BRCA1 Phosphorylation Regulates Caspase-3 Activation in UV-Induced Apoptosis

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Abstract

Apoptosis is implemented by death machinery that involves the caspase family of proteins, under the condition of a variety of stresses. Previous studies have shown that mouse embryonic stem cells deficient for caspase-3 are resistant to UV-induced apoptosis and that the active form of caspase-3 translocates to the nucleus. It has also been shown that the breast cancer tumor suppressor, BRCA1, is phosphorylated on Ser\(^{1423}\) and Ser\(^{1524}\) by the ataxia telangiectasia mutated–related kinase (ATR) after UV damage. Here, we show that activation of caspase-3 by UV is abrogated in BRCA1-mutated SNU251 and HCC1937 cells but was restored by reexpressing wild-type (wt) BRCA1, but not phosphorylation-deficient BRCA1\(^{Ser^{1423}Ala/Ser^{1524}Ala}\) (BRCA1 \(^{S1423/1524A}\)). In SNU251 cells expressing wt BRCA1, the inhibitory interaction between X-linked inhibitor of apoptosis protein (XIAP) and caspase-9 remains intact after UV, compared with cells expressing wt BRCA1. We determined that XIAP and BRCA1 interact and upon phosphorylation this complex is disrupted. Nuclear translocation of active caspase-3 is detected only when wt BRCA1 is reexpressed. Consistent with this, Rad21, a known substrate of caspase-3, is cleaved only when wt BRCA1 is expressed in vivo. These results propose a model that inhibition of BRCA1 phosphorylation leads to the abrogation of a specific form of apoptosis that is mediated by caspase-3.

Introduction

Cells are eliminated in response to increased stress by a genetically regulated apoptotic pathway. Abnormalities in apoptosis represent a major hallmark of tumorigenesis with tumor cells often demonstrating a deregulated apoptotic response.

Phosphorylation of proteins induced by genotoxic stress is integral to the DNA damage response and apoptotic pathways. BRCA1, the breast cancer tumor suppressor protein, is phosphorylated in response to cell cycle and DNA damage (1, 2). BRCA1 mutations have been found in a large number of familial breast and ovarian cancers and also in inherited breast cancers alone (3). In response to genotoxic stress, BRCA1 is phosphorylated by stress-activated kinases such as ataxia telangiectasia mutated (ATM) and ATM-related kinase (ATR; ref. 4) and is recruited to sites of DNA damage as nuclear foci that contain DNA repair proteins, including the Mre11-Rad50-NBS1 complex (5). BRCA1 has previously been implicated in many biological processes, including the DNA damage response, cell cycle regulation, transcription, and maintenance of genome stability (2, 6–8); however, the precise mechanism of its tumor suppression remains to be elucidated. Caspase-3 is one of the main executioner caspses in the apoptotic pathway. Studies involving caspase-3 knockout mice have described that the presence of caspase-3 is essential for chromatin condensation and DNA degradation in apoptosis (9). Caspase-3 is required for apoptosis under UV damage, but not for IR-induced apoptosis in mouse embryonic stem cells (9). These findings implicate caspase-3 as an integral part of the apoptotic response, with the inhibition of caspase-3 preferentially impairing the UV-induced apoptotic pathway.

Materials and Methods

Antibodies, plasmids, and adenoviruses. Primary antibodies used were rabbit polyclonal antibody to caspase-9 (Bovision, Mountain View, CA; Santa Cruz Biotechnology, Santa Cruz, CA), BRCA1 (Santa Cruz Biotechnology, Santa Cruz, CA), BRCA1 phospho-Ser\(^{1524}\) (Abcam, Inc., Cambridge, MA) and anti BRCA1 phospho-Ser\(^{1423}\) (Chemicon, Temecula, CA). A rabbit monoclonal antibody was used to anti-caspase-3 antibodies (Asp175 and 9662; Cell Signaling Technology, Beverly, MA) and mouse monoclonal antibodies to X-linked inhibitor of apoptosis protein (XIAP; Stresses Bioreagents, Victoria, British Columbia, Canada), anti-Rad21 antibody (Bethyl Laboratories, Montgomery, TX), anti-enhanced red fluorescent protein antibody (ERFP, Clontech, Mountain View, CA) and tubulin (Santa Cruz Biotechnology). Red fluorescent protein-tagged full-length wild-type (wt) BRCA1 was cloned into pEFe vector (Invitrogen, Carlsbad, CA). Phospho-BRCA1 Ser1423Ala/Ser1524Ala mutant (ERFP-BRCA1\(^{S1423/1524A}\)) was generated by site-directed PCR mutagenesis from wt EF1-BRCA1-RFP. The primers as follows: Ser1423Ala: ACAGCAGAGGCGCAGCTTCTAACAG and Ser1524Ala: AAAACTCCCAGCCTGCTATGTTT. Transfections were done with FuGENE (Roche Applied Science, Indianapolis, IN). Adenovirus for wt BRCA1 was generated by subcloning into the pADTRACK-cytomegalovirus vector, and recombinant was done in a BS5180 bacterial strain (10). The adenovirus for BRCA1\(^{S1423/1524A}\) was generated using the Adeno-X system (Clontech).

