Lack of Correlation between Caspase Activation and Caspase Activity Assays in Paclitaxel-treated MCF-7 Breast Cancer Cells

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MCF-7 human breast cancer cells are widely utilized to study apoptotic processes. Recent studies demonstrated that these cells lack procaspase-3. In the present study, caspase activation and activity were examined in this cell line after treatment with the microtubule poison paclitaxel. When cells were harvested 72 h after the start of a 24-h treatment with 100 nM paclitaxel, 37 ± 5% of the cells were nonadherent and displayed apoptotic morphological changes. Although mitochondrial cytochrome c release and caspase-9 cleavage were detectable by immunoblotting, assays of cytosol and nuclei prepared from the apoptotic cells failed to demonstrate the presence of activity that cleaved the synthetic caspase substrates LEHD-7-amino-4-trifluoromethylcoumarin (LEHD-AFC), DEVD-AFC, and VEID-AFC. Likewise, the paclitaxel-treated MCF-7 cells failed to cleave a variety of caspase substrates, including lamin A, β-catenin, gelsolin, protein kinase Cδ, topoisomerase I, and procaspases-6, -8, and -10. Transfection of MCF-7 cells with wild type procaspase-3 partially restored cleavage of these polypeptides but did not result in detectable activities that could cleave the synthetic caspase substrates. Immunoblotting revealed that caspase-8, and -3, which were proteolytically cleaved in paclitaxel-treated MCF-7/caspase-3 cells, were sequestered in a salt-resistant sedimentable fraction rather than released to the cytosol. Immunofluorescence indicated large cytoplasmic aggregates containing cleaved caspase-3 in these apoptotic cells. These observations suggest that sequestration of caspases can occur in some model systems, causing tetrapeptide-based activity assays to underestimate the amount of caspase activation that has occurred in situ.

Apoptosis is a morphologically and biochemically distinct form of programmed cell death that occurs in many cell types after exposure to toxic stimuli (1, 2). Studies performed over the past 6 years have demonstrated that aspartate-directed cysteine proteases called caspases play critical roles in the initiation and completion of this process (3–8). In particular, caspase-mediated cleavages destabilize structural components of the cytoskeleton, inactive key components of DNA repair pathways, and interrupt signal transduction pathways involved in cell survival (8). At the same time, caspases appear to activate a number of enzymes, including the caspase-activated deoxyribonuclease (9), gelsolin (10, 11), and several kinases (8). Collectively, these cleavages contribute to the stereotypic morphological and biochemical changes (1) that constitute the process of apoptosis.

Because of the importance of caspases in the apoptotic process, many laboratories are currently performing enzymatic assays to assess the activation of these proteases. In a typical assay, cells are lysed, particulate matter is sedimented, and supernatants are incubated with low molecular weight substrates that consist of a tetrapeptide or pentapeptide coupled to a fluorogenic or chromogenic leaving group (12–14). Previous results from this laboratory (13) and others (15), for example, have used these types of assays to demonstrate that multiple caspases with distinct substrate preferences are activated during the process of apoptosis.

The MCF-7 human breast cancer line has been widely utilized to study various components of the apoptotic machinery. Early studies demonstrated that apoptotic DNA degradation in this cell line generates high molecular weight DNA cleavage products but not internucleosomal fragments (16). More recently, MCF-7 cells have been utilized to investigate the cytotoxic effects initiated by ligation of various death receptors (17–21), to examine the role of ceramide in drug-induced apoptosis (22, 23), to study the actions of the anticancer drug paclitaxel (24–26) and various hormones (24, 27–32), and to evaluate the effect of cytoplasmic cytochrome c injections on subsequent cell survival (33). Many of these studies were performed under the assumption that MCF-7 cells contain a normal complement of procaspases and scaffolding molecules required for their activation. Additional studies, however, have revealed that MCF-7 cells lack procaspase-3 polypeptide (33) as a consequence of a 47-bp deletion within exon 3 of the procaspase-3 gene that alters the reading frame of the message and results in an unstable truncated polypeptide (34). Consistent with these observations, apoptotic MCF-7 cells resulting...
Caspase Activation in MCF-7 Cells

Monoclonal antibodies to caspases-2, -3, and -7 as well as β-catenin and gelsolin were purchased from Transduction Laboratories (Lexington, KY). Monoclonal antibodies to cytochrome c and polyclonal serum against ICAD were obtained from Pharmingen. Polyclonal rabbit sera that recognize protein kinase Cδ, procaspase-6, a neoptepitope at the amino terminus of the PARP substrate fragment, and an active conformation of caspase-3 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), Upstate Biotechnology, Inc. (Lake Placid, NY), CLONTECH (Palo Alto, CA), Biotrin International (Dublin, Ireland), and Cell Signaling Technology (Beverly, MA), respectively. Monoclonal antibodies against PARP, topo I, and lamin A/C, procaspase-6, and lamin B1 were kindly provided by Drs. G. Poirier (Laval University School of Medicine, Ste-Foy, Quebec), Y-C. Cheng (Yale University School of Medicine, New Haven, CT), and David Toft (Mayo Clinic, Rochester, MN), respectively. Monoclonal antibodies that recognize capase-9 and Apaf-1 were a kind gift from Y. Lazebnik (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Polyclonal sera that recognize lamin A and lamin B1 were generated and characterized as previously described (45). Rabbit antiserum that recognizes caspase-10 was raised by injecting female New Zealand White rabbits with the large subunit of recombinant human caspase-10, which was expressed as a fusion protein with glutathione S-transferase using the pGEX-2T plasmid (Amersham Pharmacia Biotech), purified by affinity chromatography on glutathione-agarose, released from the fusion protein by cleavage with thrombin, subjected to SDS-PAGE, and excised from the gel (43). Rabbit antisera that recognize the neoptepitope generated upon activation of caspase-9 (pEPD), caspase-3 (IEPD, and caspase-8 (VETD) were raised by synthesizing each peptide coupled to keyhole limpet hemocyanin prior to injection. The caspase-9 and caspase-3 neoptepitope sera were previously characterized in detail (43). Characterization of the caspase-8 neoptepitope serum is described in the legend to Fig. 4B. Affinity-purified secondary antibodies coupled to peroxidase or fluorescein were from KPL (Gaithersburg, MD).

