

Involvement of cathepsin D in chemotherapy-induced cytochrome *c* release, caspase activation, and cell death

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Abstract

Treatment of cells with chemotherapy drugs activates the intrinsic mitochondrial pathway of apoptosis and the caspase protease cascade. Recently, the lysosomal protease cathepsin D has been implicated in apoptosis caused by oxidative stress, inhibition of protein kinase C, and stimulation of the TNFR1 and Fas death receptors. However, the role of cathepsin D in chemotherapy-induced cell death has remained largely unexplored. In this report, we show that treatment of U937 leukemia cells with the chemotherapy drug etoposide (VP-16) results in cathepsin D release into the cytosol within 4 hours after initiation of drug treatment. VP-16-induced cathepsin D release was not inhibited by z-VAD-FMK or pepstatin A, suggesting that it occurred independently of the activities of caspase proteases or cathepsin D. Down-regulation of cathepsin D expression in suspension U937 cells or adherent HeLa cells using cathepsin D small interfering RNA partially inhibited cell death resulting from treatment of cells with tumor necrosis factor- α , tumor necrosis factor-related apoptosis inducing ligand, or the chemotherapy drugs VP-16, cisplatin, and 5-fluorouracil. Moreover, cathepsin D down-regulation significantly delayed cytochrome *c* release and caspase-3 activation in response to chemotherapy treatment. Incubation of isolated mitochondria with cathepsin D-treated cytosolic extracts resulted in potent release of cytochrome *c*, indicating that a cytoplasmic substrate mediates the effects of cathepsin D on mitochondria. Together, these findings show that cathepsin D plays an important role in chemotherapy-induced cell death,

and that cathepsin D lies upstream of cytochrome *c* release and caspase-3 activation in the chemotherapy-induced execution pathway. [Mol Cancer Ther 2005;4(5):733–42]

Introduction

Treatment of cells with chemotherapy drugs activates the mitochondrial-mediated intrinsic pathway of apoptosis (1, 2). The intrinsic apoptosis pathway is characterized by loss of mitochondrial membrane potential (3) and release into the cytosol of mitochondrial apoptogenic factors, including cytochrome *c* (4–6), SMAC/DIABLO (7, 8), and apoptosis-inducing factor (9). The release of apoptogenic factors into the cytosol results in activation of the caspase protease cascade. Initially, caspase-9 becomes activated, followed by activation of the executioner caspases-3, -6, and -7 (2, 10). Activated executioner caspases cleave specific cellular substrate proteins, promoting the destruction of the cell.

Although caspases are known to play a central role in apoptotic cell death, an emerging body of evidence has also implicated noncaspase proteases, including cathepsin proteases. The cathepsin proteases reside primarily in endosomes and lysosomes (11–13). Cathepsins can be divided into three categories based on their catalytic amino acid: (a) the aspartate proteases (cathepsins D and E), (b) the cysteine proteases (cathepsins B, C, F, H, K, L, O, S, T, V, W, and X), and (c) the serine proteases (cathepsins A and G). It has long been thought that the primary function of cathepsins is the terminal degradation of proteins in the lysosomal compartment. However, recent studies have determined that cathepsins B and D relocate to the cytoplasm in response to certain apoptotic stimuli, and participate in the execution of apoptosis (14, 15).

Early studies showed that inhibition of cathepsin B with CA-074-Me abrogated bile salt-induced apoptosis in hepatocytes (16). Subsequently, cathepsin B was shown to be released into the cytosol following treatment of WEHI-S fibrosarcoma cells with tumor necrosis factor- α (TNF α ; ref. 17) or treatment of non-small cell lung cancer cells with microtubule stabilizing agents (18). Inhibition of cathepsin B with pharmacologic inhibitors or antisense directed against cathepsin B mRNA-attenuated microtubule stabilizing agent- and TNF α -mediated apoptosis (17–19). Moreover, Guicciardi et al. (20) have shown that hepatocytes from cathepsin B-deficient mice are markedly resistant to TNF α .

Cathepsin D is the major intracellular aspartate protease, and is expressed in all human tissues. The cathepsin D enzyme is synthesized as a 52 kDa precursor, which undergoes processing to active single chain (48 kDa) and double chain (34 and 14 kDa subunits) forms (21–23). Mice

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that are deficient in cathepsin D die on postnatal day 26 ± 1 due to atrophy of the intestinal mucosa and consequent anorexia (24). Cathepsin D has been shown to be relocalized to the cytoplasm following treatment of cells with hydrogen peroxide (25), oxidized low-density lipoprotein (26), the quinone naphthazarin (27), and the protein kinase C inhibitor staurosporine (28, 29). Reports demonstrating participation of cathepsin D in apoptosis execution have relied heavily on the pharmacologic inhibitor pepstatin A. Treatment with this inhibitor partially inhibits apoptosis caused by bile salts, naphthazarin, TNF α , IFN- γ , sphingosine, staurosporine, Fas stimulation, and withdrawal of essential neurotrophic factor (28–34). Unfortunately, pepstatin is not entirely specific, and is also known to inhibit cathepsin E, pepsin, and renin. However, antisense-mediated inhibition of cathepsin D has served to verify a role for this enzyme in IFN- γ - and Fas-mediated apoptosis (32). Furthermore, Bidere et al. (28) have shown that down-regulation of cathepsin D using small interfering RNA (siRNA) inhibits early apoptotic events associated with staurosporine-induced apoptosis in human T lymphocytes.

Although previous reports have indicated the importance of cathepsin D during apoptosis caused by the agents and stimuli described above, less is known about the involvement of this enzyme in chemotherapy-induced apoptosis. Intriguing findings were provided by Wu et al. (35), who showed that fibroblasts derived from cathepsin D knockout mice are more resistant to Adriamycin and etoposide (VP-16). The aim of the present study was to examine the subcellular localization of cathepsin D during VP-16-induced apoptosis, and to employ siRNA-mediated down-regulation of cathepsin D to determine whether this enzyme is required for chemotherapy-induced apoptosis in both adherent and suspension cells. Using immunoblotting, we found that cathepsin D is released into the cytoplasm in chemotherapy-treated cells. Inhibition of cellular caspases had no effect on cathepsin D relocalization. Similarly, relocalization was not dependent on cathepsin D activity. Specific down-regulation of cathepsin D in adherent HeLa cells and suspension U937 cells resulted in an inhibition of cell death by VP-16, cisplatin, 5-fluorouracil, TNF α , and TNF-related apoptosis inducing ligand (TRAIL). In addition, cathepsin D down-regulation led to an inhibition of cytochrome *c* release and caspase activation in chemotherapy-treated cells. Cell-free experiments provided evidence that a cytoplasmic substrate of cathepsin D mediates the effects of this enzyme on the mitochondrial pathway of apoptosis. Taken together, these findings establish a critical role for cathepsin D in apoptosis execution caused by chemotherapy drugs with distinct mechanisms of action.

