MEDIATION OF CHEMOTHERAPY-INDUCED APOPTOSIS BY THE LYSOSOMAL PROTEASE CATHEPSIN D

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One of the most common hallmarks of cancer is dysregulation of cellular apoptotic processes. A comprehensive knowledge of the underlying mechanisms of the apoptotic machinery is vital for the identification of new drug targets and the development of innovative agents that stimulate the cell death process in cancer cells. Studies have shown that the lysosomal protease cathepsin D is important in the extrinsic apoptotic pathway stimulated by the death receptor ligands for TNFR1 and FAS, as well as by oxidative stress and the protein kinase C inhibitor staurosporine. To date, the role of cathepsin D in the chemotherapy-induced apoptotic pathway has not been characterized. This project examined the role of the lysosomal protease cathepsin D in chemotherapy-induced apoptosis of HeLa and U937 cells. The data demonstrated that following stimulation of U937 cells with the chemotherapy drug VP-16, cathepsin D was released into the cytosol approximately 4 hours after drug treatment. This release was selective for cathepsin D, as cathepsin B and the lysosomal markers LAMP and β-hexosaminidase were not released into the cytosol following VP-16 treatment. Inhibitors of caspases and

cathepsin D had no effect on cathepsin D release, demonstrating that cathepsin D release occurred independently of caspase and cathepsin D activities. Downregulation of cathepsin D expression in U937 and Hela cells using siRNA was found to inhibit cell death resulting from a variety of stimuli, including death receptor ligands, oxidative stress, PKC inhibitors, and importantly, chemotherapy drugs. In addition, U937 and HeLa cells expressing cathepsin D siRNA exhibited delayed cytochrome c release and caspase-3 activation following VP-16 treatment. Moreover, isolated mitochondria from wild-type U937 cells released cytochrome c in response to cytosolic extracts that were treated with cathepsin D, suggesting that cathepsin D acts on a cytosolic factor to induce cytochrome c release. Inhibition of caspases had no impact on cytochrome c release provoked by cathepsin D-cleaved cytosolic extract, demonstrating that caspases are not mediators of cathepsin D-induced cytochrome c release. Taken together, these results demonstrate that cathepsin D is an important component of the apoptotic pathway and that it acts via an intermediary cytosolic factor to promote cytochrome c release and caspase activation during chemotherapy-induced apoptosis.

FOREWORD

I would like to thank the people who have made my graduate student career and this dissertation possible:

To my mentor, Dr. Daniel E. Johnson, to whom I completely owe my development thus far as a scientist. I have so much gratitude for all of his guidance and support through these past 4 years. As I continue to develop as a scientist, I truly hope that I remember and practice the many lessons he has taught me about how a good scientist writes, thinks, and speaks. Thank you for never turning a deaf ear to my concerns for my project or for my future and for always keeping my eye on the goal line.

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-Fortune cookie acquired as I was
writing my thesis dissertation

Research is what I'm doing when I don't know what I'm doing.
-*Wernher von Braun*

A man should look for what is, and not for what he thinks should be.
-*Albert Einstein*

PREFACE

This project has been modified from its original manuscript to provide greater detail and a consistent format through the dissertation:

Emert-Sedlak, L, Shangary, S, Rabinovitz, A, Miranda, M, Delach, S and Johnson DE. Involvement of cathepsin D in chemotherapy-induced cytochrome c release, caspase activation, and cell death. Mol Cancer Ther 2005. *In Press*.

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1. INTRODUCTION

1.1. CANCER

It is estimated that in the year 2005, over 1.3 million new cases of cancer will be diagnosed in the United States.[1] Cancer is the cause of death for 1 in 4 Americans, and is second only to heart disease in leading causes of death in the U.S.[2] The economic impact of cancer is staggering; the National Institutes of Health estimate that overall costs for medical and morbidity-related cancer concerns were almost $190 billion in 2004.[3] Cancer is not a disease in and of itself; instead, it is, as defined by the National Cancer Institute, "a group of diseases in which abnormal cells divide without control.[4]" Proliferation of abnormal cells is dangerous because not only do they reproduce in defiance of their normal restraints, but they invade and utilize resources needed for growth of normal cells [1]. Metastatic cells develop by gradually acquiring tumor-specific genotypic mutations, leading to dysfunction of important cellular processes. While the patterns of genetic mutation may differ between the more than 100 distinct human cancer types, it is acknowledged that most cancer cells possess six general alterations in cellular physiology that allow them to surmount cellular anti-cancer defense mechanisms: "self-sufficiency in growth signals, insensitivity to growth inhibitory (antigrowth) signals, evasion of programmed cell death (apoptosis), limitless replicative

[1] [2] [3] American Cancer Society, Cancer Statistics and Figures 2005 (www.cancer.org)

[4] National Cancer Institute, US National Institutes of Health (www.cancer.gov)

potential, sustained angiogenesis, and tissue invasion and metastasis."[2] These six common traits serve as targets for development of novel anti-cancer drugs. With this in mind, it is necessary to understand the mechanisms by which cancer cells can deregulate themselves to promote their own rapid growth and immortality.

A major concern regarding metastatic cells is that they often have surmounted the cellular signals that target them for cell death. Chemotherapeutic agents are designed to elicit cell death in tumor cells. The work in this project examines the role of a novel proteolytic enzyme in chemotherapy-induced apoptosis.

1.2. APOPTOSIS

One of the major cellular safeguards against aberrant growth of abnormal cells is programmed cell death, commonly known as apoptosis. In order to maintain homeostasis of cell numbers in normal tissues, the ratio of cell proliferation to cell death must remain constant over time. In cancers, genetic aberrations disturb that balance by promoting production of oncogenes that stimulate uncontrolled cell proliferation, inhibit cell death, or both. It is critical that the biochemical pathways that render cancer cells resistant to apoptosis are understood so that treatments that target these pathways can be developed.

1.2.1. DEFINITION AND CHARACTERISTICS OF APOPTOSIS

Kerr *et al.* [3] coined the Greek term 'apoptosis' for programmed cell death, which translates as "falling leaves". Much as leaves falling from a tree die a characteristic death and are reabsorbed into the earth because they also are no longer necessary, apoptotic cells die via unique processes, and are reabsorbed because they are no longer

necessary and must make room for new cells. Apoptosis is scientifically defined as the cellular morphological process leading to deliberate controlled self-destruction [4].

Apoptosis plays a critical role in regulation and maintenance of cell homeostasis as well as a vital function in development. In the human body, mitosis produces about 100,000 cells every second, and to maintain homeostasis, an equal number of cells are eliminated [5]. During organ and limb development, apoptosis mediates digit sculpting by removing interdigital mesenchymal tissue between fingers and toes. Massive apoptosis also contributes to the deletion of unnecessary organelles and the construction of hollow structures during development [6]. Additionally, apoptosis is fundamental in the removal of cells that have sustained radiation- or drug-incurred DNA damage. Failure of these damaged cells to self-destruct can result in accumulation and proliferation of cells with genetic mutations. Therefore, metastatic cells that exhibit resistance to apoptosis are extremely dangerous.

During apoptosis, several morphological changes occur that distinguish the apoptotic process from other cell death processes, such as necrosis. First, the cell shrinks, becomes detached from neighboring cells, and loses its rigid shape. During this process, the plasma membrane remains intact [7]. Also, chromatin condenses to very dense structures and migrates to the plasma membrane while activated nuclear DNase enzymes cleave DNA into a characteristic “apoptotic ladder” [8, 9]. Phosphatidylserine, which is normally localized to the interior of the plasma membrane, becomes exposed on the outside of the membrane, and the plasma membrane begins to “bleb”, or release small membrane-bound vesicles [10, 11]. These vesicles, which contain cytosol, engulfed vesicles, and chromatin, are termed apoptotic bodies; they are rapidly engulfed by

macrophages and eliminated without initiation of an inflammatory response [8]. Figure 1.1 summarizes the morphological changes associated with apoptosis that differentiate the process from other forms of cell death.

1.2.2. APOPTOTIC PATHWAYS

In addition to the morphological changes that occur during the apoptotic process, a complex intracellular signaling network is initiated by a variety of apoptotic stimuli from inside or outside the cell. These stimuli include, but are not limited to, developmental death signals, stimulation of cell surface death receptors, deprivation of survival signals, or DNA damage incurred from chemotherapy drugs or radiation. There are two distinct apoptotic pathways that ultimately end in cell death, and though cross-talk between the pathways does occur, apoptotic stimuli typically initiate either one or the other. The extrinsic pathway, or death receptor-mediated apoptotic pathway, is evoked upon stimulation of death receptors in the tumor necrosis factor receptor family. The intrinsic pathway, or mitochondrial apoptotic pathway, is evoked by stimuli that cause DNA damage or internal damage to the cell. This section will discuss the differences between the intrinsic and extrinsic apoptotic pathways.

1.2.2.1. DEATH RECEPTOR-MEDIATED APOPTOSIS

The extrinsic death receptor-mediated apoptosis pathway is stimulated by ligand binding to members of the tumor necrosis factor receptor superfamily including TNFR-1, Fas/CD95, and TNF-related apoptosis-inducing ligand (TRAIL) receptors DR4 and DR5 [12, 13]. As a safeguard against inadvertent receptor activation, trimerization of death

Type of Cell Death	Morphological Features
Apoptosis	Cell shrinking Plasma membrane blebbing Fragmentation of DNA to form "Ladder" Externalization of phosphatidylserine Chromatin condensation Formation of apoptotic bodies No induction of inflammatory response
Necrosis	Cell swelling Precipitation of cytoplasmic contents Induction of inflammatory response

Figure 1.1. Morphological characteristics of cell death

receptors is required for activation. Binding of extracellular trimeric ligands to TNF receptors stimulates receptor activation by bringing three death receptors in close proximity, whereupon they form an active, trimerized death signaling complex [8, 11]. In their cytoplasmic tails, death receptors contain a conserved Death Domain (DD) that, when trimerized, leads to recruitment of death adaptor proteins such as TRADD (in the case of TRAIL) and FADD (in the case of Fas ligand) [13, 14]. Although death domains are absolutely essential for the transmission of apoptotic signals via the death receptor pathway, little is known about their function except that they have an extremely high propensity to form hetero- and homo-oligomers. The adaptor proteins themselves contain death domains that have a high affinity for active membrane-bound death domains. The conglomeration of trimerized ligand, receptor, and adaptor proteins is known as the death-inducing signaling complex (DISC) [15]. In addition to their N-terminal death domains, death adaptor proteins also contain C-terminal Death Effector Domains (DEDs). The N-terminal region of the zymogen form of caspase-8 protease contains a DED that binds with high affinity to the DISC [16]. Recruitment of procaspase-8 molecules to the DISC brings them in close proximity to each other, where they have the capacity to autoactivate via a process termed “proximity-induced autoactivation”. The activated caspase-8 then cleaves and activates the executioner caspase, caspase-3. Activation of executioner caspases leads to downstream proteolysis of cellular substrate proteins that will be discussed momentarily [11]. In Figure 1.2, a model of death receptor-mediated apoptosis is outlined.