Cell Culture—MCF-7 cells (kindly provided by Dr. N. Davidson, Johns Hopkins Oncology Center, Baltimore, MD) were cultured in improved minimal essential medium (Biofluids, Rockville, MD) supplemented with 5% heat-inactivated fetal bovine serum, 100 units/ml penicillin G, 100 μg/ml streptomycin, and 2 mM glutamine in a humidified atmosphere containing 5% (v/v) CO2. Untransfected MCF-7 cells (American Type Culture Collection, Manassas, VA) were cultured in minimal essential medium containing Earle’s salts, 10% (v/v) fetal bovine serum, nonessential amino acids, 1 mM sodium pyruvate, and 10 μg/ml insulin according to the supplier’s instructions. MCF-7 cells were transfected with the caspase-3 RNA, the catalytically inactive CAS3 mutant, or empty vector as described (46). After selection, cloned cell lines were cultured in the same medium containing 750 μg/ml G418.

To assess the antiproliferative effects of paclitaxel on these cells, aliquots containing 1200–1500 cells were plated in replicate 35-mm plates and allowed to adhere for 14–16 h. Cells were then treated with increasing concentrations of paclitaxel (added from a 1000-fold concentrated stock in Me:SO) for 16 h, washed, and incubated in drug-free medium for 48 h. At the completion of this incubation, nonadherent cells were removed with the culture medium, and adherent cells were released by trypsinization. Cells were sedimented at 200 × g for 10 min and processed for the various assays described below.

Whole Cell Lysates for Immunoblotting—Sedimented cells were washed once with ice-cold RPI containing 10 mM HEPES (pH 7.4 at 4°C), lysed by vigorous vortexing in 6 M guanidine hydrochloride containing 250 mM Tri-HCl (pH 8.5 at 4°C), 10 mM EDTA, 150 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride, and prepared for electrophoresis as recently described in detail (39). After protein was determined by the bicinchoninic acid method (47), lyophilized samples were resuspended at a concentration of 5 mg of protein/ml in sample buffer (4% SDS, 20% glycerol, 100 mM Tris-HCl, pH 6.8 at 21°C, and 1 mM EDTA) and heated at 65°C for 20 min. Aliquots containing 50 μg of total cellular protein were subjected to SDS-PAGE on gels with 5–15% (w/v) acrylamide gradients, transferred to nitrocellulose or polyvinylidene difluoride, and probed with antibodies using standard techniques (13).

Cell Fractionation, Caspase Assays, and Affinity Labeling—Subcellular fractions were prepared at 4°C by a minor modification of recently

The abbreviations used are: TNF-α, tumor necrosis factor-α; DEVD- AFC, aspartylglutamylvalinylaspartyl-7-amino-4-trifluoromethylcoumarin; ICAD, inhibitor of caspase-activated deoxyribonuclease.

from treatment with TNF-α or staurosporine were reported to lack activity that cleaves DEVD-AFC, a substrate of active caspase-3 (35). Despite this lack of caspase-3 protein and activity, cleavage of all caspase substrates except α-fodrin was initially reported to be normal in MCF-7 cells (35).

Previous studies from our laboratory have demonstrated that paclitaxel, a chemotherapeutic agent that is widely used in the clinical treatment of breast cancer (36, 37), induces apoptosis in MDA-MB-468 breast cancer cells (38, 39). This process of paclitaxel-induced apoptosis was accompanied by release of cytochrome c from mitochondria; activation of caspases-3, -6, and -7; and cleavage of all caspase substrates examined, including PARP, lamin A, lamin B1, focal adhesion kinase, and topo I (39, 40). Previous studies (24, 40) have demonstrated that MCF-7 cells also undergo paclitaxel-induced apoptosis. Preliminary results, however, indicated that cleavage of topo I, the single caspase substrate examined in our earlier study, was undetectable in paclitaxel-treated MCF-7 cells (40).