Materials and Methods

Reagents

TNF and TRAIL ligand were purchased from PeproTech, Inc. (Rocky Hill, NJ). VP-16, cisplatin, 5-fluorouracil, cycloheximide, DMSO, β -hexosaminidase substrate

(4-methylumbelliferyl-*N*-acetyl- β -D-glucosaminide dehydrate), and anti- β -actin antibody were obtained from Sigma (St. Louis, MO). Caspase-3 substrate (Ac-DEVD-AFC) was obtained from BD PharMingen (San Diego, CA). The caspase-3 inhibitor DEVD-FMK was purchased from Calbiochem (San Diego, CA) and pancaspase inhibitor z-VAD-FMK was from Calbiochem. Cathepsin D inhibitor pepstatin A was from Roche Applied Biosciences (Indianapolis, IN). Polyclonal anti-cathepsin D and polyclonal anti-cathepsin B antibodies were obtained from Calbiochem, and anti-caspase-3 and anti-caspase-8 antibodies were from Cell Signaling Technology (Beverly, MA). Polyclonal anti-cathepsin E antibody was obtained from Santa Cruz (Santa Cruz, CA). Monoclonal anti-cytochrome *c* was purchased from BD PharMingen, and monoclonal anti-cytochrome *c* oxidase IV from Molecular Probes (Eugene, OR). Chemotherapy drugs were dissolved in DMSO as 1,000 \times stocks and stored at -20°C .

Cells

Wild-type HeLa cells were maintained in 5% CO $_2$ at 37°C in DMEM (Mediatech, Herndon, VA) containing 10% fetal bovine serum, 100 $\mu\text{g}/\text{mL}$ penicillin-streptomycin, and 0.5 $\mu\text{g}/\text{mL}$ amphotericin B (Fungizone). Transfected HeLa cells harboring cathepsin D siRNA constructs were additionally supplied with 250 $\mu\text{mol}/\text{L}$ hygromycin B (Calbiochem). Wild-type U937 cells were maintained in 5% CO $_2$ at 37°C in RPMI medium (BioWhitaker, Walkersville, MD) containing 10% fetal bovine serum, 100 $\mu\text{g}/\text{mL}$ penicillin-streptomycin, and 0.5 $\mu\text{g}/\text{mL}$ amphotericin B. U937 clones containing cathepsin D siRNA were supplemented with 250 $\mu\text{mol}/\text{L}$ hygromycin B.

Chemotherapy and Death Ligand Treatment, and Assay of Cell Death

Prior to induction of apoptosis, log-phase U937 cells and HeLa cells were plated at a density of 8×10^5 cells/mL in complete medium. To induce apoptosis, cells were treated at 37°C with chemotherapy drugs or death ligands for up to 48 hours. Chemotherapy drugs were added from stocks at a 1:1,000 dilution, giving a final concentration of DMSO diluent of 0.1%. Cisplatin was added to a concentration of 10 $\mu\text{mol}/\text{L}$, 5-fluorouracil to 5 $\mu\text{mol}/\text{L}$, and TRAIL to 2 $\mu\text{g}/\text{mL}$. In the case of VP-16, U937 cells were treated with 2 $\mu\text{mol}/\text{L}$ VP-16; HeLa cells were treated with 100 $\mu\text{mol}/\text{L}$ VP-16 due to greater resistance in these cells. HeLa cells were treated with chemotherapy drugs 24 hours after replating. For TNF α -induced apoptosis, cells were simultaneously treated with 10 ng/mL TNF α and 10 $\mu\text{g}/\text{mL}$ cycloheximide. Experiments involving treatment with chemotherapy drugs always included control cells treated with an equal concentration of DMSO (0.1%). At designated time points, aliquots of cells were removed for preparation of cytosolic fractions, or for determination of cell viability. Cell viability was determined by trypan blue exclusion. For each data point, a minimum of 100 cells were counted, and all counts were done in triplicate. Error bars represent SD. Control cultures always maintained >97% viability throughout the course of the experiment.

Cytosolic Isolation and Cathepsin D Relocalization

To assess subcellular relocalization of cathepsin D, immunoblotting was done using cytosolic extracts. Cytosolic extracts were prepared from 20×10^6 DMSO- or VP-16-treated U937 cells. Cells were collected by centrifugation at 1,300 rpm for 6 minutes at 4°C, then washed twice in ice-cold PBS. The final cell pellets were resuspended in 400 μ L of lysosomal resuspension buffer [20 mmol/L HEPES (pH 7.4), 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 1 mmol/L EDTA, 250 mmol/L sucrose, 1 mmol/L DTT, 1.5 mmol/L phenylmethylsulfonyl fluoride, 3 μ g/mL leupeptin, and 20 μ g/mL aprotinin], and homogenized on ice by 20 strokes in a type B Dounce homogenizer (Wheaton, Millvale, NJ). The homogenates were subjected to microcentrifugation for 30 minutes at 4°C and 14,000 rpm, and the resulting supernatants were further clarified by a second microcentrifugation for 30 minutes. Protein concentrations were determined by Bio-Rad Protein assay dye reagent and 25 μ g of protein was used for SDS-PAGE gel electrophoresis and immunoblotting analysis of cathepsin D.

Construction of Cathepsin D siRNA and Transfected Cell Lines

Appropriate sequences for use in siRNA constructs were identified in human cathepsin D mRNA using the Ambion (Austin, TX) siRNA target-finder program. Oligonucleotides containing desired sequences for hairpin loop formation and a 21-nucleotide cathepsin D target sequence were designed according to instructions provided by Ambion. For the 144 cathepsin D siRNA, single-stranded oligonucleotides capable of forming siRNA hairpin loops and encoding sequences complementary to cathepsin D mRNA at position 144 (5'-AGGCCCCGUCUCAAAGUACUU-3') were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA). The oligonucleotides also contained *Hind*III and *Bam*HI at their 5' and 3' ends, respectively. The two single-stranded oligonucleotides were mixed at a concentration of 1 μ g/ μ L each, denatured for 3 minutes at 90°C, then annealed for 1 hour at 37°C. The annealed DNA was cleaved with *Bam*HI and *Hind*III and ligated into the corresponding sites of the p*Silencer* 2.1-U6 hygro siRNA expression vector (Ambion), which contains a human U6 RNA polymerase III promoter and a hygromycin B resistance gene. The ligated DNA was transformed into DH5- α bacteria (Invitrogen, Carlsbad, CA) and plasmid DNA was purified (WizardPLUS minipreps, Promega, Madison, WI). DNA sequencing was used to verify the orientation and sequence of the siRNA construct. Sequence-verified plasmid DNA was then transfected into HeLa and U937 cells using GenePORTER transfection reagent (Gene Therapy Systems, San Diego, CA) according to the manufacturer's instructions. Twenty-four hours after transfection, hygromycin B (250 μ mol/L) was added to the cells. Clonal cell lines were isolated by limiting dilution in selection media. Transfection of p*Silencer* vector containing a sequence that shares only limited homology with known human sequences (provided by Ambion) was used as a control.