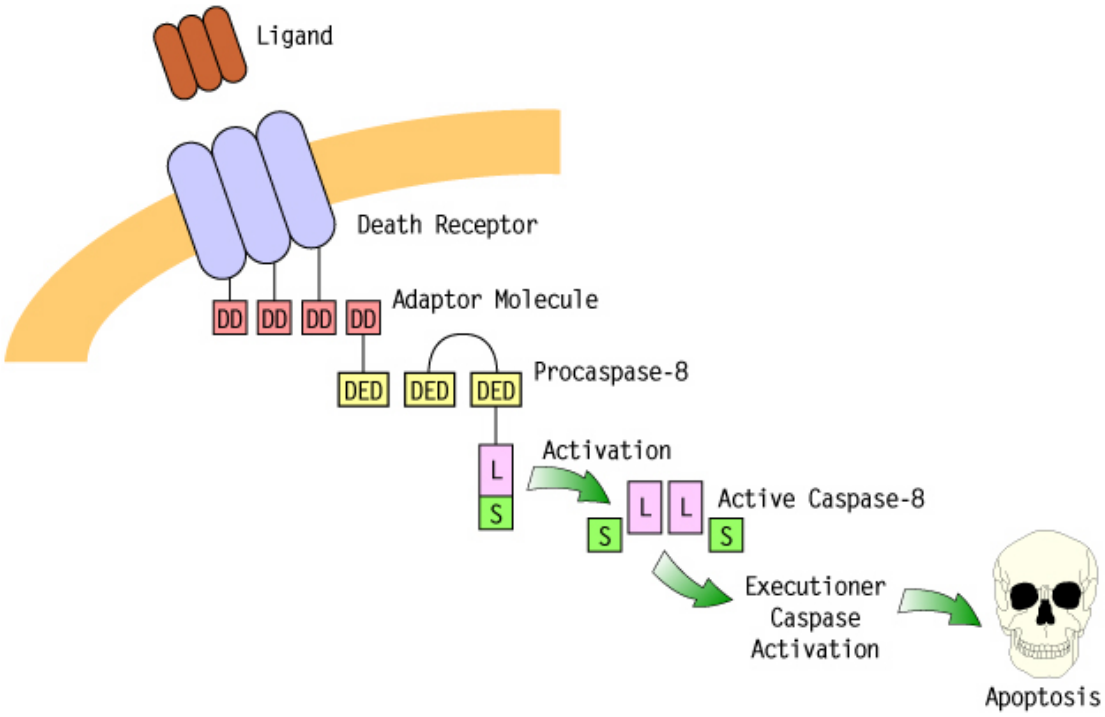


Figure 1.2. The extrinsic death receptor-mediated apoptotic pathway. The death receptor-mediated pathway is initiated by death ligands such as TNF-α, TRAIL, or Fas/APO-1. Trimerization of the receptors is required for formation of the death-inducing signaling complex (DISC). Trimeric death domains (DDs) recruit adaptor molecules with death effector domains (DEDs) that subsequently recruit procaspase-8. See text for details.

1.2.2.2. MITOCHONDRIA-MEDIATED APOPTOSIS

The intrinsic mitochondrial-mediated apoptosis pathway is initiated by signals that originate within the cell, such as from DNA damage induced by chemotherapeutic agents or radiation. These agents induce mitochondrial dysfunction by provoking a reduction in mitochondrial membrane potential and release of mitochondrial apoptogenic proteins into the cytosol [8, 17]. One such apoptogenic factor is cytochrome c. Cytochrome c functions in normal cells as an electron carrier in the mitochondrial respiratory electron-transport chain [1, 18]. During apoptosis, cytochrome c is released from mitochondria into the cytosol. As mentioned previously, multiple safeguards prevent accidental initiation of apoptosis; therefore, cytochrome c release is tightly regulated. Regulation is controlled in part by the Bcl-2 family of apoptosis regulatory proteins, which contains both pro- and anti-apoptotic members. Following an apoptotic stimulus, some pro-apoptotic factors are activated via oligomerization (e.g. Bax and Bak) or via cleavage (e.g. Bid). Anti-apoptotic factors (e.g. Bcl-2 and Bcl-X_L) are inactivated via cleavage or via binding of regulatory proteins that suppresses their inhibitory function [19]. Bcl-2 and Bcl-X_L reside in the outer mitochondrial membrane and inhibit cytochrome c release. Figure 1.3 contains a current list of known Bcl-2 family members. In addition to regulation by the Bcl-2 family, Boehning *et al.* [20] and Mattson *et al.* [21] have also shown that release of cytochrome c from the mitochondria requires calcium efflux from the endoplasmic reticulum.

Through hydrolysis of dATP, cytosolic cytochrome c binds to and provokes oligomerization of the monomeric adaptor molecule Apaf-1 [22]. The cytochrome c-

Bcl-2 family members		
Anti-Survival	Bad	Bak
	Bax	Bcl-G
	Bfk	Bid
	Bik	Bim
	Blk	Bmf
	BNip/3	Bok
	BPR	Egl-1
	HRK/DP5	Mil-1
	Nip 1/3	Noxa
	Puma	
Pro-Survival	A1	Bcl-2
	Bcl-W	Bcl-XL
	Boo/Diva	Mcl-1
	Bcl-XS	

Figure 1.3. The Bcl-2 protein family

Apaf-1 complex then binds the zymogen form of caspase-9, forming a complex known as the apoptosome. Formation of the apoptosome evokes catalytic activation of caspase-9, a process necessary for chemotherapy-induced apoptosis. Once activated, caspase-9 cleaves and activates executioner caspases, including caspase-3 and -7. Activation of the executioner caspases culminates in cell death [22]. Figure 1.4 depicts a representation of the intrinsic apoptosis pathway.

1.3. PROTEASES IN APOPTOSIS

Signaling cascades that occur during both the intrinsic and extrinsic apoptosis pathways ultimately culminate in the activation of proteolytic enzymes that are responsible for the degradation of proteins and concomitant destruction of the cell. These proteases, known as caspases, are the most well-characterized apoptotic enzymes, as they are vital for apoptotic execution following most stimuli. This section addresses the role of caspases in the apoptotic pathway as they are currently understood.

1.3.1. CASPASES

Caspases are cytosolic cysteine proteases; specifically, their catalytic activity is dependent on a cysteine residue in a highly conserved QACXG sequence (with X being R,Q, or G) [23, 24]. Caspase-mediated cleavage is also highly specific, always following an aspartate residue found within a common motif (e.g. DEVD or IETD). The proenzyme forms of all caspases contain an N-terminal prodomain, followed by a large and a small subunit. During activation, the prodomain is cleaved and degraded, while the

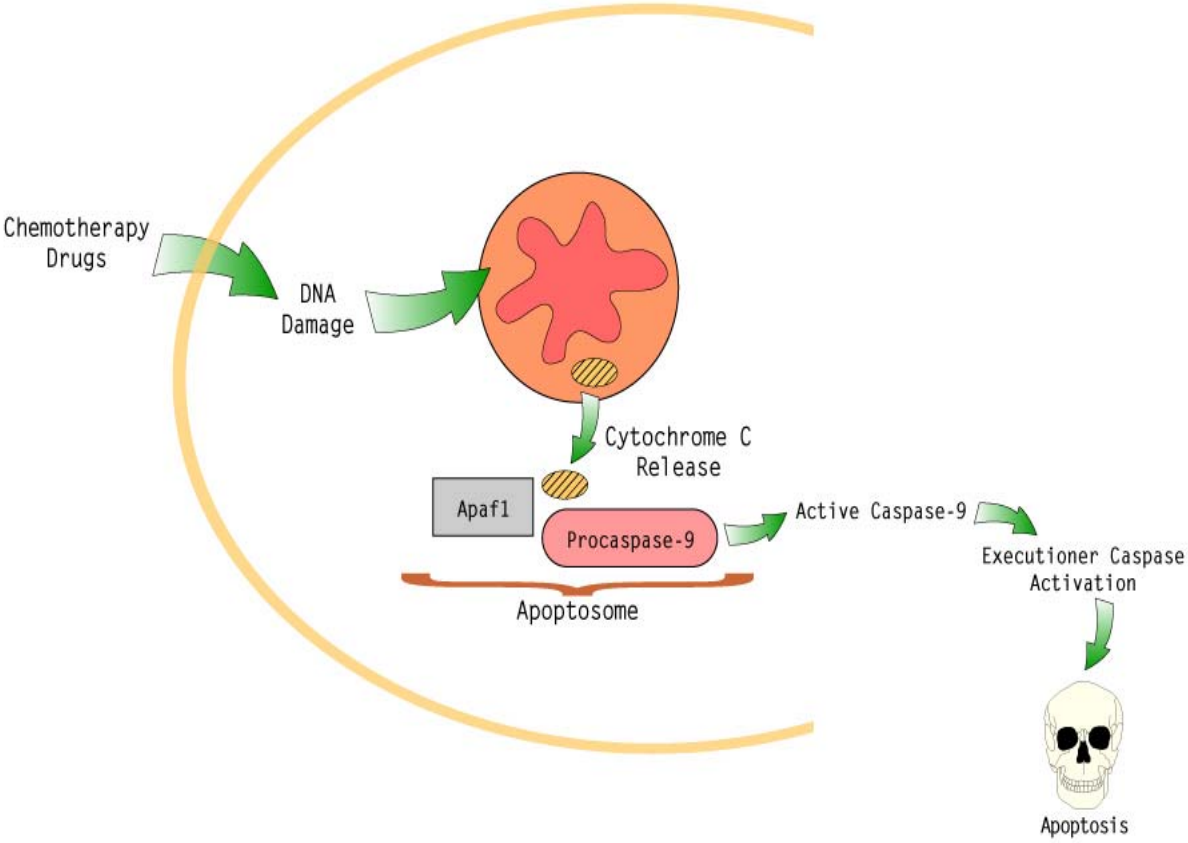


Figure 1.4. The intrinsic mitochondrial apoptosis pathway. The intrinsic mitochondrial pathway can be initiated by DNA damage caused by chemotherapeutic agents, radiation, etc. Mitochondria are stimulated to release cytochrome c, which complexes with Apaf-1 and procaspase-9 to form the 'apoptosome', which initiates activation of caspase-9 and the caspase cascade. See text for full details.

large and small subunits heterodimerize to form a complex containing two large subunits and two small subunits [23].

The caspase family consists of two subtypes: the initiators and the executioners. The initiator caspases are caspases-2 and -9 (for the intrinsic pathway) and caspases-8 and -10 (for the extrinsic pathway). They are activated following binding of ligand to death receptor or following cytochrome c release and apoptosome complex formation [25]. Activated initiator caspases cleave and activate executioner caspases (caspases-3, -6, -7, and -12), so called because they elicit cell death through cleavage of substrates that are critical to the normal function of the cell. Initiator caspases differ structurally from the executioner caspases in that they have a very long prodomain that contains essential sequences for adaptor binding, while the prodomains of executioner caspases are short and act solely to inhibit caspase activation [23, 25].

Caspases are an integral part of the apoptotic cascade because they cleave over 60 substrate proteins that are necessary for normal cellular function. This extensive list of substrates includes proteins necessary for DNA metabolism (caspase-activated DNase), cytoskeletal scaffold proteins (lamins, gelsolin), cell cycle regulators (Cdk inhibitors), repair and housekeeping proteins (poly-ADP-ribose polymerase (PARP)), and signaling molecules (MEKK1, MAP3Ks) [17, 25-27]. Additionally, both pro- and anti-apoptotic members of the Bcl-2 family that are activated early in apoptosis serve as downstream caspase substrates. Caspase-mediated cleavage of the Bcl-2 proteins functions as part of a positive feedback loop to further enhance caspase activity and accelerate the cell death process [23, 25, 26]. Figure 1.5 comprises a partial list of caspase substrates relevant to my study.

