Deficiency of Caspase-3 in MCF7 Cells Blocks Bax-mediated Nuclear Fragmentation but not Cell Death

Shunsuke Kagawa, Jian Gu, Tsuyoshi Honda, Timothy J. McDonnell, Stephen G. Swisher, Jack A. Roth, and Bingliang Fang

Departments of Thoracic and Cardiovascular Surgery [S. K., J. G., S. G. S., J. A. R., B. F.] and Molecular Pathology [T. J. M.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030, and Department of Obstetrics and Gynecology, Yamanashi Medical University, Yamanashi 409-3898, Japan [T. H.]

ABSTRACT

Caspase-3 plays a critical role in a proteolytic cascade within the apoptosis signal pathway; this enzyme is commonly activated by numerous death signals and cleaves a variety of important cellular proteins. Using caspase-3-deficient MCF7 cells and clones stably transfected with the caspase-3 gene (MCF7/Casp3), we evaluated the role of caspase-3 in Bax-induced apoptosis. Bax overexpression induced cell death in both parental MCF7 cells and MCF7/Casp3 cells. The introduction of the caspase-3 gene did not change the rate of cell death. Caspase-3-deficient parental MCF7 cells, however, failed to undergo morphological nuclear and DNA fragmentation, whereas MCF7/casp3 cells displayed intact nuclear dismantling and DNA fragmentation. Caspase-3 deficiency, however, did not affect Bax-induced levels of poly(ADP-ribose) polymerase cleavage, caspase-6 activation, and lamtin B cleavage. Together, these results suggest that a deficit in caspase-3 is not sufficient to block Bax-induced cell death.

INTRODUCTION

Apoptosis is a genetically regulated mechanism of cellular suicide that plays a crucial role in development and in homeostasis. It is typically accompanied by the activation of a class of death proteases called caspases and widespread biochemical and morphological changes. Caspases are now known to be essential components of cell death pathways. They are constitutively and ubiquitously expressed and are usually coactivated by proteolytic processing in response to death signals. In fact, caspases are arranged in a proteolytic cascade that serves to transmit and amplify death signals (1). Caspases contribute to the drastic morphological changes associated with apoptosis by proteolyzing a number of key substrates, including the structural proteins such as α-fodrin (2) and gelsolin (3), p21-activated kinase, PAK2 (4), and focal adhesion kinase (5).

Among the caspases, caspase-3 stands out because it is commonly activated by numerous death signals and cleaves a variety of important cellular proteins including PARP (3) and DFF (DFF45; Refs. 6 and 7). But several reports have raised questions recently about whether caspase-3 is required in apoptosis. Woo et al. (8) reported that, in embryonic stem cells, caspase-3 is required for apoptosis induced by UV but not apoptosis induced by gamma irradiation. Thus, the necessity of caspase-3 seems to be dependent on the apoptosis-inducing event. McCarthy et al. (9) reported that apoptosis induced by c-Myc, EIA, and Bak could not be blocked by pan-caspase inhibitor z-VAD. Janicke et al. (10) also demonstrated that neither TNF-α nor staurosporine-induced apoptosis requires caspase-3. Therefore, although caspase-3 is involved in most cases of apoptosis, it is a questionable whether it is a necessary component of the apoptotic signal pathway.

The Bax gene, a member of the Bcl-2 family and an apoptosis promoter, regulates the release of cytochrome c from mitochondria (11), and its forced expression is known to lead to the activation of caspases and to programmed cell death (12–14). However, it is controversial whether caspases are required for Bax-induced apoptosis. Both caspase-dependent cell death (15, 16) and caspase-independent cell death (17) mediated by Bax have been reported. Because several caspase-3-like proteases exist, it is even uncertain whether caspase-3 is absolutely required in Bax-mediated cell death.

Using a binary adenoviral vector system, we have demonstrated recently that direct transfer of the Bax gene to cancer cells elicited extensive apoptosis in vitro and suppressed tumor growth in vivo (14, 18). In brief, the system contains two adenoviral vectors. One of these vectors contains a human Bax cDNA under the control of a minimal synthetic promoter comprising five Gal4-binding sites and a TATA box, which is dormant in 293 packaging cells, thus avoiding the toxic effects of the Bax gene on the 293 cells and allowing vector (Ad/GT-Bax) production. The expression of the Bax gene can be induced

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2 To whom requests for reprints should be addressed, at Department of Thoracic and Cardiovascular Surgery, The University of Texas M. D. Anderson Cancer Center, Box 109, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 794-4039; Fax: (713) 794-4669; E-mail: bfang@mdanderson.org.

