The Nuclear Receptor Interacting Factor-3 Transcriptional Coregulator Mediates Rapid Apoptosis in Breast Cancer Cells through Direct and Bystander-Mediated Events

Sharmistha Das, Jerome C. Nwachukwu, Dangsheng Li, Anthony I. Vulin, Sonia Martinez-Caballero, Kathleen W. Kinnally, and Herbert H. Samuels

Departments of Pharmacology and Medicine, New York University School of Medicine, and Department of Basic Sciences, College of Dentistry, New York University, New York, New York

Abstract

We previously reported that amino acids 20 to 50 of nuclear receptor interacting factor-3 mediates rapid apoptosis in breast cancer cell lines but not in cells derived from other tissues. We refer to this short region as death domain-1 (DD1). Small interfering RNA studies indicated that DD1-mediated apoptosis is caspase-2 dependent. In this study, we examined DD1-mediated apoptosis in more detail and generated stable caspase-2 knockdown breast cancer cells. These cells are resistant to DD1-mediated apoptosis. Time-lapse movies suggested that DD1-mediated apoptosis also leads to a "bystander effect." We found that within 5 h of DD1 expression, breast cancer cells release a factor(s) into the medium that leads to apoptosis of naive breast cancer cells or DD1-resistant cells (e.g., HeLa). The DD1-expressing caspase-2 knockdown cells also release a factor(s) that kills other cells, indicating that this effect is not dependent on the apoptogenic process. The bystander effect seems dependent on the production of reactive oxygen species (ROS). These and other studies indicate that DD1 expression in breast cancer cells leads to at least two death signals: one involving the rapid production of ROS and/or other soluble factors that directly or indirectly leads to a bystander effect and a second caspase-2-dependent process that leads to apoptosis in cells in which DD1 is expressed. [Cancer Res 2007;67(4):1775–82]

Introduction

Caspase-initiated programmed cell death or apoptosis is a fundamental process in growth and development as well as a process that is targeted in the treatment of various tumors. Mammalian cells express many caspases that have been categorized as "initiator" caspases (e.g., caspase-2, caspase-8, caspase-9, and caspase-10) and "effector" caspases (e.g., caspase-3, caspase-6, and caspase-7; refs. 1, 2). The initiator caspases contain an extended NH2-terminal domain called caspase-recruitment domain (CARD) or death effector domain (DED). CARD or DED acts to bring the initiator caspases in close proximity, leading to a conformational change to form activate caspase (2). This active initiator caspase then cleaves and activates effector caspases, which leads to cleavage of a wide variety of protein components in the cell. The initiator-effector caspase cascade is best exemplified by the extrinsic pathway involving surface membrane death receptors [e.g., Fas, tumor necrosis factor-α (TNF-α), and TNF-related apoptosis-inducing ligand; ref. 3].

Apoptosis also occurs through an intrinsic pathway initiated by intracellular stress signals, such as DNA damage and radiation (3). The intrinsic pathway involves changes in permeability of the mitochondrial outer membrane to release apoptogenic factors. The proapoptotic proteins Bax and/or Bak oligomerize to form the mitochondrial apoptosis–induced channel, which permits the release of cytochrome c and presumably a number of mitochondrial proteins such as apoptosis-inducing factor (AIF), Smac/DIABLO, and EndoG (4–6). After release, AIF translocates to the nucleus and is thought to lead to cleavage of DNA into large fragments. The released cytochrome c complexes with the WD-40 repeats of Apaf-1, which leads to oligomerization of Apaf-1 that recruits pro-caspase-9 through its CARD domain (7). This structure (apoptosome) activates pro-caspase-9, which then activates effector caspses such as caspase-3.

Although caspase-2 is considered to be an initiator caspase, until recently its role in apoptosis has been considered minor because caspase-2 knockout mice exhibit only minor phenotypic changes (8). In addition, unlike caspase-8 or caspase-9, active caspase-2 does not seem to cleave other caspases, leading to a proteolytic cascade (9). Although caspase-2 had long been thought to act downstream of mitochondria, recent studies indicate an important role for caspase-2 in stress-induced mitochondrial permeability. Studies using a small interfering RNA (siRNA) against caspase-2 mRNA indicated that reduction in caspase-2–mediated apoptosis mediated by cisplatin, etopside, or UV irradiation in a number of cell types (10). Caspase-2–mediated changes in mitochondrial permeability can occur without activation of caspase-9, although these changes in mitochondrial permeability lead to the release of cytochrome c, which activates the caspase-9 pathway (11, 12).