Caspase Substrate	Function of Substrate in Normal Cells
PARP	DNA repair enzyme
DNA-PK_{cs}	DNA double-strand break repair
Protein kinase C	Cleaved to active form in apoptosis
Lamins	Nuclear envelope proteins
Fodrin	Membrane-assoc. cytoskeletal protein
Rb	Cell cycle regulator
D4-GDP dissociation inhibitor	Rho GTPase inhibitor
IL-1β	Proinflammatory cytokine
Gelsolin	Activated during apoptosis to degrade actin
Bcl-2 family members	Aid in apoptotic progression
Other caspases	Aid in apoptotic progression
Inhibitor of caspase-activated DNase	Releases DNase during apoptosis

Figure 1.5. Examples of caspase substrate proteins and their functions.

Because caspases play such a vital role in apoptosis, great efforts have been invested in elucidating their mechanistic role in apoptosis. However, much evidence has arisen to suggest that other protease families also play an active role in apoptosis. The cathepsin proteases are one such family. Cathepsins were overlooked for many years because their residence in the lysosomal compartment appeared to isolate them from apoptotic processes in the cytosol and mitochondria. It is now known, however, that cathepsins are not alienated from cell death signaling cascades; in fact, some of them play a noteworthy role in cell death induced by various stimuli. Before expounding on the role of lysosomal proteases in apoptosis, however, the role of the lysosomal compartment in normal cells will be discussed.

1.4. FUNCTION OF THE LYSOSOMES IN NORMAL CELLS

Proteins that have been targeted for degradation are sent to the lysosomes, thus making the lysosomal compartment a cellular "garbage disposal". The lysosomal compartment contains about 40 different types of acid hydrolases, comprised of proteases, nucleases, lipases, glycosidases, and sulfatases [1]. Lysosomes are responsible for a variety of degradative processes. They degrade phagocyte-engulfed particles and microorganisms (endocytosis and phagocytosis), cellular organelles that have exceeded their lifespan and become obsolete (autophagy), and targeted proteins that contain a selective signaling motif (often KFERQ) on their surface [1].

The lysosome is defined as "the terminal organelle of the endocytic pathway and one that is devoid of recycling receptors [28, 29]". Substrates are acquired for endocytic degradation via complex signaling networks mediated by PI-3 kinase and small G-protein

cascades as well as by actin [28]. Proteins are first endocytosed into early "sorting" endosomes, where they are either shuttled back to the plasma membrane for recycling (e.g. receptor-ligand complexes) or are transferred into endosome carrier vehicles (ECVs) that transport the protein to the late endosomes. From the late endosomes, proteins are carried to the lysosomes, where a fusion of the endosomal/lysosomal compartments mediates delivery of the protein substrate to the hydrolytic enzymes [30]. Figure 1.6 outlines the stages of the endocytic pathway. At each stage, the pH of the organelles drops considerably, culminating in a pH of about 5 in the lysosomal compartment. Acidification is important for optimal activity of lysosomal membrane transporters. Additionally, most lyosomal hydrolases are activated (often by autoactivation) and exhibit optimal activity at acidic pH [28].

Newly synthesized lysosomal enzymes are sequestered in the lysosomal compartment via unique mannose 6-phosphate (M6P) groups that attach to the termini of *N*-linked oligosaccharides [1]. The lysosomal hydrolases are heavily glycosylated structures that are recognized by the M6P receptor proteins, which bind and direct packaging of lysosomal enzymes into clathrin-coated transport vehicles that deliver them to the late endosomes/lysosomes [31, 32]. In this manner, the acid hydrolases of the lysosomes are sequestered from the rest of the cell.

Because hydrolytic enzymes could trigger massive cellular destruction in the cytosol, lysosomes must be tightly regulated to control the influx and efflux of proteins. All lysosomal enzymes exhibit maximal activity at an acidic pH, which the lysosomal compartment provides by utilizing an ATP-driven H^+ pump to maintain interior

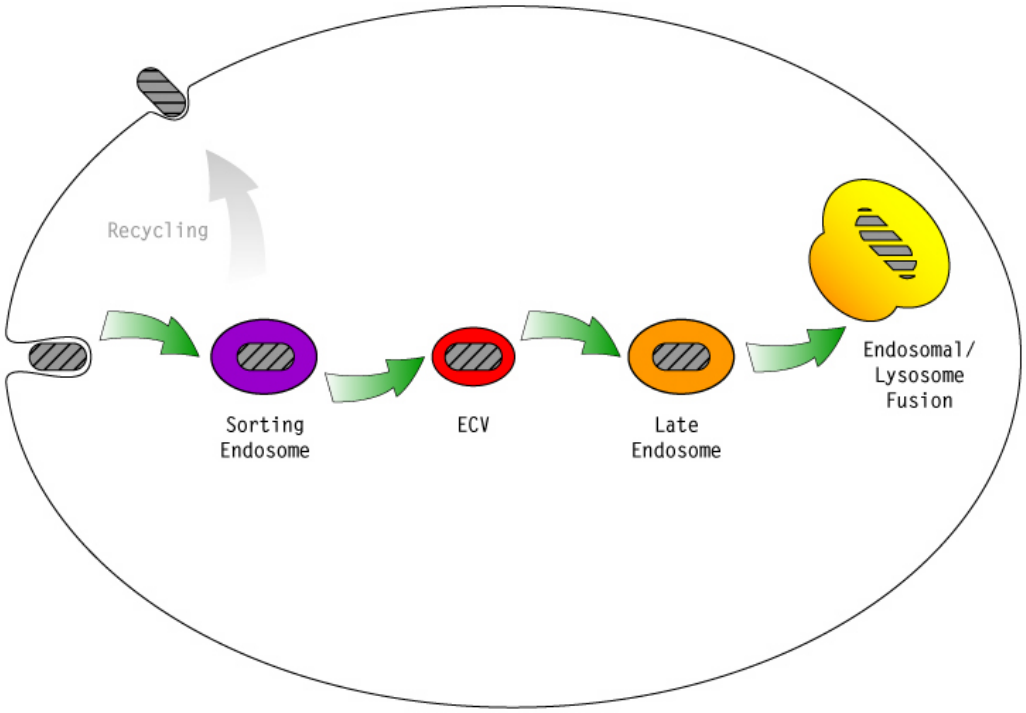


Figure 1.6. Stages of the endocytic pathway. Endocytosed particles are first taken to the 'sorting' endosome, where they may be recycled or passed on to the endosomal carrier vehicle (ECV). The ECV shuttles the cargo to the late endosome, which then fuses with the lysosome for delivery to the hydrolytic enzymes. The color change in compartments (purple-red-orange-yellow) represents a decrease in pH with progression to each vesicle.

acidification [33]. As a precaution against lysosomal enzymes causing fortuitous damage to the cell, the lysosomal membrane is specifically designed to tightly guard against accidental leakage of hydrolytic enzymes. Almost all lysosomal membrane proteins are extensively glycosylated, which not only protects them against the digestive properties of the acidic interior, but also serves to mediate transport across the membrane [34, 35]. In order to pass across these heavily glyscosylated membranes, proteins must possess distinct recognition signals that target them for internalization. The membrane acts in the same way to prevent release of hydrolases from the compartment. As an additional safeguard against accidental cellular digestion, many of the lysosomal enzymes are completely or nearly inactivated at neutral pH; therefore, if they are inadvertently released into the cytosol, most can do little damage to the cell [1]. An exception to this rule is the cysteine protease family, of which several members are highly active at neutral pH, posing a potential hazard to the cell if relocalized. As a prime example of how dangerous lysosomal protease activation outside of the compartment can be, secreted cysteine cathepsins have been implicated in cancer progression by degrading the extracellular matrix around malignant cells, allowing tumor cell dissemination from the primary tumor [36]. Therefore, regulation of the localization of lysosomal proteases is critical for normal cellular function.

As mentioned previously, there are several types of hydrolytic enzymes present in the lysosomes. Proteins that have been targeted to the lysosomes for degradation are digested by peptidases, also known as proteinases or proteases [37, 38]. Though lysosomal proteases represent only a small fraction of the total cellular protein, the

cathepsin proteases account for up to 40% of the lysosomal hydrolytic enzyme content in the cell.

1.5. THE CATHEPSIN FAMILY

As with all proteases, the 15 members of the cathepsin family are characterized according to their catalytic site. In addition to their role in digestion of unessential cellular material, the cathepsins represent a family of proteases with extraordinarily diverse functions. The 11 cysteine cathepsins (B, C, F, L, K, V, S, X/Z, H, W, and O) are by far the largest cathepsin subfamily and are the most well-characterized [36]. Cathepsin B plays a role in migration of cells following wound healing and in the inflammatory response [39]. Cathepsin L functions in secretory vesicles to process pro-hormones for production of the neurotransmitter enkephalin [40].

The serine cathepsins (A and G) are less well-characterized. Cathepsin A possesses a function that is independent from its enzymatic activity. In addition to its role in proteolytic degradation, procathepsin A acts as a protection protein by binding and stabilizing β-galactosidase in the lysosomes [41]. Cathepsin G cleaves and inactivates proteins involved in blood clotting, plays a role in neutrophil response to a variety of bacteria, and also is important in tissue remodeling during wound healing [42].

While the aspartate cathepsins (D and E) share considerable homology in their amino acid sequences, their cellular function and localization is very different. Cathepsin E is necessary for the MHC Class I antigen processing pathway in dendritic cells. However, its mechanism of action is unknown [43, 44]. Unlike the other cathepsins, cathepsin E is not localized exclusively to the lysosomal compartment; localization and

expression of cathepsin E differs between cell types [45]. Cathepsin D, which is localized solely in the lysosomal/endosomal compartments in normal cells, has been implicated in inflammatory processes and tumor invasion, and is vital for development of the intestinal mucosa and lymphoid cells [46]. As cathepsin D is the focus of my study, it will be discussed in detail.

Figure 1.7 contains a current list of the cathepsin proteases. Despite their varying functions, cathepsin proteases share one common feature: they degrade proteins that have been targeted for destruction via the endocytic pathway. Because these enzymes are usually completely sequestered within the lysosomal compartment, the concept of an extra-lysosomal role was given little credence. Recent evidence, however, has shed light on roles for several members of the cathepsin family in the apoptotic process.

