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Activation of Caspase-3 and c-Jun NH_2-terminal Kinase-1 Signaling Pathways in Tamoxifen-induced Apoptosis of Human Breast Cancer Cells[1]

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ABSTRACT

Tamoxifen (TAM) is widely used in the treatment of breast cancer. The cytostatic effects of TAM have been attributed to the antagonism of estrogen receptor (ER) and inhibition of estrogen-dependent proliferative events. However, the mechanism by which TAM is also effective against certain ER-negative breast tumors remains to be elucidated. Here we report that TAM induced the activity of caspase-3-like proteases in ER-negative breast cancer cell lines MDA-MB-231 and BT-20, as evidenced by the cleavage of fluorogenic tetrapeptide substrate and of poly(ADP-ribose) polymerase. The activation of caspase-3-like proteases preceded TAM-induced chromatin condensation and nuclear fragmentation, the typical apoptotic morphologies. Pretreatment of cells with a specific inhibitor of caspase-3, acetyl-Asp-Glu-Val-Asp-aldehyde, or with a general inhibitor of caspases, benzyloxycarbonyl-Val-Ala-Asp-fluoromethyl ketone, prevented TAM-induced apoptosis. TAM also stimulated c-Jun NH_2-terminal kinase (JNK) 1 activity, and interfering with the JNK pathway by overexpressing a DN JNK1 mutant attenuated TAM-induced apoptosis. In addition, treatment of cells with a lipid-soluble antioxidant vitamin E blocked TAM-induced caspase-3 and JNK1 activation as well as apoptosis, whereas water-soluble antioxidants *N*-acetyl L-cysteine and glutathione had little effect. Thus, this study demonstrates that TAM induces apoptosis in ER-negative breast cancer cells through caspase-3 and JNK1 pathways, which are probably initiated at the cell membrane by an oxidative mechanism.

INTRODUCTION

TAM,[4] a nonsteroidal triphenylethylene derivative, has been used extensively in the treatment of both advanced and early-stage breast cancer (1). Recently, the efficacy of TAM in reducing the incidence of breast cancer in a high-risk population was established by a National Cancer Institute study that began in 1992 (2). TAM is a potent ER antagonist, and its pharmacology has been reviewed extensively (3). Inhibition of ER by TAM blocks the secretion of polypeptide growth factors, such as epidermal growth factor and transforming growth factor α, leading to growth arrest of ER-positive cells. In addition to its cytostatic effects mediated through the ER, TAM has also been shown to be cytotoxic to both ER-positive and ER-negative cells (4, 5), and such a cytotoxic effect is believed to be mediated by the induction of apoptosis (6, 7). Although several signaling intermediates, such as PKC (8, 9), transforming growth factor β (10), and c-myc (11), have been implicated, the precise molecular mechanism of TAM-induced apoptosis remains unclear.

One of the key events in apoptosis is the activation of a cascade of intracellular cysteine proteases known as caspases (12). To date, at least 13 caspases have been identified in mammalian cells (13). These have been classified into three families, depending on their functions in the caspase cascade (14). The caspases with a death effector domain comprise caspase-8 and -10. They are associated with the cell membrane DRs of TNF-α, Fas, or TNF-related apoptosis-inducing ligand via their death domains. The caspases with a caspase recruiting domain include caspase-1, -4, -5, -2, and -9. Finally, the effector or downstream caspases include caspases 3, 6, and 7. They are activated by death effector domain caspases or by caspases with a caspase recruiting domain by cleavage at specific tetrapeptide residues. Caspase-3 (15), a downstream caspase, has been shown to play a pivotal role in the terminal, execution phase of apoptosis induced by diverse stimuli. On proteolytic activation by upstream caspases, caspase-3 is able to cleave a variety of substrates, including PARP (15), DNA fragmentation factor (16), PKC-θ (17), and others. The cleavage of various substrates contributes to the typical morphological and biochemical features observed in apoptosis. Because of the diversity of its substrates, caspase-3 is thought to be a general mediator of physiological as well as stress-induced apoptosis.

JNK1 is a member of the of mitogen-activated protein kinase family that has been shown to regulate a number of physiological and pathological processes, including chemical-induced cell transformation (18) and apoptosis (19, 20). JNK1 is strongly activated by anticancer (21–23) and chemopreventive (24, 25) agents. JNK1 activation by cytotoxic agents was shown to be mediated by oxidative or mechanical stress in the form of ROS or microtubule perturbations, respectively (21, 22). The molecular ordering of JNK1 and caspase pathways seems to be stimulus dependent. Induction of caspase activity by the anticancer drugs etoposide and camptothecin has been shown to be mediated by JNK1 activation (26), whereas in doxorubicin- or death ligand-induced apoptosis, caspase activation appears to be independent of the JNK pathway (27). Thus, although a growing body of evidence suggests that both caspase and JNK are important mediators of apoptosis induced by various agents, the relationship between these two pathways remains elusive.

In this study, we investigated the role of caspase and JNK in TAM-induced apoptosis. Our results showed that TAM-induced apoptosis in breast cancer cell lines MDA-MB-231, BT-20, and MCF-7 was mediated by both caspase-3 and JNK1.

