BRCA1-Induced Apoptosis Involves Inactivation of ERK1/2 Activities

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Running Title: Role of ERK1/2 in BRCA1-induced apoptosis
SUMMARY

Mutation in the BRCA1 gene is associated with an increased risk of breast and ovarian cancer. Recent studies have shown that the BRCA1 gene product may be important in mediating responses to DNA damage and genomic instability. Previous studies have indicated that overexpression of BRCA1 can induce apoptosis or cell cycle arrest at the G2/M border in various cell types. While the activation of JNK kinase has been implicated in BRCA1-induced apoptosis, the role of other members of the MAPK family in mediating the cellular response to BRCA1 have not yet been examined. In this study, we monitored the activities of three members of the MAPK family (ERK1/2, JNK, p38) in MCF-7 breast cancer cells and U2OS osteosarcoma cells following their exposure to a recombinant adenovirus expressing wild type BRCA1 (Ad.BRCA1). While overexpression of BRCA1 in MCF-7 cells resulted in arrest at the G2/M border, BRCA1 expression in U2OS cells induced apoptosis. Although BRCA1 induced JNK activation in both cell lines, there were marked differences in ERK1/2 activation in response to BRCA1 expression in these two cell lines. BRCA1-induced apoptosis in U2OS cells was associated with no activation of ERK1/2. In contrast, BRCA1 expression in MCF-7 cells resulted in the activation of both ERK1/2 and JNK. To directly assess the role of ERK1/2 in determining the cellular response to BRCA1, we used dominant negative mutants of MEK1 as well as MEK1/2 inhibitor PD98059. Our results indicate that inhibition of ERK1/2 activation resulted in increased apoptosis following BRCA1 expression in MCF-7 cells. Furthermore, BRCA1-induced apoptosis involved activation of JNK, induction of Fas-L/Fas interaction, and activation of caspases 8 and 9. The studies presented in this report indicate that the response to BRCA1 expression is determined by the regulation of both the JNK and ERK1/2 signaling pathways in cells.
INTRODUCTION

Breast cancer remains the most common cancer affecting women in the Western world. Although most breast cancers are sporadic, approximately 10% are inheritable and associated with mutations in at least two loci, BRCA1 and BRCA2. The BRCA1 gene is located at position 17q21 of the human genome, and mutations in this gene are associated with an increased risk of development of breast and ovarian cancer (1). The BRCA1 gene encodes a protein of 1864 amino acid residues that is primarily located in the nucleus (2). The BRCA1 gene product contains several domains that may effect its interaction with many other cellular proteins. The N-terminus includes a domain presumably involved in the formation of homodimers and heterodimers (with the BARD1 protein) and a ring finger domain that is involved in interaction with BAP1 and E2F-1; the middle portion of BRCA1 contains a nuclear localization signal and domains that can bind to c-Myc, p53, pRB and the DNA repair proteins RAD50 and RAD51; The C-terminus includes two BRCT domains that interact with multiple factors involved in transcriptional regulation, including CtIP, p300, p53, pRB, CBP, BRCA2, RNA Pol II and RNA helicase A (3, 4). Recent studies suggest that the BRCA1 protein may be involved in regulating numerous cellular functions including DNA damage repair, gene transcription, chromosome segregation, cell cycle arrest and apoptosis (4).

Several studies suggest a role for BRCA1 in DNA repair. First, BRCA1 interacts with RAD51, a human homologue of the yeast RecA protein involved in ds-DNA break repair (5, 6). In vitro, BRCA1 can associate with the RAD50-MRE11-NBS1 complex, a functional unit implicated in homologous recombination, non-homologous end joining and meiotic recombination. In irradiated cells, BRCA1 is recruited to this complex where it likely plays a role in DNA repair (7). Moreover, ectopic expression of BRCA1 decreases cellular sensitivity to radiation and increases the efficiency of DNA break repair (8). Second, BRCA1 interacts with proteins involved in mismatch repair, mainly MSH2, MSH3 and MSH6. Furthermore, the
association of BRCA1 with MSH2 and MSH6 was found to be essential for transcription-coupled DNA repair (9). A recent study by Wang et al. provided evidence for the existence of a large BRCA1-containing complex that incorporates factors involved in various types of DNA repair. The identified complex was found to contain BRCA1 and the DNA repair factors MSH2, MSH6, MLH1, ATM, BLM, RAD50, MRE11, NBS1 and replication factor C (9). These results suggest that BRCA1 might provide a scaffold that functions in coordinating multiple activities required for the maintenance of genomic integrity and the fidelity of DNA replication (9).

Besides DNA repair function, numerous studies also suggest that BRCA1 might play an important role in transcriptional regulation. BRCA1 physically interacts with key enzymes involved in transcription, mainly RNA polymerase II and RNA helicase A, suggesting that it might be a component of the transcriptional machinery (10–12). BRCA1 also associates with several factors known to act as transcriptional activators or repressors, such as E2F1, c-myc, p53, p300, CBP, CtIP, and pRB (3). Finally, overexpression of BRCA1 can enhance or repress transcription of specific genes (3). The mechanism of BRCA1 involvement in regulation of gene transcription is not clear. Although BRCA1 can interact with subunits of the RNA polymerase complex, there is little evidence to support its role as a universal component of the transcription machinery. For instance, BRCA1 protein has very little effect on gene transcription driven by the Jun, Fos, USF or Gal4 factors (13–15). It is possible that the role of BRCA1 in gene transcription is restricted to a small subset of genes and factors that are involved in either cell proliferation or programmed cell death. A role for BRCA1 in these processes is supported by recent studies showing that the ectopic expression of BRCA1 can cause either a cell cycle arrest, typically at the G2/M transition (16, 17), or the induction of apoptosis (18–20). The mechanisms that control the fate of BRCA1-transfected cells (apoptosis or cell cycle arrest) have not yet been defined.
Mitogen-activated protein kinases (MAPK) are key components of at least three signal transduction pathways that impact cell proliferation, survival and differentiation. On the basis of their sequence similarities and the nature of their upstream activators, the MAPKs can be grouped into three subfamilies: ERK, JNK/SAPK and p38. The first class includes the kinases, ERK1 and ERK2, whose activation by mitogens leads to the induction of cyclin D1 and the initiation of cell cycle progression (21). Furthermore, activation of the Raf/MEK/ERK pathway was found to be essential for cell survival and for proliferation (21). In contrast, JNK/SAPK and p38 kinases are primarily activated by stress signals (such as protein synthesis inhibitors, UV irradiation and DNA damaging agents) and their activation tends to inhibit proliferation and/or to compromise survival (21). JNK signaling pathway has been suggested to play a crucial role in BRCA1-mediated apoptosis. Harkin et al. showed that the induction of apoptosis in U2OS cells by over-expressed BRCA1 is dependent upon functional JNK (18). Thangaraju et al. described similar findings in MCF-7 cells and further delineated the events taking place both upstream and downstream of JNK activation (20). Their results showed that BRCA1 expression resulted in the sequential involvement of H-Ras, MEKK4, JNK, Fas-L/Fas interaction and caspase-9 activation and that removal of mitogens was required for the induction of BRCA1-induced apoptosis in MCF-7 breast cancer cells (20). This latter observation suggests that the balance between ERK1/2 activity and that of JNK/p38 kinases may influence the response of cells following overexpression of BRCA1. Similar findings were described in other systems, including the induction of apoptosis in PC12 cells following removal of NGF (22).