Whole Cell Extract Preparation

Whole cell extracts were prepared for immunoblotting experiments of cathepsin D and caspase-3 and for enzymatic assays of caspase-3. Cells (5×10^6) were harvested by centrifugation at 1,300 rpm for 6 minutes at 4°C, then washed in PBS (4°C). Cell pellets were lysed in 75 μ L of cell lysis buffer [50 mmol/L Tris (pH 8), 5 mmol/L EDTA, 150 mmol/L NaCl, 0.5% NP40], incubated on ice for 10 minutes, then centrifuged at 14,000 rpm for 10 minutes at 4°C. Supernatants were removed and protein concentrations were assessed with the Bio-Rad protein dye concentrate. Proteins (25 μ g per lane) were electrophoresed on 12.5% SDS-PAGE gels, followed by immunoblotting for cathepsin D, caspase-3, caspase-8, or β -actin. Enzymatic assays for caspase-3 using whole cell extracts were done as described below.

Immunoblotting

For immunoblotting experiments proteins (25 μ g per lane) were electrophoresed on 12.5% SDS-PAGE gels, then transferred to nitrocellulose membranes for 3 hours at 40 V. Membranes were blocked in TBST [50 mmol/L Tris (pH 8.0), 0.15 mol/L NaCl, 0.1% Tween 20] containing 5% dry milk for 1 hour at room temperature, followed by a quick wash in TBST. All primary antibodies, with the exception of anti-cathepsin B, were diluted 1:1,000 in TBST containing 1% bovine serum albumin, then incubated with membranes overnight at 4°C. Anti-cathepsin B was used at a 1:250 dilution. Following overnight incubation, membranes were washed once in TBST for 15 minutes, followed by three washes in TBST for 5 minutes each. Membranes were then incubated for 1 hour at room temperature with horseradish peroxidase-conjugated secondary antibody (Promega) diluted 1:4,000 in TBST containing 1% dry milk. The membranes were then washed once in TBST for 15 minutes, followed by three washes of 5 minutes each. The washed membranes were dried, then developed using Western lightning renaissance enhanced chemiluminescence reagent (Perkin-Elmer, Boston, MA).

Caspase-3 Enzymatic Assays

For caspase-3 enzymatic assays, 20 μ g of whole cell extract was added to reaction buffer containing 20 mmol/L Hepes (pH 7.5), 100 mmol/L NaCl, 10% glycerol, 5 mmol/L DTT, 1 mmol/L phenylmethylsulfonyl fluoride, to achieve a total reaction volume of 100 μ L. To this, Ac-DEVD-AFC was added to a concentration of 1 μ mol/L and the reaction incubated for 2 hours at 37°C. Cleavage of the substrate was assessed by measuring fluorescence at $Ex_{\lambda} = 400$ nm and $Em_{\lambda} = 505$ nm using a fluorescence spectrometer (Perkin-Elmer). For enzymatic determination, the data are presented as the mean \pm SD of three independent assays.

Cytochrome c Release Assays

To examine cytochrome c release in intact cells following treatment with VP-16, cells (10×10^6) were collected by centrifugation, washed with PBS (4°C), then resuspended at a density of 1×10^6 cells/mL in complete media. Cells were then incubated in the absence ($t = 0$) or presence of 2 μ mol/L VP-16 at 37°C for varying lengths of time.

Cytosolic extracts were then prepared and proteins quantified as described above. Cytosolic proteins (25 μ g per lane) were electrophoresed on 13% SDS-PAGE gels, transferred to nitrocellulose, and probed with anti-cytochrome *c*.

To assess the release of cytochrome *c* from mitochondria in cell-free reactions, mitochondria were first isolated from 10×10^6 U937 cells. Cells were collected by centrifugation at 1,300 rpm for 5 minutes at 4°C, washed in PBS (4°C), and again subjected to centrifugation. The cell pellets were resuspended in 100 μ L of resuspension buffer [20 mmol/L Hepes (pH 7.4), 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 1 mmol/L EDTA, 250 mmol/L sucrose, 1 mmol/L DTT, 1.5 mmol/L phenylmethylsulfonyl fluoride, 3 μ g/mL leupeptin, 20 μ g/mL aprotinin, and 700 μ g/mL digitonin], incubated on ice for 2 minutes, then subjected to centrifugation at 14,000 rpm for 30 minutes at 4°C. The mitochondria-containing pellets were washed twice with PBS (4°C) and resuspended in 100 μ L of resuspension buffer lacking digitonin. For cytochrome *c* release assays, isolated mitochondria (50 μ g per reaction) were incubated in the absence or presence of cathepsin D (Calbiochem) or cytosolic extract for 1 hour at 37°C in a total volume of 50 μ L of resuspension buffer. Pepstatin A was added to a final concentration of 100 μ mol/L 5 minutes prior to the addition of cathepsin D or cytosolic extract. Following 1 hour of incubation, reactions were subjected to centrifugation at 14,000 rpm for 30 minutes at 4°C. The supernatants were removed and subjected to another round of centrifugation. From each final supernatant, 15 μ L was electrophoresed on a 13% SDS-PAGE gel and analyzed by immunoblotting for cytochrome *c*.

Results

Lysosomal Release of Cathepsin D During Chemotherapy-Induced Apoptosis

In untreated, healthy cells, cathepsin D is localized to the lysosomal and endosomal compartments, where it functions to cleave and degrade cellular proteins (11–13). However, the activation of caspase proteases occurs primarily in the cytoplasm, and it is unclear how a protease that is restricted to lysosomes/endosomes could participate in apoptosis execution (36). Thus, several studies have investigated and found that cathepsin D is relocalized to the cytoplasm during apoptosis resulting from oxidative stress or treatment with the protein kinase C inhibitor staurosporine (25–29). The subcellular relocalization of cathepsin D during chemotherapy-induced apoptosis has not been reported. We reasoned that release of cathepsin D from the lysosomes would be likely if this enzyme plays a significant role in chemotherapy-induced cell death. To test this possibility, we did immunoblotting of cytosolic extracts from cells treated for varying lengths of time with VP-16 or DMSO control (Fig. 1A). By 4 hours, cathepsin D was detected in the cytosol of VP-16-treated cells. The levels of cytosolic cathepsin D continued to increase, even up to 18 hours. By contrast, only minor levels of cytosolic cathepsin

