reconstitution of BRCA1 in HCC1937 cells can rescue the radioresistant phenotype, although this had not

Fig. 5. BRCA1 is a differential modulator of chemotherapy induced apoptosis. Dose-response curves comparing the IC50 concentrations of paclitaxel and etoposide in the HCC-BR18 (A) and HCC-BR mixed (C) cells reconstituted with exogenous wild-type BRCA1 compared with HCC-EV2 (A) and HCC-EV3 (C) cells reconstituted with empty vector. The HCC-BR18/HCC-BR mixed and HCC-EV2/EV3 cells were treated with a range of concentrations of (i) paclitaxel and (ii) etoposide. The HCC-BR18 and HCC-BR mixed cells display a marked increase in sensitivity (5000-fold and 7000-fold, respectively) to paclitaxel compared with the HCC-EV2 and HCC-EV3 cells and conversely display a marked resistance to etoposide (100-fold and >1000-fold, respectively) compared with the HCC-EV2 and HCC-EV3 cells. IC50 values for the individual chemotherapeutic agents were calculated from the respective sigmoidal dose response curves. (C), HCC-BR18/HCC-BR mixed; (A), HCC-EV2/HCC-EV3). PARP cleavage assays demonstrating the differential effects of paclitaxel and etoposide on the induction of apoptosis in the HCC-BR18 and HCC-BR mixed cells compared with the HCC-EV2 and HCC-EV3 cells. The HCC-BR18 and HCC-BR mixed cells exhibit marked PARP cleavage after treatment with paclitaxel (B, Lane 4; D, Lane 6) but are resistant to apoptosis induced by etoposide (B, Lane 6; D, Lane 4). In contrast the HCC-EV2 and HCC-EV3 cells are resistant to apoptosis induced by paclitaxel (B, Lane 5; D, Lane 5) but are sensitive to apoptosis induced by etoposide (B, Lane 5; D, Lane 3). Untreated HCC-BR18/HCC-BR mixed (B, Lane 1; D, Lane 1) and HCC-EV3/HCC-EV3 (B, Lane 2; D, Lane 2) lysates are included as a control. The identical blots were reprobed with α-tubulin as a control for loading in each case.
been linked to inhibition of apoptosis (2, 18, 19). The mechanism through which BRCA1 rescues the resistant phenotype in response to these agents has yet to be defined; however, it is likely to be linked to the activation of BRCA1 dependent G2/M arrest and or DNA repair pathways (5). In support of this hypothesis, BRCA1 has been shown to be essential for activation of the Chk1 kinase and regulation of the G2/M checkpoint after DNA damage (22). Similarly, ATM-dependent phosphorylation of BRCA1 on serine 1423 has been shown to be required for BRCA1-induced activation of the G2/M checkpoint after ionizing radiation (10). In light of the above studies, our observation that BRCA1 can mediate G2/M arrest in response to etoposide and bleomycin suggests that BRCA1 plays an important role in the regulation of the G2/M checkpoint after exposure of breast cancer cells to a range of different DNA-damaging agents. Many studies have also suggested that BRCA1 is required for the efficient repair of DNA damage and, in particular, double-strand breaks in DNA. BRCA1 has been shown to colocalize at sites of DNA damage with RAD51 and RAD50, which are involved in homologous recombination and non-homologous end joining, respectively (3, 4). In addition, ES cells from BRCA1 knockout mice exhibit a defect in the repair of double-strand breaks by homologous recombination (5). Therefore, it is possible that BRCA1 functions to couple G2/M arrest with repair pathways, thereby mediating survival after DNA damage. The exact mechanism through which BRCA1 regulates DNA repair pathways is unclear, but may involve its ability to function as a transcriptional coactivator or repressor. BRCA1 has been shown to mediate the induction of genes...
involved in nucleotide excision repair in a p53-independent manner after exposure to UV-irradiation (8). In addition, it has been demonstrated that exogenous expression of BRCA1 mediates the induction of growth arrest and DNA repair genes in a p53-dependent manner in the absence of DNA damage (28). Because the HCC1937 cell line also harbors mutant p53, our data would suggest that BRCA1-mediated inhibition of apoptosis in response to DNA damage and conversely induction of apoptosis in response to spindle poisons occurs independently of a fully functional p53 protein.

Therefore, in summary, the data presented suggest that BRCA1 functions as a molecular determinant of response to a range of different chemotherapeutic agents and, in particular, functional BRCA1 may be required for an effective apoptotic response to paclitaxel chemotherapy. With the recent adoption of paclitaxel containing regimens for potentially curable breast cancer (29) it is likely that determinants of response to this agent will become increasingly important. Therefore, we suggest that BRCA1 expression level could be considered as a potential predictive marker for response to chemotherapy in both sporadic and inherited breast cancer.

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