3 The abbreviations used are: PARP, poly(ADP-ribose) polymerase; DFF, DNA fragmentation factor; TNF, tumor necrosis factor; EM, electron microscopy; GT, GAL4/TATA; GV16, GAL4/VP16 fusion protein; PGK, 3-phosphoglycerate kinase; XTT, 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide; FACS, fluorescence-activated cell sorting.
by coinfecting the Ad/GT-Bax virus with the second adenoviral vector in the binary system (Ad/GPK-GV16). Ad/GPK-GV16 contains a synthetic transactivator, consisting of a fusion protein comprised of a Gal4 DNA-binding domain and a VP16 activation domain under the control of a constitutively active PGK promoter, a housekeeping gene promoter from the mouse PGK gene. To characterize the role of caspase-3 in Bax-mediated cell death, we compared cell death in a caspase-3-deficient human breast cancer cell line (MCF7) and in its stable caspase-3 transfectants after treatment with the Bax-expressing adenoviral vectors. Our results showed that caspase-3 is required for nuclear apoptotic change but not for the cell death triggered by Bax overexpression, thus demonstrating that Bax can mediate caspase-3-independent cell death. Our results also suggest that a downstream deficit in the Bax-mediated apoptotic pathway may not necessarily block Bax-mediated cell death.

**MATERIALS AND METHODS**

**Cell Lines and Plasmids.** Human breast cancer cell line MCF7 obtained from Dr. K. Hunt’s laboratory (The University of Texas M. D. Anderson Cancer Center) was grown in RPMI 1640 supplemented with 10% FBS and antibiotics. MCF7/neo and MCF7/Casp3 cell lines were generated by transfecting parental MCF7 cells with pCDNA3.1 and pCDNA3.1/Casp3 vectors, respectively, using FUGENE-6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN). Plasmid pCDNA3.1/Casp3 was constructed by inserting human caspase-3 cDNA (Cambridge Bioscience Ltd., Cambridge, United Kingdom) into the EcoRI-XbaI site of a pCDNA3.1(+) vector (Invitrogen, Carlsbad, CA). Cells were then cultured in medium supplemented with geneticin (G418; Life Technologies, Inc., Grand Island, NY) at a dose of 0.5 mg/ml for 4 weeks. After 2 weeks selection in the G418-containing medium, single-cell clones were selected and further cultured in medium supplemented with G418 at a dose of 0.5 mg/ml for 4 weeks. Caspase-3 protein expression in established MCF7/casp3 cell lines was confirmed by Western blot analysis and immunostaining. The MCF7/Casp3 that expressed the highest level of caspase-3 protein was used for additional experiments.

**Adenoviruses.** Adenoviral vectors Ad/GPK-GV16, Ad/GT-LacZ, and Ad/GT-Bax were constructed and characterized as described previously (14, 19). The expansion, purification, titration, and quality analysis of all vectors used were performed at the vector core facility of our institution as described previously (18). All viral preparations were found to be free of the E1+ adenovirus by PCR assay (20) and free of endotoxin by testing with a Limulus amebocyte lysate endotoxin detection kit (BioWhittaker, Walkersville, MD). The tier determined by the absorbency of dissociated virus at A260 (one A260 unit = 10^12 viral particles/ml) was used in this study.

**In Vitro Gene Transfer.** As determined in preliminary experiments, cells were coinfected with Ad/GT-Bax or Ad/GT-LacZ and Ad/GPK-GV16 at a ratio of 1:1. The optimal multiplicity of infection was determined by infecting each cell line with Ad/GT-LacZ + Ad/GPK-GV16 and assessing the expression of β-galactosidase via 5-bromo-4-chloro-3-indolyl-β-D-galactoside staining. The multiplicity of infection that resulted in ~50% of cells being stained blue, 4000 viral particles/cell for all MCF7 cell lines, was used in subsequent experiments. Unless otherwise specified, Ad/GT-LacZ + Ad/GPK-GV16 was used as the vector control for Ad/GT-Bax + Ad/GPK-GV16. Cells treated with PBS only were used as a mock control.