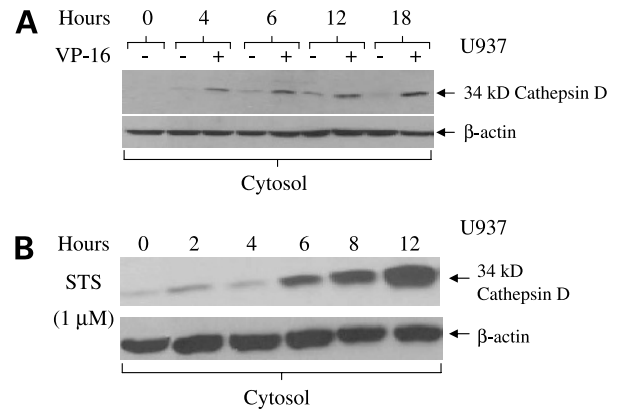


Figure 1. Chemotherapy drugs promote subcellular relocalization of cathepsin D. **A**, U937 cells were treated with 0.1% DMSO (–) or 2 μ mol/L VP-16 (+), and cytosolic extracts were prepared at the indicated time points. Proteins (25 μ g per lane) were electrophoresed on a 12.5% SDS-PAGE gel, and subjected to immunoblotting with anti-cathepsin D polyclonal antibody. The blot was stripped and reprobed with anti- β -actin to show equal loading. Cathepsin D was first detected in the cytosol after 4 h of treatment. **B**, U937 cells were treated with 1 μ mol/L staurosporine (STS) for varying lengths of time, and appearance of cathepsin D in cytosolic extracts was examined by immunoblotting.

D were detected in cells treated with DMSO. The form of cathepsin D that was released into the cytoplasm following VP-16 treatment was the 34 kDa active enzyme form. The 52 kDa proenzyme form of cathepsin D did not relocalize to the cytoplasm in response to VP-16 (data not shown). Cathepsin D release in U937 cells also was not restricted to the VP-16 stimulus because a similar time course of cathepsin D release was seen following treatment with 1 μ mol/L staurosporine (Fig. 1B).

Chemotherapy-Induced Cathepsin D Relocalization Occurs Independently of Caspase or Cathepsin D Activity

Caspase proteases are known to be critical mediators of chemotherapy-induced apoptosis. Therefore, we sought to determine whether VP-16-induced release of cathepsin D was dependent on the activation of cellular caspases. Pretreatment of U937 cells with 50 μ mol/L of the pancaspase inhibitor z-VAD-FMK prior to addition of VP-16 was sufficient to abrogate drug-induced caspase-3 activation (Fig. 2A). However, z-VAD-FMK did not prevent the release of cathepsin D into the cytosol (Fig. 2A). Thus, a caspase-independent pathway leads to cathepsin D release during VP-16-induced apoptosis.

In related experiments, we examined the impact of pepstatin A on cathepsin D release from lysosomes. Pepstatin A is an inhibitor of aspartic cathepsin proteases, as well as renin and pepsin. Unexpectedly, treatment of cells with 50 μ mol/L pepstatin A, a dose commonly used to inhibit cellular cathepsin D, caused enhanced cathepsin D release in the VP-16-treated cells (Fig. 2B). Although it is unclear why pepstatin A stimulated cathepsin D release, these results showed that VP-16-induced cathepsin D relocalization was not dependent on cathepsin D activity.

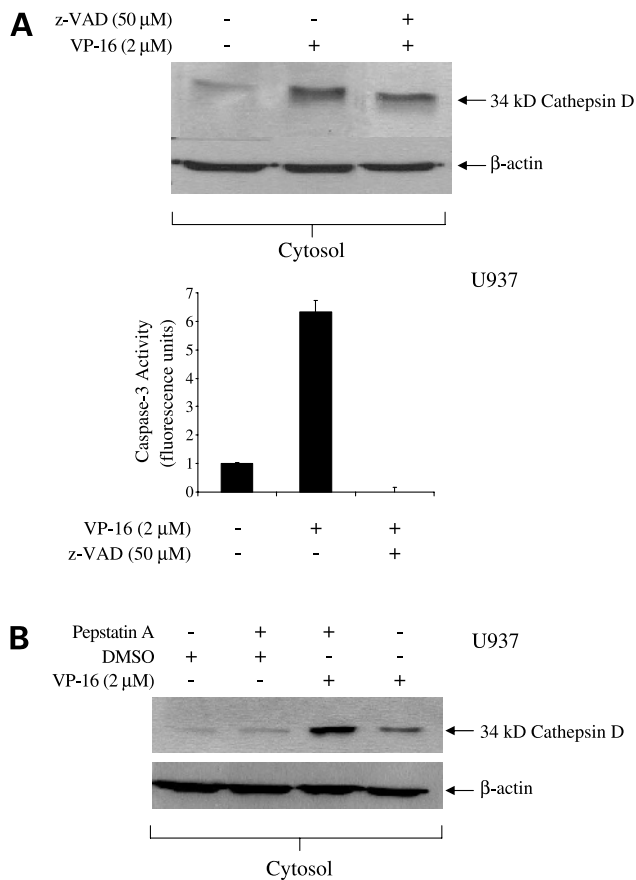


Figure 2. Chemotherapy-induced release of cathepsin D from lysosomes occurs independently of caspase and cathepsin D activities. **A**, U937 cells were pretreated for 1 h at 37°C in the absence or presence of 50 μ mol/L of the pancaspase inhibitor z-VAD-FMK. Cells were then incubated with 2 μ mol/L VP-16 or 0.1% DMSO (-) for 24 h, followed by preparation of cytosolic extracts. Immunoblotting was used to detect cytosolic cathepsin D, as described in Fig. 1. To verify efficient inhibition of caspases by z-VAD-FMK, VP-16-induced release of cathepsin D into the cytosol was not affected. **B**, U937 cells were pretreated for 24 h at 37°C with 50 μ mol/L pepstatin A, or were left untreated. Cells were then treated with DMSO or VP-16 for 24 h, followed by preparation of cytosolic extracts. Immunoblotting was used to detect the presence of cytosolic cathepsin D.