MATERIALS AND METHODS

Cell Culture, Chemicals, and Treatment. Human breast cancer cell lines MCF-7 and BT-20 were obtained from American Type Culture Collection. The human breast cancer cell line MDA-MB-231 was provided by Dr. Carol Westbrook (Department of Hematology and Oncology, College of Medicine, University of Illinois at Chicago, Chicago, IL). MCF-7 and BT-20 were maintained in MEM, and MDA-MB-231 was maintained in RPMI 1640. Both

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[4] The abbreviations used are: TAM, tamoxifen; ER, estrogen receptor; PKC, protein kinase C; JNK, c-Jun NH_2-terminal kinase; PARP, poly(ADP-ribose) polymerase; DAPI, diamidinophenylindole; AMC, 7-amino-4-methyl coumarin; V-E, vitamin E; NAC, *N*-acetyl L-cysteine; Ac-DEVD-CHO, acetyl-Asp-Glu-Val-Asp-aldehyde; z-VAD-fmk, benzyl-Val-Ala-Asp-fluoromethyl ketone; DR, death receptor; TNF, tumor necrosis factor; Ac-DEVD-MCA, Acetyl-Asp-Glu-Val-Asp-methylcoumaryl-7-amide; Ac-YVAD-MCA, Acetyl-Tyr-Val-Ala-Asp-methylcoumaryl-7-amide; Ac-VEID-MCA, Acetyl-Val-Glu-Ile-Asp-methylcoumaryl-7-amide; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; TBS, Tris-buffered saline [20 mM Tris-HCl (pH 7.4) and 8 grams/liter NaCl]DN, dominant negative; GSH, glutathione; ROS, reactive oxygen species; X-gal, 5-bromo-4-chloro-3-indoyl-β-O-galactoside.

media were supplemented with 10% FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin. TAM, NAC, V-E (α-tocopherol), and DAPI were obtained from Sigma (St. Louis, MO). The fluorogenic tetrapeptide substrates of caspase-3 (Ac-DEVD-MCA), caspase-1 (Ac-YVAD-MCA), and caspase-6 (Ac-VEID-MCA) were obtained from Peptides International (Louisville, KY), and the substrates of caspase-8 (Acetyl-Ile-Glu-Thr-Asp-methylcoumaryl-7-amide (Ac-IETD-MCA) and caspase-9 (Acetyl-Leu-Glu-His-Asp-trifluoromethylcoumaryl-7-amide (Ac-LEHD-FCA) and caspase inhibitors Ac-DEVD-CHO and z-VAD-FMK were purchased from Calbiochem (San Diego, CA). Before TAM treatment, cells (at approximately 80% confluence) were cultured in serum-free MEM. For pretreatment, the cells were incubated with the indicated agents for 1 h before challenge with TAM.

MTS Assay for Cell Viability. Cells were plated at a density of 10^4 cells/well into 96-well plates and cultured overnight before challenge with a series of concentrations of TAM for 24 h. Cell viability was determined by CellTiter^{96} Non-Radioactive Cell Proliferation Assay Kit (Promega, Madison, WI) using MTS as substrate.

Nuclear Staining Assay. After treatment, detached cells were collected in the medium, and the attached cells were harvested by trypsinization and combined with the detached fraction. Cells were centrifuged at 1000 × *g* for 5 min, washed once with PBS, and fixed in a solution of methanol:acetic acid (3:1) for 30 min. The fixed cells were placed on slides and stained with 2 μg/ml DAPI for 15 min. Excess dye was washed off with PBS. Nuclear morphology was observed under a fluorescence microscope.

Internucleosomal DNA Fragmentation Assay. After treatment, cells were harvested as described above. The cell pellet was resuspended in a lysis buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.5% Triton X-100. Cell lysate was left on ice for 30 min. DNA was extracted by adding an equal volume of neutral phenol:chloroform:isoamyl alcohol mixture (pH 8.0; Fisher Scientific) and precipitated with 0.1 volume of 5 M sodium chloride and 2 volumes of 100% ethanol at −20°C overnight. The DNA sample was dissolved in TE buffer [10 mM Tris (pH 8.0) and 1 mM EDTA] and treated with 1 μg/ml RNase at 37°C for 2 h. DNA fragments were resolved by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining.

Caspase Activity Assay. After treatment, cells were washed twice with ice-cold PBS and harvested as described previously (28). The catalytic activity of caspases was measured with their fluorogenic substrate (28). Briefly, 10 μg of total protein, as determined by the Bio-Rad (Richmond, VA) protein assay, was incubated with 200 μM fluorogenic peptide substrates Ac-DEVD-MCA, Ac-YVAD-MCA, Ac-VEID-MCA, Ac-IETD-MCA, or Ac-LEHD-FCA in a 50-μl assay buffer at 37°C for 2 h. The release of AMC was measured with a spectrofluorometer (PerSeptive Biosystems, Inc., Framingham, MA) at an excitation wavelength of 360 nm and an emission wavelength of 460 nm. The release of 7-amido-4-trifluoromethylcoumarin was monitored at 360/530 nm.