We have been interested in understanding the mechanism of retinoid-mediated inhibition of breast cancer cell growth (13, 14). We recently examined the role of a nuclear hormone receptor coactivator that we cloned (NRIF3), which is selective for thyroid hormone and retinoid receptors on the retinoid inhibition of growth (13, 14) of several breast cancer cell lines (15). Surprisingly, we found that expression of NRIF3 rapidly leads to apoptosis (within 5 h of expression) independent of retinoid incubation. We mapped the region of NRIF3, which was sufficient to lead to apoptosis to an ~30 amino acid region of the protein (amino acids 20–50) and refer to this region as death domain-1 (DD1; ref. 15).

Interestingly, expression of DD1 [alone or as a green fluorescent protein (GFP) fusion] in a wide variety of breast cancer cell lines resulted in apoptosis (15). This effect seems selective for breast
cancer cells because expression of DD1 in a wide variety of cell lines derived from other cell types, including HeLa cells, did not lead to cell death. DD1-mediated apoptosis occurred in the presence of a pan-caspase inhibitor (zVAD-fmk), which initially suggested that the apoptogenic response was caspase independent. However, zVAD-fmk has a much lower affinity for caspase-2 than that for other caspases (16), which raised the possibility that caspase-2 might be the initiator caspase involved. This conclusion was supported by the finding that an siRNA directed against caspase-2 mRNA (10) abrogated the effect of DD1 on mediating apoptosis in breast cancer cells (15).

In this study, we have further examined the mechanism(s) of DD1-mediated apoptosis of breast cancer cells. We found that expression of DD1 not only leads to rapid apoptosis but also leads to a “bystander effect” resulting from the rapid release of a factor(s) into the medium that mediate apoptosis in cell lines that do not exhibit apoptosis when DD1 is expressed (e.g., HeLa). We also generated a stable knockdown of caspase-2 using retroviral-mediated RNA interference in the T47D breast cancer cell line. We document that these cells are resistant to DD1-mediated apoptosis, further indicating an important role for caspase-2 in this process. Although these cells do not exhibit apoptosis when DD1 is expressed, they also release a factor(s) that leads to apoptosis of other cell lines, indicating that production of these factors is independent of the apoptotic cascade. We provide evidence that several reactive oxygen species (ROS) are produced within 5 h of DD1 expression. When the formation of ROS is blocked in DD1-expressing breast cancer cells, the cells still die but the medium does not lead to apoptosis when transferred to other cells. This indicates that DD1 expression in breast cancer cells leads to at least two death signals: one that involves the rapid production of ROS and/or other factors that directly or indirectly lead to a bystander effect resulting in the death of nearby cells, and a second caspase-2–dependent process that leads to apoptosis in cells in which DD1 is expressed.

Materials and Methods

Plasmids and chemicals. Plasmids expressing GFP-DD1 and the GFP–nuclear localization signal (GFP-NLS) control have been previously described (15). The GFP-DD1 vector and the GFP-NLS control vector express nuclear-localized GFP, in which the NLS is derived from the SV40 T antigen (17). N(G)-nitro-l-arginine methyl ester (lNAME) and rat monoclonal antibody 11B4 against caspase-2 were obtained from Alexis Biochemicals (San Diego, CA); apocynin, zVAD-fmk, and zVDVAD-fmk were from Calbiochem (San Diego, CA); NONOates were from Cayman Chemical (Ann Arbor, MI); and superoxide dismutase and catalase were from Sigma (St. Louis, MO). The TNF-α antibody (Remicade) was a gift of the Rheumatology Division, Department of Medicine, New York University School of Medicine. A pSIREN-RetroQ RNA interference vector (Clontech, Mountain View, CA) was used to express a short hairpin RNA (shRNA) to silence caspase-2 in T47D cells. GATC CGCGTGGT GGAGGAAATTTCAAGAGATATTCCGCTCAACAACAGCTTTTTG and its complementary strand with TAATTCGCTCAACAACAGCTTTTTG were used for transfection, the donor cells were transformed, and the pSIREN-RetroQ (shRNA-caspase-2) plasmids were transformed. In addition to the correct DNA sequence above (A-series), we also identified a vector that contained four mutations that were presumed to have been introduced by the bacteria as a result of a recombination event. This vector served as a control (B-series). The retrovirus was obtained from the medium of a Phoenix packaging cell line that was transfected with the pSIREN-RetroQ plasmids.