1.5.1. CATHEPSINS IN APOPTOSIS

Lysomotropic agents are lipophilic bases that induce lysosomal destabilization by accumulating in the lysosomal lumen, disrupting the membrane in a manner similar to detergents. Firestone *et al.* [47-49] demonstrated that selective lysomotropic rupture of the lysosomal compartment induced cell death. Their description of the morphological changes in the dying cells could be categorized as classic apoptosis. More recently, studies have shown that addition of lysomotropic agents such as the antibiotics norfloxacin and ciprofloxacin induce apoptosis in HeLa cells via activation of the proapoptotic mitochondrial proteins Bax and Bak [50, 51]. Additionally, the detergents sphingosine and O-methyl-serine dodecylamide hydrochloride (MSDH) induce lysosomal rupture and subsequent apoptosis in Jurkat leukemic and J774 macrophage-

Cathepsin	Type	Inhibitors
Cathepsin A	Serine	Boc-VF-NHO-Bz-*p*Cl
Cathepsin B	Cysteine	Leupeptin, Ac-LVK, z-FG
Cathepsin C	Cysteine	N/A
Cathepsin D	Aspartate	Pepstatin A
Cathepsin E	Aspartate	Pepstatin A
Cathepsin F	Cysteine	N/A
Cathepsin G	Serine	Boc-VF-NHO-Bz-*p*Cl
Cathepsin H	Cysteine	Stefins
Cathepsin K	Cysteine	1,3-Bis(N-carbobenzoyloxy-L-leucyl)amino Acetone 1,3-Bis(CBZ-Leu-NH)-2-propanone
Cathepsin L	Cysteine	z-FF, z-FG
Cathepsin O	Cysteine	N/A
Cathepsin S	Cysteine	z-FG, z-FL
Cathepsin V	Cysteine	N/A
Cathepspin W	Cysteine	N/A
Cathepsin X/Z	Cysteine	N/A

Figure 1.7. Cathepsin Proteases

like cells, as well as in HeLa epithelial cells and AG1518 fibroblasts [51, 52].

Even with this evidence, however, many assumed the role of lysosomes was restricted to an autophagic role (type II programmed cell death) in the late stages of cell death. Autophagy is characterized by morphological changes that differ from apoptosis (type I programmed cell death). During apoptosis, cytoplasm and chromatin condense, inducing cell shrinkage. During autophagy, cytoplasmic constituents are fragmented and distinct autophagic vacuoles are formed [53]. Additionally, apoptotic cells fragment into small apoptotic bodies, while an autophagic cell stays intact throughout the cell death process [54]. Both type I and type II programmed cell death are completed by lysosomal degradation of cellular constituents [54]. Dying cells can undergo both type I and II programmed cell death simultaneously; therefore, it is possible that the role of lysosomes is not mutually exclusive to one type of cell death [55].

Recent evidence points to a distinct role for lysosomal proteases in apoptosis. Several lysosomal storage disorders arise as a result of a lack or accumulation of lysosomal enzymes [56]. Interestingly, many of these diseases are characterized by either an inhibition or an exacerbation of cell death, suggesting that lysosomal enzymes may be important for the apoptotic process [56]. Stoka *et al.* [57] demonstrated that while the cathepsin proteases did not directly cleave caspase proteases, lysosomal extracts induced the cleavage of the proapoptotic factor Bid to its active form. Cleaved Bid is capable of promoting cytochrome c release and subsequent activation of caspases. Incubation of mitochondria with Bid that had been cleaved by lysosomal extracts *in vitro* was found to induce cytochrome c release, suggesting that cathepsins might play a role in caspase

activation by acting through Bid. The effects on Bid by the cathepsins, especially cathepsin D, will be discussed in Chapter 5.

In vivo assays have also demonstrated that lysosomal proteases are important for apoptosis. Yuan *et al.* [58] developed a M1-t-p53 myeloid cell line with a temperature-sensitive p53 protein that was active at 32 °C. Using these cells, they showed that p53-induced apoptosis was initiated by lysosomal destabilization and release of lysosomal contents. Also, Zang *et al.* [59] showed that labeling of lysosomes in HL-60 cells using the synthetic retinoid CD437 induced leakage of lysosomal contents. The cathepsin D inhibitor pepstatin A prevented formation of free radicals and apoptosis following treatment with CD437, suggesting a direct role for the cathepsins in CD437-induced apoptosis. Reports have also revealed a role for lysosomal proteases in apoptosis induced by supraoptimal activation of T lymphocytes [60], Hsp70 depletion [61], and photodamage [62]. Taken together, these studies suggest that lysosomal enzymes may play an active role in various types of apoptosis.

1.5.2. CATHEPSIN B AND CATHEPSIN L IN APOPTOSIS

Of all the lysosomal enzymes, the cysteine cathepsins (specifically cathepsin B) are the most well-characterized. Cathepsin B is synthesized as a 38 kD zymogen that is ubiquitously expressed in high levels [63]. Proteolytic cleavage generates a 31 kD mature form, followed by subsequent processing to 27 kD and 5 kD fragments that are linked via interchain disulfide bonds [64]. In addition to its role in proteolytic degradation, cathepsin B has been implicated in extracellular matrix remodeling [65]. Cathepsin B can be secreted, where it promotes tumor migration and invasion by

degrading extracellular matrix proteins such as collagen, laminin, and proteoglycans [66]. Additionally, studies have shown that several apoptotic stimuli evoke relocalization of cathepsin B to the cytoplasm during cell death. For instance, the microtubule-stabilizing agents (MSAs) paclitaxel, epothilone B, and discodermolide all induce relocalization of cathepsin B to the cytosol, accompanied by activation of the enzyme [67]. Additionally, this study demonstrated that inhibition of cathepsin B with CA-074 Me and zFA-FMK protected H460 lung cancer cells from death induced by the aforementioned drugs.

Several reports have shown that cathepsin B is also important in cell death induced by the death receptor ligand TNF-α. Antisense-mediated downregulation of cathepsin B in WEHI-S leukemic cells, as well as treatment with the cathepsin B inhibitors CA-074-Me and zFA-FMK, significantly protected against cell death induced by either TNF-α or TRAIL [68]. TNF-α-induced cytochrome c release, caspase activation, and apoptosis were markedly diminished in cathepsin $B^{-/-}$ mice following stimulus with TNF-α [64, 69]. In line with this, cathepsin B was relocalized from the lysosomes to the cytosol following TNF-α treatment [64, 68, 70, 71]. The selective release of cathepsin B from lysosomes has also been noted for bile salt-induced apoptosis [69], TNF-α-mediated hepatocyte apoptosis [69, 72], and supraoptimal activation of T lymphocytes [60]. Evidence suggests that the release of cathepsin B into the cytosol is caspase dependent [64, 68]. Interestingly, forced overexpression of cathepsin B, resulting in protein expression levels similar to those found in tumors, did not sensitize the cells to TNF-α-mediated apoptosis, suggesting that the basal levels of cathepsin B are sufficient to initiate the stimulus for apoptotic signaling [73].

Cathepsin B initiates an apoptotic cascade following treatment with TNF-α and bile salts or after supraoptimal activation of T lymphocytes, resulting in the downstream activation of the executioner caspases. The mechanism of cathepsin B-induced apoptosis remains unknown. However, it is understood that while cathepsin B can directly activate the inflammatory caspases, caspase-1 and caspase-11, it cannot directly activate proapoptotic caspases [57, 74]. This evidence suggests that cathepsin B likely acts on an intermediary factor to induce caspase activation.

Preliminary studies suggest that the cysteine protease cathepsin L may also be involved in apoptotic events. Cathepsin L, like cathepsin B, can be secreted in response to growth factors, transformation, and tumor promoters [75]. Cathepsin L and cathepsin B are also important in neurological development, as deficiencies of the two proteases are lethal early in life [65]. While a definitive proapoptotic role of cathepsin L has not been elucidated, some studies have shown that activation of caspase-3 is enhanced *in vitro* by cathepsin L, implying that cathepsin L may be involved in the process of caspase-3 activation [76, 77].

1.6. CATHEPSIN D

Cysteine cathepsins are not the only potential mediators of apoptosis present in the lysosomes. Over the past 10 years, evidence has implicated a role for the aspartate protease cathepsin D in apoptosis induced by several types of stimuli. The section that follows outlines the current knowledge of cathepsin D and its role in normal cells as well as in apoptotic cells.

1.6.1. STRUCTURE AND EXPRESSION

Cathepsin D, like all lysosomal proteases, undergoes a variety of processing steps during its synthesis and maturation. It is made as a preproenzyme that is targeted to the endoplasmic reticulum (ER) via a 20 amino acid N-terminal signal peptide sequence. This sequence interacts with a signal recognition particle that induces receptor-mediated transport of cathepsin D across the endosomal membrane [78]. Because this prepeptide sequence is removed cotranslationally by a luminal signal peptidase, the cathepsin D preproenzyme is never detected in cells [37, 38]. Similar to other cathepsins, a 44-residue prodomain keeps procathepsin D inactive during transport and is removed sometime before final transport to the mature lysosomes. Following cleavage of the prodomain, a single-chain active form of cathepsin D is generated. Further processing results in an active light-chain/heavy-chain form of the enzyme in human tissues. Late in the biosynthesis process, cathepsin D also undergoes proteolytic "trimming", during which it loses another seven amino acids between the heavy and light chains as well as two amino acids at the carboxy terminus. As this processing occurs following a significant pH drop in the vesicle (a step that often accompanies fusion of a lysosome with a phagosome), it is believed that it occurs as a final enzymatic activation step [37]. Figure 1.8 depicts the structure of the cathepsin D protein and cleavage/activation sites.

The cathepsin D protein also acquires a high-mannose oligosaccharide chain on each chain during translation [38]. Phosphorylation of these chains occurs in the early Golgi by a lysosome-specific phosphotransferase enzyme, *N*-acetylglucosamine 1-phosphate [79]. The removal of the *N*-acetylglucosamine then exposes the phosphate as a

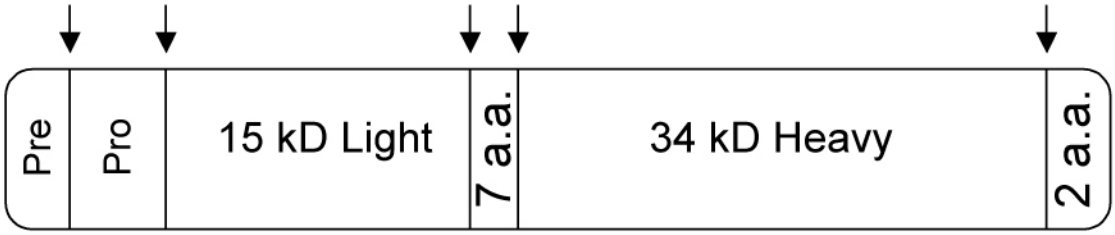


Figure 1.8. Structure of cathepsin D. The cathepsin D protein is comprised of a predomain, a prodomain, a 15 kD light chain, and a 34 kD heavy chain. During the course of biosynthesis and activation, proteolytic processing cleaves the single chain preprocathepsin D enzyme to an active double chain form. Additional proteolytic processing of 7 amino acids between the heavy and light chains and 2 amino acids at the carboxyl terminus are needed for full activation of the enzyme. Arrows depict processing sites. See text for details on the function of each domain.

recognition signal for binding to mannose-6-phosphate (M6P) receptors in the late Golgi, which targets the enzyme to the late endosomes.

Cathepsin D that is localized in the lysosomes associates with the membrane via a M6P-independent mechanism. Though the significance of this membrane interaction is unclear, it has been shown that while procathepsin D is membrane-bound, active enzyme is soluble [80]. Therefore, membrane dissociation may be associated with activation of the protease [81].