Caspase-3 activity assay and Annexin V staining. Caspase-3 activity was determined using the TACS fluorescent caspase-3 detection assay IB-FAM kit (Trevigen, Inc., Gaithersburg, MD). SNU 251 cells were transfected with either wt BRCA1 or phospho-deficient BRCA1\(^{S1423/1524A}\). After 48 hours, cells were UV irradiated (100 mJ/cm\(^2\)). Four hours after UV, cells were analyzed by either flow cytometry or fluorescence microscopy. Annexin V staining and propidium iodide staining was done according to the instructions of the manufacturer (Roche Applied Science). Results were analyzed using FACS Calibur (BD Biosciences, San Jose, CA).

Cell culture. Human normal mammary epithelial cells, HeLa, U2OS, and 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS, Invitrogen), SNU251 and HCC1937 cells were grown in RPMI 1640 also supplemented with 10% FBS (Invitrogen). UV irradiation was administered using the GS gene linker UV chamber (Bio-Rad, Hercules, CA).

Glutathione S-transferase pull-down assay. Purification of glutathione S-transferase (GST) BRCA1 constructs was described previously (11). 293T cells were transfected with 3 μg of each GST construct or pEBG vector alone and incubated for 48 hours. Briefly, 1 mg of total cell lysate was incubated with glutathione beads and samples were rotated overnight at 4°C in
NET-N buffer. After extensive washing with NET-N buffer [20 mmol/L Tris-HCl (pH 8.0), 100 mmol/L NaCl, 1 mmol/L EDTA, 0.5% NP40, 100 mmol/L NaF; and 200 μmol/L sodium orthovanadate], the samples were loaded in 12% SDS-PAGE and blotted with anti-XIAP or anti-GST antibodies.

Immunoblot and immunoprecipitation analysis. Cells were lysed in EBC buffer [50 mmol/L Tris-HCl (pH 8.0), 120 mmol/L NaCl, 0.5% NP40, 100 mmol/L NaF; 200 μmol/L sodium orthovanadate, 100 μg/mL phenylmethylsulfonyl fluoride, 2 μg/mL leupeptin, and 2 μg/mL aprotinin]. Protein concentration in cell lysates were determined using the Bio-Rad protein assay kit. Sixty micrograms of whole cell extract were loaded per lane and separated by 6%, 7.5%, or 12% SDS-PAGE. Transfer to Immobilon-P membrane (Millipore, Billerica, MA) was done using a semidy transfer method (Trans-Blot; Bio-Rad) in 25 mmol/L Tris base, 192 mmol/L glycine, and 10% methanol for 1.5 hours at 15 V. Membranes were blocked in 1% nonfat dry milk in PBS/0.05% Tween and incubated with primary antibodies and horseradish peroxidase–conjugated secondary antibodies (The Jackson Laboratory, Westgrove, PA) followed by enhanced chemiluminescence detection. For immunoprecipitation, 1 mg of total cell lysate was incubated with the specific antibody overnight at 4°C followed by either protein A or protein G Sepharose beads (Sigma, St. Louis, MO). The samples were washed with NET-N buffer and subjected to SDS-PAGE.

Small interfering RNA assay. The following small interfering RNAs (siRNA) directed against BRCA1 were generated by Dharmacon (Lafayette, CO): (a) 5’-GGACCCGUGCCCAAAAG-3’ and (b) 5’-UCACAGGUCUUUAUGUA-3’. Briefly, U2OS cells were seeded at 30% confluence the day before transfection and cells were transfected with FuGENE (Roche Applied Science). Twenty-four hours later, transfections were repeated as before. Lysates were collected after a further 48 hours and subjected to SDS-PAGE.