Time-lapse imaging. T47D cells were cultured and then transfected to express GFP-DD1 on 25-mm glass coverslips and mounted in sealed Rose chambers as described by Khodjakov and Rieder (18). Cells were maintained at 37°C during time-lapse experiments. Images were taken every 10 min for 48 h with time-lapse video differential interference contrast microscopy (DIC)/fluorescence microscopy using either a Spot RT monochome Camera (Diagnostic Instruments, Inc., Sterling Heights, MI) or a ORCA ER Digital CCD camera model CF42-95-12ERG (Hamamatsu Photonics, Bridgewater, NJ) on NIKON Eclipse TE300 or TE2000-U microscopes. Spot RT software or Simple PCI software (Compix, Inc., Sewickley, PA) controls the Uniblitz Shutter (model VMMD1 or VMM-D3). Montages of pictures were prepared through Photoshop v.6.0 or 7.0.

Cell culture and transfection conditions. Breast cancer cell lines were maintained in DMEM (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; refs. 13–15). HeLa cells were cultured in DMEM supplemented with either 10% FBS or 10% Hyclone defined/supplemented bovine calf serum. For most of the transient transfection studies, cells were plated at a density of 3 × 10⁶ per well (250-μL medium) on coverslips in 48-well tissue culture plates. About 20 h later, the cells were transfected with indicated plasmid(s) using LipofectAMINE 2000 (Life Technologies) according to the manufacturer's protocol. Generally, the amount of plasmid used in transfections was 25 ng for GFP-DD1 or GFP-NLS. After transfection, cells were incubated in 150 to 175 μL DMEM/10% FBS medium for 5 h. The medium was then centrifuged and transferred to naive breast cancer cells or to HeLa cells, and the recipient cells were incubated for –18 h. In some experiments (where indicated), 1.5 h after transfection, the donor cells were washed six times with fresh medium; 5 h later, the medium was transferred to naive cells. In certain studies, the medium collected –5 h after transfection was indirectly assessed for NO production using an assay from Active Motif (Carlsbad, CA), which measures nitrite and nitrate accumulation. Both the donor and recipient cells were analyzed for apoptosis by terminal deoxyribonucleotide transferase–mediated nick-end labeling (TUNEL) assay (In situ Cell Death Detection kit, TMR red, Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's protocol. Cells were also stained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize nuclei. Cells were then mounted on slides, examined by fluorescence microscopy, and digitally imaged. The details of experiments with NONOates and other compounds indicated above are described in the figure legends.

To generate stable caspase-2 knockdown cells, T47D cells were transfected with pSIREN-RetroQ (shRNA-caspase-2) retrovirus (A-series) or the retrovirus with mutations in the DNA sequence specifying the shRNA (B-series). After puromycin selection (2 μg/mL), pooled cells were examined for caspase-2 levels by Western blotting with 11B4 monoclonal antibody (see Fig. 2). To isolate individual clones, the A-series cells were plated onto 96-well plates with an inoculum of approximately one cell per well. Clones were expanded and caspase-2 levels were estimated by Western blotting with 11B4 monoclonal antibody. Mouse embryo fibroblasts derived from a caspase-2 knockout mouse (19) served as a control for caspase-2 knockdown.

To generate T47D cells devoid of mitochondrial DNA, cells were continuously cultured with 200 ng/mL ethidium bromide-100 μg/mL pyruvate and 50 μg/mL uridine as a pyrimidine source, as described previously (20, 21). This selectively inhibits mitochondrial DNA replication but not chromosomal DNA replication and leads to inhibition of the mitochondrial electron transport system (20). The requirement for a pyrimidine source stems from the finding that mitochondrial DNA transport is required for the function of an enzyme in the pyrimidine biosynthetic pathway (20). After 30 days, cells were tested for mitochondrial DNA depletion by incubating the cells with medium containing 200 ng/mL ethidium bromide-100 μg/mL pyruvate but no uridine. Without uridine, the cells died within 24 h, indicating depletion of mitochondrial DNA.