To further define the potential roles played by p38 and ERK1/2 in controlling the BRCA1-induced apoptotic response in MCF-7 and U2OS cells, we have monitored the activities of the two kinases following BRCA1 overexpression. We have also examined the effects of MEK1/2 specific inhibitor PD98059 and a dominant negative mutant of the MEK1 kinase on the cellular response to BRCA1 overexpression. Studies in this report suggest that activation of ERK1/2
inhibits BRCA1-induced apoptosis in MCF-7 breast cancer cells and indicates an important role for this pathway in determining the fate of cells following BRCA1 expression.

EXPERIMENTAL PROCEDURES

Cell culture and drug treatment—The human breast cancer cell line MCF-7 and the human osteosarcoma cell line U2OS were obtained from ATCC (Manassas, VA). Cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum in an atmosphere of 5% CO₂. Log-phase cells (1 X 10⁶ cells per 100 mm dish) were incubated in medium containing PD98059 (CALBIOCHEM, San Diego, CA) dissolved in DMSO. For control, parallel cultures incubated in medium containing the same amount of DMSO (final concentration 0.05%) without drug. Pre-treatment with PD98059 involved a one-hour exposure to the drug just prior to viral infection. For serum starvation experiments, log phase cells were grown in regular culture medium (containing 10% serum) until 60-70% confluency. The cells were then washed once with serum-free DMEM and then changed to medium containing 0.1% serum. Cells were maintained under the serum starvation condition for 24 hours prior to further analysis.

Adenoviral vectors and adenoviral infections—BRCA1 cDNA was obtained from Dr. J. S. Humphrey (Cell Biology and Metabolism Branch, NCI). The Ad.BRCA1 vector was generated by splicing the full-length BRCA1 cDNA into an adenoviral shuttle vector downstream from the CMV promoter. The resulting shuttle vector was co-transfected along with Ad5 viral DNA (Quantum Biotechnologies Inc., Montreal, Canada) into 293 cells using calcium phosphate precipitation method (Life Technologies Inc., Gaithersburg, MD). Individual adenoviral plaques were isolated and amplified in 293 cells. Plaques screening for BRCA1 sequences was performed by PCR using the forward primer 5’ –ATT CAC CCT TGG CAC AGG TGT C-3’ and the reverse primer 5’ AGC TCT GGG AAA GTA TCG CTG TC-3’. A recombinant adenovirus was identified (Ad.BRCA1) that contained full length BRCA1 cDNA. Sequencing of the
recombinant adenovirus indicated that the inserted cDNA was wild type and full length (Myriad Genetic Laboratories, Salt Lake City, UT).

Recombinant adenovirus Ad.MEK1(dn) was obtained from Dr. J. Han (The Scripps Research Institute, La Jolla, CA). In Ad.MEK1(dn), the MEK1 cDNA has been altered and two crucial serine residues located in the catalytic domain (Ser-217 and Ser-221) were replaced by alanines. The resulting MEK1 mutant has dominant negative activity and can be used to block the activation of ERK1/2 by wild type MEK1 (23, 24).

Log-phase cells were infected at 100 pfu/cell with either Ad.BRCA1 or Ad.Control, an empty vector that shares the same backbone as Ad.BRCA1 but carries no gene insert. In experiments involving two adenoviruses, Ad.MEK1(dn) was always transferred to the cells first (at 100 pfu/cell), 18 hours prior to the second infection with the Ad.BRCA1 virus. In these experiments, samples were then collected at the indicated time points following the second infection.

Cell cycle analysis—The cells were harvested, washed with PBS, and fixed in 70% ethanol. FACS analysis for DNA content was performed using FACSCalibur instrument (Beckon Dickinson, Mountain View, CA) by measuring the intensity of the fluorescence produced by propidium iodide, as recommended by the manufacturer.

TdT mediated dUTP-biotin end labeling (or TUNEL assay)—Seventy-two hours following infection with either Ad.BRCA1 or Ad.Control, cells were harvested for TUNEL assay. Cells were briefly washed twice with PBS and TUNEL assay performed using the MEBSTAIN Apoptosis Kit (Medical and Biological Laboratories, Japan) as recommended by the manufacturer. Results were analyzed by flow cytometry using the Cell Quest Software and the FACSCalibur instrument (Becton Dickinson, Mount View, CA).

DAPI staining—DAPI staining was performed as described previously (25). Briefly, cells (50,000) were resuspended in 100 µl PBS and bovine serum albumin (BSA) was added to a final concentration of 30%. The cell suspension was placed in a cytocentrifuge and spun at
1000 rpm for 5 min. The resulting slides were air dried for 15 min and washed with PBS two
times. The cells on the slides were fixed with 4% paraformadehyde for one hour at 4\(^\circ\)C, washed
three times with PBS and then stained with DAPI (2.5 \(\mu\)g/ml in 0.05M phosphate buffer, pH 7.2)
in the dark for one hour at room temperature. The stained cells were washed with PBS and
examined by fluorescence microscopy. Apoptotic cells were identified by condensation and
fragmentation of nuclei (26). Percentage of apoptotic cells was calculated as the ratio of
apoptotic cells to the total cells counted. At least 800 cells were counted from each sample.