Down-Regulation of Cathepsin D Using siRNA

To determine whether cathepsin D is important for chemotherapy-induced apoptosis, we used siRNA technology (37, 38) to down-regulate expression of cathepsin D protein. Three distinct siRNA constructs were generated, each targeting different 21-nucleotide sequences in the coding region of human cathepsin D mRNA. The targeting sequences were subcloned into the p*Silencer* plasmid downstream from the U6 RNA polymerase III promoter element, and the resulting constructs were transfected into cells. In order to address the role of cathepsin D in both suspension and adherent cells, constructs were transfected into both U937 and HeLa

cells. Following transfection, stable clonal cell lines were isolated and analyzed. Two of the siRNA constructs, targeting codons 73 to 77 and 218 to 222, failed to significantly inhibit cathepsin D expression (data not shown). Similarly, a control siRNA construct encoding a sequence that shares only limited homology with known human sequences, also did not affect cathepsin D expression (Fig. 3B and C). By contrast, siRNA targeting codons 50 to 55, termed 144 siRNA (Fig. 3A), potently down-regulated cathepsin D levels in both cell lines (Fig. 3B and C). Densitometric scanning revealed that cathepsin D was down-regulated by >95% in the HeLa and U937 cells transfected with the 144 cathepsin D siRNA (data not shown). The specificity of cathepsin D down-regulation by the 144 siRNA construct was verified by immunoblotting for caspase-8, caspase-3, and β -actin. None of these control proteins were down-regulated by the 144 siRNA construct in HeLa (Fig. 3B) and U937 cells (Fig. 3C and data not shown). In addition, we examined

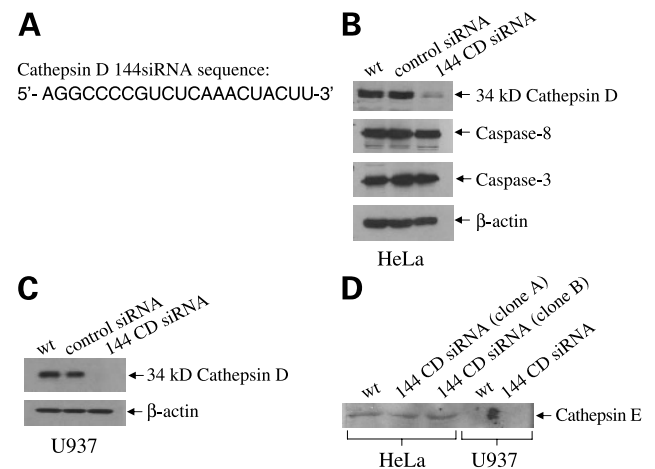


Figure 3. Down-regulation of cathepsin D in HeLa and U937 cells using siRNA. **A**, the 21-nucleotide cathepsin D 144 siRNA sequence targeting codons 50 to 55 in human cathepsin D mRNA. **B**, cathepsin D protein expression is down-regulated in clonal HeLa cells transfected with p*Silencer* plasmid containing the cathepsin D (CD) 144 siRNA sequence (see Materials and Methods). Control lane, clonal HeLa cell line transfected with p*Silencer* plasmid containing a control sequence that bears only limited homology with all known sequences in the human genome. Whole cell extracts from wild-type HeLa cells, control-p*Silencer*/HeLa cells, and 144 siRNA-p*Silencer*/HeLa cells were subjected to immunoblotting with anti-cathepsin D, anti-caspase-8, anti-caspase-3, or anti- β -actin. The data show specific down-regulation of cathepsin D protein in HeLa cells harboring the 144 siRNA construct. **C**, cathepsin D expression is also down-regulated in clonal U937 cells transfected with p*Silencer* plasmid containing the cathepsin D (CD) 144 siRNA sequence. Whole cell extracts from wild-type U937 cells, control-p*Silencer*/U937 cells, and 144 siRNA-p*Silencer*/U937 cells were subjected to immunoblotting with anti-cathepsin D or anti- β -actin. Additional immunoblotting experiments showed no down-regulation of caspase-3 or caspase-8 in U937 cells harboring the 144 siRNA construct (data not shown). **D**, whole cell extracts from two clonal HeLa cell lines (clones A and B) and one clonal U937 cell line expressing the 144 siRNA were subjected to immunoblot analysis for the aspartic cathepsin protease, cathepsin E. Cathepsin E was not down-regulated by cathepsin D 144 siRNA in HeLa cells. Expression of cathepsin E was not detected in parental, wild-type U937 cells.