Results

We previously reported that expression of amino acids 20 to 50 of NRII3 (DD1) was sufficient to lead to rapid apoptosis by TUNEL
with vectors expressing GFP-DD1 or a GFP-control. Five hours after apoptosis in wild-type T47D and A1-9 cells. Cells were transfected shows a study that examined the effect of DD1 on mediating the A-series and the B-series cells. Figure 2

A

(B-series). After puromycin selection, the level of pro-caspase-2 expressing a shRNA with mutations in the target sequence shRNA directed against caspase-2 (A-series) or with a retrovirus mRNA. T47D cells were infected with retrovirus expressing the pSIREN RetroQ) expressing a shRNA directed against caspase-2 mRNA. T47D cells were infected with retrovirus expressing the shRNA directed against caspase-2 (A-series) as well as a retrovirus where the shRNA contained four base mismatches (B-series). Cells were selected with 2 µg/mL puromycin, and the pooled population of cells was examined for pro-caspase-2 expression. T47D cells infected with the retrovirus pSIREN RetroQ expressing a shRNA directed against pro-caspase-2 mRNA (see Materials and Methods for sequence; A-series) as well as a retrovirus where the shRNA expression by Western blotting.

Knockdown of pro-caspase-2 in T47D cells by retroviral-mediated shRNA expression. To dissect the mechanism of DD1-mediated apoptosis in breast cancer cells, we sought to generate a stable cell line of T47D cells that is resistant to DD1-mediated apoptosis. Such a cell line would allow for biological and biochemical studies of DD1 activity, which would be precluded in wild-type cells that rapidly die after DD1 expression. To generate cells resistant to DD1-mediated cell death, we generated T47D cells with stable knockdown of caspase-2 using a retroviral vector (pSIREN RetroQ) expressing a shRNA directed against caspase-2 mRNA. T47D cells were infected with retrovirus expressing the shRNA directed against caspase-2 (A-series) or with a retrovirus expressing a shRNA with mutations in the target sequence (B-series). After puromycin selection, the level of pro-caspase-2 was determined by Western blotting in the pooled populations of the A-series and the B-series cells. Figure 2A shows the A-series cells exhibiting a marked reduction of pro-caspase-2 compared with the B-series or the parent T47D cells. Extracts from mouse embryonic fibroblasts derived from wild-type mice and from caspase-2 knockout mice (19) document that the protein recognized in the Western blot for caspase-2 (Fig. 2A). To isolate individual clones from the A-series, cells were plated onto 96-well plates with an inoculum of approximately one cell per two wells. Clones were expanded and pro-caspase-2 levels were estimated by Western blotting (Fig. 2B). One clone (A1-9), which had the lowest levels of pro-caspase-2 (>90% reduction), was chosen for further study.

A1-9 cells are resistant to DD1-mediated apoptosis. Figure 3 shows a study that examined the effect of DD1 on mediating apoptosis in wild-type T47D and A1-9 cells. Cells were transfected with vectors expressing GFP-DD1 or a GFP-control. Five hours later, cells were examined for apoptosis by TUNEL assay (Fig. 3). As previously found (15), cells expressing the GFP-control showed no response (not shown). The A1-9 cells were found to be resistant to DD1-mediated apoptosis, whereas the parental T47D cells exhibited apoptosis within 5 h of DD1 expression. In previous studies in T47D cells, we noted that only the GFP-DD1–expressing cells underwent apoptosis (15). In Fig. 3 and other studies, however, we noted that cells that did not express DD1 also exhibited apoptosis. Figure 3 shows results with high transfection efficiency of GFP-DD1 (experiment a) and a study with lower transfection efficiency in which cells other than DD1-expressing cells undergo apoptosis (experiment b). The study in Fig. 3 used a much lower ratio of medium to cells than our prior study, suggesting that the DD1–expressing cells might release a factor(s) into the medium that leads to apoptosis in cells that do not express DD1. Such an effect might explain why cell 3 in Fig. 1 exhibited apoptosis without expressing GFP-DD1.

DD1-expressing breast cancer cells release a soluble factor(s) that mediates apoptosis of breast cancer and HeLa cells. GFP-DD1 was expressed in T47D cells, and 1.5 h after transfection the medium was removed and the cells were washed with serum-free medium six times to remove any residual plasmid. Five hours later, the medium was transferred to new T47D monolayers and incubated overnight. A TUNEL assay of the recipient T47D cells (Fig. 4A) indicated widespread apoptosis. Although the transfected cells were washed six times to remove plasmid, the recipient cells occasionally exhibited very low levels of GFP-DD1 expression. To exclude the possibility that the low levels of expression of GFP-DD1 in the recipient T47D cells resulted in apoptosis, we also carried out medium transfer experiments from
transfected T47D cells to recipient HeLa cells because expression of DD1 in HeLa cells does not lead to apoptosis (15). Interestingly, such medium transfer experiments lead to the apoptosis of HeLa cells (Fig. 4A). An examination of 15 independent medium transfer experiments indicated that the extent of apoptosis of the HeLa cells varied from 62% to 85%, with most experiments showing >70% apoptosis. Such variation may reflect the extent of DD1-mediated expression in the T47D cells or differences in the number of cells transfected. Although expression of DD1 in caspase-2-deficient A1-9 cells does not lead to apoptosis, the cells also release a factor(s) into the medium that leads to apoptosis of wild-type T47D cells or HeLa cells (Fig. 4A). Apoptosis of the HeLa cells was blocked by addition of either zVAD-fmk (a pan-caspase inhibitor) or zVDVAD-fmk (a caspase-2 inhibitor) to the medium just before transfer (Fig. 4B).