Results

Caspase-3 activation requires BRCA1 phosphorylation after UV. U2OS and HeLa cells both expressing wt BRCA1 were UV irradiated. Similarly, SNU251 and HCC1937 cells, which both express functionally inactive BRCA1 with a truncated BRCA1 COOH-terminal region, were also subjected to UV damage. Immuno blot analysis using a specific antibody for cleaved caspase-3 shows that in the absence of wt BRCA1, caspase-3 is not activated (Fig. 1A). Similarly, in U2OS cells transfected with BRCA1 siRNA resulting in a reduction in the level of BRCA1 expression, caspase-3 activation was not observed after UV (Fig. 1B). We and others have previously shown that DNA damage
induced phosphorylation of BRCA1 is important for its localization and function (1). A number of kinases have been identified to be involved in BRCA1 phosphorylation, including ATM, ATR, Chk2, and Aurora-A (4, 6, 12). ATM and Chk2 phosphorylate BRCA1 following IR at several phosphorylation sites including Ser-988, Ser-1387, Ser-1423, Ser-1457 and Ser1524 (1, 12). BRCA1 phosphorylation following UV and hydroxyurea is ATM independent and is mediated by ATR at overlapping sites to ATM, including Ser1423 and Ser1524 (4). In this study, we examined whether phosphorylation of BRCA1 is also important for its role in apoptosis.

As previously stated, BRCA1 is phosphorylated at Ser1423 and Ser1524 following UV (Fig. 1C; ref. 4). We generated an adenovirus expressing either wt BRCA1 or a phosphorylation-deficient form of BRCA1 at both residues by carrying out site-directed mutagenesis substituting Ala for Ser. This phosphodeficient mutant BRCA1 (BRCA1S1423A/1524A) allowed us to investigate the possible role of BRCA1 phosphorylation in UV-induced apoptosis. SNU251 and HCC1937 cells were infected with either Ad-wt BRCA1 or Ad-BRCA1S1423A/1524A for 48 hours. Normal mammary epithelial cells were also treated with UV. Upon reexpression of wt BRCA1 in SNU251 and HCC1937 cells, caspase-3 activation was restored after UV. Interestingly, in the absence of BRCA1 phosphorylation at Ser1423 and Ser1524 following UV irradiation, caspase-3 was not activated (Fig. 2). To confirm this abrogation of caspase-3 activity, we used a fluorescent caspase-3 detection assay by flow cytometry or fluorescence microscopy.
activation, we detected caspase-3 activity using the TACS fluorescent caspase-3 detection assay. This assay can detect active caspase-3 using a specific caspase-3 inhibitor coupled to a green fluorochrome. Cells that contain the bound caspase-3 inhibitor can be analyzed by flow cytometry (Fig. 2B) and also by fluorescence microscopy (Fig. 2C). Figure 2B shows that in SNU251 cells expressing BRCA1<sub>S1423A/1524A</sub>, caspase-3 activity only slightly increased from 2.1% to 3.4% after UV damage. However, upon reintroduction of wt BRCA1, caspase-3 activation was restored, with an increase from 2.1% to 8.4% after UV.

**BRCA1 phosphorylation-dependent nuclear translocation of caspase-3 after UV damage.** Recently, it has been reported that active caspase-3 is translocated to the nucleus during apoptosis induced by treatment with both etoposide and anti-Fas antibody with actinomycin D (13). In the present study, with the TACS fluorescent caspase-3 detection assay, nuclear localization of active caspase-3 was also observed in SNU251 cells expressing BRCA1<sub>S1423A/1524A</sub> after UV damage.
caspase-3 was detected only when wt BRCA1 was expressed in SNU251 cells (Fig. 2C). In BRCA1 S1423/1524A expressing cells, although a basal level of active caspase-3 is weakly detected in the nucleus, caspase-3 activity is not increased upon UV damage. Taken together, these results strongly suggest that phosphorylation of BRCA1 at Ser1423 and Ser1524 is required for caspase-3 activation after UV damage.