In view of the widespread use of MCF-7 cells as a model system for the study of apoptosis, we have reexamined the relationship between caspase activation, substrate cleavage, and detection of caspase activity in MCF-7 cells. Using a wide range of immunological probes, we demonstrate in the present study that paclitaxel treatment in this cell line is accompanied by release of cytochrome c to cytosol, proteolytic cleavage of procaspases-9 and -7, and digestion of PARP and ICAD, two polypeptides that can be cleaved by either caspase-3 or caspase-7 (41, 42). In contrast, cleavage of additional polypeptides, including topo I, the laminas, β-catenin, gelsolin, protein kinase Cδ, and procaspases-6, -8, and -10, is undetectable after paclitaxel treatment. Transfection of procaspase-3 into MCF-7 cells partially restores the paclitaxel-induced cleavage of this second group of polypeptides. Interestingly, however, cleavage of tetrapeptide substrates such as DEVD-AFC, LEHD-AFC, and VEID-AFC remains extremely low after paclitaxel treatment of procaspase-3-transfected MCF-7 cells. Further studies using an antisera that recognizes a neoptepitope at the carboxyl terminus of the caspase-3 large subunit (43) indicate that procaspase-3 is cleaved appropriately in these cells but is not released to the cytosol. Instead, it accumulates in a detergent- and salt-resistant fraction that sediments at low speed. These observations not only provide additional evidence that caspase-3 is required for the cleavage of a variety of nuclear and cytoplasmic substrates but also indicate that sequestration of cleaved caspases can result in underestimation of enzyme activity when caspase assays are performed using widely accepted methods.

EXPERIMENTAL PROCEDURES

Materials—Reagents were obtained from the following suppliers: paclitaxel from Sigma; ECL and SuperSignal™ ULTRA enhanced chemiluminescent reagents from Amershams Biosciences, Inc., and Pierce; purified recombinant caspases-3 and -7 from Pharmingen (San Diego, CA); DEVD-AFC from BioMol (Plymouth Meeting, PA); VEID-AFC, LEHD-AFC, IETD-AFC, YVAD-AFC, and zVAD(Ome)-fmk from Enzyme Systems Products (Dublin, CA); and zEKBioD-aomk (13) from the Peptide Institute (Osaka, Japan).

The abbreviations used are: TNF-α, tumor necrosis factor-α; DEVD- AFC, aspartylglutamylvalinylaspartyl-7-amino-4-trifluoromethylcoumarin; EGFP, enhanced green fluorescent protein; LEHD-AFC, leucinylglutamylhistidylaspartyl-7-amino-4-trifluoromethylcoumarin; YVAD-AFC, tyrosylvalinylglutaminylaspartyl-7-amino-4-trifluoromethylcoumarin; PARP, polyADP-ribose) polymerase; PBS, calcium- and magnesium-free phosphate-buffered saline; topo, topoisomerase; VEID-AFC, valinylglutaminylvalinylaspartyl-7-amino-4-trifluoromethylcoumarin; zEKBioD-aomk, N-(N’-benzoylcarbonylglutamyl-N’-biotinyl) aspartic acid [(2,6-dimethylbenzoyl)oxy]methylketone; zVAD(Ome)-fmk; N-(N’-benzoylcarbonylvalinylalanyl) aspartic acid (O-methylester) fluoromethylketone; ICAD, inhibitor of caspase-activated deoxyribonuclease.
Caspase Activation in MCF-7 Cells

Caspase-3 Deficiency in MCF-7 Cells but Not Other Breast Cancer Cell Lines—Previous studies have demonstrated that MCF-7 cells lack caspase-3 (33, 34). To assess the possibility that down-regulation or deletion of caspase-3 or other components of the core apoptotic machinery might be a common feature in breast cancer cells, expression of Apaf-1 and procaspase-3, -6, -7, -8, and -9 was examined in 10 breast cancer cell lines (Fig. 1). Results of this analysis confirmed that procaspase-3 was undetectable in MCF-7 cells (top panel, lane 5). In contrast, procaspase-3 as well as Apaf-1 and procaspases-6, -7, -8, and -9 were detectable in all breast cancer cell lines examined. Cytochrome c varied widely among the cell lines (bottom panel), possibly reflecting differences in mitochondrial content, but was also universally detectable.

Paclitaxel-induced Apoptosis in MCF-7 Cells—In subsequent experiments, the response of MCF-7 cells to paclitaxel was examined. Initial experiments indicated that treatment of MCF-7 cells with paclitaxel induced apoptosis in a concentration- and time-dependent manner. In these experiments, cells were incubated with increasing concentrations of paclitaxel for 24 h, an exposure that mimics a widely utilized schedule for clinical administration of this agent (36). Cells were then returned to drug-free medium for 48 h and harvested. By 48 h after removal of paclitaxel, 37 ± 5% of MCF-7 cells had detached from their substratum. As indicated in Fig. 2, A and B, paclitaxel also induced typical apoptotic features, including chromatin condensation and nuclear fragmentation, in a dose-dependent manner. As was the case with MDA-MB-468 cells (39), paclitaxel-treated MCF-7 cells displaying an apoptotic morphology were found exclusively in the detached cell population. Treatment of MCF-7 cells with the broad spectrum caspase inhibitor zVAD-fmk during the 48 h after the removal of paclitaxel suppressed the appearance of apoptotic morphological features (Fig. 2C) as well as detachment of cells from the substratum (Fig. 2D), suggesting that caspases play a critical role in the development of these paclitaxel-induced changes.