DD1 expression in T47D cells leads to the rapid production of nitric oxide. Nitric oxide (NO) has been reported to lead to apoptosis in some cell lines, including breast cancer lines (22–27). Therefore, we explored whether DD1 expression leads to NO production. NO might function as the apoptotic factor released into the medium after DD1 expression. Alternatively, because NO has a short half-life, it may function as an intracellular signal that leads to the production of a factor(s) (e.g., bioactive peptide or oxidized lipid) that then leads to apoptosis. Breast tumors as well as MCF7 cells have been reported to express endothelial NOS (eNOS; refs. 26–28). We found that the both the parent T47D cells and the A1-9 cells also express eNOS (Fig. 5). Western blotting studies did not detect an induction of inducible NOS in these cells (not shown). T47D cells were transfected to express GFP-DD1 or the GFP-control. Five hours later, the medium was indirectly assayed for NO by measuring the concentration of nitrite and nitrate, which accumulated in the medium. T47D cells expressing DD1 were found to produce a large amount of NO within 5 h of DD1 expression (Table 1). A similar effect of DD1 on NO production was also found with MCF7 and SKBR3 breast cancer cells. HeLa cells showed no increase in NO production. Interestingly, DD1-expressing A1-9 cells showed no increase in NO production (Table 1). Thus, it is unlikely that the production of NO directly leads to apoptosis when the medium is transferred to HeLa cells. However, it is possible that NO may be a signal that leads to apoptosis of NO-producing breast cancer cells.

Elevation of NO levels using NONOates leads to apoptosis of T47D cells but not HeLa or A1-9 cells. To examine whether NO might lead directly or indirectly to apoptosis, we examined the effect of spermine NONOate (29), which releases NO when added to the medium, on a number of cell lines. Spermine NONOate (10 μmol/L) resulted in apoptosis of T47D cells (Fig. 6) but to a generally lesser extent than with expression of DD1, which resulted in similar levels of NO production. The NONOate did not lead to apoptosis of the caspase-2 knockdown A1-9 cells (Fig. 6). Also, the NONOate had no effect on HeLa cells (Fig. 6). Similar results were found using propylamine NONOate. This finding, along with the observation that the medium of DD1-expressing A1-9 cells, which does not exhibit an increase in NO, leads to apoptosis of HeLa cells, indicates that the cells release at least one other apoptogenic factor. Although elevation of NO using NONOates can elicit apoptosis of T47D cells, the medium from these cells does not lead to apoptosis when transferred to HeLa cells (not shown). Thus, although DD1 expression in T47D cells leads to an increase in NO production, NO per se does not fully account for the observed bystander effect.

Inhibition of ROS protects recipient HeLa cells but not donor T47D cells from apoptosis. We also considered the possibility that expression of DD1 in T47D cells leads to the rapid production of other ROS, such as superoxide, H₂O₂, or peroxynitrite, which forms through reaction of NO with superoxide. To explore this, we examined the effect of factors that interfere with these pathways on T47D cell apoptosis and apoptosis of HeLa cells.
after incubation with the T47D cell medium. The factors we studied were L-NAME (30, 31), an inhibitor of NOS activity, which would block NO formation and, as a result, peroxynitrite formation (24, 32, 33); apocynin (34, 35), which inhibits superoxide formation by blocking assembly of the superoxide-generating enzyme NADPH oxidase, which would also inhibit peroxynitrite formation; superoxide dismutase, which catalyzes the breakdown of superoxide into H2O2 and O2; and catalase, which converts H2O2 to H2O and O2 (36).