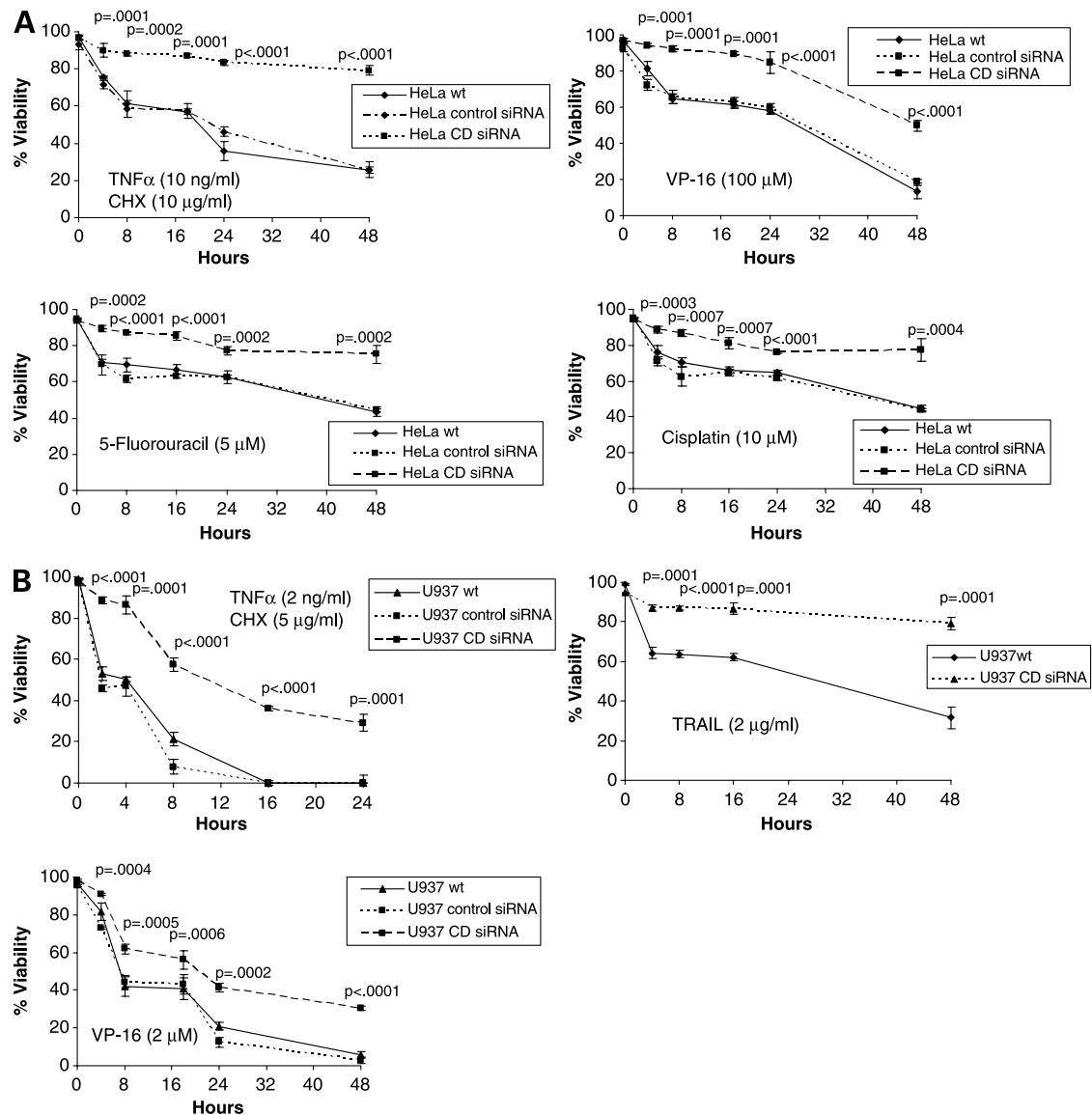


Figure 4. Chemotherapy- and death ligand-induced cell death is inhibited by siRNA-mediated down-regulation of cathepsin D. **A**, HeLa cells were exposed for varying lengths of time to 10 ng/mL TNF α + 10 μ g/mL cycloheximide, 100 μ mol/L VP-16, 5 μ mol/L 5-fluorouracil, or 10 μ mol/L cisplatin. At the indicated time points, aliquots of cells were removed and assayed for viability by trypan blue exclusion. For each data point, quadruplicate counts were performed using a minimum of 100 cells per count. Bars, SD. Control, untreated cultures always maintained >97% viability up to 48 h. Each viability experiment was performed a minimum of four times, with similar results obtained each time. The statistical significance of differences seen at each time point was determined using the VassarStats web site (<http://faculty.vassar.edu/lowry/VassarStats.html>) and Microsoft Excel data analysis tools by comparing HeLa cells expressing cathepsin D 144 siRNA versus HeLa cells expressing control siRNA. As depicted, statistically significant differences (P values ranging from 0.001 to <0.0001) were observed at all time points. **B**, U937 cells were treated with 2 ng/mL TNF α + 5 μ g/mL cycloheximide, 2 μ g/mL TRAIL, or 2 μ mol/L VP-16 for varying lengths of time and cell viabilities determined as in (**A**). Statistical significance was determined as described in (**A**) by comparing cells expressing cathepsin D siRNA with cells expressing the control siRNA, with the exception of TRAIL treatment, where cells expressing cathepsin D siRNA were compared with wild-type cells. Statistically significant differences were detected at each time point, with P values ranging from 0.0006 to <0.0001. Each viability experiment was done a minimum of thrice (TNF α and VP-16) or twice (TRAIL), with similar results obtained each time.

expression of cathepsin E, the other known aspartic cathepsin. The levels of this enzyme were unaffected in two different HeLa cell clones (144A and 144B) expressing the 144 siRNA construct (Fig. 3D). Expression of cathepsin E was not seen in either wild-type or transfected U937 cells (Fig. 3D).

Down-Regulation of Cathepsin D Provides Protection against Chemotherapy Drugs

In preliminary experiments, we examined the impact of siRNA-mediated down-regulation of cathepsin D on apoptosis caused by the death ligands TNF α and TRAIL. Previous studies using pepstatin A or antisense directed

against cathepsin D mRNA have indicated a role for cathepsin D in apoptosis mediated by TNF α and Fas (32), whereas the role of this enzyme in TRAIL-induced apoptosis has not been reported. As shown in Fig. 4A, down-regulation of cathepsin D in HeLa cells led to significantly delayed TNF α -induced cell death, relative to that observed in wild-type HeLa cells or HeLa cells transfected with control siRNA. Similar results were seen following cathepsin D down-regulation in U937 cells (Fig. 4B). In both HeLa and U937 cells, cells transfected with control siRNA were found to behave nearly identically to wild-type cells in these and subsequent analyses (Figs. 4 and 5). Although cathepsin D down-regulation provided only partial protection against TNF α , our results are similar to the partial protection others have observed using pepstatin A or antisense (32). Moreover, we found that cathepsin D down-regulation also provided substantial protection against TRAIL-induced cell death (Fig. 4B). Thus, involvement of cathepsin D may be a common feature in death receptor-mediated apoptosis pathways.

To determine whether cathepsin D is important in chemotherapy-induced cell death, HeLa and U937 cells expressing the 144 cathepsin D siRNA were subjected to treatment with VP-16, 5-fluorouracil, or cisplatin (Fig. 4A and B). Down-regulation of cathepsin D afforded statistically significant protection against VP-16 throughout the 48-hour time course of cell death in both HeLa and U937 cells. Again, the observed protection was only partial, indicating that whereas cathepsin D is important for efficient cell killing, it is not absolutely required for death to occur. Parental U937 cells were remarkably resistant to cisplatin and 5-fluorouracil, precluding study of these drugs in this cell line. However, in HeLa cells, cathepsin D down-regulation was found to inhibit both cisplatin- and 5-fluorouracil-induced cell death (Fig. 4A). Taken together, these experiments show an important role for cathepsin D in chemotherapy-induced cell death.

Down-Regulation of Cathepsin D Inhibits VP-16-Induced Caspase Activation

Experimental inhibition of caspase proteases is known to significantly inhibit chemotherapy-induced cell death. Experiments shown in Fig. 2A, however, indicated that cathepsin D release into the cytosol in chemotherapy-treated cells was not dependent on caspase activities. To determine whether cathepsin D is important for caspase activation during apoptosis caused by chemotherapy drugs, we examined caspase-3 activation in VP-16-treated cells expressing the cathepsin D siRNA (Fig. 5). Down-regulation of cathepsin D by the 144 cathepsin D siRNA was found to significantly attenuate VP-16-induced activation of caspase-3 in both U937 and HeLa cells. After 16 hours of VP-16 treatment, down-regulation of cathepsin D caused a 31% reduction in caspase-3 activity in U937 cells and a 55% reduction in activity in HeLa cells (Fig. 5). As in Fig. 4, wild-type cells and cells expressing control siRNA behaved nearly identically (data not shown). The importance of caspase-3 for VP-16-induced apoptosis

was shown in Fig. 5C, wherein the caspase-3 inhibitor DEVD-FMK was shown to markedly inhibit VP-16-induced cell death. Taken together, our findings indicate that cathepsin D acts upstream of the caspase cascade to promote caspase activation during chemotherapy-induced apoptosis.