Failure to Detect Caspase Activity in MCF-7 Cytosol Despite Caspase Activation—In an attempt to determine which
caspases might be involved, cytosol and nuclei prepared from paclitaxel-treated MCF-7 cells were assayed for caspase activity using various fluorogenic substrates. In these experiments, paclitaxel-treated MDA-MB-468 cells served as a positive control. Activities that cleaved LEHD-AFC (a preferred substrate of caspase-9) (8), DEVD-AFC (a well characterized substrate of caspases-3 and -7), and VEID-AFC (a preferred substrate of caspase-6) were readily detected in cytosol and nuclei from nonadherent (apoptotic) MDA-MB-468 cells (Fig. 3, A–C). In contrast, activities that cleaved these substrates did not detectably increase after paclitaxel treatment of MCF-7 cells. In additional experiments, cleavage of YVAD-AFC (a preferred caspase-1 substrate), IETD-AFC (a preferred caspase-8 substrate), and zVAD-AFC (a broad spectrum caspase substrate) was also undetectable in cytosol and nuclei from paclitaxel-treated MCF-7 cells. Mixing experiments (Fig. 3D) failed to provide any evidence that cytosol from apoptotic paclitaxel-treated MCF-7 cells contains caspase inhibitor or other substances that interfered with the tetrapeptide-based fluorogenic assays. Instead, caspase activity was absent from the cytosol. Consistent with this conclusion, affinity labeling with zEK(bio)D-aomk, a reagent that reacts with a variety of active caspases (13, 54), also failed to detect active caspase species in MCF-7 cytosol or nuclei (Fig. 3E). Collectively, these results raised doubts about the conclusion that caspases participate in paclitaxel-induced apoptosis in this model system.

In an attempt to resolve this apparent inconsistency, we examined caspase activation and substrate cleavages in paclitaxel-treated MCF-7 cells. Previous studies (39, 55) demonstrated that paclitaxel treatment results in mitochondrial release of cytochrome c followed by activation of a caspase-9-initiated proteolytic cascade. Consistent with this model, cytochrome c was readily detected in cytosol prepared from MCF-7 cells that had detached from their substratum after paclitaxel treatment (Fig. 4A, upper panel, lane 6). Although lower amounts of cytochrome c were consistently detected in cytosol from apoptotic MCF-7 cells compared with MDA-MB-468 cells (Fig. 4A, lanes 3 and 6), this difference appeared to reflect the lower expression of cytochrome c in MCF-7 cells (Fig. 4B). Nevertheless, the appearance of cytochrome c in the cytosol was accompanied by cleavage of procaspase-9 (Fig. 4A, lanes 3, 5, and 6). Like- wise, the appearance of cytochrome c in the cytosol was accompanied by cleavage of procaspase-7 (Fig. 4C, third panel). Additional studies using antibodies that recognize other procaspases (Fig. 4C) or cleaved caspase species (Fig. 4D), however, revealed substantial differences between the two cell lines. In particular, cleavage of procaspase-6 was diminished in MCF-7 cells (Fig. 4C, fourth panel). Moreover, cleavage of procaspase-8 (Figs. 4C, fifth panel) and procaspase-10 (Fig. 4D, third panel) to active caspase species (Fig. 4D, second and third...
was also markedly diminished. Thus, only caspase-9 and possibly caspase-7 appeared to be activated in MCF-7 cells. To provide further evidence for caspase activation in paclitaxel-treated MCF-7 cells, we examined cleavage of a variety of polypeptide substrates. Paclitaxel treatment of MDA-MB-468 cells resulted in cleavage of PARP, ICAD, topo I, lamin B1, lamins A and C, β-catenin, gelsolin, and protein kinase C (Fig. 5A, lane 3). For many of these substrates, discrete fragments that had the apparent molecular weights of previously reported caspase cleavage products (56) were detected (Fig. 5A, arrows, and data not shown). A different pattern emerged in paclitaxel-treated MCF-7 cells. PARP and ICAD were both cleaved after paclitaxel treatment (Fig. 5A, lane 6). The resulting 89-kDa PARP fragment reacted with an anti-neoepitope serum raised against the peptide GIDE (Fig. 5A, second panel), confirming that at least one caspase capable of cleaving the sequence DEVD†GIDE (44) was activated in paclitaxel-treated MCF-7 cells. In contrast, cleavage of topo I, lamin B1, lamins A and C, β-catenin, gelsolin, and protein kinase Cβ was undetectable in these cells.

To confirm that the altered pattern of substrate cleavages was due to the absence of caspase-3, MCF-7 cell extracts were treated with caspase-3 or caspase-7 (Fig. 5B). The addition of active caspase-3 to these lysates resulted in cleavage of PARP, topo I, lamin B1, lamin A, β-catenin, gelsolin, protein kinase Cβ, and procaspase-6 (Fig. 5B, lane 2, and data not shown). In contrast, cleavage of PARP but not the other substrates was observed when lysates were treated with caspase-7 (Fig. 5B, lane 3). Thus, the selective cleavage of some caspase substrates but not others in paclitaxel-treated MCF-7 cells (Fig. 5A) appears to be explained by the activation of caspase-7 but not caspase-3 in these cells. Nonetheless, these results failed to explain the inability to detect active caspase species using activity assays and affinity labeling procedures (Fig. 3).