**Antibodies and Immunoblotting**—Antibodies against PARP, BRCA1 and Bcl-2 are
mouse monoclonal antibodies that recognize full length PARP, the amino acids 1-304 of
BRCA1, and the amino acids 41–54 of Bcl-2 (Oncogene Research Products, Boston, MA).
Antibody Bcl-S/L (S-18) is an affinity-purified rabbit IgG antibody that recognizes the amino
terminus of both Bcl-X\(_S\) and Bcl-X\(_L\). Mouse monoclonal antibody against phospho-JNK (P-JNK)
recognizes the amino acids 183-191 of JNK in which the thr-183 and tyr-185 are
phosphorylated. Mouse monoclonal antibody against phospho-ERK1/2 (P-ERK1/2) binds to a
short amino acid sequence of ERK1 and ERK2 that includes the phosphorylated Tyr-204.
Antibodies against JNK and ERK1/2 are rabbit polyclonal IgG that recognize either the full-
length polypeptides of JNK1 and JNK2 or full length polypeptides of ERK2 and to a lesser
extent ERK1. The latter two antibodies were chosen because they react with both
phosphorylated and unphosphorylated forms of their target proteins. Both anti-Fas ligand and
anti-Fas antibodies are rabbit IgG recognizing amino acids 100-278 of Fas ligand and carboxy
terminus of Fas respectively. The two antibodies against caspase-8 and -9 can recognize both
the cleaved p10 catalytic subunit of the caspases and their full-length precursor forms. Anti-
caspase-8 antibody is a goat polyclonal IgG and anti-caspase-9 is a rabbit polyclonal IgG. To
confirm that equal amounts of protein were loaded, an affinity purified anti-actin goat IgG was
also used for quantitating actin protein levels presented on all immunoblots. All antibodies were
purchased from Santa Cruz Biotechnology (Santa Cruz, CA) unless specified. Preparation of
cell lysates, SDS-PAGE and Western blot analyses were performed as described previously (27).

RESULTS

Ectopic expression of BRCA1 induces apoptosis in U2OS cells but not in MCF-7 cells—

To investigate the effects of BRCA1 expression on cell growth, a recombinant adenovirus containing full-length BRCA1 cDNA (Ad.BRCA1) was generated as described in the Experimental Procedures. Ad.BRCA1 was used to infect U2OS cells, a human osteosarcoma cell line, and MCF-7 cells, a human breast cancer cell line. As controls, the same two cell lines were either left uninfected or infected with Ad.Control, an empty vector that shares the same backbone as Ad.BRCA1. Three days after infection, samples were analyzed for BRCA1 expression, cell cycle progression and apoptosis. Western blot analysis using an anti-BRCA1 antibody showed that BRCA1 expression was only detected in U2OS and MCF-7 cells infected with Ad.BRCA1 and that the protein was undetectable in those cells left uninfected (data not shown) or infected with Ad.Control (Fig.1A).

To investigate the effects of BRCA1 overexpression on apoptosis, TUNEL assays were performed. In U2OS cells, overexpression of BRCA1 resulted in a marked shift in the intensity of the fluorescence produced by dUTP incorporation, indicative of apoptosis (Fig. 1B). Only minor differences were seen between Ad.Control-infected cells and uninfected cells (Fig. 1B). In contrast, no shifts in fluorescence were observed in MCF-7 breast cancer cells following infection with Ad.BRCA1, compared to the fluorescence intensity in uninfected MCF-7 cells or Ad.Control-infected MCF-7 cells (Fig. 1B).

To confirm these results and quantitate the incidence of apoptosis, both U2OS and MCF-7 cells were infected with Ad.BRCA1 at the indicated doses. Following 72 hours of incubation at 37°C, the cells were stained with DAPI and analyzed by microscopy for apoptosis
as described in Experimental Procedures. As shown in Figure 2A, overexpression of BRCA1 in U2OS cells resulted in accumulation of cells with condensed and fragmented nuclei, indicative of apoptosis. BRCA1-induced apoptosis in U2OS cells was dose-dependent, with the highest incidence of apoptosis (66% of all cells) observed when cells were infected with 200 pfu/cell Ad.BRCA1 (Fig. 2C, open bars). In contrast, Ad.BRCA1 infection in MCF-7 cells did not result in apoptosis (Fig. 2C, solid bars).

To further confirm that programmed cell death was responsible for BRCA1-induced cytotoxicity in U2OS, Western blot analyses were performed on cell lysates from MCF-7 and U2OS cells infected for 72 hours with 100 pfu/cell of Ad.BRCA1 or Ad.Control virus. As shown in Figure 2B, Western blot analysis demonstrates that the level of full-length PARP protein in U2OS cells was decreased approximately 10 fold in Ad.BRCA1-infected cells relative to Ad.Control-infected cells. In contrast, no changes in full-length PARP protein levels were observed in Ad.BRCA1-infected MCF-7 cells (Fig. 2B). These results are consistent with the results from TUNNEL assays and DAPI staining (Fig. 1B and Fig. 2A) and indicate that the overexpression of BRCA1 induces apoptosis in U2OS cells but not in MCF-7 cells.