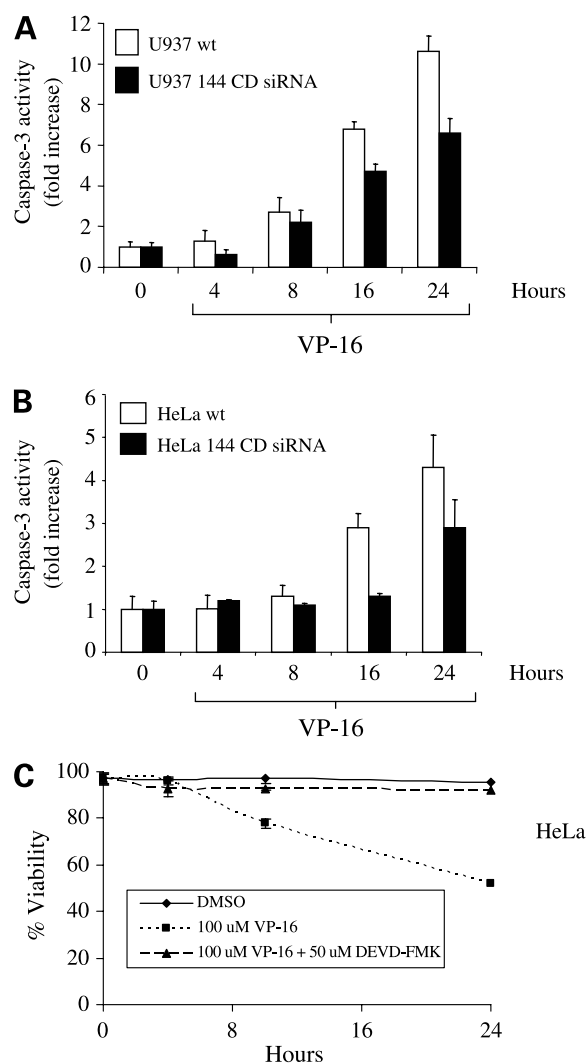


Figure 5. Down-regulation of cathepsin D inhibits VP-16-induced caspase-3 activation. **A**, wild-type U937 cells and U937 cells expressing cathepsin D 144 siRNA were seeded at a density of 1×10^6 cells/mL, then treated with 2 μ M VP-16 at 37°C. At varying time points, whole cell extracts were prepared and caspase-3 activities determined as described in Materials and Methods. Each assay was done in triplicate, and plotted values indicate the fold increase in activity relative to untreated ($t = 0$) samples. Bars, SD. The experiments were done thrice, with similar results obtained each time. **B**, wild-type HeLa cells and HeLa cells expressing cathepsin D 144 siRNA were seeded at a density of 5×10^5 cells per plate in 60-mm plates, treated with 100 μ M VP-16, and analyzed as described in (A). **C**, HeLa cells were plated at a density of 1×10^5 cells/mL. Forty-eight hours after plating, cells were treated with 50 μ M DEVD-FMK for 1 h prior to treatment with 100 μ M VP-16 or DMSO. Cell viabilities were assessed by trypan blue exclusion assay. For each data point, triplicate counts were done using a minimum of 100 cells per count. Bars, SD.

Cathepsin D Promotes Cytochrome *c* Release by Acting on a Cytosolic Protein(s)

The activation of caspase-3 during chemotherapy-induced apoptosis occurs downstream of cytochrome *c* release from the mitochondria (4, 10). To determine whether cathepsin D plays a role in chemotherapy-induced cytochrome *c* release, we compared the time courses of cytochrome *c* release for VP-16-treated cultures of U937 cells expressing cathepsin D siRNA versus U937 cells expressing control siRNA (Fig. 6A). As shown, down-regulation of cathepsin D resulted in substantially reduced cytochrome *c* release following 16 or 24 hour treatment with 2 $\mu\text{mol/L}$ VP-16. This suggested that cathepsin D plays a role in enhancing cytochrome *c* release during VP-16-induced apoptosis.

We next sought to determine whether cathepsin D acts directly on mitochondria to promote cytochrome *c* release, or, instead, acts on cytosolic components which then mediate the effects of cathepsin D on cytochrome *c* release.

For these experiments, mitochondria-enriched extracts (also containing some endosomes/lysosomes and nuclei) were isolated from wild-type U937 cells, then incubated with either cathepsin D or cytosolic extract from U937 cells. Cytochrome *c* release was then assessed by immunoblotting (Fig. 6B). Incubation of the isolated mitochondria with cytosol alone (lane 2) resulted in no induction of cytochrome *c* release. Incubation with cathepsin D (lane 3) resulted in a modest (roughly 4-fold) induction of cytochrome *c* release. Remarkably, preincubation of cytosolic extract for 1 minute with cathepsin D prior to addition to the isolated mitochondria resulted in a 28-fold increase in cytochrome *c* release (lane 4), an effect that was completely inhibited by pepstatin A (lane 5). This finding indicates that cathepsin D contributes to cytochrome *c* release by first cleaving a cytosolic protein (or proteins). The cleaved protein, or the pathway initiated by the cleaved protein, then acts upon the mitochondria to cause cytochrome *c* release.