Effect of Caspase-3 Transfection on Caspase-mediated Cleavages, Caspase Activity, and Drug Sensitivity—To determine whether the lack of caspase-3 in MCF-7 cells contributed to the inability to detect cleavage of fluorogenic substrates, MCF-7 cells were stably transfected with wild type procaspase-3 or a catalytically inactive C163S mutant. Indistinguishable results were obtained with two separate clones, designated MCF-7/wt1 and MCF-7/wt2, each of which expressed wild type pro-
caspase-3 at levels comparable with those of MDA-MB-468 cells (Fig. 6A, inset). Expression of procaspase-3 did not alter the sensitivity of MCF-7 cells to paclitaxel as assessed using colony-forming assays (Fig. 6A) or morphological assessment of drug-induced apoptosis (Fig. 6B). Moreover, expression of procaspase-3 did not alter the release of cytochrome c to the cytosol (Fig. 6C, upper panel) or the amount of cleaved caspase-9 detected in apoptotic MCF-7 cells (Fig. 6C, lower panel). After paclitaxel treatment, procaspase-3 was quantitatively cleaved in procaspase-3 transfectants (Fig. 6D). Cleavage of procaspase-6, topo I, lamin B1, lamin A, β-catenin, gelsolin, and protein kinase C8 was restored in transfectants containing wild type procaspase-3 (Fig. 6D and data not shown), although the cleavages were somewhat less complete than those observed in MDA-MB-468 cells (cf. Fig. 5A). Despite the cleavage of these substrates, cytosol from transfectected cells did not contain detectable DEVD-AFC cleavage activity after paclitaxel treatment (Fig. 6E). Identical results were obtained when DEVD-AFC was replaced with LEHD-AFC or VDID-AFC.2

Sequestration of Cleaved Caspases after Activation in MCF-7 Cells—In a final series of experiments, several approaches were utilized to resolve the apparent discrepancy between caspase activation, as assessed by substrate cleavages (Figs. 5A and 6D), and the lack of detectable caspase activity in cytosol or nuclei of paclitaxel-treated MCF-7 cells (Figs. 3 and 6E). Whole cell lysates and cytosol were prepared from procaspase-3-transfected cells before and after paclitaxel treatment. Immunoblot analysis revealed that procaspase-3 was readily detected in cytosol of nonapoptotic cells (Fig. 7, lanes 3, 4, 6, 7, 9, and 10). As expected, procaspase-3 levels were diminished in the apoptotic cells (Fig. 7, lanes 2, 5, 8, and 11). When the same blot were probed with a previously characterized antisemur (43) raised against IETD, the peptide present at the C terminus of the caspase-3 large subunit (8), species corresponding to the 17- and 20-kDa forms of the caspase-3 large subunit were readily detected in the whole cell lysates (Fig. 7, lane 2, and data not shown). In contrast, cleaved caspase-3 species were not detectable in cytosol from apoptotic MCF-7 transfectants (Fig. 7, lanes 5, 8, and 11). Similarly, a previously characterized antisemur that recognizes the 35- and 19-kDa forms of active caspase-9 large subunit (43, 57) detected cleaved caspase-9 in whole cell lysates (Fig. 7, lane 2) but not in cytosol from transfected MCF-7 clones (Fig. 7A, lanes 5, 8, and 11).

To assess the fate of cleaved caspase species in these cells, MCF-7/wt1 cells were lysed under hypotonic conditions and subjected to differential centrifugation (58) in order to isolate fractions enriched in nuclei plus cytoskeleton (48), mitochondria, lysosomes plus endoplasmic reticulum, and cytosol. MDA-MB-468 cells treated in a similar fashion provided a basis for comparison. In nonapoptotic MDA-MB-468 cells, procaspases-3 and -9 were predominately found in the cytosol (Fig. 8A, lower panel, lanes 13 and 14). After induction of apoptosis with paclitaxel, cleaved caspase species predominated in the nucleus plus cytoskeleton fraction (Fig. 8A, lower panel, lane 6), although small amounts were also observed in cytosol and the other cell fractions (Fig. 8A, lower panel, lanes 9, 12, and 15).

T. J. Kottke and S. H. Kaufmann, unpublished results.
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Fig. 5. Selective cleavage of some substrates in paclitaxel-treated MCF-7 cells and in vitro. A, after MCF-7 cells were treated with 100 nM paclitaxel (+) or diluent (−) for 24 h, washed, and incubated for 48 h in drug-free medium, whole cell lysates were prepared. Aliquots containing 50 μg of protein were subjected to SDS-PAGE, transferred to nitrocellulose, and probed with reagents that recognize the indicated polypeptides. Paclitaxel-treated MDA-MB-468 cells served as a positive control for substrate cleavages. Arrows, cleavage products that correspond in molecular weight to caspase-generated fragments described in previous papers (56). The same samples shown in Fig. 4 were utilized for this experiment, which is representative of three independent experiments. B, selective cleavage of some caspase-3 substrates and not others by caspase-7. Cytosol (280,000 × g supernatant) and nuclei (800 × g pellet) from MCF-7 cells were combined and treated with buffer, purified recombinant caspase-3, or purified recombinant caspase-7. After a 30-min incubation at 37 °C, samples were diluted with 3× SDS sample buffer and subjected to SDS-PAGE. Blots were probed with reagents that recognize the indicated polypeptides.