Western Blotting Analysis of PARP Cleavage. Cells were harvested in a lysis buffer as described previously (28). Fifty μg of total cytosolic protein, as determined by Bio-Rad protein assay, were resolved in 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane (Millipore) using a semidry transfer system (Fisher Scientific). The membrane was blocked with 5% nonfat dry milk in TBS for 1 h at room temperature and then incubated with 2 μg/ml primary polyclonal antihuman PARP antibody (Upstate Biotechnology Inc.) in TBS (containing 3% nonfat milk) at 4°C overnight. The membrane was washed three times with TBS and blotted with a horseradish peroxidase-conjugated secondary antibody (1:2000 dilution; Santa Cruz Biotechnology) at room temperature for 1 h and then washed in TBS three times. The protein was visualized using enhanced chemiluminescence system (Amersham).

***In Vitro* Immunocomplex Kinase Assay for JNK1 Activity.** The JNK1 assay was performed as described previously (24). Briefly, after cell harvesting, equal amounts of protein were incubated with rabbit anti-JNK1 antiserum (Ab101) for 2 h at 4°C in the presence of protein A-Sepharose 4B conjugate. The immunocomplexes were precipitated by centrifugation at high speed for 1 min and washed twice with lysis buffer and twice with kinase buffer. Kinase reactions were initiated by adding 30 μl of kinase assay buffer containing 10 μg of GSH *S*-transferase-c-Jun(1–79) fusion protein, 2 μCi of [γ-^{32}P]ATP, and 20 μM ATP. After incubation for 30 min at 30°C, the reactions were terminated by adding 10 μl of 4× Laemmli buffer and by heating at 94°C for 5 min. The phosphorylation products were resolved in 10% SDS-PAGE and visualized by autoradiography.

Transient Transfection Cell Death Assay. Cells were plated in 6-well plates 12 h before transfection at a density of 1.5×10^5 cells/well. Cells were cotransfected with the indicated plasmids by using calcium phosphate precipitation method. After removing the transfection medium, cells were incubated in culture medium for 12 h and then treated with 2.5 μM TAM for 24 h. Cells were then washed with PBS and fixed in 1% paraformaldehyde for 5 min on ice. The fixed cells were washed once in PBS and stained overnight in a solution containing 1 mM $MgCl_2$, 10 mM $K_4Fe(CN)_6$, 10 mM $K_3Fe(CN)_6$, and 1 mM X-gal in PBS. The β-galactosidase-expressing cells (blue-stained cells) were counted under a light microscope from five randomly chosen fields. Cell survival was determined as follows: (percentage of blue-stained cells in treated group/percentage of blue-stained cells in untreated group) × 100% (25).

RESULTS

TAM-induced Cytotoxicity in Breast Cancer Cells Is Due to Induction of Apoptosis. We compared the cell viability of TAM-treated ER-positive MCF-7 and ER-negative BT-20 and MDA-MB-231 breast cancer cells using the MTS assay. Treatment with TAM caused a dose-dependent reduction in cell survival. Regardless of their ER status, the three cell lines showed a similar IC_{50} (approximately 9–10 μM; Fig. 1). Because the clinically relevant concentrations of TAM are close to 5 μM (29), we used the concentration of 5 μM for all subsequent treatments.

It is possible that the decreased cell viability as determined by MTS assay could be due to either cell growth arrest or cell death. We next examined whether TAM at toxic concentrations induced cell death. MDA-MB-231 cells were treated with 5 μM TAM for various time periods. Cell death was assayed by two methods, trypan blue staining, which determines membrane integrity, and DAPI staining, which detects nuclear morphology. TAM-induced cell death was a relatively late event, occurring only after 24 h of treatment with 5 μM TAM. The number of both the trypan blue-positive cells and the condensed and fragmented nuclei increased with TAM treatment (Fig. 2, *A* and *B*), indicating that TAM-induced cell death was due to apoptosis in ER-negative MDA-MB-231 cells. This was further confirmed by an independent method, the DNA fragmentation assay (Fig. 2*C*). Similar results were obtained when ER-negative BT-20 cells were treated with TAM (data not shown).

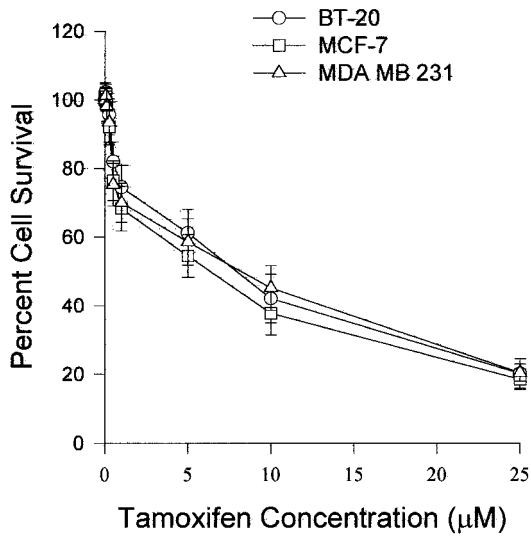

Fig. 1. Determination of the IC_{50} value of TAM in breast cancer cells. ER-positive MCF-7 cells and ER-negative MDA-MB-231 and BT-20 cells were plated in 96-well plates in triplicate and, after attachment, treated with TAM at various concentrations for 24 h. The medium was removed, and the cells were treated with MTS assay reagents as described in the manufacturer's protocol. The absorbance of the formazan product formed was measured at 340 nm. The percentage of survival was equal to the following formula: (absorbance of treated cells/absorbance of untreated cells) × 100. Data shown are the means of two independent experiments; *bars*, SE.