It is clear that sequestration of lysosomal hydrolases is vital for safeguarding the cell from self-digestion. A variety of precautionary measures target and maintain cathepsin D within lysosomes, including specific signaling peptides, M6P-receptor targeting, and cathepsin D-membrane association [38]. Despite these safeguards, transformed cells such as MCF-7 breast cancer cells have the ability to dissociate cathepsin D from the membrane in a M6P-independent manner [38, 82]. In these cells, cathepsin D is secreted as a proenzyme, where it can degrade intact basement membranes and contribute to the metastatic potential of tumors [83, 84]. Evidence also suggests that secretion of procathepsin D enhances cell proliferation in some types of ovarian tumors [85]. These data suggest that the secreted proform of cathepsin D can be quite detrimental in several types of cancers.

Cathepsin D is necessary for normal tissue homeostasis [46]. Cathepsin $D^{-/-}$ mice are not impaired in their ability to undergo lysosomal bulk proteolysis, as cysteine proteases can elicit a compensatory mechanism for this loss. However, although they develop normally for about two weeks, $CD^{-/-}$ mice soon exhibit intestinal atrophy and a

significant decrease in the number of T and B lymphocytes of the spleen and thymus. Therefore, cathepsin D is essential both in the development of adult epithelial cells of the gut as well as the development of T and B lymphocytes in central and peripheral lymphoid organs [46].

Cathepsin D is expressed in high levels in all cell types and accounts for as much as 11% of the total lysosomal enzyme content [78]. Cathepsin D, which is a member of the pepsin proteinase family, is a bilobed molecule that is primarily comprised of β-sheets and contains a deep active site cleft in which Asp-215, Asp-32, and Serine-35 are critical residues [46, 86]. Cathepsin D prefers to cleave substrates between dipeptide bonds (usually Phe-Phe) that are flanked by bulky hydrophobic amino acids at a pH optimum between 2.8 and 4 [87]. As I will show, cathepsin D maintains enzymatic activity up to a pH of 7.0.

1.6.2. CATHEPSIN D IN APOPTOSIS

The possibility that lysosomal cathepsin D plays an important role in the cell death pathway has come as a surprise to the apoptosis research community. In 1996, Deiss *et al.* [88] were attempting to isolate positive mediators of interferon (IFN)-γ-induced cell death utilizing a strategy in which random genes were inactivated following transfection with antisense cDNA expression libraries. The clones containing cDNA inserts that protected against IFN-γ-induced cell death were rescued and sequenced for identification. One of the isolated cDNAs was found to correspond to a fragment of human cathepsin D cDNA. In the same study, cathepsin D antisense RNA and the cathepsin D inhibitor pepstatin A were shown to protect HeLa cells from Fas/APO-1-, TNF-α,- and IFN-γ-

mediated cell death. Another study showed that pepstatin A elicited protection from TNF-α-induced cell death in L929 fibroblasts [89].

Apoptosis can also be induced by the quinone naphthazarin through production of superoxide radicals [90, 91]. In a series of studies, Roberg *et al.* [90-92] demonstrated through immunohistochemical methods that cathepsin D was relocalized to the cytosol following a short exposure to oxidative stress. It was postulated that the production of hydroxyl radicals destabilized the lysosomal membranes through lipid peroxidation and provoked a generalized leakage of the lysosomal contents into the cytosol. Pepstatin A also prevented oxidative stress-induced cytochrome c release and loss of mitochondrial membrane potential ($\Delta\Psi_m$) in cardiomyocytes [90, 92]. Additionally, oxidative stress-mediated caspase activation was inhibited in cardiomyocytes that were pretreated with pepstatin A [93]. It is important to note, however, that although pepstatin A did protect against oxidative stress-induced apoptosis, it did not prevent cytoplasmic relocalization of cathepsin D [93]. These results suggest that cathepsin D acts as an upstream factor that regulates cytochrome c release and subsequent caspase activation following oxidative stress. In support of this, Roberg *et al*. [94] demonstrated that microinjection of cathepsin D into human fibroblasts induced apoptosis via cytochrome c release and caspase activation. Similarly, pepstatin A protected alveolar epithelial cells against apoptosis induced by the fibrogenic agent bleomycin [95].

Research has also suggested that cathepsin D may be important in ceramide-induced apoptosis. Ceramide is an intracellular signaling molecule that transduces the effects of exogenous stimuli such as growth factors or cytokines for cellular functions such as proliferation or apoptosis [96, 97]. Ceramide acts as a mediator in the apoptotic process

induced by cytotoxic cytokines. Although a definitive role for cathepsin D in ceramide-mediated apoptosis has not been established, it has been demonstrated that ceramide binds to procathepsin D, resulting in autocatalytic proteolysis of cathepsin D to its active double-chain form [96].

Additionally, a role for cathepsin D has been suggested for apoptosis caused by the broad-spectrum protein kinase inhibitor staurosporine. Treatment of human foreskin fibroblasts with pepstatin A inhibited staurosporine-induced cytochrome c release, caspase activation, and apoptosis [98]. Following staurosporine treatment of T lymphocytes, cathepsin D triggered Bax activation followed by subsequent release of the mitochondrial apoptogenic protein AIF-1 [99, 100]. AIF-1 contributes to apoptosis in a caspase-independent manner. The fact that cathepsin D may play a proapoptotic role in both a caspase-dependent and a caspase-independent pathway suggests that cathepsin D may participate in more than one signaling cascade that culminates in apoptosis or that its mechanism of action during apoptosis may be cell- or stimulus-dependent.

In 1998, a study by Wu *et al.* [101] showed that cathepsin D protein expression increased following etoposide treatment in ML1 cells. This same study demonstrated that cathepsin D-deficient fibroblasts were partially protected against etoposide-induced cell death. Though these data suggest that cathepsin D might also mediate chemotherapy-induced apoptosis, the role of cathepsin D in the chemotherapy-induced apoptotic pathway has remained largely unexplored.

1.7. STATEMENT OF PROBLEM AND HYPOTHESIS

The apoptotic cascade represents a complex process that culminates in the destruction and degradation of the cell. One characteristic of many cancer cells is their ability to evade apoptosis, thus maintaining an immortal status. Many mediators of the apoptotic cascade have been well characterized. However, as the incidence of chemotherapeutic drug-resistance rises, the discovery and characterization of novel apoptosis regulatory proteins that have potential to become drug targets is vital. Lysosomal proteases, particularly the aspartate protease cathepsin D, have been implicated in apoptosis induced by a variety of stimuli, including death receptor ligands, oxidative stress, and protein kinase C inhibitors. Very little is known about the role of cathepsin D in chemotherapy-induced apoptosis, but preliminary evidence suggests that it may be a potential apoptotic mediator of chemotherapy drugs as well. The hypothesis of this project is that cathepsin D is important for chemotherapy-induced apoptosis. Because it would be difficult for a sequestered lysosomal enzyme to directly mediate apoptosis signal transduction, these studies will first examine the subcellular localization of cathepsin D following chemotherapy-treatment. This project will also examine the impact of siRNA-mediated cathepsin D downregulation on chemotherapy-induced apoptosis in two different cell lines. Finally, this project will attempt to shed light on the molecular mechanism of cathepsin D action in the chemotherapy-induced apoptotic pathway.

2. MATERIALS AND METHODS

2.1. MATERIALS

Polyclonal antibodies to cathepsin D and cathepsin B, hygromycin B, and the caspase inhibitor z-VAD-FMK were purchased from Calbiochem (San Diego, CA). Caspase-8 inhibitor (z-IETD-FMK), cathepsin B inhibitor (z-FA-FMK) and cathepsin L inhibitor (z-FF-FMK) also were from Calbiochem. Pepstatin A was obtained from Roche Applied Sciences (Indianapolis, IN). Polyclonal anti-caspase-3 antibody and monoclonal anti-caspase-8 antibody were purchased from Cell Signaling Technology (Beverly, MA). Polyclonal anti-cathepsin E antibody was purchased from Santa Cruz (Santa Cruz, CA). Monoclonal anti-cytochrome c oxidase IV antibody was obtained from Molecular Probes (Eugene, OR). Polyclonal anti-Smac antibody was purchased from R&D Systems (Minneapolis, MN). Monoclonal β-actin antibody, VP-16, cisplatin, 5-fluorouracil, cycloheximide, naphthazarin, and DMSO were from Sigma (St. Louis, MO). Staurosporine was purchased from Upstate Biotechnology (Lake Placid, NY). Sphingosine was obtained from EMD Biosciences (San Diego, CA). Monoclonal cytochrome c antibody and the caspase-3 substrate Ac-DEVD-AFC were obtained from BD Pharmingen (San Diego, CA). The cathepsin D substrate MoCAc-Gly-Lys-Pro-Ile-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 was purchased from Peptides International (Louisville, KY). The β-hexosaminidase substrate, 4-methylumberriferyl-N-acetyl-β-D-glucosaminide dehydrate, was purchased from Sigma. TRAIL and TNF-α were from PeproTech, Inc (Rocky Hill, NJ).

2.2. CELL CULTURE

Wild-type U937 cells were cultured in RPMI media (BioWhittaker) containing 10% fetal bovine serum, 100 μg/ml penicillin-streptomycin, and 0.5 μg/ml amphotericin B (fungizone). U937 clones transfected with siRNA constructs were supplemented with 250 μM hygromycin B (Calbiochem) with every media change. Wild-type HeLa cells were cultured in DMEM media (Mediatech) containing 10% fetal bovine serum, 100 μg/ml penicillin-streptomycin, and 0.5 μg/ml amphotericin B. HeLa clones containing cathepsin D siRNA were supplemented with 250 μM hygromycin B. All cells were maintained at 37 °C in the presence of 5 % CO_2.

2.3. INDUCTION OF CELL DEATH

One day prior to drug treatment, HeLa cells were plated in 24 well plates at a density of 3 x 10^5 cells/ml. Wells were washed with PBS before addition of the drug-containing media. For drug treatment in U937 cells, cells were harvested by centrifugation at 1,300 rpm for 5 minutes at 4 °C. Following a wash with PBS, U937 cells were resuspended in RPMI media to a concentration of 8 X 10^5 cells/ml. To induce U937 cell death, cells were incubated at 37 °C with the following drugs: 2 μM VP-16, 2 μg/ml TRAIL, 1 μM staurosporine, or 1 μM napthazarin. For TNF-α-induced apoptosis, U937 cells were treated simultaneously with 10 ng/ml TNF-α and 10 μg/ml cycloheximide. To induce apoptosis in HeLa cells, cells were incubated at 37 °C with the following drugs: 100 μM VP-16, 5 μM 5-fluorouracil, 10 μM cisplatin, 1 μM staurosporine, 1 μM napthazarin, or 10 μM sphingosine. HeLa cells were also treated

with 10 ng/ml TNF-α and 10 μg/ml cycloheximide concomitantly. Drugs were utilized at a 1:1000 dilution in media. All drug stock solutions were dissolved in DMSO; therefore, control samples in experiments were treated with 0.1% DMSO. Untreated HeLa and U937 cells always exhibited greater than 97% viability throughout experiments involving cell death. At the designated time points, aliquots of cells were taken for viability analysis. In the case of HeLa cells, media was removed from the wells and the wells were washed with PBS. The wells were then treated with 200 μl trypsin/EDTA for a sufficient length of time to release the cells from the plate (about 1 minute). The cellular mixtures of media, PBS, and trypsin were centrifuged at 2,000 rpm for 4 minutes at room temperature, and the pellets were resuspended in 100 μl PBS. Cell viabilities were assessed by adding an equal volume of trypan blue exclusion dye and counting a minimum of 100 cells to assess dye uptake. In the viability counts, the ratio of clear translucent cells (excluding dye) to total cells indicated the percentage of viable cells; clear cells represented viable cells and blue cells represented dead or dying cells. All counts were done in triplicate; error bars represent standard deviations.