Consistent with these results, activity that cleaved DEVD-AFC was detectable in fractions that contained cytosol, nuclei plus cytoskeleton, or mitochondria from apoptotic MDA-MB-468 cells (Fig. 8B). It is important to note, however, that the level of activity did not precisely parallel the immunoreactive caspase-3 fragments (Fig. 8A), raising the possibility that part of the cleaved caspase species present in fractions containing mitochondria and nuclei plus cytoskeleton might be inhibited.

Results obtained with MCF-7/wt1 cells were different in a number of ways. First, more procaspase-3 and procaspase-9 were detected in particulate fractions even before the induction of apoptosis (Fig. 8A, upper panel, lanes 4, 7, and 10). Second, the cleaved caspase species were detected exclusively in fractions containing nuclei plus cytoskeleton and mitochondria after treatment with paclitaxel (Fig. 8A, upper panel, lanes 6 and 9). Thus, the failure to detect appropriately cleaved caspase species in the cytosol of MCF-7/wt1 cells (Fig. 7) does not reflect their destruction during the cell fractionation procedure but rather their sequestration in subcellular fractions that are sedimented during the preparation of cytosol. On the other hand, activity capable of cleaving DEVD-AFC was barely detectable (Fig. 8B) even in the MCF-7/wt1 fractions containing the largest amounts of cleaved caspase species (Fig. 8A). Identical results were obtained using LEHD-AFC and VEID-AFC. Mixing experiments indicated that the nucleus/cytoskeleton fraction from MCF-7/wt1 cells failed to inhibit purified recombinant caspase-3 or DEVD-AFC cleavage activity in cytosol from paclitaxel-treated MDA-MB-468 cells (data not shown; identical to Fig. 3D), ruling out a diffusible inhibitor in this fraction as well.

In further experiments, the localization of caspase-3 within MCF-7 cells was examined morphologically using two complementary approaches. MDA-MB-468 cells again served as a basis for comparison. In the first approach, cells were transiently transfected with cDNA encoding procaspase-3 fused at its C terminus to EGFP. In nonapoptotic MDA-MB-468 cells, this fusion protein was diffusely distributed throughout the cytoplasm but largely excluded from nuclei (Fig. 9A). This pattern was distinct from the distribution of EGFP alone, which was distributed throughout the cells including the nuclei (data not shown). After paclitaxel treatment, EGFP-labeled caspase-3 was still distributed diffusely in apoptotic cells (Fig. 9B), although it was also concentrated in punctate aggregates in some cells. The distribution of the procaspase-3/EGFP fusion protein in MCF-7 cells paralleled that of MDA-MB-468 cells prior to induction of apoptosis (Fig. 9D). After paclitaxel treatment, however, the vast majority of the cleaved caspase-3/EGFP was detected in large cytoplasmic aggregates with little diffuse cytoplasmic labeling (Fig. 9E).

To evaluate the possibility that these observations resulted from the use of transient transfection or the fusion construct, MDA-MB-468 cells and MCF-7/wt1 cells were stained using an antiserum that recognizes only cleaved caspase-3. Control experiments indicated that nonapoptotic cells failed to stain with this serum. After paclitaxel treatment, most apoptotic MDA-MB-468 cells displayed diffuse labeling (short arrow, Fig. 9C). Although some punctate aggregates were observed (long arrow, Fig. 9C), these were generally smaller than the aggregates observed after procaspase-3/EGFP transfection (Fig. 9B). Apoptotic MCF-7/wt1 cells, however, displayed striking punctate staining (Fig. 9F), consistent with the formation of cytoplasmic aggregates. Additional fractionation experiments (Fig. 9G) demonstrated that these cleaved caspase-3 species were resistant to extraction with neutral detergent or 0.5 M NaCl but instead required ≥ 4 M urea for efficient solubilization. Although we noted that the caspase inhibitor XIAP was also present in the 5000 × g pellet and displayed a similar extraction profile (Fig. 9G), the requirement for denaturing conditions to solubilize these polypeptides precluded immunoprecipitation studies to determine whether the two polypeptides were physically associated.

DISCUSSION

In the present study, we have compared the apoptotic response triggered by paclitaxel in MDA-MB-468, MCF-7, and caspase-3-transfected MCF-7 cells. Results of this analysis revealed that paclitaxel treatment of MCF-7 cells results in release of cytochrome c to cytosol, proteolytic cleavage of pro-
caspases-9 and -7, and digestion of some caspase substrates but not others. Surprisingly, activities that cleave widely utilized low molecular weight model substrates were undetectable in cytosol and nuclei from these apoptotic cells. Transfection with wild type procaspase-3 partially restored the apoptotic digestion of caspase-3 substrates but did not restore detectable activities that cleave low molecular weight substrates. Instead, appropriately cleaved caspases-9 and -3 were sequestered in a sedimentable fraction and were not active when assayed. These results have important implications for current understanding of the fate of activated caspases in apoptotic epithelial cells and for interpretation of assays that are widely used to study caspase activation during apoptosis.