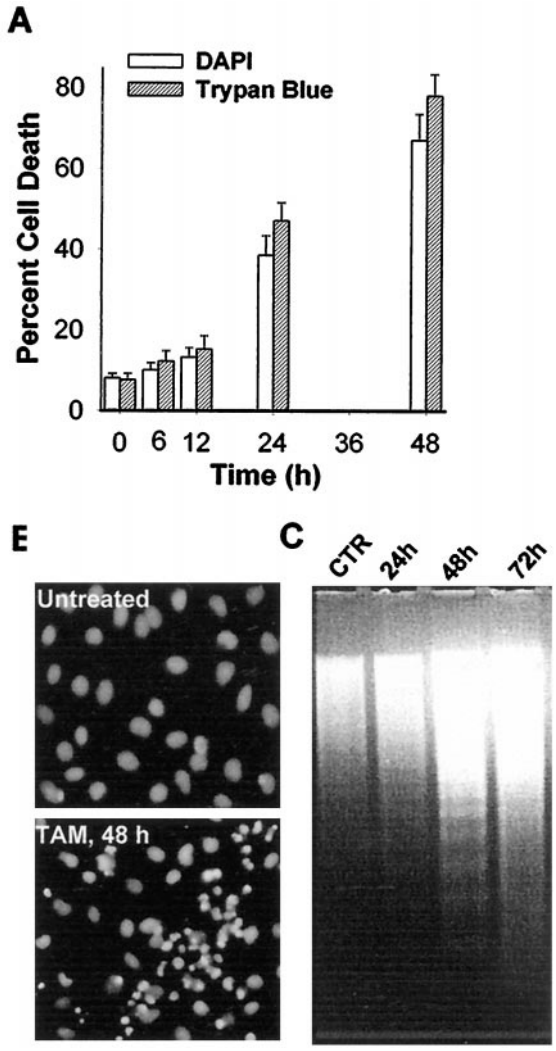

Fig. 2. Induction of apoptosis in breast cancer cells by TAM. *A*, determination of cell death by trypan blue and DAPI staining. After treatment with 5 μM TAM for indicated time periods, MDA-MB-231 cells were harvested. A portion of cells was stained with 0.1% trypan blue solution, and the remaining portion was fixed and stained with DAPI as described above. Trypan blue-stained cells were counted as blue or white cells in a hemocytometer, and DAPI-stained nuclei were counted as normal or condensed nuclei. Percentage of death was determined by the percentage of trypan blue-positive cells and condensed nuclei. Data shown are means of three independent experiments; *bars*, SE. *B*, nuclear condensation. MDA-MB-231 cells were treated with either 0.1% DMSO or 5 μM TAM for 24 h. Cells were harvested and fixed in methanol:acetic acid (3:1) for 15 min. The fixed cells were treated with 1 μg/ml DAPI for 15 min, and the nuclei were examined by fluorescence microscopy. *Arrows*, apoptotic cells with condensed nuclei. *C*, genome digestion. Cells were treated with 5 μM TAM for indicated time periods. Cellular DNA was extracted and analyzed by agarose gel electrophoresis.

TAM Induces the Activity of Caspase-3-like Proteases, Which Precedes Apoptosis. To measure the activation of caspases in TAM-treated cells, we took advantage of different fluorogenic tetrapeptide substrates, Ac-IETD-AMC, Ac-LEHD-AFC, Ac-YVAD-AMC, Ac-VEID-AMC, and Ac-DEVD-AMC, which have been shown to be specific for caspase-8, -9, -1, -6, and -3, respectively. As shown in Fig. 3*A*, TAM at the apoptosis-inducing concentration (5 μM) strongly stimulated caspase-3-like activity in MDA-MB-231 cells. Under similar conditions, TAM also activated caspase-8, -9, and-6, although to a lesser extent. Furthermore, the activation of these caspases preceded the onset of apoptosis as determined by DAPI and trypan blue staining. Similar results were obtained in BT-20 cells (data not shown). However, in MCF-7 cells, we did not detect any increased activity of caspase-3 and -6 after TAM treatment, although caspase-8 and -9 were activated to a level similar to that seen in MDA-MB-231 and BT-20 cells (data not shown). In contrast to a large peak of caspase-3 activity, little caspase-1 activity was observed throughout the experiment in either of these cell lines.