2.4. CYTOSOLIC ISOLATION AND CATHEPSIN D RELOCALIZATION

To assess subcellular relocalization of cathepsin D following chemotherapy treatment in wild-type U937 cells, 20 X 10^6 U937 cells treated with either DMSO, TNF-α + cycloheximide, staurosporine, or VP-16 were harvested by centrifugation at 1,300 rpm for 5 minutes at 4 °C, followed by 2 washes with ice-cold PBS. The cell pellets were resuspended in 400 μl resuspension buffer (20 mM HEPES, pH 7.4, 10 mM KCl,

1.5 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose, 1 mM DTT, 1.5 mM PMSF, 3 μg/ml leupeptin, and 20 μg/ml aprotinin) and homogenized on ice by 20 strokes with a Wheaton Type B Dounce homogenizer (Kontes Glass, Vineland NJ). Cell homogenates were centrifuged at 14,000 rpm for 30 minutes at 4 °C. The supernatants, which contained the cytosolic fraction, were then subjected to another 30 minute centrifugation for further clarification. The pellets, which consisted of a crude heavy-membrane fraction that contained isolated lysosomes, were purified further by two washes in resuspension buffer and microcentrifugation at 14,000 rpm for 10 minutes at 4 °C. Bio-Rad protein assay dye reagent (Bio-Rad Labs, Hercules, CA) was used to determine protein concentrations in both the pellet and supernatant fractions. 25 μg/lane of protein was used for immunoblotting with anti-cathepsin D.

2.5. ISOLATION OF MITOCHONDRIA

To isolate mitochondria from wild-type U937 cells, 20 X 10^6 cells were centrifuged at 1,300 rpm for 5 minutes at 4 °C, washed with PBS, and centrifuged again. The cell pellets were resuspended in 400 μl resuspension buffer (20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 250 mM sucrose, 1 mM DTT, 1.5 mM PMSF, 3 μg/ml leupeptin, and 20 μg/ml aprotinin) and subjected to 20 strokes in a Type B Dounce homogenizer. The homogenates were then centrifuged for 30 minutes at 14,000 rpm at 4 °C. The enriched mitochondrial pellets were washed twice with cold resuspension buffer, then resuspended in 100 μl of resuspension buffer. The supernatants, containing cytosolic proteins, were further clarified by two rounds of

centrifugation at 14,000 rpm for 30 minutes at 4 °C. Protein concentrations were estimated using Bio-Rad protein dye reagent; 50 μg of mitochondrial protein and 10 μg of cytosolic protein were used for cell-free cytochrome c-release assays.

2.6. CATHEPSIN D siRNA CONSTRUCTION AND LIGATION INTO p*SILENCER* VECTOR

siRNA sequences suitable for targeting of cathepsin D mRNA were identified using the Ambion siRNA target finder (www.ambion.com). Five oligonucleotides were designed, containing appropriate 21-nucleotide sense and antisense sequences separated by a hairpin loop, according to Ambion recommendations (See chapter 4 for specific details). Oligonucleotides were synthesized by Integrated DNA Technologies, Inc. Oligonucleotides contained *Hind III* and *Bam HI* restriction sites at their 5' and 3' ends, respectively. Single-stranded oligonucleotides were annealed by combining 1 μg/μl of homologous oligonucleotides, denaturing for 3 minutes at 90 °C, and annealing for 1 hour at 37 °C. The annealed oligonucleotides were cleaved by *Hind III* and *Bam HI* restriction enzymes, then ligated into the corresponding sites of the Ambion p*Silencer* 2.1-U6 hygro vector by incubating oligonucleotides with vector (1 part oligonucleotide to 40 parts vector) for 3 hours at room temperature in the presence of 5 units of T4 DNA ligase.

2.7. TRANSFORMATION AND CONSTRUCT PREPARATION

For each siRNA target sequence, 40 ng DNA from p*Silencer* vector containing an siRNA sequence was incubated with competent DH5-α bacteria for 40 minutes at 4 °C. Samples were heat-shocked for 2 minutes at 42 °C, then incubated at room temperature for 5 minutes. The bacteria were then incubated with LB media for 1 hour and plated onto LB plates containing 50 mg/ml ampicillin for incubation overnight at 37 °C. Plasmid DNA was purified from bacterial colonies (Promega WizardPLUS minipreps), and DNA sequencing was used to verify that the siRNA sequences were correctly incorporated into the p*Silencer* vector.

2.8. TRANSFECTION OF p*SILENCER* CONSTRUCTS INTO U937 AND HELA CELLS

Plasmid DNAs were transfected into HeLa and U937 cells using the GenePORTER 2 Transfection reagent (Gene Therapy Systems). DNAs (4 μg/sample) were transfected into cells at a density of 2 x 10^6 cells/ml in a 6-well plate under serum-free conditions. Fresh growth media containing 20% serum was added 3 hours post-transfection. Hygromycin B was added to a concentration of 250 μM after 48 hours. Cells were maintained for 3 weeks in hygromycin B-containing media, with media being replenished every 3 days. Surviving clones were isolated by limiting dilution (U937) or cloning cylinders (HeLa) and expanded in media containing hygromycin B. The sequence that was complementary to the cathepsin D gene at nucleotide 144 (5'-

AGGCCCCGUCUCAAAGUACUU -3') proved the most effective at silencing cathepsin D in both HeLa and U937 cells (See Chapter 4).

2.9. IMMUNOBLOTTING

Following SDS-PAGE electrophoresis on 16 cm gels, the gels were transferred to nitrocellulose membrane at 45 V for 3 hours. For mini-gels, gel transfer to nitrocellulose was done at 100 V for 1 hour. Membranes were blocked in 5% dry milk/TBST (50 mM Tris, pH 8.0, 0.15 M NaCl, 0.1% Tween-20) for 1 hour, followed by a quick rinse in TBST. Primary antibodies were diluted in TBST containing 1% bovine serum albumin and incubated overnight at 4 °C with the membrane. Antibody dilutions are listed in Appendix B. Following incubation with primary antibody, the membranes were washed for 15 minutes with TBST, followed by three washes of 5 minutes each. Secondary antibody, diluted 1:4000 in TBST containing 1% milk, was then added to the membrane for 1 hour at room temperature. Secondary antibody for β–actin was used at a 1:5000 dilution. Following incubation with secondary antibody, the membranes were washed for 15 minutes in TBST, followed by three 5 minute washes. Membranes were then dried and developed using the Western Lightning Renaissance ECL reagent.

2.10. PREPARATION OF WHOLE CELL EXTRACTS

To prepare whole cell extracts for enzymatic cleavage assays or for immunoblotting, 5 X 10^6 cells were subjected to centrifugation at 4 °C for 5 minutes at 1,300 rpm, followed by a wash with PBS. Cell pellets were resuspended in 75 μl lysis

buffer (50 mM Tris, pH 8, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40), incubated on ice for 10 minutes, then centrifuged at 4 °C for 10 minutes at 14,000 rpm. The protein concentration of the supernatant was quantified using Bio-Rad Protein Dye Reagent. 25 μg of protein per lane was used for immunoblotting, and 20 μg of protein per sample was used for enzymatic cleavage assays.

2.11. CHEMOTHERAPY-INDUCED CYTOCHROME C RELEASE ASSAYS

For investigation of cytochrome c release in chemotherapy-treated U937 cells, 20 X 10^6 cells were treated with 2 μM VP-16 or an equivalent amount of DMSO (0.1%) over a time course of 24 hours. At designated time points, cells were harvested by centrifugation at 1,300 rpm for 5 minutes at 4 °C, followed by a wash in cold PBS. Cytosolic fractions were isolated and quantified as described in Section 2.4. Cytosolic proteins (25 μg/lane) were electrophoresed on 13% SDS-PAGE gels, transferred to nitrocellulose membranes, and immunoblotted for cytochrome c. To assess release of cytochrome c from isolated mitochondria in cell-free assays, mitochondria were isolated from untreated wild-type U937 cells as described in Section 2.5. Proteins (50 μg/sample) from the mitochondria-containing pellets were incubated with 2 μg cathepsin D in the presence or absence of 10 μg of cytosolic extract in a total of 50 μl for 1 hour at 37 °C. Inhibitors were used at the following concentrations: 100 μM pepstatin A, 100 μM z-VAD, 100 μM IETD, 100 μM Cathepsin B inhibitor, and 100 μM Cathepsin L inhibitor. Samples were centrifuged at 14,000 rpm for 30 minutes at 4 °C, and the supernatants

were subjected to further similar centrifugation for 30 minutes. Supernatant proteins (15 μl/lane) were electrophoresed on 13% SDS-PAGE gels and subjected to immunoblotting for cytochrome c.

2.12. ENZYMATIC CLEAVAGE ASSAYS

For caspase-3 enzymatic assays, 20 μg of whole cell extracts were added to reaction buffer containing 20 mM HEPES, pH 7.5, 100 mM NaCl, 10% glycerol, 5 mM DTT, and 1 mM PMSF to achieve total reaction volumes of 100 μl. Ac-DEVD-AFC substrate (1 μM) was then added and the reactions were incubated at 37 °C for 2 hours. Cleavage of the substrate was measured at Ex_{λ}= 400 nm and Em_{λ}=505 nm using an L55 luminescence/fluorescence spectrometer (Perkin-Elmer). To measure cathepsin D activity, 5 μg of cytosolic extracts (Fig 3.6) or 5 μg of whole cell extracts (Fig 4.5B) were incubated with fluorogenic cathepsin D substrate (MoCAc-Gly-Lys-Pro-Ile-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2) for 1 hour at 37 °C. Cleavage of this substrate was analyzed by fluorescence spectroscopy at Ex_{λ}=328 nm and Em_{λ}=393 nm. Cytosolic extracts (20 μg) were analyzed for β-hexosaminidase activity by assessing cleavage of the fluorogenic substrate 4-methylumberriferyl-N-acetyl-β-D-glucosaminide dehydrate in reaction volumes of 200 μl of 100 μM NaAc, pH 4.0. Cleavage of the substrate was measured at Ex_{λ}=364 nm and Em_{λ}=488 nm. Error bars represent the standard deviations of three independent assays.

3. CATHEPSIN D UNDERGOES RELOCALIZATION TO THE CYTOSOL FOLLOWING CHEMOTHERAPEUTIC DRUG TREATMENT

3.1. INTRODUCTION

Lysosomal proteolytic enzymes normally function within cells to degrade proteins that are targeted to the lysosomal compartment [1]. The pH of the lysosomal compartment is approximately 5.0, and proteases that reside there are often only active at low pH [1]. Maximal lysosomal enzyme activity at an acidic pH is a protective measure for cells; if these enzymes were to leak into the cytosol, they could digest the entire cell. However, because they are not typically active at cytosolic pH (~7.2), if accidental leakage occurs, the presence of lysosomal proteases in the cytosol usually does little damage [1].