Previous studies from our laboratory (39) and others (55) have suggested that paclitaxel induces apoptosis by activating the cytochrome c/Apaf-1/caspase-9 pathway. In paclitaxel-treated MDA-MB-468 cells, however, we also observed proteolytic cleavage of procaspase-8 and procaspase-10 (Fig. 4, C and D). Some studies have raised the possibility that anticancer drugs might induce apoptosis through a death receptor pathway (59, 60), whereas other studies have suggested that procaspase-8 might be activated downstream of caspase-3 in cyto-
Figs. 7-9. Failure to detect cleaved caspase species in cytosol of caspase-3-transfected MCF-7 cells. The indicated clone of MCF-7 cells was treated for 24 h with 100 nm paclitaxel (+) or diluent (−), washed, and incubated for 48 h in drug-free medium. Aliquots containing 50 μg of total cellular protein (lanes 1 and 2) or cytosolic protein (228,000 × g supernatant; lanes 3–11) prepared from adherent (A) or nonadherent (F) cells were subjected to SDS-PAGE followed by immunoblotting with monoclonal antibodies that recognize procaspases-9 and -3 or previously characterized antisera that specifically recognize cleaved forms of caspases-9 and -3 (43). An antiserum that recognizes heat shock protein 90 (HSP90) was utilized to confirm loading of cytosolic protein in lanes 3–11. Results are representative of three independent experiments.

A variety of additional polypeptides are cleaved in paclitaxel-treated MDA-MB-468 cells but not MCF-7 cells (Fig. 5A). These differences were observed before cleavage (Fig. 4C) and presumed activation (41) of caspase-7 in apoptotic MCF-7 cells. Transfection of MCF-7 cells with procaspase-3 resulted in apoptosis-associated cleavage of these caspase substrates (Fig. 6D). Even though caspases-3 and -7 have similar abilities to cleave the tetrapeptide substrate DEVD-AFC (62, 63), these observations suggest that the substrate specificities of these enzymes toward macromolecular peptides are overlapping but nonidentical. Consistent with this conclusion, recombinant caspase-7 was unable to cleave a variety of caspase-3 substrates, including topo I, protein kinase Cα, gelsolin, β-catenin, and procaspase-6, when added to MCF-7 cell lysates (Fig. 5B). These results indicate that cleavages of certain substrates require active caspase-3.

These observations differ from the conclusions of Jänne et al. (35), who reported that all caspase substrates except fodrin were cleaved normally in TNF-α- or staurosporine-treated MCF-7 cells. It is possible that these conflicting conclusions might reflect differences in the apoptotic response of MCF-7 cells from different sources (21, 64), the use of different stimuli, or the examination of different substrates. The present observations, however, are in agreement with the more limited results of Tang and Kidd (65) and Kirsch et al. (26), who noted that cleavages of gelsolin and Bcl-2 are diminished in apoptotic MCF-7 cells, and with the report of Zheng et al. that lamin B1 is not cleaved during CD95-mediated apoptosis in caspase-3−/− hepatocytes (66). Our observations not only expand the list of caspase-3-dependent substrates, but also confirm that caspase-7 is unable to cleave these substrates in vitro.

Tang and Kidd (65) have reported that additional apoptotic changes, including detachment of cells from the substratum and fragmentation of nuclei into apoptotic bodies, are also absent from staurosporine- or TNF-α-treated MCF-7 cells as a consequence of their caspase-3 deficiency (65). Interestingly, the absence of these features of the cell death pathway appears to depend on the stimulus or the strain of MCF-7 cells analyzed. In our hands, paclitaxel treatment results in detachment of the parental MCF-7 cells from their substratum (Fig. 2D). The ability of zVAD-fmk to inhibit this process (Fig. 2D) suggests that detachment might depend on the activity of another caspase in these caspase-3-deficient cells. Likewise, paclitaxel-treated MCF-7 cells undergo nuclear fragmentation when they become apoptotic (Fig. 2, A and B; Ref. 25) despite the absence of caspase-3. These results are consistent with the previous demonstration of nuclear fragmentation in other caspase-3-deficient cells (67).

Despite the presence of cleaved caspase-9 (Fig. 4D), as well as cleavage of a number of presumed caspase substrates (Fig. 5A), we were unable to detect activities capable of cleaving the tetrapeptide substrates DEVD-AFC, VEID-AFC, or LEHD-AFC in cytosol or nuclei from paclitaxel-treated MCF-7 cells (Fig. 3, A–C). These results were in marked contrast to the ~50-fold increases in cleavage activities observed in the same experiments using cell fractions from MDA-MB-468 cells. Although others have reported detectable levels of DEVD-AFC cleavage activity after treatment of MCF-7 cells with hydrogen peroxide (68), TNF-α (23, 27, 69), or agonistic anti-Fas antibodies (69), it is important to stress that these levels were extremely low in comparison with other lines (69). Our subsequent experiments indicated that the paucity of DEVD-AFC cleavage activity was not due to the presence of a soluble inhibitor or interfering substance (Fig. 3D). In addition, cytosolic DEVD-AFC cleavage activity was not restored by transfection of MCF-7 cells with procaspase-3 (Fig. 6E). Instead, the paucity of cytosolic cleavage activity appeared to reflect, at least in part, altered trafficking of cleaved caspase-3 and -9.
species. Fractionation studies demonstrated that these species were recovered in fractions that sedimented at $5000 \times g$ rather than in cytosol (Figs. 7 and 8A). Two different morphological approaches (Fig. 9, E and F) demonstrated that cleaved caspase-3 species were present in cytoplasmic aggregates. This is in contrast to the more diffuse localization of active caspase-3 seen in MDA-MB-468 cells, particularly MDA-MB-468 cells expressing physiological levels of procaspase-3 (Fig. 6A, inset, and Fig. 9C). Collectively, these experiments provide evidence for trafficking of the active caspase species in MCF-7 cells to a subcellular compartment that resists solubilization under the conditions usually employed to isolate cytosol, a process we term "sequestration."