To provide further evidence for the activation of caspases, we measured the cleavage of PARP, an endogenous substrate of caspase-3, whose cleavage is considered to be a hallmark of apoptosis. Treatment of MDA-MB-231 cells with TAM (5 μM) resulted in cleavage of PARP to an 85-kDa fragment (Fig. 3*B*). The cleavage of PARP was inhibited by pretreatment of cells with Ac-DEVD-CHO, a caspase-3-specific inhibitor, or with z-VAD-FMK, a general inhibitor of caspases. The lipid-soluble antioxidant V-E also inhibited TAM-induced PARP cleavage (Fig. 3*B*).

Caspase Inhibitors Ac-DEVD-CHO and z-VAD-fmk Block the Induction of Apoptosis by TAM. The above-mentioned results indicate that caspases are activated in TAM-treated cells. To define the role of such caspase activation in TAM-induced apoptosis, we treated MDA-MB-231 cells with caspase inhibitors before challenge with TAM. The broad specificity caspase inhibitor, z-VAD-fmk (25 μM), abrogated the induction of apoptosis by TAM as measured by the number of fragmented nuclei and trypan blue-positive cells (Fig. 4*A*). Similar results were obtained in both MCF-7 and BT-20 cells (data not shown). The caspase-3-specific inhibitor, Ac-DEVD-CHO (50 μM), also attenuated TAM-induced apoptosis in MDA-MB-231 cells (Fig. 4*A*). The caspase inhibitors also abrogated caspase-3 activity in MDA-MB-231 cells (Fig.

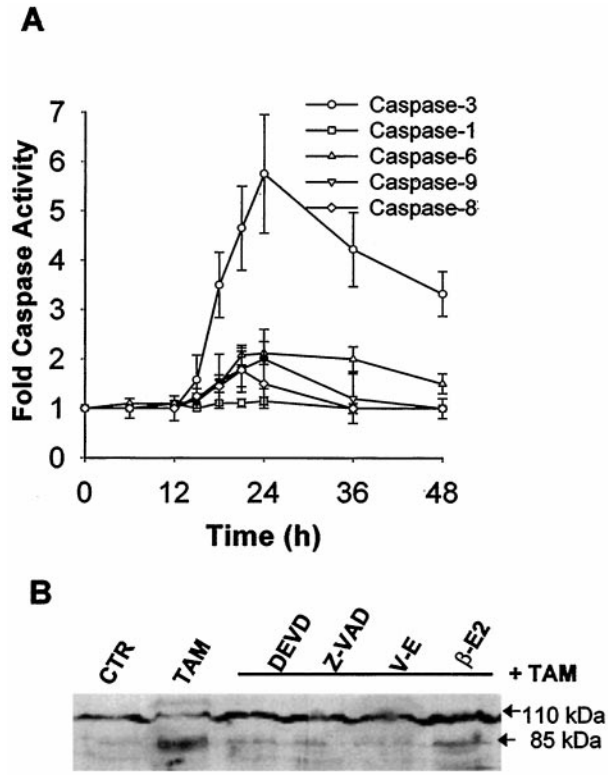

Fig. 3. *A*, induction of caspase activity by TAM. MDA-MB-231 cells were treated with 5 μM TAM for the indicated time periods and harvested and lysed in caspase lysis buffer. Enzymatic activity of caspase-1-, caspase-3-, caspase-6- and caspase-9-like proteases was determined by incubation of 10 μg of total protein with fluorogenic substrates Ac-YVAD-MCA, Ac-DEVD-MCA, Ac-VEID-MCA, and Ac-LEHD-AFC, respectively, for 2 h at 37°C. The release of AMC or AFC was monitored spectrofluorometrically (360/460 nm and 360/530 nm, respectively). Fold induction in caspase activity was calculated as a ratio of the fluorescence of TAM-treated samples to that of untreated samples. Data shown are the means of three independent experiments; *bars*, SE. *B*, cleavage of PARP by TAM and the effect of caspase inhibitors and V-E. MDA-MB-231 cells were treated with 5 μM TAM for 24 h either alone or in the presence of 50 μM Ac-DEVD-CHO, 50 μM z-VAD-fmk, or 0.5 mM V-E. Cells were harvested, and PARP cleavage was analyzed by Western blotting as described in "Materials and Methods." The blot shown is from one of three independent experiments.

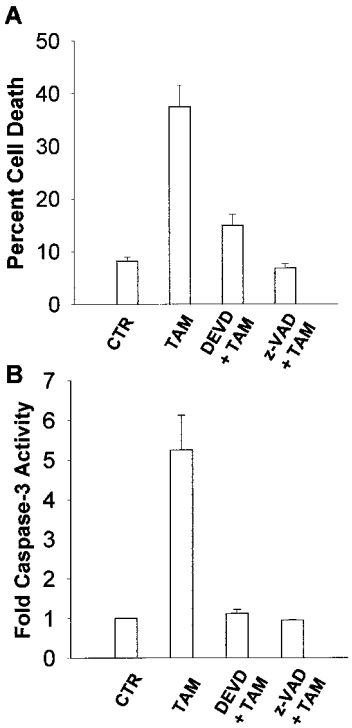

Fig. 4. Effects of caspase inhibitors on caspase-3-like activity and apoptosis induced by TAM. *A*, inhibition of TAM-induced apoptosis by caspase inhibitors. MDA-MB-231 cells were pretreated with 50 μM of either caspase-3 inhibitor Ac-DEVD-CHO or general caspase inhibitor z-VAD-fmk for 1 h, followed by 5 μM TAM for 24 h, and the percentage of cell death was assessed by both DAPI and trypan blue staining as described in the Fig. 2 legend. Data shown are the means of three independent experiments; *bars*, SE. *B*, abrogation of caspase-3-like activity by caspase inhibitors. Cells were treated as described above. The enzymatic activity of caspse-3-like proteases was determined as described in *A*.