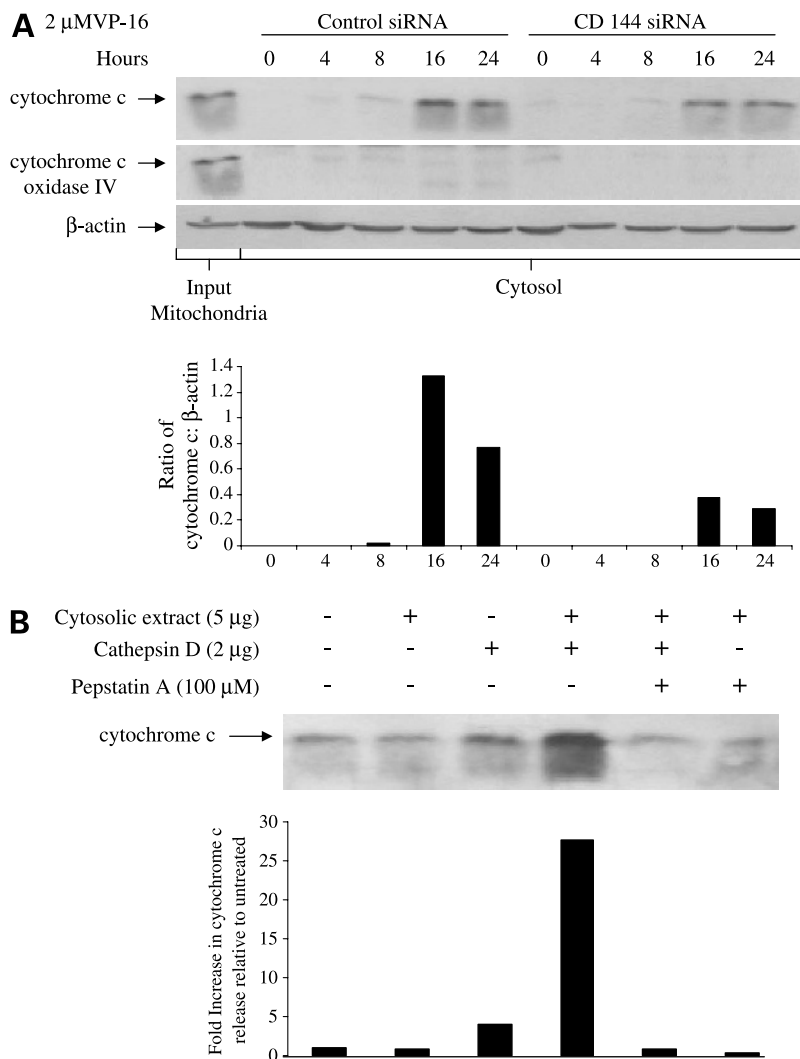


Figure 6. Cathepsin D promotes cytochrome *c* release. **A**, U937 cells were treated with 2 $\mu\text{mol/L}$ VP-16 and cytosolic extracts were prepared at the indicated time points, then analyzed by immunoblot with anti-cytochrome *c* antibody to assess release of cytochrome *c* into the cytosol. Densitometry was used to calculate the ratio of cytochrome *c* release to β -actin signal in each lane. The blot was stripped and re probed for the mitochondrial protein cytochrome *c* oxidase IV to show the integrity of the mitochondria and the specificity of the cytochrome *c* release. Probing with anti- β -actin was used to verify equal loading. **B**, isolated U937 mitochondria (50 μg) were prepared as described in Materials and Methods, then incubated for 1 h at 37°C with U937 cell cytosolic extract (5 μg) alone (lane 2), cathepsin D (2 μg) alone (lane 3), or cytosolic extract preincubated for 1 min with cathepsin D (lane 4). Following incubation, supernatants were subjected to two rounds of centrifugation (30 min at 14,000 rpm), and immunoblotting of the clarified supernatants was used to assess cytochrome *c* release. Densitometry was used to quantify the fold increase in cytochrome *c* release relative to that observed with untreated mitochondria. Pepstatin A (100 $\mu\text{mol/L}$) was used to show the importance of the cathepsin D enzymatic activity.

Discussion

The role of caspase proteases during apoptosis stimulated by chemotherapy drugs is well established. By contrast, little is known about the potential involvement of cathepsin proteases in chemotherapy-induced apoptosis. Our findings show that cathepsin D, the major lysosomal aspartic protease, is important for efficient induction of cell death by chemotherapy drugs with different mechanisms of action. Chemotherapy-induced cell death was associated with release of cathepsin D from lysosomes, and this release was not dependent on caspase or cathepsin D activities. Although cathepsins D and B have been shown to be relocalized from lysosomes to cytoplasm in response to a variety of apoptotic stimuli, the mechanism(s) responsible for this release remains poorly understood (14, 15). A number of possible mechanisms have been proposed, including increased cytosolic levels of reactive oxygen species, sphingosine, or Ca^{2+} (15). The involvement of caspase proteases in this relocalization process seems unlikely, because we found that caspase inhibition had no effect on cathepsin D release.

Efforts to examine the role of cathepsin D in various apoptotic processes have relied heavily on the pharmacologic inhibitor pepstatin A. However, potential nonspecific inhibition of other proteases complicates interpretation of studies using this inhibitor. In a similar fashion, pharmacologic inhibitors of caspase proteases have been shown to nonspecifically inhibit cathepsins (39, 40), raising the possibility that the role of cathepsins may have been overlooked in defining many "caspase-dependent" processes. To evaluate the role of cathepsin D in chemotherapy-induced apoptosis, we employed siRNA technology to achieve highly specific down-regulation of the cathepsin D protein. Down-regulation of the enzyme was found to inhibit apoptosis caused by the death ligands TNF α and TRAIL, and the chemotherapy drugs VP-16, 5-fluorouracil, and cisplatin. These findings confirm the role of cathepsin D in death receptor-mediated apoptosis, and establish an important role in chemotherapy-induced apoptosis. Similarly, Bidere et al. (28) have used siRNA targeting a different region of cathepsin D mRNA and shown that cathepsin D is involved in the early stages of apoptosis induction resulting from inhibition of protein kinase C.

A major question that remains unresolved concerns the mechanism of apoptosis execution that is activated by the cathepsin proteases. Our experiments using cathepsin D siRNA indicate that cathepsin D plays a significant role in promoting caspase activation in chemotherapy-treated cells. It is possible that cathepsins directly cleave and activate caspase proteases. Vancompernelle et al. (41) have reported cleavage of procaspase-1 and procaspase-11 by cathepsin B in cell-free reactions. However, caspases-1 and -11 are not typically involved in apoptotic cell death processes, and it is unclear whether cathepsin B ever cleaves these caspases in intact cells. Moreover, Stoka et al. (42) have reported that purified cathepsins B, H, K, L, S, and X all fail to directly cleave the executioner caspases,

procaspase-3 and procaspase-7. It is more likely that cathepsins promote caspase activation by stimulating mitochondrial events which are known to precede caspase activation during chemotherapy-induced cell death. Our studies show that down-regulation of cathepsin D results in impairment of cytochrome *c* release in chemotherapy-treated cells, implicating a role for cathepsin D in promoting cytochrome *c* release. Others have shown that treatment of cells with lysosomal damaging agents, such as quinolone antibiotics or lysosomotropic detergents, causes the release of cytochrome *c* (43, 44). Moreover, cathepsin B has been shown to stimulate cytochrome *c* release from mitochondria (45).

The mechanism(s) whereby cathepsins promote cytochrome *c* release warrants further investigation. Some studies have suggested that Bid, a member of the Bcl-2 protein family, may act to mediate the effects of cytosolic cathepsin B on mitochondria (42, 46). During death receptor-mediated apoptosis, Bid is known to be cleaved by caspase-8 to produce a proapoptotic fragment that migrates to the mitochondria and promotes cytochrome *c* release (47, 48). Interestingly, recent studies have shown that incubation of full-length Bid with lysosomal extracts or purified cathepsins B, H, L, S, or K results in Bid cleavage following Arg⁶⁵ or Arg⁷¹, near the known caspase-8 cleavage/activation site (Asp⁵⁹; ref. 46). Moreover, Bid fragments produced by treatment with these cathepsins or with lysosomal extracts promote cytochrome *c* release from isolated mitochondria (42, 46). Although Bid remains a possible mediator of the effects of certain cathepsins, other cathepsins, including cathepsin D, do not exhibit any capacity to directly cleave the Bid protein (46). Our demonstration that cathepsin D-treated cytosolic extracts potently induce cytochrome *c* release from isolated mitochondria argues that enzymatic cleavage of an unknown cytosolic mediator is an important first step in the cathepsin D-to-mitochondria signaling pathway. The elucidation of this signaling pathway will broaden our understanding of chemotherapy-induced apoptosis, and may provide possible sites for therapeutic intervention in chemotherapy resistant cancers.

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