**BRCA1 overexpression results in the induction of Fas and of its ligand in both U2OS and MCF-7 cells**—Fas-L/Fas interactions have previously been shown to play an important role in mediating BRCA1-dependent apoptosis (20). In order to confirm the role of Fas-L/Fas in mediating BRCA1-induced apoptosis, we monitored the levels of Fas-L and Fas following the over-expression of BRCA1 in both U2OS and MCF-7 cells. As shown in the Figure 3, over-expression of BRCA1 resulted in an increase in both Fas-L and Fas expression in both cell lines. Furthermore, in both cells lines, the induction of Fas by BRCA1 was found to be much greater than the effect on Fas-L (11-fold versus 1.5-fold). Since BRCA1 expression does not result in apoptosis in MCF-7 cells (Fig. 1 and Fig. 2), the induction of Fas-L and Fas by BRCA1 expression is apparently not sufficient to activate the programmed cell death pathway in these cells.
BRCA1 effect on JNK kinase and ERK1/2 kinases in U2OS and MCF-7 cells—Harkin et al. recently showed that BRCA1-induced apoptosis in U2OS cells is associated with JNK kinase activation (18). However, the roles of other members of MAPK family, mainly p38 and ERK1/2, were not investigated. Since recent studies have suggested that the activation of ERK1/2 kinases can induce survival signals that can inhibit apoptosis (22), we therefore investigated the roles of p38 and ERK1/2 in BRCA1-induced apoptosis. Following infections of U2OS and MCF-7 cells with Ad.BRCA1, Western blot analysis were performed using antibodies against the phosphorylated forms of JNK, p38 and ERK1/2 from cell lysates at various time points after infection. As shown in Figure 4, BRCA1 expression induced phosphorylation of JNK in both cell lines. However, BRCA1 expression had no detectable effect on the phosphorylation status of p38 (data not shown). Furthermore, in U2OS cells the BRCA1-induced increase in phosphorylation of JNK was preceded by a rapid dephosphorylation of ERK1/2 (Fig. 4, P-JNK and P-ERK1/2). This effect was observed within 16 hours following Ad.BRCA1 infection. In contrast, BRCA1 expression in MCF-7 cells induced phosphorylation of ERK1/2 as well as phosphorylation of JNK. Activation of both JNK and ERK1/2 was noted in MCF-7 cells within 16 hours following infection with Ad.BRCA1 (Fig. 4, P-JNK and P-ERK). Since BRCA1 expression resulted in apoptosis in U2OS but not in MCF-7 cells, these results suggested that the activation of ERK1/2 signaling might play a role in inhibiting BRCA1-induced apoptosis in MCF-7 cells.

Inhibition of ERK1/2 phosphorylation by PD98059 promotes BRCA1-induced apoptosis in MCF-7 cells—PD98059, a specific inhibitor of MEK1/2 (28), was used to study the role of ERK1/2 signaling in regulating the effects of BRCA1 expression on the survival and proliferation of MCF-7 cells. Figure 5A represents the results obtained following incubating MCF-7 cells with 100 pfu/cell Ad.BRCA1 for 72 hours in the presence of increasing concentration of PD98059 as described in the Experimental Procedure. As shown in Figure 5A, ERK1/2 phosphorylation was inhibited in Ad.BRCA1 infected cells incubated in the presence of the inhibitor PD98059 at concentration >10 µM and maximal inhibition was achieved at 50µM (Fig. 5A). The maximal
inhibition produced by PD98059 corresponded to a 4.2-fold decrease in ERK1/2 phosphorylation, a decrease similar to that produced by serum starvation of uninfected MCF-7 cells (Fig. 5A, lanes 5 and 6), a condition known to down-regulate ERK1/2 phosphorylation (21). Similar effects of PD98059 on ERK1/2 phosphorylation was observed in Ad.Control virus infected MCF-7 cells (data not shown).

To examine the effect of ERK1/2 inhibition on the induction of apoptosis in Ad.BRCA1 infected MCF-7 cells, cells were infected with either Ad.Control or Ad.BRCA1 (100 pfu/cell) for 72 hours in the presence of increasing concentrations of PD98059 and then stained with DAPI as described previously. As shown in Figure 5B, incubation of Ad.Control infected MCF-7 cells with increasing concentrations of PD98059 did not result in apoptosis (Fig 5B, solid bars). In contrast, incubation of Ad.BRCA1-infected MCF-7 cells in the presence of PD98059 did result in a marked increase in apoptotic cell death (Fig 5B, open bars). Moreover, an increase in apoptosis was observed in Ad.BRCA1-infected MCF-7 cells incubated with 10 µM PD98059 and was maximal (50%) in cells incubated with 100 µM drug. The magnitude of BRCA1-induced apoptosis in MCF-7 cells following incubation with increasing doses of PD98059 correlated with the relative inhibition of ERK1/2 (Fig. 5A). It should be noted that serum starvation of MCF-7 cells, which resulted in marked inhibition of ERK1/2 phosphorylation (Fig. 5A, lane 6), also resulted in increased BRCA1-induced apoptosis (Fig, 5B).

The time course of the effect of ERK1/2 inhibition and the induction of apoptosis following Ad.BRCA1 infection was also examined. In these experiments, MCF-7 cells were exposed to PD98059 (100 µM) and infected one hour later with either Ad.BRCA1 or Ad.Control virus (100 pfu/cell). At various time points following incubation at 37°C, cells were analyzed for ERK1/2 phosphorylation and apoptosis. As shown in Figure 5C, incubation of MCF-7 cells for 24 hours with 100µM PD98059 resulted in complete inhibition of ERK1/2 phosphorylation in both Ad.Control and Ad.BRCA1 infected cells. Furthermore, incubation of MCF-7 cells with
PD98059 did not influence the total level of ERK1/2 and had no effect on BRCA1-induced JNK phosphorylation (Fig. 5C).

When the PD98059-treated MCF-7 cells were analyzed for apoptosis using DAPI staining, only cells infected with Ad.BRCA1 displayed condensed and fragmented nuclei, indicative of apoptosis (Fig. 5D, open diamond). Apoptosis was first detected as early as 24 hours following Ad.BRCA1 infection of PD98059-treated MCF-7 cells and was maximal at 72 hours. MCF-7 cells incubated with either PD98059 alone (inverted open triangle) or Ad.BRCA1 alone (solid square) showed no evidence of apoptosis (Fig. 5D). Taken together, these data (Fig. 5, A, B, C, and D) suggest a correlation between inhibition of ERK1/2 phosphorylation and induction of apoptosis following BRCA1 overexpression in MCF-7 cells.

To confirm the induction of apoptosis in these cells, protein samples collected from the 48-hour time point were analyzed by Western blot to assess the integrity of PARP [poly (ADP-ribose) polymerase] protein. The cleavage of PARP by caspases, a hallmark of apoptosis, occurs during the execution phase of programmed cell death (29, 30). Using an antibody against full length PARP, we found that the level of intact PARP protein was decreased 2.5-fold in Ad.BRCA1 infected MCF-7 cells exposed to PD98059 (Fig. 5E). In the absence of PD98059, expression of BRCA1 had little effect on the level of intact PARP protein. Treatment of Ad.Control infected cells with inhibitor failed to lower the level of intact PARP in the Ad.Control-infected cells (Fig. 5E). Collectively, these results (Fig. 5) suggest that inhibition of the ERK1/2 signaling pathway can sensitize MCF-7 cells to induction of apoptosis following overexpression of BRCA1.