T47D cells were preincubated with L-NAME (30 nmol/L) for 1 h and then transfected with a vector expressing GFP-DD1. Five hours later, the medium was transferred to HeLa cells that were incubated overnight. Both the T47D cells and the HeLa cells that were incubated overnight were assessed for apoptosis by TUNEL assay (Fig. 7). Assay of NO indicated that L-NAME preincubation inhibited NO production by the T47D cells. L-NAME, however, had little or no effect on blocking apoptosis of T47D cells, further indicating that NO production per se is not a major apoptogenic factor for T47D cells. However, L-NAME preincubation prevented HeLa cells from undergoing apoptosis when cultured with the T47D cell medium (Fig. 7). Similar results were found using apocynin, catalase, or superoxide dismutase alone or in combination. There was no protection of T47D cells but each or all of these factors protected HeLa cells from apoptosis when the cells were incubated overnight with the T47D cell medium (Fig. 7). In contrast, when apocynin, superoxide dismutase, or catalase were added to the medium 5 h after T47D cells were transfected to express DD1, the compounds did not protect the recipient HeLa cells from apoptosis. Thus, the compounds do not directly protect HeLa cells from undergoing apoptosis but seem to act by blocking the T47D cell production of ROS, which then directly or indirectly [through the production of another factor(s)] mediates apoptosis when incubated with HeLa cells.

Because catalase incubation with DD1-expressing T47D cells led to protection when the medium was transferred to HeLa cells, we examined the effect of H2O2 by directly adding it to the T47D cell medium (100 μmol/L) followed by transfer of the medium to HeLa cells 5 h later. H2O2 led to apoptosis of T47D cells but incubation of HeLa cells with this medium has no effect (not shown). Also, addition of 100 μmol/L H2O2 directly to the HeLa cells was also without effect. These findings indicate that although H2O2 may lead to apoptosis of T47D cells, at least one other signal is required to generate a factor(s) that leads to apoptosis of HeLa cells.

The findings with L-NAME and apocynin suggest that the formation of ROS might occur independent of oxidative pathways in mitochondria. To assess whether the generation of ROS might be dependent on mitochondrial electron transport, we generated T47D cells with deleted mitochondrial DNA using ethidium bromide and uridine as described for other cell lines (20, 21). The mitochondrial genome encodes 13 proteins involved in mitochondrial oxidative phosphorylation (37), and these proteins are not synthesized in cells lacking mitochondrial DNA. These cells were transfected with vectors expressing the GFP-control or GFP-DD1. After 5 h, the medium was transferred to HeLa cells. The results were the same as found for wild-type T47D cells (not shown), which supports the notion that the increase in ROS formed in DD1-expressing cells occurs independent of mitochondrial electron transport.

Figure 4. DD1-expressing T47D and A1-9 cells release a factor(s) into the medium that leads to apoptosis of T47D and HeLa cells. A, cells in the “donor” column were transfected with a GFP-DD1 expression vector. After 1.5 h, the medium was removed and the cells were washed six times with fresh serum-free medium. The cells were then incubated with serum-containing medium for an additional 5 h, and the medium was then transferred to naive recipient T47D or HeLa cells, which were then incubated overnight. TUNEL assay and DAPI stain for the recipient T47D and HeLa cells. B, addition of 100 μmol/L zVAD-fmk (pan-caspase inhibitor) or 20 μmol/L zVDVAD-fmk (caspase-2 inhibitor) protects the HeLa cells from the apoptogenic factor(s) released from the donor DD1-expressing T47D cells.
Last, we considered the possibility that DD1 expression might lead to the production of TNF-α (directly or through pathways modulated by ROS), which is known to mediate apoptosis of breast cancer cells as well as HeLa cells. Monoclonal antibody (Remicade) against TNF was added to the medium (final concentration 10 μg/mL) before the expression of DD1 and again 5 h later when the medium was transferred to HeLa cells. The TNF-α antibody did not protect either cell type from apoptosis, suggesting that TNF-α is not involved in the bystander effect mediated by DD1 expression.

Discussion

We previously reported that a 30-amino-acid DD1 sequence from the NRII3 thyroid hormone and retinoid X receptor coactivator can lead to a rapid and profound apoptosis of a wide variety of breast cancer cell lines but not a wide variety of cell lines derived from other tissues (15). The first suggestion that caspase-2 might be involved came from the finding that zVAD-fmk, a pan-caspase inhibitor (except for caspase-2), did not protect T47D cells from DD1-mediated apoptosis (15). We further explored this possibility by using an siRNA reported to knock down caspase-2 in human cells and found that knockdown of caspase-2 abrogated DD1-mediated apoptosis of T47D cells (15).