While the present studies were being prepared for publication, other groups reported that DEVD-AFC cleavage activity was detectable in cytosol prepared from procaspase-3-transfected MCF-7 cells after apoptosis was triggered with prolonged nocodazole or cisplatin treatments.2 Similar results were obtained when the procaspase-3-transfected cells of Faliero and Lazebnik (71) were subjected to the same analysis.2 Collectively, these observations suggest that the results shown in Figs. 6–8 are not unique to paclitaxel-induced apoptosis or the transfected MCF-7 isolates we initially studied.

The results presented in Figs. 6–9 are consistent with the observations of McFarlane et al. (73), who reported that exogenous epitope-tagged caspases were bound to cleaved cytokeratins after treatment of MCF-7 cells with high concentrations of TNF-\(\alpha\)-related apoptosis-inducing ligand, an activator of the death receptor pathway. The present results, however, extend the observations of McFarlane et al. in a number of ways. First, our experiments have shown that trapping of cleaved caspases-3 and -9 in a particulate fraction can also occur after activation of the mitochondrial pathway. Second, we have shown that this trapping occurs independent of the use of epitope tags to follow caspase species. Third, we have examined the effect of this trapping on caspase activity in situ and in subcellular fractions prepared from apoptotic MCF-7 cells. These experiments demonstrate that transfection of MCF-7 cells with procaspase-3 partially restores the cleavage of a

![Caspase localization and activity in MCF-7/wt1 and MDA-MB-468 cells.](image-url)
number of cellular polypeptides in situ (Fig. 6D) but does not restore the ability of subcellular fractions to cleave tetrapeptide substrates (Figs. 6E and 6B).

Further evaluation revealed that the cleavage of caspase-3 substrates after paclitaxel treatment was less extensive in transfected MCF-7 cells than in MDA-MB-468 cells (cf. Fig. 5A and 6D) even though the transfected MCF-7 cells express as much pro-caspase-3 as MDA-MB-468 cells (Fig. 6A, inset). This disparity raises the possibility that caspase-3 might be inhibited or inactivated in situ before its substrate cleavages are complete. Consistent with this possibility, we have observed that DEVD-AFC cleaving activity is at the limit of detection even when MCF-7/wt1 or MCF-7/wt2 fractions containing properly cleaved caspases-3 and -9 are assayed (Fig. 6B, nuclei plus cytoskeleton and mitochondria). It is possible that the inability to detect caspase enzymatic activity when fractions from parental MCF-7 cells are examined (Fig. 3) also reflects a physiological process that serves to limit caspase activity in certain cell types.

The observation that cleaved caspases can be sequestered and possibly inhibited has two potentially important implications for methods that are currently utilized to assess caspase activation. First, methods that involve preparation of cytosol by enzymatic assay or immunoblotting will potentially miss caspase activation. The MCF-7 line represents the extreme of this phenomenon, but our results in MDA-MB-468 cells indicate that a substantial portion of the cleaved caspase species in this cell line are also found in the nuclear/cytoskeleton fraction (Fig. 8A). Second, methods that rely solely upon cleavage of tetrapeptide-based substrates also have the potential to underestimate caspase activation. Both the appearance of properly cleaved caspase species on immunoblots (Figs. 4D, 7, and 8A) and the cleavage of caspase substrates in situ (Figs. 5A and 6D) clearly indicate that caspases have been activated in MCF-7 and MCF-7/wt1 cells even though similar amounts of pro-caspase-9 have been cleaved in the two cell lines (Fig. 4C). The requirement for denaturing conditions to solubilize XIAP (Fig. 9G) provides a potential explanation for the inability to detect a soluble caspase inhibitor in MCF-7 cell fractions (Fig. 3D and data not shown). On the other hand, this requirement for denaturing conditions to solubilize XIAP and cleaved caspase species (Fig. 9G) also precluded coimmunoprecipitation experiments designed to determine whether XIAP and cleaved caspases were physically associated. Thus, the conclusion that caspases in MCF-7 cells are inhibited in addition to being sequestered must be considered tentative. We also cannot at present rule out the possibility that caspase-3 and -9 are bound to other inhibitory polypeptides in the sedimentable fraction. In either case, the fact that the inhibition of activity observed in these cellular fractions is accompanied by diminished cleavage of caspase-3 substrates in situ compared with other cell lines raises the possibility that the inhibition of caspase activity observed in MCF-7 and MCF-7/wt1 cells might reflect a physiological process that serves to limit caspase activity in certain cell types.
Caspase Activation in MCF-7 Cells

AFC, and LEHD-AFC activity in MCF-7 low speed pellets is not due to trivial technical difficulties. Instead, these observations raise the possibility that inhibition of caspase activity might occur in some subcellular fractions. This limitation must be kept in mind when negative assays for caspase activation are encountered.

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Lack of Correlation between Caspase Activation and Caspase Activity Assays in Paclitaxel-treated MCF-7 Breast Cancer Cells

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