4*B*). These results demonstrate that TAM induces apoptosis in breast cancer cells in a caspase-dependent manner.

TAM Stimulates JNK1 Activity, Which Is Not Affected by Caspase Inhibitors. To measure the JNK1 activity in TAM-treated cells, we performed *in vitro* immunocomplex kinase assays. JNK1 activity began to increase after treatment with 0.5 μM TAM for 1 h and reached a maximum level after treatment with 10 μM TAM. However, the induced JNK1 activity declined when the concentration of TAM reached more than 25 μM (Fig. 5*A*). Thus, the activation of JNK1 by TAM is dose dependent. JNK1 activation by TAM is also time dependent, with maximum activation being achieved 1 h after treatment with 5 μM TAM (Fig. 5*B*). This earlier kinetics of JNK1 activation as compared with caspase activation suggests that JNK1 is either upstream or independent of caspases. Consistent with this notion, pretreatment of cells with caspase inhibitor z-VAD-fmk or Ac-DEVD-CHO had no significant effect on JNK1 activation by TAM (Fig. 5*C*).

Blockade of JNK1 Pathway Attenuates TAM-induced Apoptosis. Activation of JNK1 is essential in radiation-induced (20) or ceramide-induced (30) apoptosis. To examine whether JNK1 activation has a similar role in TAM-induced apoptosis, we transfected MDA-MB-231 cells with the expression vectors for wild-type JNK1 or a DN JNK1 mutant, JNK1(APF), along with a vector encoding β-galactosidase (pCMV-βgal). The transfected cells were treated with TAM (5 μM) for 24 h and then assayed for cell death by staining with a β-galactosidase substrate, X-gal. The survival rate of TAM-treated transfected cells was calculated as the percentage of blue cells in the treated group divided by the percentage of blue cells in the untreated group (25). As shown in Fig. 6*A*, overexpression of DN JNK1(APF) prevented cell death induced by TAM. The JNK1 activity in DN JNK1-transfected cells was not induced in response to TAM treatment (Fig. 6*B*). This result suggests that JNK1 participates in the regulation of TAM-induced apoptosis.

V-E Blocks TAM-induced Caspase-3 and JNK1 Activation and Inhibits Apoptosis. Cytotoxicity of TAM has been attributed to the generation of oxidative stress (9, 31). Accordingly, we examined the role of oxidative stress in the TAM-induced caspase and JNK1 signaling pathways and apoptosis. Pretreatment of MDA-MB-231 cells with lipid-soluble antioxidant V-E (α-tocopherol) prevented TAM-induced activation of caspase-3-like proteases, and JNK1 and also abolished TAM-induced apoptosis (Fig. 7, *A*—*C*). In contrast, water-soluble antioxidants NAC or GSH had little effect. Similar results were obtained in MCF-7 and BT-20 cells (data not shown). These data suggest that oxidative stress generated in the cell membrane is responsible for the initiation of JNK1 and caspase pathways that ultimately lead to apoptosis in TAM-treated breast cancer cells.

DISCUSSION

TAM has been effectively used in the treatment of breast cancer for more than three decades, yet the molecular mechanisms underlying TAM-induced cytotoxicity in breast cancer remain elusive. The purpose of this study was to investigate the role of caspases and JNK1 in TAM-induced apoptosis in ER-positive and ER-negative breast cancer cells and the possible signaling intermediates in the two pathways. To provide a plausible mechanism for the observed clinical efficacy of TAM in treatment of breast cancer, we used TAM at a concentration of 5 μM in breast cancer cells, which is close to the breast tumor concentration achieved in patients (29). As confirmed by two different methods, nuclear staining and genome digestion, this concentration of TAM did indeed induce apoptotic cell death in breast cancer cells. The induction of apoptosis by TAM was preceded by induction of the activity of caspase-3-like proteases, which was proven by two lines of evidence. TAM treatment resulted in (*a*) a time-dependent increase in the release of AMC from the fluorogenic caspase-3-specific substrate, Ac-DEVD-MCA, and (*b*) in cleavage of PARP, an endogenous substrate of caspase-3, to its