Dominant negative MEK1 induces BRCA1-dependent apoptosis in MCF-7 cells—To confirm the role of the ERK1/2 signaling pathway in the regulation of BRCA1-induced apoptosis in MCF-7 cells, we used an adenoviral vector expressing a dominant negative mutant of MEK1 [Ad.MEK1(dn)], as described in Experimental Procedures. To examine the effect of MEK1(dn) expression on ERK1/2 phosphorylation, MCF-7 cells were infected with increasing
concentrations of Ad.MEK1(dn) (0 to 200 pfu/cell) and Western blot analysis was performed at 24 hours following infection. As shown in Figure 6A, infection of MCF-7 cells with Ad.MEK1(dn) resulted in marked inhibition of phosphorylation of ERK1/2 in a dose-dependent manner (Fig. 6A). Maximal inhibition of ERK1/2 phosphorylation (> 90% inhibition) was observed in cells that were infected with 200 pfu/cell of the Ad.MEK1(dn) virus (Fig. 6A).

To assess the effect of dominant negative MEK1 expression on the response of MCF-7 cells to BRCA1 overexpression, cells were infected with increasing doses of Ad.MEK1(dn) for 18 hours followed by exposure to 100 pfu/cell of either Ad.Control or Ad.BRCA1. Forty-eight hours after infection with Ad.BRCA1 or control virus, the incidence of apoptosis and the level of phosphorylated ERK1/2 were measured by DAPI staining and Western blot analysis, respectively. As shown in Figure 6B, exposure of MCF-7 cells to Ad.MEK1(dn) followed by infection with Ad.BRCA1 resulted in a marked increase in apoptotic cells (Fig. 6B, open bars). In contrast, infection with Ad.MEK1(dn) had no effect on cells exposed to Ad.Control virus. Furthermore, the incidence of the apoptosis in Ad.BRCA1-infected cells increased with increasing doses of Ad.MEK1(dn) virus (Fig. 6B, open bars) and the subsequent degree of inhibition of ERK1/2 phosphorylation (Fig. 6A, p-ERK1/2). Once again, these results suggest a relationship between the magnitude of ERK1/2 inhibition and the induction of apoptosis by BRCA1 overexpression in MCF-7 cells (Fig. 6, A and B).

To confirm the correlation between the inhibition of ERK1/2 phosphorylation by MEK1(dn) and the induction of apoptosis by BRCA1 overexpression in MCF-7 cells, a time-course experiment was also performed. MCF-7 cells were first infected with either the Ad.MEK1(dn) or Ad.Control viruses (100 pfu/cell). Following incubation at 37°C for eighteen 18 hours, the cells were then exposed to Ad.BRCA1 or Ad.Control virus (100 pfu/cell). At various time points following the second infection, the cells were harvested and analyzed for ERK1/2 phosphorylation and apoptosis. As shown in Figure 6C, Western blot analysis revealed that expression of the dominant negative mutant of MEK1 diminished the level of phosphorylated-
ERK1/2 in both Ad.Control and Ad.BRCA1 infected MCF-7 cells, but had no effect on the total level of ERK1/2 protein. As shown in Figure 6D, DAPI staining indicated that apoptosis developed following infection with Ad.MEK1(dn) and Ad.BRCA1 beginning at 24 hours and reached a maximum at 72 hours (open diamond). During this time frame, cells that were exposed to either MEK1(dn) alone (inverted open triangle) or BRCA1 alone (solid square) showed no evidence of apoptosis. To confirm the induction of apoptosis in these cells, protein lysates were prepared from cells harvested after 48 hours following the second viral infection and Western blot analysis performed using anti-PARP antibody. The results shown in Figure 6C, demonstrated a marked decrease in the level of intact PARP protein only in MCF-7 cells exposed to both Ad.BRCA1 and Ad.MEK1(dn) (Fig. 6C, lane 4). These observations are consistent with previous results obtained by incubating MCF-7 cells with the inhibitor PD98059 (Fig. 5) and support the hypothesis that the inhibition of ERK1/2 is required in MCF-7 cells for the induction of apoptosis following BRCA1 overexpression.

Inhibition of ERK1/2 enhances BRCA1-induced activation of caspases in MCF-7 cells—A previous study had shown that BRCA1 expression in serum-depleted MCF-7 cells resulted in the activation of caspases-8 and -9 (20). Although the status of ERK1/2 signaling had not been investigated in that study, studies have indicated that serum depletion in other cells resulted in down-regulation of ERK1/2 activities (31). To test whether caspases-8 and -9 are activated in Ad.BRCA1-infected MCF-7 cells following inhibition of the MAPK kinase pathway, cell lysates were probed with antibodies against both caspases. As shown in Figure 7, treatment of MCF-7 cells with Ad.BRCA1 alone, Ad.MEK1(dn) alone or PD98059 did not by itself result in any detectable decrease in uncleaved, inactive caspase 8 precursor level. In contrast, inhibition of ERK1/2 activity by treatment of MCF-7 cells with PD98059 or Ad.MEK1(dn) resulted in a marked decrease in the level of uncleaved, inactive precursor of caspase-8 in Ad.BRCA1 infected MCF-7 cells. Similar results were obtained when probing for the activated form of caspase-9. As shown in Figure 7, activated caspase-9 was only detected in MCF-7 cells that
were treated with Ad.BRCA1 in combination with either PD98059 or Ad.MEK1(dn). These observations indicate that both caspase-8 and -9 are activated in MCF-7 cells during BRCA1-induced apoptosis and that their activation is dependent upon inhibition of ERK1/2 signaling.