The disruption of the X-linked inhibitor of apoptosis protein–caspase-9 inhibitory complex requires BRCA1 phosphorylation. It has been previously shown that caspase-9 is responsible for the cleavage of caspase-3 following UV (14). We next investigated the mechanism involved in this caspase-3 inactivation by initially analyzing the activation status of caspase-9. As shown in Fig. 2A, caspase-9 is similarly cleaved following UV in both SNU251 and HCC1937 cells expressing either wt BRCA1 or BRCA1 S1423/1524A. Therefore, we determined that inactivation of caspase-3 by BRCA1 S1423/1524A is occurring downstream of caspase-9 cleavage. It has been shown that members of the caspase family, including caspase-3, caspase-7, and caspase-9 are kept inactive by their direct interaction with XIAP (14). Upon apoptotic stress, cytochrome c is released from the mitochondria and binds to Apaf-1. Apaf-1, in turn, binds to procaspase-9, leading to the cleavage of caspase-9. An inhibitory association with XIAP regulates the activation of caspase-9 following its cleavage (14). Upon dissociation of this complex, caspase-3 is cleaved by caspase-9 and initiates the downstream apoptotic process. We next examined whether the mechanism of caspase-3 inactivation in the absence of BRCA1 phosphorylation involved XIAP. We carried out a coimmunoprecipitation assay between XIAP and cleaved caspase-9 in the presence of either wt BRCA1 or BRCA1 S1423/1524A in SNU251 cells before and after UV damage (Fig. 3A). Our results clearly show that cleaved caspase-9 remains associated with XIAP upon UV damage in cells expressing BRCA1 S1423/1524A. In contrast, this interaction is dissociated after UV in cells expressing wt BRCA1. These results implicate the increased interaction of XIAP and caspase-9 as the mechanism abrogating caspase-3 cleavage in BRCA1 S1423/1524A cells, suggesting that BRCA1 phosphorylation is involved in the dissociation of the XIAP–caspase-9 complex.

BRCA1 and X-linked inhibitor of apoptosis protein form a complex. We next investigated the possible interaction between BRCA1, XIAP, and caspase-9. In Fig. 3B, we show that BRCA1 associates with XIAP in normally growing cells. Interestingly, upon UV damage, the association between BRCA1 and XIAP is impaired. Figure 3B illustrates that in SNU251 cells expressing BRCA1 S1423/1524A, the BRCA1-XIAP complex remains intact after UV damage. Taken together, these results strongly suggest that phosphorylation of BRCA1 is required for the dissociation of the XIAP-BRCA1 complex and this dissociation is involved in the regulation of the XIAP–caspase-9 inhibitory interaction. Next, we determined the XIAP-binding region of BRCA1. BRCA1 amino acids 1 to 324; 260 to 553; 502 to 802; 758 to 1,064; 1,005 to 1,313; 1,314 to 1,863; and 1,314 to 1,600 were expressed as GST fusion proteins and we did a GST pull-down assay. As shown in Fig. 3C, the BRCA1-XIAP interaction region was mapped to amino acids 1,314 to 1,600. These results suggest that BRCA1 phosphorylation at Ser1423 and Ser1524 interrupts its interaction with XIAP and subsequently results in the XIAP–caspase-9 dissociation. Coimmunoprecipitation of BRCA1 and caspase-9 was also determined. Wt or BRCA1 S1423/1524A was expressed in SNU251 cells and treated with UV. Interaction of BRCA1 and cleaved caspase-9, not procaspase-9, was detected only when wt BRCA1 was expressed in SNU251 cells after UV treatment (Fig. 3D; data not shown). Detailed studies of how BRCA1 regulates XIAP and caspase-9 are in progress.