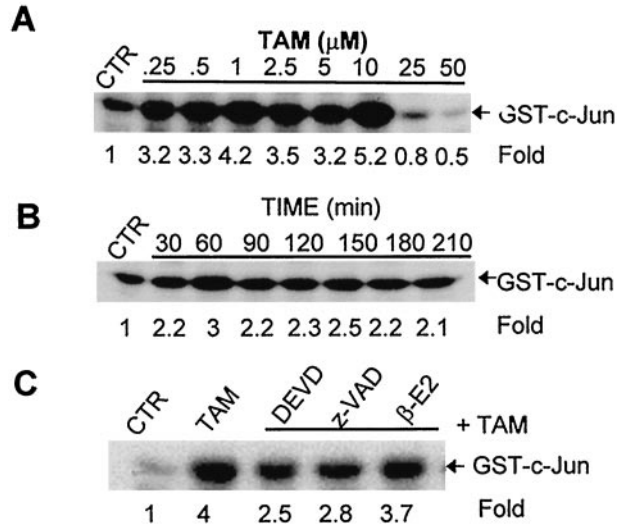

Fig. 5. Activation of JNK1 by TAM and the effect of caspase inhibitors on JNK1 activity. *A*, dose-response of JNK1 activation by TAM. MDA-MB-231 cells were treated for 1 h with different concentrations of TAM. Cells were harvested, and JNK1 activity was determined by an *in vitro* immunocomplex kinase assay as described in "Materials and Methods." *B*, kinetics of JNK1 activation by TAM. Cells were treated with 5 μM TAM for different time periods, and JNK1 activity was determined as described above. *C*, effect of caspase inhibitors on TAM-induced JNK1 activity. Cells were pretreated for 30 min with 50 μM Ac-DEVD-CHO or z-VAD-FMK, followed by 5 μM TAM for 1 h. JNK1 activity was determined as described previously. All data shown are from one of three independent experiments.

85-kDa degradation product. The role of caspase-3 in TAM-induced apoptosis was further substantiated by our results showing that Ac-DEVD-CHO, the synthetic peptide inhibitor of caspase-3, blocked TAM-induced apoptosis. The absence of caspase-3 activation in MCF-7 cells raised the possibility that TAM induces apoptosis via a caspase-independent pathway in ER-positive MCF-7 cells. But even other stress stimuli, such as UV radiation, TNF-α, or anticancer drugs like camptothecin and etoposide that act independent of the ER and are known to induce apoptosis via the caspase cascade, failed to activate the caspase-3 in MCF-7 cells (data not shown). One explanation of the observed phenomenon is provided in two recent reports (32, 33) that document the absence of caspase-3 activity in MCF-7 cells due to a mutation in exon 3 of the *CASP-3* gene. This may also explain the absence of genome digestion in MCF-7 cells. Thus, other caspases may be operative in TAM-induced apoptosis in MCF-7 cells. This was supported by our results that the general caspase inhibitor z-VAD-fmk completely blocked TAM-induced apoptosis in MCF-7 cells (data not shown). The activation of caspase-8 by TAM in breast cancer cell lines is intriguing, suggesting that TAM, like other anticancer drugs, may induce the expression of DRs or apoptotic ligands such as TNF-α, Fas ligand, or TNF-related apoptosis-inducing ligand or signal through death domain-containing adapter proteins (27, 34). Caspase-9 can be activated by caspase-8 or activated independently by apoptotic protease-activating factor 1 on binding of cytochrome *c* released from the mitochondria (Ref. 35 and the references therein). Indeed, the involvement of mitochondria in the proapoptotic action of TAM was implicated by a time-dependent reversal of both the calcium-induced mitochondrial membrane depolarization and calcium release in TAM-treated rat liver microsomes, suggesting that the effect was on the permeability transition pore (36). The activation by TAM of the effector caspases, caspase-3 and -6, could then be explained by proteolytic cleavage by these activated upstream caspases. Caspase-1, however, did not seem to play a role in TAM-induced apoptosis because no activation was detected under experimental conditions. Thus, DR- or mitochondria-mediated activation of the caspase cascade may be a potential mechanism underlying TAM-induced apoptosis in breast cancer.