Most apoptosis regulatory proteins reside within the cytosol or the mitochondria; therefore, the complex execution processes of cell death also occur there. As discussed earlier, chemotherapeutic drugs initiate release into the cytosol of various apoptogenic proteins, including cytochrome c, SMAC, or AIF-1, as a result of activation of proapoptotic factors and inhibition of antiapoptotic factors [8]. In the cytosol, cytochrome c induces an apoptotic cascade by initiating formation of the apoptosome, which activates caspases and ultimately culminates in destruction of the cell. The cytosol provides the main stage for cell execution. Though lysosomal proteases cleave most rigorously in an acidic environment, cathepsin D retains some activity up to a pH of 7.0, as will be demonstrated. Studies have shown that cellular activation of the proapoptotic factor Bax, which occurs after treatment with chemotherapy drugs, initiates acidification

of the cytosol to a pH between 6.3 and 6.8 [102]. Therefore, it is possible that cathepsin D may retain activity in the cytosol of apoptotic cells, thus participating in cell death.

Studies have shown that cathepsin D is relocalized to the cytosol during apoptosis induced by oxidative stress [91, 92]. I have hypothesized that the lysosomal protease cathepsin D plays an important role in chemotherapy drug-induced cell death. Since processes critical to chemotherapy-induced apoptosis occur in the cytosol, I have further hypothesized that treatment of cells with chemotherapy drugs initiates relocalization of cathepsin D from lysosomes to the cytosol. The experiments described in this chapter were designed to test this hypothesis.

3.2. RESULTS

This chapter investigates the subcellular localization of cathepsin D during chemotherapy-induced apoptosis. Throughout this project, I have used etoposide, also known as VP-16, as the chemotherapy drug of choice to treat human monocytic U937 leukemic cells or epithelial HeLa cells. VP-16 is commonly used as an anticancer drug, as it elicits cell death in a variety of cancers, is relatively inexpensive, and can easily be used in combination with other chemotherapies [103, 104]. VP-16 contributes to the cellular production of double-stranded DNA breaks by stabilizing a normally transient DNA-topoisomerase II complex [105, 106]. The results in this chapter show that cathepsin D was detected in the cytosol as early as four hours following VP-16 treatment of cells. Release of cathepsin D occurred prior to release of cytochrome c from the mitochondria or activation of caspase proteases. Release also appeared to be selective for cathepsin D, as lysosomal marker proteins and cathepsin B were not detected in the

cytosol following VP-16 treatment. The pancaspase inhibitor z-VAD-FMK did not prevent cathepsin D release, nor did the cathepsin D inhibitor pepstatin A. Therefore, cathepsin D relocalization to the cytosol was not dependent on caspase or cathepsin D activities. By placing this normally sequestered lysosomal enzyme in an ideal location to contribute to the apoptosis process, these results support the hypothesis that cathepsin D is important for chemotherapy-induced apoptosis.

3.2.1. CATHEPSIN D IS ACTIVE AT CYTOPLASMIC pH.

Cathepsin D is normally localized within a compartment with a pH of approximately 5.0, and not surprisingly, it is most active at acidic pH. I have hypothesized that cathepsin D is released into the cytosol during chemotherapy-induced apoptosis. Since the ability of cathepsin D to retain activity at the cytosolic pH of an apoptotic cell (typically pH 6.3-6.8) is critical to support a potential role for this enzyme in apoptosis execution, it was necessary to ascertain whether cathepsin D retains activity under a variety of pH conditions. To examine this, U937 whole cell lysates (25 μg/sample) were incubated with purified cathepsin D in buffers of varying pH for 1 hour at 37 °C, followed by immunoblotting with anti-caspase-8 antibody to assess the ability of cathepsin D to cleave full-length caspase-8. Previous studies from our lab have shown that cathepsin D cleaves procaspase-8, though at sites distinct from the normal autoactivation cleavage sites. Figure 3.1 depicts cathepsin D cleavage of caspase-8 at pH 4.5, 6.5, or 7.0. At pH 4.5, cathepsin D completely degraded full-length caspase-8. At pH 6.5, the approximate pH of the cytosol during apoptosis, cathepsin D completely cleaved full-length caspase-8 to fragments of 44 kD and 47 kD. At pH 7.0, cleavage of

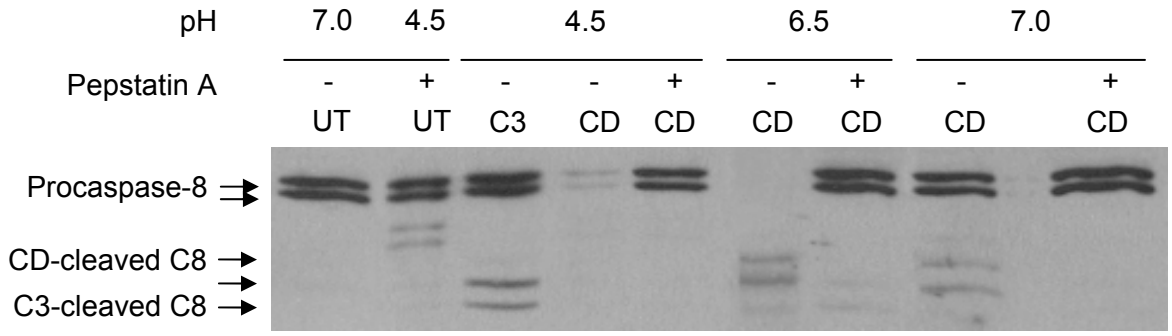


Figure 3.1. Cathepsin D cleaves caspase-8 under various pH conditions. Whole cell lysates were prepared from 5 x 10^6 wild-type U937 cells and incubated with either 0.2 μg caspase-3 or 2 μg cathepsin D in the presence or absence of 100 μM pepstatin A under a variety of pH conditions. Proteins (25 μg/lane) were electrophoresed on a 10% SDS-PAGE gel and subjected to immunoblotting for caspase-8. Cathepsin D maintains the ability to cleave caspase-8 up to pH 7.0.

full-length caspase-8 by cathepsin D was only partial, resulting in both full-length and fragmented caspase-8. Cleavage of caspase-8 under all three pH conditions was inhibited completely by 100 μM pepstatin A. These results suggest that although enzyme activity is greatest in the highly acidic environment of the lysosome, cathepsin D does maintain the ability to cleave proteins at the cytosolic pH of an apoptotic cell.

3.2.2. CATHEPSIN D RESIDES WITHIN THE LYSOSOMES OF UNTREATED CELLS.

Studies in this chapter were designed to investigate whether cathepsin D undergoes relocalization from the lysosomal compartment to the cytosol following chemotherapy drug treatment. Therefore, it was necessary to establish that cathepsin D is contained within lysosomes of healthy untreated U937 cells and to verify that the methodology used in this chapter was sufficient to achieve desired subcellular fractionation. To accomplish these goals, a cytosolic fraction and a lysosome-enriched membrane fraction were prepared from untreated U937 cells using a subcellular fractionation method that involves homogenization of cells in a Dounce Type B tissue grinder followed by centrifugation (see Materials and Methods). The Western blot for cathepsin D in Figure 3.2 shows that cathepsin D was contained in lysosomes. Lysosomal-associated membrane protein (LAMP-1), a marker of lysosomal integrity, was not detected in the cytosolic fraction; therefore, there was no lysosomal contamination in the cytosol. Likewise, immunoblotting for β-actin, a cytosolic protein, showed that there was no cytosolic contamination in the lysosomal fraction. Therefore,

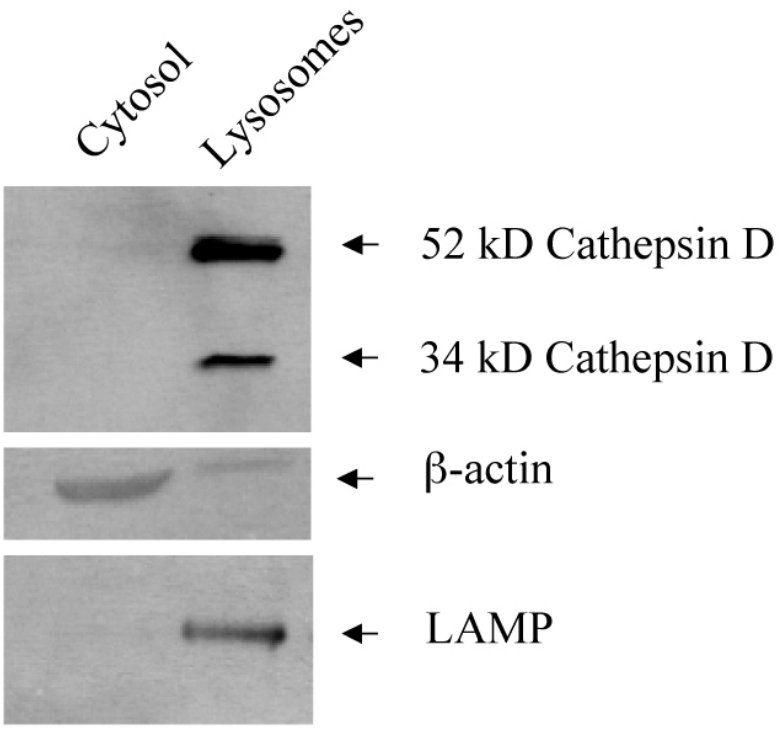


Figure 3.2. Cathepsin D is located in the lysosomes of untreated U937 cells. Cytosolic and lysosomal fractions were isolated from 20 x 10^6 wild-type U937 cells as described in Materials and Methods. Proteins (25 μg/lane) were electrophoresed on a 12.5% SDS-PAGE gel and subjected to immunoblotting for cathepsin D, β-actin, or LAMP-1. Cathepsin D was detected predominantly in the lysosomes of untreated cells.

the fractionation method we employed was suitable to examine chemotherapy-induced cathepsin D relocalization.