Recent studies have shown that members of the Bcl-2 family play a key role in controlling caspase activation during apoptosis (32–35) and that Bcl-2 and Bcl-x\textsubscript{L} are negative regulators of caspase activation. To examine the possible role of Bcl-2 and Bcl-x\textsubscript{L} in BRCA1-induced apoptosis in MCF-7 cells, samples were probed using antibodies against both proteins (Fig. 7). The results indicate that the levels of both Bcl-2 and Bcl-x\textsubscript{L} were decreased in Ad.BRCA1 infected MCF-7 cells treated with either PD98059 or Ad.MEK1(dn). Although exposure to Ad.MEK1(dn) alone resulted in a decrease in Bcl-2 protein level, this decrease was not associated with a similar change in Bcl-x\textsubscript{L} level. Treatment with PD98059 by itself or exposure to Ad.BRCA1 alone did not produce detectable changes in the levels of either Bcl-x\textsubscript{L} or Bcl-2. These results suggest that the apoptosis induced by the inhibition of ERK1/2 signaling in BRCA1-infected MCF-7 cells involves both the activation of the caspases-8 and -9 and the down-regulation of both Bcl-2 and Bcl-x\textsubscript{L}.

**DISCUSSION**

Evidence continues to suggest an important role for BRCA1 in the cellular response to DNA damages or loss of genomic integrity. Previous studies have shown that overexpression of BRCA1 protein can elicit either cell cycle arrest (16, 17) or the induction of apoptosis (18, 19) (20), depending on the cell type, and that BRCA1-induced apoptosis involves the activation of JNK. Furthermore, expression of a dominant negative mutant of JNK can block the apoptotic process triggered by BRCA1 overexpression (18). Other studies by Thangaraju et al. have shown that BRCA1-induced apoptosis in serum-depleted MCF-7 cells also involves JNK phosphorylation, Fas-L/Fas interaction and caspase-9 activation (20).
Although JNK activation may be necessary for BRCA1-induced apoptosis, the possible role of other members of the MAPK kinase family, including ERK1/2, in BRCA1-induced apoptosis had not been previously examined. ERK1/2 signaling has been previously shown to promote cell growth and survival and inhibit the induction of apoptosis elicited by the stress signals transmitted through JNK and p38 (22, 36). Furthermore, the observation that the apoptotic response promoted by BRCA1 overexpression in MCF-7 cells was apparently dependent on serum depletion suggested a possible role for ERK1/2 in determining the cellular response to BRCA1 (20).

In this report, we investigated the role of ERK1/2 and p38 in BRCA1-dependent apoptosis in U2OS and MCF-7 cells. Our results confirmed that overexpression of BRCA1 protein in U2OS osteosarcoma cells resulted in a rapid induction of JNK phosphorylation and was associated with induction of apoptosis. Similarly, BRCA1 overexpression in MCF-7 cells also resulted in a rapid induction of JNK phosphorylation. These studies as well as results by Thangaraju et al. (20) suggest that while JNK phosphorylation may be required for BRCA1-induced apoptosis, it is not sufficient by itself to induce apoptosis and that other factors must influence the fate of cells following BRCA1 expression. Since BRCA1 expression had no effect on the level of p38 phosphorylation in both U2OS and MCF-7 cells, signaling via this pathway is apparently not involved in the cellular response to BRCA1 overexpression.

Previous studies have shown that the induction of apoptosis by BRCA1 overexpression is associated with an increase in Fas-L/Fas interactions and activation of caspases-8 and -9 (18, 20). In this report we show that overexpression of BRCA1 in both U2OS and MCF-7 cells resulted in increased Fas and Fas ligand expressions (Fig. 3). While the induction of Fas and Fas ligand by BRCA1 in U2OS cells resulted in activation of caspase-8 and -9 and apoptosis, the increase in Fas-L/Fas levels following BRCA1 expression in MCF-7 cells did not result in activation of caspases and is not associated with apoptosis. Thus, the induction of Fas and the
activation of JNK following BRCA1 overexpression in MCF-7 cells are not by themselves sufficient to induce apoptosis.

Although BRCA1 expression results in JNK activation in both U2OS cells and MCF-7 cells, expression of BRCA1 produced different effects on ERK1/2 phosphorylation in these two cell lines. In U2OS cells, BRCA1 expression was associated with a reduction in the phosphorylation of ERK1/2, while BRCA1 expression in MCF-7 cells resulted in increased phosphorylation of ERK1/2 (Fig. 4). Other studies have also suggested that the activity of ERK1/2 relative to that of JNK can influence the propensity of cells to undergo apoptosis (22). Indeed, in this report we show that inhibition of the ERK1/2 using three different methods (PD98059, dnMEK1, and serum depletion) all resulted in enhanced apoptosis in MCF-7 cells following BRCA1 expression. These studies provide evidence of the importance of this signal transduction pathway in determining cell survival following BRCA1 expression.

Recent studies have shown that induction of apoptosis by the Fas-L/Fas interaction can proceed through redundant pathways and that cell lines can be classified as type I or II on the basis of the pathways utilized (activation of caspase-3 vs. activation of caspase-8 and -9) (37). Since MCF-7 cells lack caspase-3 (37), apoptosis in these cells must be dependent upon activation of caspase-8 and -9, and this activation apparently proceeds following release of cytochrome c from mitochondria. Previous studies using inhibitors specific to these caspases have shown that both caspase-8 and -9 are required for BRCA1-induced apoptosis in serum-depleted MCF-7 cells, and that caspase-9 was downstream of activation of caspase-8 (20).

Results in this report suggest that JNK activation and increased Fas expression in MCF-7 cells does not result in caspase-8 or -9 activation and apoptosis unless ERK1/2 is inhibited. Thus, activation of ERK1/2 following BRCA1 expression in MCF-7 cells grown in medium containing serum apparently inhibits apoptosis. Since BRCA1 induces Fas ligand and Fas interaction in MCF-7 cells grown in medium containing serum, the activation of ERK1/2 must interfere with the activity of the death receptor (the Fas ligand/Fas complex) and the subsequent
activation of downstream caspase-8. At the level of the mitochondria, release of cytochrome c and the activation of the apoptotic cascade can be blocked by increased expression of anti-apoptotic members of the Bcl-2 family, such as Bcl-2 and Bcl-xL (33, 37). In our studies inhibition of ERK1/2 in MCF-7 cells led to decreased expression of Bcl-2 and Bcl-xL following Ad.BRCA1 infection, indicating a possible role for these genes in the regulation of apoptosis by ERK1/2 in MCF-7 cells.