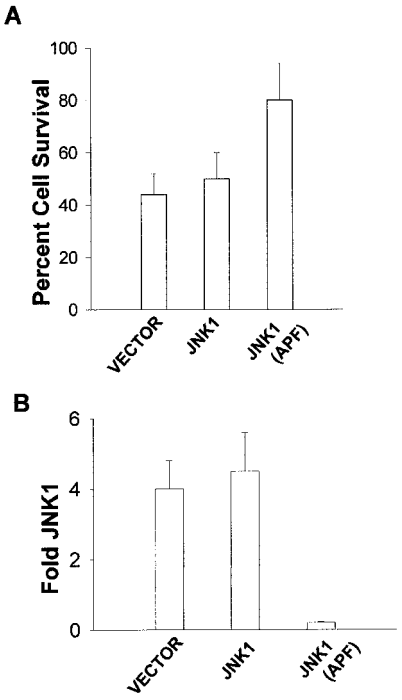

Fig. 6. Attenuation of TAM-induced apoptosis by blocking the JNK pathway. *A*, effect of DN JNK1 on TAM-induced cell death. MDA-MB-231 cells were cotransfected in duplicate with pCMV-βgal (2 μg) and wild-type JNK1-expressing- or DN JNK1 [JNK1(APF)]-expressing plasmid vectors (3 μg) using the calcium phosphate method. After transfection, the cells were incubated in culture medium for 12 h and were either left untreated or treated with 2.5 μM TAM for 24 h. After fixing and staining with X-gal, the β-galactosidase-positive cells (blue-stained cells) were counted under a microscope from five randomly chosen fields. Cell survival was determined as follows: (percentage of blue-stained cells in the treated group/percentage of blue-stained cells in the untreated group) $\times$ 100%. The data presented are the means $\pm$ SD of three experiments. *B*, effect of DN JNK1 on TAM-induced JNK1 activation. The JNK1 activity in MDA-MB-231 cells transfected with either wild-type or DN JNK1 was determined by an *in vitro* immunocomplex kinase assay as described in "Materials and Methods." Average data from three experiments are presented.

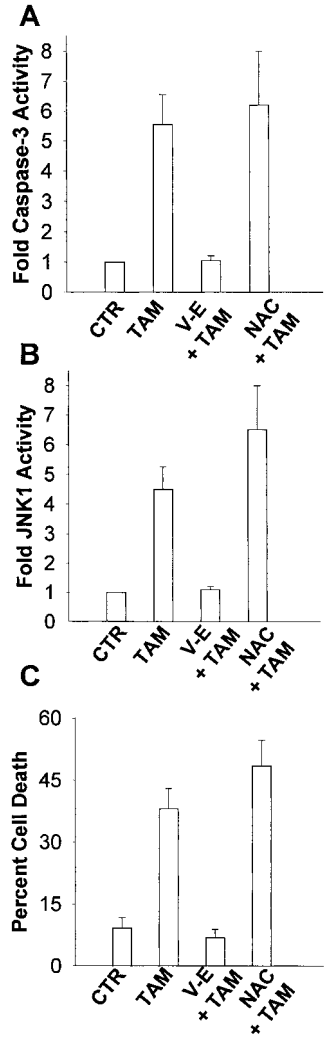

Fig. 7. Effect of lipid- and water-soluble antioxidants on TAM-induced caspase-3 and JNK1 activity and cell death. *A*, effect of V-E and NAC on caspase-3 activity. MDA-MB-231 cells were pretreated for 1 h with 0.5 mM V-E or 20 mM NAC, followed by 5 μM TAM for 24 h. Caspase-3 activity was determined as described previously. *B*, effect of V-E and NAC on JNK1 activity. Cells were pretreated for 30 min with V-E or NAC, followed by 5 μM TAM for 1 h. JNK1 activity was determined as described previously. *C*, effect of V-E and NAC on cell death. Cells were pretreated for 1 h with V-E or NAC, followed by 5 μM TAM for 24 h. The percentage of cell death was assessed as described in the Fig. 2*A* legend. All data shown are the means of three independent experiments; *bars*, SE.

TAM also activated JNK1 under conditions that induced apoptosis. Kinetically, the JNK1 activation was a much earlier event as compared with caspase activation and apoptosis. Furthermore, neither of the caspase inhibitors significantly blocked JNK1 activation, although DN JNK1, at least partially inhibited TAM-induced apoptosis. This suggests that JNK1 is upstream of the caspase cascade. Thus, our results indicate that both the caspase and JNK pathways are required in TAM-induced apoptosis in breast cancer cells because interfering with either pathway blocked apoptosis. Importantly, the lipid-soluble antioxidant V-E also blocked both JNK1 and caspase-3 activation and completely abolished TAM-induced cell death, implicating the involvement of oxidative stress. Two possi-

bilities exist for generation of oxidative stress by TAM: either autoxidation of the phenolic moiety, producing superoxides and hydrogen peroxide through redox cycling, or local effects in the membrane due to TAM partitioning into lipid bilayer. Ferlini *et al.* (31) demonstrated the generation of ROS in Jurkat and A2780 ovarian cancer cells treated with TAM. In the current study, the water-soluble antioxidants NAC and GSH failed to block caspase or JNK activation and cell death. Thus, generation of ROS alone may not be sufficient to activate the signaling pathways leading to apoptosis. V-E, on the other hand, inhibits cell membrane oxidation (37). Hence, membrane oxidation by TAM seems to play an important role in caspase and JNK signaling and apoptosis. In this regard, the actions of TAM may be similar in part to UV radiation. Therefore, it will be interesting to test whether TAM-induced ceramid accumulation (38), PKC translocation (39), or other second messengers contribute to the activation of JNKs and caspases by TAM. A recent study demonstrating protective effect of V-E against TAM-induced lysis of erythrocytes suggests that disruption of structural characteristics of biomembranes and release of peripheral membrane-, cytoskeleton-, or membrane-bound cytosol proteins by TAM contributes to its anticancer action (42).

In summary, it is tempting to speculate that TAM, by virtue of its high lipophilicity and partitioning in the cell membrane, generates a transmembrane signal transduction cascade leading to the early activation of JNK1 and subsequent activation of caspases. The caspase-dependent apoptotic effect of TAM may be of clinical relevance in determining sensitivity *versus* resistance of human breast cancer to TAM.

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