**Biochemical Analysis.** Cell viability was assessed by XTT assay using the Cell Proliferation Kit II (Roche Molecular Biochemicals) according to the manufacturer’s protocol. Cell death was quantified by the Annexin V binding assay, which detects apoptotic cells that have exposed phosphatidylserine, a surface marker of apoptotic cells. Treated cells were incubated with phycoerythrin-conjugated Annexin V (PharMingen, San Diego, CA) in binding buffer [10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM CaCl2] according to the manufacturer’s protocol. Phycoerythrin-positive cells were analyzed by FACS analysis. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside staining of cultured cells was performed as described previously. Western blot analysis was performed as described previously (14), using the following primary antibodies: anti-PARP (4C10–5; PharMingen); anti-caspase-3 (65906E; PharMingen); anti-pro-caspase-8 (B9–2; PharMingen); anti-caspase-8 p20 (c-20; Santa Cruz Biotechnology, Santa Cruz Biotechnology, CA); anti-actin (AC-15; Sigma Chemical Co., St. Louis, MO); anti-caspase-6 (606–691; Upstate biotechnology, Lake Placid, NY); and anti-lamin B (NA12; Oncogene Research Products, Boston, MA). FACS analysis of cellular DNA content was performed on day 3 as described previously (18).

**Immunocytochemical Staining of Caspase-3.** Cells were seeded on two-chamber Falcon culture slides (Becton Dickinson Labware, Franklin Lakes, NJ) and then fixed in 4% formalin. Fixed cells were stained with rabbit polyclonal anti-caspase-3 antibody (65906E; PharMingen) using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer’s protocol.

**Electron Microscopic Analysis.** Cells were plated 1 day before infection. The cells were then infected with adenovirus as mentioned above. Three days later, culture media containing floating (dead) cells were collected into clean tubes. Cells attached to culture dishes were also collected by brief trypsinization and transferred into new tubes. Cells were then centrifuged at 2000 × g for 3 min and washed with serum-free culture medium twice. The cell pellets were then fixed with a fixative containing 2% paraformaldehyde and 3% glutaraldehyde in a 0.1 M cacodylate buffer (pH 7.2). The fixed specimens were processed and photographed at our institution’s High-Resolution Electron Microscopy Core Facility.

**Colorimetric Caspase-6 Activity Assay.** Four days after viral treatment, cells were incubated in cell lysis buffer for caspase assay (PharMingen) and centrifuged at 10,000 × g for 5 min to remove cell debris. The protein concentration of the supernatants (cytosolic extracts) was measured. Extract samples (50 μg each) were incubated with colorimetric caspase-6 substrate Ac-VEID-p-nitroaniline (Calbiochem, San Diego, CA) in assay buffer [50 mM HEPES (pH 7.4), 100 mM NaCl, 0.1% 3-[4-chloromidophenyl]-3dimethylaminomino]-1-propanesulphonate, 10 mM DTT, and 0.1 mM EDTA] on 96-well plates overnight. The absorbency at 405 nm was then measured in a microplate reader.
Caspase-3 in Bax-mediated Apoptosis of cells by 5 days. This difference was significant (contrast, treatment with Bax-overexpression vectors killed 50% of control adenoviral vectors caused no cell killing by day 5. In gene transfer in MCF7 cells. Treatment of MCF7 cells with measured the cell-killing effects of adenovirus-mediated whether caspase-3 is required for Bax-mediated cell killing, we XT-T data were analyzed using an unpaired Student’s t
value for cells treated with Ad/GT-Bax (red) was significantly different from the value for those treated with PBS (black) or Ad/GT-LacZ + Ad/PGK-GV16 (black, *P < 0.01). B, cleavage of PARP and activation of caspase-8 in MCF7 cells. Results are from Western blot analysis of PARP and caspase-8. Lane 1, PBS; Lane 2, Ad/PGK-GV16 + Ad/GT-LacZ; Lane 3, Ad/PGK-GV16 + Ad/GT-Bax. Protein loading was normalized using β-actin as a loading control.

Statistical Analysis. The significance of difference in the XTT data were analyzed using an unpaired Student’s t test (two-tailed). P < 0.05 were regarded as significant.

RESULTS

Bax Overexpression Induces Apoptosis in MCF7 Cells. MCF7 cells are deficient in caspase-3 expression because of a deletion mutation in exon 3 of their caspase-3 gene (10). To test whether caspase-3 is required for Bax-mediated cell killing, we measured the cell-killing effects of adenovirus-mediated Bax gene transfer in MCF7 cells. Treatment of MCF7 cells with control adenoviral vectors caused no cell killing by day 5. In contrast, treatment with Bax-overexpression vectors killed 50% of cells by 5 days. This difference was significant (P < 0.01; Fig. 1A). At day 3 after infection, Western blot analysis showed cleavage of PARP, a biochemical hallmark of apoptosis, and processing of caspase 8 into a small active fragment (Fig. 1B). The mechanism of the caspase-8 activation after the Bax gene overexpression is not yet clear. The results suggested that certain apoptotic events occurred in MCF-7 cells in the absence of caspase-3.