In summary, our results indicate that BRCA1 induces apoptosis in U2OS cells but not in MCF-7 cells grown in medium containing serum. While BRCA1 expression results in JNK activation in both cell lines, the effects on ERK1/2 activities differed in U2OS and MCF-7 cells following infection with Ad.BRCA1. ERK1/2 inhibition studies in MCF-7 cells indicate an important role for this pathway in protecting cells from BRCA1-induced apoptosis. Further studies will be required to fully understand the effects of BRCA1 expression on the activity of ERK1/2 in different cells and the role of signal transduction pathways in regulating the effects of BRCA1 expression on cell cycle inhibition and apoptosis.
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The abbreviations used are: DMEM, Dulbecco’s Modified Eagle’s Medium; DAPI, 4, 6-diamidino-2-phenylindole; FACS, fluorescence-activated cell sorting; Fas, the cell surface receptor that also designated Apo-1 or CD95; Fas-L, Fas ligand; JNK, c-Jun N-terminal kinase; P-JNK, phosphorylated – c-Jun N-terminal kinase; MAPK, mitogen activated protein kinase; ERK, extracellular signal regulated protein kinase; P-ERK, phosphorylated-extracellular signal regulated protein kinase, MEK1(dn), dominant negative mitogen activated protein kinase 1; NGF, nerve growth factor; PARP, poly (ADP-ribose) polymerase; PI, propidium iodide; TUNEL assay, TdT mediated dUTP-biotin end labeling assay; Ad, adenovirus.
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FIG. 1. **Overexpression of BRCA1 induces apoptosis in U2OS cells but not in MCF7 cells.** Uninfected U2OS cells and MCF-7 cells or cells infected with Ad.BRCA1 or Ad.Control at 100 pfu/cell were incubated at 37°C for three days, and then harvested and washed with PBS. The cell samples were divided into two portions for analysis. **A,** one half of the samples were lysed and 100 μg of protein lysates analyzed for BRCA1 expression by Western blot using an anti-BRCA1 antibody. Protein loading was confirmed by monitoring the levels of actin by immunoblotting using an anti-actin antibody. **B,** the other half of the samples were analyzed for apoptosis by TUNEL assay (TdT-mediated dUTP-biotin nick end labeling assay) as described in the Experimental Procedures.

FIG. 2. **Determination of BRCA1-mediated apoptosis by DAPI staining.** **A** and **B,** MCF-7 and U2OS cells were infected with Ad.BRCA1 or Ad.Control at 100 pfu/cell and incubated for 3 days. **A,** One portion of each cell sample (50,000 cells) was analyzed for apoptosis by DAPI staining and fluorescence microscopy as described under Experimental Procedure. **B,** The other portion of each sample was lysed and 100 μg of protein lysate was immunoblotted for PARP protein level using an anti-PARP antibody (PARP). Protein loading was confirmed by western blot using an anti-actin antibody (Actin). **C,** MCF-7 (solid bars) and U2OS (open bars) cells were infected with Ad.BRCA1 at the indicated doses. Following 3 days of incubation at 37°C, cells were harvested and analyzed for apoptosis by DAPI staining as described above. Percentage of apoptosis is shown as the mean ± S.D. of quadruplicate samples.

FIG. 3. **BRCA1 overexpression results in the induction of both Fas and the Fas ligand.** U2OS and MCF-7 cells were treated as described in Figure 1. Cells were harvested, lysed and
examined by Western blot analysis (100 µg protein per sample) for Fas (Fas) and the Fas ligand (Fas-L) using specific antibodies. To confirm equal loading of all lanes, duplicate samples were probed with an anti-actin antibody (Actin).

FIG. 4. Determination of phosphorylation of JNK and ERK1/2 in U2OS cells and MCF-7 cells upon overexpression of BRCA1. U2OS cells and MCF-7 cells were infected with Ad.BRCA1 at 100 pfu/cell and incubated at 37°C for the times indicated. The cells were harvested, lysed, and the level of phosphorylated-JNK (P-JNK) or phosphorylated-ERK1/2 (P-ERK1/2) determined by immunoblotting with a relevant phosphorylation specific antibody. Parallel sets of protein samples were probed with antibodies against JNK (JNK), ERK1/2 (ERK1/2), or actin (Actin) to assess the total level of each protein.

FIG. 5. MEK1/2 Inhibition with PD98059 induces apoptosis in MCF-7 cells that overexpress BRCA1. A, MCF-7 cells were pre-incubated with PD98058 at the indicated doses for one-hour and then incubated with Ad.BRCA1 at 100 pfu/cell. After 24 hours incubation at 37°C, the cells were collected, lysed, and the level of phosphorylated-ERK1/2 (P-ERK1/2) determined by western blot with a phosphorylation specific antibody. The level of total ERK1/2 protein (ERK1/2) in each sample was determined by immunoblotting using an anti-ERK1/2 antibody. For serum starvation experiment, log-phase growing cells were washed once with DMEM and the medium was changed to DMEM containing 0.1% serum and the cells incubated at 37°C for 24 hours prior to infection with Ad.BRCA1 at 100 pfu/cell. Infected cells were incubated in the same medium (containing 0.1% serum) for an additional 24 hours and then collected for analysis of ERK1/2 phosphorylation. B, MCF-7 cells were either pre-incubated with PD98059 at the indicated dose for one hour or incubated in medium containing 0.1% serum for 24 hours as described above. The treated cells were then infected with Ad.BRCA1 (open bars) or Ad.Control (solid bars) at 100 pfu/cell, incubated for additional 72 hours at 37°C. The cells
were then harvested and analyzed for apoptosis by DAPI staining. The percentage of cells undergoing apoptosis is expressed as the mean ± S.D. of quadruplicate samples. C, cells were treated with PD98059 (100 µM), or as a control, DMSO (0.5%) for one-hour prior to infection with Ad.BRCA1 or Ad.Control at 100 pfu/cell. Twenty-four hours following infection, the cells were harvested, lysed and the levels of phosphorylated–ERK1/2 (P-ERK) and phosphorylated-JNK (P-JNK) determined by Western blot with appropriate antibodies. Parallel sets of protein samples were quantitated for total protein levels of JNK (JNK), ERK1/2 (ERK1/2), or actin (Actin) by Western blot using relevant antibodies. D, cells were treated as described above (C) and incubated for the times indicated. The treatments were carried out with DMSO+Ad.Control (●), DMSO+Ad.BRCA1 (■), PD98059+Ad.Control (▼), or PD98059+Ad.BRCA1 (◇). Following treatment, the cells were collected and analyzed for apoptosis by DAPI staining as described previously. The percentage of cells undergoing apoptosis is shown as the mean ± S.D. of quadruplicate samples. E, cells were treated as described above (C) and incubated for 48 hours at 37°C. The levels of full-length PARP were determined by Western blot analysis using a specific anti-body (PARP). Protein loadings were confirmed by measuring actin levels in the immunoblot using an anti-actin antibody (Actin).