In this study, we have further examined the mechanism(s) leading to DD1-mediated apoptosis. To further establish a role for caspase-2 in DD1-mediated apoptosis, we generated stable T47D cell lines expressing a shRNA against caspase-2 mRNA by retroviral-mediated transformation. Studies with the clone having the lowest level of pro-caspase-2 by Western blotting (A1-9) indicated that the cells were resistant to DD1-mediated apoptosis. Studies with other caspase-2 knockdown cells of the A-series showed similar results (not shown). Our previous studies with DD1 expressed apoptosis by Annexin V staining or TUNEL assay. To further examine this, we visualized the fate of the DD1-expressing breast cancer cells using time-lapse fluorescence microscopy (Fig. 1). The GFP-DD1–expressing cells exhibit blebbing and shrinkage typically found in apoptotic cell death. In the time-lapse movie, we noted that some cells that did not express DD1 seem to undergo apoptosis, suggestive of a bystander-like effect.

We previously found that only GFP-DD1–expressing breast cancer cells exhibited apoptosis (15). However, when we decreased the amount of medium relative to the cell surface area in this study, we noted that some T47D cells that did not express GFP-DD1 also exhibited apoptosis. This suggested a bystander effect in which the DD1-expressing T47D cells released a factor(s) into the medium that mediates apoptosis of T47D cells that do not express DD1. To study this further, we examined whether this medium could lead to apoptosis of naive recipient T47D cells. These recipient T47D cells exhibited apoptosis, indicating the release of a factor(s) into the medium by the DD1-expressing T47D cells (Fig. 4). To exclude the possibility that low levels of DD1 expression occurred in the recipient T47D cells although the donor T47D cells were washed six times, we used HeLa cells as the recipient cell line because expression of DD1 does not lead to apoptosis of HeLa cells (15). Interestingly, we found that the medium collected 5 h after expressing DD1 in T47D cells leads to apoptosis of HeLa cells. Unlike T47D cells, A1-9 cells do not undergo apoptosis when DD1 is expressed. However, the medium from the DD1-expressing A1-9 cells also leads to apoptosis when cultured with T47D or HeLa cells (Fig. 4). This indicates that the factor(s) released into the medium in DD1-expressing breast cancer cells is independent of the development of apoptosis of the donor cells.

We considered NO as a possible apoptogenic factor because T47D cells were found to produce NO within several hours of DD1 expression. However, NO does not mediate apoptosis of HeLa cells because elevation of NO using NONOates does not result in apoptosis of HeLa cells and is a weaker mediator of apoptosis of T47D cells compared with DD1 expression. We considered the possibility that DD1 expression rapidly leads to the production of NO.

Table 1. Expression of DD1 leads to the production of NO in a number of breast cancer cell lines

<table>
<thead>
<tr>
<th>Cells</th>
<th>NO production (μmol/L)</th>
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<tbody>
<tr>
<td>T47D</td>
<td></td>
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<tr>
<td>Untreated</td>
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<tr>
<td>GFP-control</td>
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<tr>
<td>GFP-DD1</td>
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</tr>
<tr>
<td>A1-9</td>
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<td>Untreated</td>
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<td>GFP-control</td>
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<tr>
<td>GFP-DD1</td>
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</tr>
<tr>
<td>HeLa</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>0.21</td>
</tr>
<tr>
<td>GFP-control</td>
<td>0.13</td>
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<tr>
<td>GFP-DD1</td>
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<td></td>
</tr>
<tr>
<td>Untreated</td>
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</tr>
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<tr>
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<tr>
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<tr>
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</table>