MCF7 Cells Stably Transfected with the Human Caspase-3 Gene. In comparison with cell lines we have tested previously (18), MCF7 cells showed an apparent delay in cell death. We speculated that this trait might be attributable to caspase-3 deficiency because caspase-3 is one of the common executor proteases in the apoptotic process. Thus, we introduced human caspase-3 cDNA into MCF7 cells and then selected stably transfectant clones. Among the caspase-3 transfectant MCF7 subclones, the one that expressed the highest levels of caspase-3 protein in >50% of its population as detected by immunohistochemical assay (data not shown) was further characterized. Subsequent Western blot analysis showed that this caspase-3 transfectant, which we termed MCF7/Casp3, expressed caspase-3 protein, whereas parental cells (MCF7/Par) and control vector transfectant cells (MCF7/Neo) did not (Fig. 2A). Treatment of the MCF7/Casp3 cells with an adenovirus expressing Bax resulted in correct processing of caspase-3.

Restoration of Caspase-3 in MCF7 Does Not Affect Cell Killing by Bax. The apoptosis profiles of MCF7/Par, MCF7/Neo, or MCF7/Casp3 cells were compared after treatment with Bax-expressing adenoviral vectors. Treatments with PBS or control vectors were used as experimental controls. Interestingly, restoration of caspase-3 did not increase the cleavage of PARP or the cell-killing effects of the Bax gene. The XTT assay showed that the kinetics of cell killing induced by Bax in all three lines were similar (Fig. 2B). The same was observed when the annexin V binding assay was used to quantify dead cells (Fig. 2C). Similar percentages of apoptotic cells (36.4–36.8%) were observed in all three lines after treatment with the Bax gene, regardless of the status of caspase-3. Thus, restoration of caspase-3 in MCF7 cells apparently did not change the cell-killing effects of the Bax gene.

Caspase-3 Is Required in Apoptosis-related Nuclear Events. These findings in turn raised a question about the importance of caspase-3 in the execution of apoptosis. Thus, apoptotic DNA fragmentation was tested by flow cytometric assay. As this assay showed, Bax overexpression preferentially produced more DNA fragmentation in MCF7/Casp3 cells (24.2% of cells were in the subdiploid fraction) than it did in MCF7/Par (7.5%) or MCF7/Neo (14.1%) cells (Fig. 3A).

To closely observe the cellular events occurring during the process of apoptosis in these cell lines, EM was performed. In brief, cells were infected with adenoviral vectors, cultured for 72 h, and then examined by EM after negative staining to reveal cytoplasmic and nuclear structures. In dead MCF7/Par and MCF7/Neo cells (Fig. 3B), the cytoplasm had been destroyed and had developed many intracellular vacuoles. Although nuclei of these cells showed condensed chromatin, their nuclear envelopes were kept relatively intact and maintained their shape. In contrast, in MCF7/Casp3 cells, the whole cellular component was disassembled and digested. Thus, in the absence of caspase-3, MCF7 cells underwent aberrant nuclear and cytoplasmic destruction after Bax gene overexpression; in its presence, they underwent nuclear fragmentation and further digestion of...
Caspase-3 was required for the nuclear dismantling seen during Bax-mediated cell death but not for the cell death itself. Caspase-6 Activation and Lamin B Cleavage Remain the Same in Caspase-3-positive and -negative Cells. Caspase-6 is reportedly responsible for the digestion of nuclear substrates such as the lamins (21, 22), which are the components of the inner nuclear membrane. To further investigate whether activation of caspase-6 and cleavage of lamins were partly responsible for the differences in nuclear morphology seen between MCF7/Neo cells and MCF7/Casp3 cells, caspase-6 activation and lamin B cleavage were analyzed in the cell lines after treatment with the Bax gene. Both Western blot analysis and caspase activity assay showed that caspase-6 was activated by overexpression of the Bax gene in both MCF7/neo and MCF7/Casp3 (Fig. 4A). Moreover, overexpression of the Bax gene resulted in degradation of lamin B, regardless of the status of the caspase-3 gene in these cells (Fig. 4B). These results suggested that activation of caspase-6 and degradation of lamin B may not have been responsible for the differences in nuclear morphology between MCF7/Neo and MCF7/Casp3 cells.