FIG. 6. Dominant negative MEK1 expression inhibits endogenous MEK1 activity and induces apoptosis in MCF-7 cells expressing exogenous BRCA1. A, MCF-7 cells were infected with Ad.MEK1(dn) at the doses indicated. Following incubation for 24 hours at 37°C, cells were collected and analyzed for phosphorylated-ERK1/2 (P-ERK) by Western blot analysis. Protein loading was confirmed by immunoblotting for level of total ERK1/2 (ERK1/2) using a specific antibody. B, cells were first infected with Ad.MEK1(dn) at the doses indicated for 18 hours, followed by infection with either Ad.BRCA1 (open bars) or Ad.Control (solid bars) at 100 pfu/cell. The cells were then incubated for an additional 48 hours at 37°C and then
analyzed for apoptosis by DAPI staining. The percentage of cells undergoing apoptosis is expressed as the mean ± S.D. of quadruplicate samples. C, cells were infected first with Ad.MEK1 or Ad.Control at 100 pfu/cell and incubated for 18 hours followed by infection with Ad.BRCA1 or Ad.Control at 100 pfu/cell. Cells were then incubated for an additional 48 hours, and then harvested cell lysates were immunoblotted for phosphorylated-ERK1/2 (P-ERK1/2) and PARP (PARP) using appropriate antibodies as described in Experimental Procedures. Parallel sets of protein lysates were examined for total of ERK1/2 (ERK1/2) and actin (Actin) protein levels by Western blot with relevant antibodies. D, cells were treated as described above and incubated for additional times as indicated. The treatments were carried out with Ad.Control+Ad.Control (●), Ad.Control+Ad.BRCA1 (■), Ad.MEK1(dn)+Ad.Control (▽), or Ad.MEK1(dn)+Ad.BRCA1 (◇). Resulting cells were collected and analyzed for apoptosis by DAPI staining. The percentage of cells undergoing apoptosis is shown as the mean ± S.D. of quadruplicate samples.

FIG. 7. MEK1 inhibition induced BRCA1-dependent apoptosis correlates with activation of caspase 8 and caspase 9. MCF-7 cells were treated and cell lysates prepared as described in Fig.5 and Fig.6. One hundred micrograms of cell lysates were separated by SDS-PAGE, and protein levels of caspase 8 precursor (caspase 8 precursor), active caspase 9 (active caspase 9), Bcl-2 (Bcl-2), and Bcl-x\textsubscript{L} (Bcl-x\textsubscript{L}) were determined by Western blot analysis using appropriate antibodies as described previously. To confirm equal protein loading, actin protein levels were quantitated in a parallel set of protein lysates (Actin).
Fig 1A, Yan et al

**MCF7**
- **BRCA1**
- **Actin**

**U2OS**
- **BRCA1**
- **Actin**
B

**MCF7**

- Uninfected
- Ad.Control
- Ad.BRCA1

**U2OS**

- Uninfected
- Ad.Control
- Ad.BRCA1

Cell count vs. Log-dUTP-FITC for MCF7 and U2OS cells. Fig 1B, Yan et al.
Fig 2A, Yan et al
Fig 2B, Yan et al
Fig 2C, Yan et al
Fig. 3, Yan et al.
Fig 4, Yan et al.

| U2OS | MCF7 |
|------|------|
| 0 16 24 48 | 0 16 24 48 |

- Time (hr)
- P - JNK
- JNK
- P-ERK1/2
- ERK1/2
- Actin

Fig 4, Yan et al.
Fig 5A, Yan et al
Fig 5B, Yan et al
|           | Ad. Control | Ad. BRCA1 | PD98059 | P-ERK1/2 | ERK1/2 | P-JNK | JNK | Actin |
|-----------|-------------|-----------|---------|----------|--------|------|-----|-------|
| -         | -           | -         |         |          |        |      |     |       |
| +         | +           | +         |         |          |        |      |     |       |

Fig 5C, Yan et al
% apoptotic cells

24 hr                48 hr                72 hr

Fig 5D, Yan, et al
**Fig 5E, Yan et al**

|       | Ad.Control | Ad.BRCA1 |
|-------|------------|----------|
| PD98059 | -  +       | -  +     |
| PARP  |            |          |
| Actin |            |          |
A

| Ad.MEK1(dn) (pfu/cell) |
|------------------------|
| 0  | 50  | 100 | 200 |

- P-ERK1/2
- ERK1/2

Fig 6A, Yan et al
Fig 6C, Yan et al

C

| Ad. Control | Ad. MEK1(dn) |
|------------|-------------|
| -          | -           | Ad.BRCA1 |
| +          | +           |          |
| P-ERK1 / 2 |             |
| ERK1 / 2   |             |
| PARP       |             |
| Actin      |             |

1  2  3  4
Fig. 6D, Yan et al
Fig 7, Yan et al.

|                | DMSO | PD98059 |
|----------------|------|---------|
| Control        | -    | +       |
| MEK1(dn)       | -    | +       |

- Ad.BRCA1
  - Caspase 8 precursor
  - Active Caspase 9
- Bcl-2
- Bcl-xL
- Actin
BRCA1-induced apoptosis involves inactivation of ERK1/2 activities
Ying Yan, John P. Haas, Min Kim, Magdalene K. Sgagias and Kenneth H. Cowan

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