Lack of BRCA1 phosphorylation impairs UV-induced apoptosis. Both BRCA1 and caspase-3 have been implicated as important regulators of apoptosis. Therefore, we investigated whether this inactivation of caspase-3 regulated by expression of BRCA1 S1423/1524A was influencing the apoptotic response in BRCA1 mutant cell lines. Annexin V staining was used to analyze the apoptotic populations of cells expressing either wt or BRCA1 S1423/1524A (Fig. 3E). SNU251 cells expressing a control LacZ adenovirus exhibited a small increase in apoptosis after UV, increasing from 10.1% to 11.7%. In comparison, cells expressing wt BRCA1 showed a much greater increase in apoptosis after UV treatment with the apoptotic cell population increasing from 10% to 22.7%. Interestingly, upon expression of BRCA1 S1423/1524A, similar to control cells, only a slight increase in the apoptotic cell population was observed in cells after UV, from 11.2% to 13.2%. We further studied whether any known substrates of caspase-3 is cleaved in a BRCA1-dependent manner. It has been shown that Rad21 is cleaved into a 64 kDa product by caspase-3 under conditions of cell stress, such as etoposide and UV (15, 16). 293T cells were transfected with either an empty vector or ERFP-BRCA1 S1423/1524A–expressing vector. After 2 days, cells were treated with UV and Rad21 was immunoblotted. A cleaved product of 64 kDa Rad21 was detected only in parental cells and was not detected in BRCA1 S1423/1524A-expressing cells (Fig. 4). Taken together, these results indicate that expression of BRCA1 S1423/1524A mutant form is inhibitory for the activation of caspase-3 pathway and show an important role for BRCA1 phosphorylation at these sites in the UV-induced apoptotic pathway.

Discussion

Many studies have implicated BRCA1 as a mediator of apoptosis. BRCA1 COOH-terminal mutations of BRCA1 have been shown to
confer increased cellular sensitivity to ionizing radiation and the chemotherapy drug paclitaxel (17). However, further studies have shown that expression of BRCA1 has been shown to induce apoptosis in response to different stresses, including substratum detachment, ionizing radiation, and treatment with anticancer agents (18). This notion of BRCA1 as a differential regulator of apoptosis depending on the kind of the cellular insult has previously been hypothesized, suggesting that BRCA1 infers cellular sensitivity to apoptosis by antimicrotubule agents and cellular resistance to a range of DNA-damaging agents (19). In this study, we provide evidence that loss of BRCA1 phosphorylation results in increased cellular survival after UV irradiation.

The ATR kinase is responsible for phosphorylation of BRCA1 after UV with expression of catalytically inactive ATR, resulting in the abrogation of the UV-induced phosphorylation of BRCA1 (4). After IR, ATM is involved in the immediate phosphorylation of BRCA1 at different sites, including Ser1387 and Ser1457 (4). These differences in DNA damage may also contribute to the role of BRCA1 in apoptosis regulation.

The reported role of caspase-3 in breast cancer has been conflicting. O’Donovan et al. (20) suggest that the rate of apoptosis measured by caspase-3 activation was higher in breast cancer compared with nonmalignant breast tissue. However, in contrast, caspase-3 deficiency and down-regulation have frequently been detected in breast cancer (21). Most recently, it has been shown that loss of caspase-3 may be involved in the promotion of tumor development, not only by apoptosis inhibition but also by stimulation of tumor growth (22). Taken together, these findings implicate an important role for caspase-3 in the suppression of breast cancer development.

Recent studies have shown that Rad21, a cohesin component, is cleaved by caspase-3 or caspase-3-like activity under conditions of DNA damage, such as etoposide and UV (15). These studies have shown that Rad21 undergoes cleavage after these treatments, producing the COOH-terminal 64 kDa cleavage product. Interestingly, this product translocates to the cytoplasm and induces apoptosis (15). In the present studies, we observed that Rad21 is cleaved into a 64 kDa product under UV treatment in a BRCA1 phosphorylation-dependent manner, indicating the physiologic role of BRCA1 in activating caspase-3. We also observed that the COOH-terminal 64 kDa product of Rad21 accumulates in the cytoplasm in UV-treated cells by immunocytochemical analysis (data not shown).

In summary, we have established that BRCA1 functions as a regulator of apoptosis in response to UV irradiation. In particular, BRCA1 phosphorylation at Ser1423 and Ser1524 is required for caspase-3 activation following UV damage. We have delineated a BRCA1 phosphorylation-dependent mechanism of caspase-3 activation, through the dissociation of the XIAP-BRCA1 interaction and inhibition of the XIAP–caspase-9 complex. We speculate that perhaps BRCA1 is acting as a scaffold protein for XIAP, and dissociation of BRCA1 from XIAP is necessary for abrogation of the caspase-9–XIAP complex. This study proposes caspase-3 inactivation due to loss of BRCA1, as a mechanism for breast cancer development, with the regulation of caspase-3 by BRCA1 further clarifying the role of BRCA1 as a tumor suppressor.

Acknowledgments

Received 6/14/2005; revised 8/12/2005; accepted 10/10/2005.

Grant support: NIH grants CA79892 and CA90631 (T. Ouchi).

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