**DISCUSSION**

Accumulating evidence shows that a broad range of classical triggers of apoptosis induce programmed cell death with necrotic morphology characterized by cytoplasmic vacuolation and minimal nuclear changes when caspase activities are inhib-

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**Fig. 2.** A, expression of caspase-3 in MCF7 cells and stable transfectants and activation of caspase-3 and PARP cleavage in MCF7/Casp3 cells. Whole-cell lysates were subjected to Western blot analysis. Lane 1, MCF7/Par; Lane 2, MCF7/Neo; Lane 3, MCF7/Casp3. B, MCF7/Par, MCF7/Neo, and MCF7/Casp3 cells all lost cell viability to a similar extent in response to Bax overexpression. Cell viability was determined by colorimetric XTT assay after infection. In all MCF7 subclones, cell viability after treatment with Ad/GT-Bax + Ad/PGK-GV16 (▲) was significantly different from that after treatment with PBS (●) or Ad/GT-LacZ + Ad/PGK-GV16 (●; P < 0.01). C, the percentage of apoptotic cells was determined by Annexin V binding assay. Values were expressed as an average of the results from the two independent experiments.
ited (reviewed in Ref. 23). The MCF7 cell line, which lacks caspase-3 expression, is known to undergo cell death in response to stimulation of TNF-α, staurosporine, and other agents (7, 10). Those previous findings have led to the hypothesis that caspase-3 is necessary for apoptosis-related nuclear events, and especially DNA fragmentation via activation of DFF45/ICAD, but may or may not be essential for cell death itself (8, 10, 24, 25). To test this hypothesis, we studied the role of caspase-3 in Bax-mediated apoptosis of MCF7 cells.

We and others have shown previously that overexpressed Bax kills a variety of cells (14, 15) by regulating the release of cytochrome c from mitochondria (13) and activating the caspase cascade (15, 16). Now, our present results demonstrate that caspase-3 is not required for Bax-mediated cell death itself. Restoration of the caspase-3 gene into MCF7 cells increased the percentage of “sub-G1” cells but not the cell death measured by other parameters, including the cleavage of PARP, the preferred substrate of caspase-3 (7). This suggests that, in terms of cell death, mammalian cells may compensate well for their lack of the caspase-3 gene, so that the cell death process could be completed even if caspase-3 is deficient. In fact, several reports have implicated non-caspase proteases in apoptosis. For example, calcium-dependent enzymes called calpains are known to cleave the cytoskeletal protein fodrin and cause membrane blebbing (26). The lysosome protease cathepsin D is required for apoptosis induced by IFN-γ, Fas/APO-1, and TNF-α (27). Yet, the fact that several types of proteases are simultaneously activated during apoptosis has made it difficult to characterize the role of any single enzyme in cell death. Nevertheless, our results suggest that a downstream deficit in the Bax-mediated apoptosis pathway may not be sufficient to block cell death caused by overexpression of the Bax gene.

The dramatic difference in nuclear morphology between caspase-3-positive and -negative cells in the present study supported the role of caspase-3 in apoptosis-related nuclear event proposed by others (8–10, 25). Although the responsible mechanism has not been delineated, some studies have suggested that caspase-3 is required for activation of DFF40 by its cleavage of DFF45 (25, 28), which in turn is required for DNA fragmentation. Our observation of relatively intact nuclei in dead, caspase-3-deficient cells suggests that the extent of cell death could have been underestimated when assayed by nuclear morphology or DNA fragmentation. In fact, in terms of the percentage of apoptotic cells (i.e., subdiploid cells) determined by FACS analysis, there was a dramatic difference between caspase-3-positive and -negative cells after treatment with Bax gene-expressing vectors, although annexin V binding assays and XTT assays of actual cell death showed no difference. Thus, simul-
Simultaneous measurement of several parameters was required to determine cell death in these cells.

Although the mechanism responsible for the difference in nuclear morphology in dead MCF7/Par, MCF7/Neo, and MCF7/Casp3 cells remains to be delineated, the results of our studies on caspase-6 activation and lamin B degradation suggest that cleavage of these two molecules may not account for the morphological difference. Lamins are major peripheral proteins of the nuclear envelope; their polymerization generates a nuclear lamina that forms a fenestrated proteinous mesh network between the chromatin and the inner nuclear membrane. Both lamin A and lamin B are known substrates of caspase-6 (21, 22, 29), and their degradation is one proposed mechanism of nuclear morphological change during apoptosis (29, 30). Because activation of caspase-6 is reportedly dependent on activation of caspase-3 (1, 21, 31), the deficit of caspase-3 in MCF7 cells could theoretically result in deficient caspase-6 activation and lamin cleavage. However, our results showed that caspase-3 can be activated even in the absence of caspase-3. Thus, unlike for other isolated cellular components, a cellular mechanism may exist that compensates the function of caspase-6 for caspase-3 activation.

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Caspase-3 in Bax-mediated Apoptosis


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