NOTE: T47D, HeLa, A1-9, MCF7, and SKBR3 cells were cultured in 48 Spot multwell plates (150 μL medium). Cells were then transfected to express GFP-DD1 or the GFP-control. Untransfected cells (untreated) were also examined. Five hours after the cells were transfected, the medium was assayed for NO production in duplicate or triplicate as described in Materials and Methods. Expression of DD1 resulted in a marked increase in NO production in T47D, MCF7, and SKBR3 breast cancer cells but not in HeLa cells. The caspase-2 knockdown A1-9 cells also showed no increase in NO production when DD1 was expressed.
ROS, which have been reported to result in apoptosis in breast cancer and other cell lines (38). These factors include H$_2$O$_2$, superoxide, and peroxynitrite. Peroxynitrite can form through reaction of superoxide and NO and may be highly toxic to certain cells (36, 39, 40). We explored these possibilities using specific inhibitors or factors that catalytically reduce their levels. L-NAME at 30 nmol/L selectively inhibits eNOS found in breast cancer cells and blocks DD1-mediated NO production. L-NAME preincubation did not prevent DD1-mediated apoptosis of T47D cells, but it protected HeLa cells incubated with the donor T47D cell medium. Similar results were found when the DD1-expressing T47D cells were preincubated with superoxide dismutase, catalase, or apocynin, which inhibits superoxide formation from NADPH oxidase and, thus, may block the formation of peroxynitrite from NO and superoxide. However, addition of these factors to the medium 5 h after DD1 expression just before transfer of the medium to HeLa cells does not block apoptosis of HeLa cells, indicating that the toxic factor that accumulates in the medium is not likely H$_2$O$_2$ or superoxide. In addition, direct addition of H$_2$O$_2$ to HeLa cells has no effect. Furthermore, although H$_2$O$_2$ can lead to apoptosis of T47D cells, transfer of this medium to HeLa cells has no effect. Thus, although H$_2$O$_2$ can lead to apoptosis of breast cancer cells, the production of one or more additional signals are necessary to confer apoptosis on HeLa cells.

Taken together, our findings suggest that DD1 expression in T47D cells rapidly leads to the production of NO, superoxide, H$_2$O$_2$, and peroxynitrite (and possibly other ROS). Although it is possible that these ROS may directly lead to the apoptosis of cells that do not express DD1, the very short half-lives of these ROS suggest that the bystander effect found in the medium transfer experiments is mediated by a soluble factor that is stimulated or modified by one or more of the ROS. Apoptosis is not required for the bystander effect to occur because similar results were obtained with the medium of DD1-expressing of A1-9 cells, which do not undergo apoptosis. Interestingly, although superoxide dismutase, catalase, apocynin, or L-NAME alone or in combination block the production of factors that lead to apoptosis of the recipient HeLa cells, they do not block DD1-mediated apoptosis of T47D cells that do not express DD1 (Fig. 7). Thus, expression of DD1 in T47D cells results in rapid production of NO, superoxide, H$_2$O$_2$, and peroxynitrite, which may lead to bystander apoptosis of neighboring cells.

**Figure 6.** Elevation of NO leads to apoptosis of T47D cells but not A1-9 cells or HeLa cells. T47D, A1-9, or HeLa cells were incubated with 10 μmol/L spermine NONOate, which generates NO when added to the medium (half-life for NO release at 37 °C is ~ 40 min). Five hours after addition of spermine NONOate, the cells were analyzed by TUNEL assay, and the nuclei were revealed by staining (DAPI).

**Figure 7.** Inhibition of ROS protects the recipient HeLa but not the donor T47D cells from apoptosis. T47D cells were preincubated for 30 min with 30 nmol/L L-NAME (a selective eNOS inhibitor at 30 nmol/L), 30 μmol/L apocynin (an NADPH-oxidase inhibitor), 1,200 units/mL catalase, or 1,200 units/mL superoxide dismutase. Cells were then transfected to express GFP-DD1. Five hours later, the medium was transferred to recipient HeLa cells that were incubated overnight. Both the donor and recipient HeLa cells were analyzed for apoptosis by TUNEL assay. Each factor protects the recipient HeLa cells but not the donor T47D cells from apoptosis.
in at least two apoptotic pathways: one that is caspase-2 dependent and ROS independent, and a second resulting from the production of one or more ROS that directly or indirectly leads to a bystander effect. The finding that inhibition of ROS production protects HeLa cells but not T47D cells that do not express DD1 suggests that the DD1-expressing T47D cells also release another factor(s) that may selectively kill breast cancer cells. Our future studies will focus on identifying the factor(s) that DD1 interacts with in breast cancer cells, which initiates both the DD1-mediated, cell-specific, and bystander apoptotic pathways.

Acknowledgments

References

Correction: Mechanisms of NRIF3-Mediated Apoptosis in Breast Cancer

In the article on mechanisms of NRIF3-mediated apoptosis in breast cancer in the February 15, 2007 issue of Cancer Research (1), the bottom DAPI panel of Figure 4A was printed incorrectly; the corrected figure appears below.

![Corrected Figure 4A](image_url)

The Nuclear Receptor Interacting Factor-3 Transcriptional Coregulator Mediates Rapid Apoptosis in Breast Cancer Cells through Direct and Bystander-Mediated Events

Sharmistha Das, Jerome C. Nwachukwu, Dangsheng Li, et al.


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