3.2.3. CATHEPSIN D RELOCALIZATION OCCURS FOLLOWING TNF-α OR STAUROSPORINE TREATMENT

Several studies have indicated that cathepsin D is important for cell death induced by stimuli such as oxidative stress, PKC inhibitors, and photodynamic therapy [92]. It has also been shown that cathepsin D is important for cell death induced by death receptor ligands such as TNF-α, IFN-γ or Fas ligand [88]. Studies involving death ligands utilized antisense RNA and the pharmacologic inhibitor of cathepsin D, pepstatin A, to show that inhibition of cathepsin D also inhibited apoptosis. These investigations have not, however, investigated cathepsin D localization following death receptor stimulation. Because the importance of cathepsin D in TNF-α-induced apoptosis has already been established, I predicted that cathepsin D would relocalize to the cytosol following TNF-α treatment. Therefore, I treated U937 cells for 18 hours with 10 ng/ml TNF-α (concurrently with 10 μg/ml cycloheximide to sensitize cells to TNF-α) [99, 107], isolated cytosolic fractions, and probed for cathepsin D. Figure 3.3.A shows that, as expected, cathepsin D was released into the cytosol of TNF-α-treated U937 cells, but not in DMSO-treated control cells. Likewise, cathepsin D has been shown to be important in apoptosis induced by the protein kinase C inhibitor staurosporine [99]. When U937 cells were treated with 1 μM staurosporine for varying lengths of time, cathepsin D was detected in the cytosol as early as 6 hours following treatment, with levels continuing to increase up to 12 hours (Figure 3.3.B). These results confirmed my expectation that, in

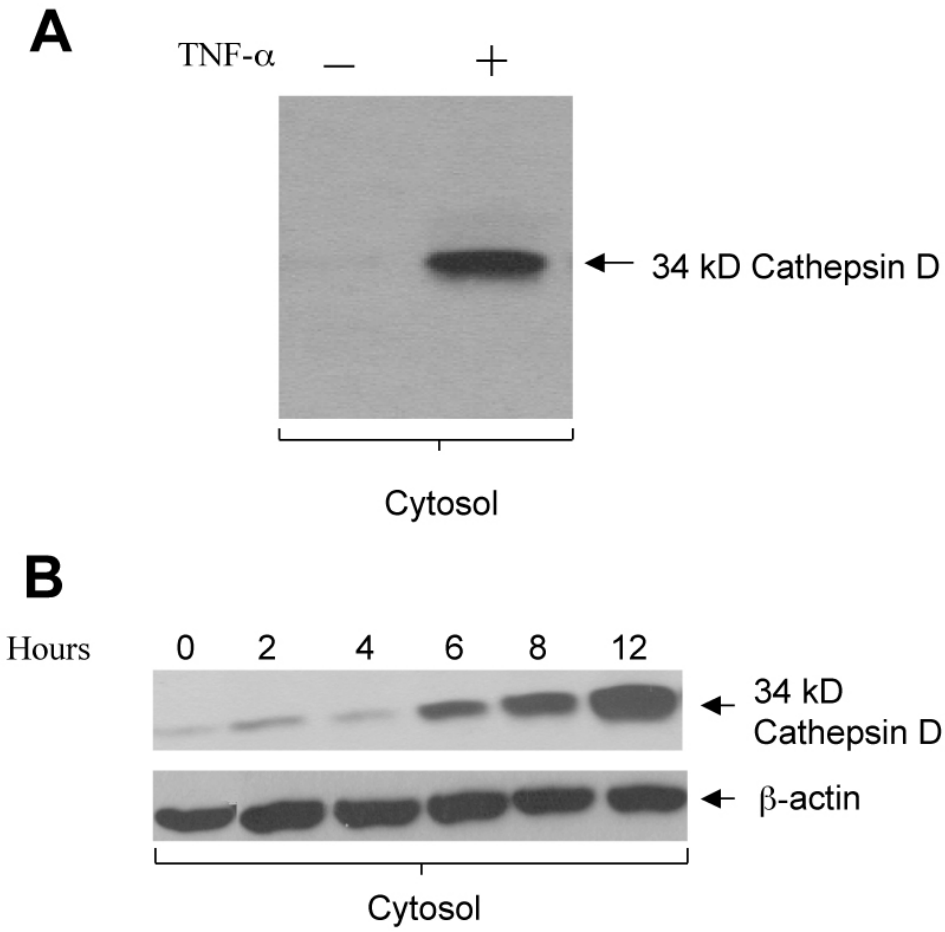


Figure 3.3. Cathepsin D is released into the cytosol following TNF-α or staurosporine treatment. *A,* 20 X 10^6 U937 cells were treated with 10 ng/ml TNF-α + 10 μg/ml cycloheximide for 18 hours and harvested to obtain a cytosolic fraction. An immunoblot for cathepsin D shows that cathepsin D was released into the cytosol following TNF-α treatment. *B,* U937 cells were treated with 1 μM staurosporine for varying times, harvested to obtain cytosolic fractions, and subjected to immunoblotting for cathepsin D. Cathepsin D was released into the cytosol 6 hours following staurosporine treatment and levels increased up to 12 hours.

U937 cells, cathepsin D relocalized to the cytosol during apoptosis [97].

3.2.4. CATHEPSIN D IS RELOCALIZED TO THE CYTOSOL DURING CHEMOTHERAPY DRUG TREATMENT.

If cathepsin D is important for chemotherapy-induced apoptosis and yet remained completely in the lysosomal compartment following drug treatment, I would need to establish an argument that the lysosome plays a direct role in the apoptotic process. This seems unlikely; trafficking across the lysosome's unique membrane is tightly controlled to protect the cell from the interior digestive components. However, cathepsin D relocalization to the cytosol following chemotherapy treatment would place the enzyme in a prime location to play an important role in cell death.

To test whether chemotherapy treatment induces the release of cathepsin D into the cytosol, U937 cells were treated with 2 μM VP-16 for 18 hours, and a cytosolic and lysosome-enriched fraction were obtained via subcellular fractionation. Following 18 hours of VP-16 treatment, a substantial amount of cathepsin D was detected in the cytosol (Figure 3.4). Because such a large amount of cathepsin D protein is expressed in these cells, it was difficult to discern a loss of cathepsin D in the drug-treated lysosomal fraction via immunoblotting (data not shown).

To investigate the kinetics of cathepsin D release during chemotherapy treatment, U937 cells were treated with 2 μM VP-16 for varying lengths of time, followed by subcellular fractionation. Figure 3.5 shows a Western blot of cytosolic fractions from both VP-16-treated and DMSO-treated samples. Cathepsin D was detected in the

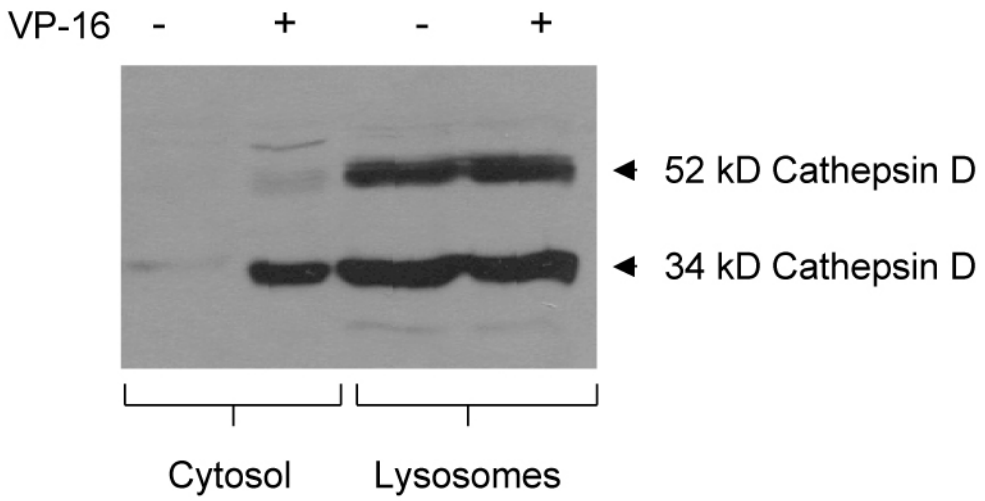


Figure 3.4. Cathepsin D relocalizes from the lysosomes to the cytosol following VP-16 treatment. U937 cells ($20x10^6$ cells) were treated with 2 μM VP-16 or 0.1% DMSO for 18 hours, and cytosolic and lysosomal fractions were isolated. Proteins (25 μg/lane) were electrophoresed on a 10% SDS-PAGE gel and subjected to immunoblotting with anti-cathepsin D antibody. The active fragment of cathepsin D is released into the cytosol following VP-16 treatment.

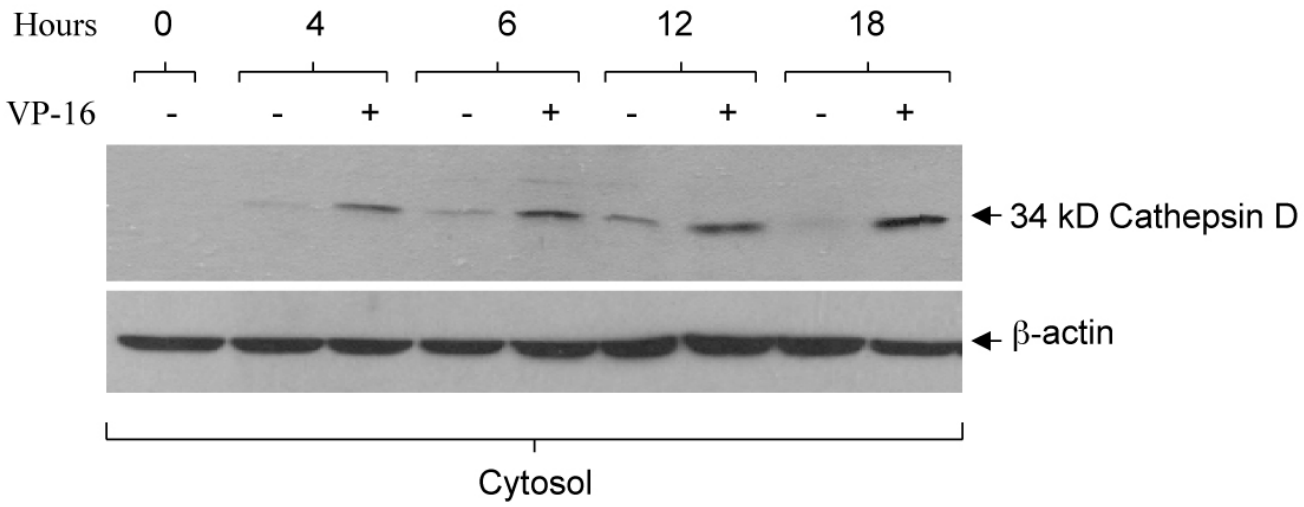


Figure 3.5. Cathepsin D is released into the cytosol over 18 hours of VP-16 treatment. Wild-type U937 cells were treated with 2 μM VP-16 over a period of 18 hours. At the indicated time points, cytosolic fractions were prepared from 20 x 10^6 cells. Cytosolic fractions (25 μg/lane) were electrophoresed on a 10% SDS-PAGE gel and immunoblotted for cathepsin D. Cathepsin D was first released into the cytosol after 4 hours of VP-16 treatment.

cytosol as early as 4 hours following VP-16 treatment, and the levels of cytosolic cathepsin D continued to rise out to 18 hours following drug treatment. A β-actin immunoblot is shown as a loading control. A small amount of cathepsin D was also detected in some DMSO-treated samples; this phenomenon may not be surprising, as DMSO is known to damage cellular membranes by increasing membrane fluidity [108, 109].

In support of the Western blotting data, a cathepsin D activity assay revealed that cathepsin D activity was increased in the cytosol of VP-16-treated cells relative to that in DMSO-treated control cells (Figure 3.6). Following 18 hours of treatment with 2 μM VP-16 or 0.1% DMSO, cytosolic fractions were obtained from U937 cells. Cathepsin D activity in the cytosol was assessed by incubation of cytosolic samples with a fluorogenic cathepsin D substrate (MoCAc-Gly-Lys-Pro-Ile-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2) for 1 hour at 37 °C. Cleavage of this substrate, measured by fluorescence at Ex_λ= 328 nm and Em_λ=393 nm, is depicted in Figure 3.6. Cytosolic cathepsin D activity, shown in fluorescence units, was found to increase in response to VP-16 treatment in U937 cells. Unfortunately, a rather high level of cathepsin D substrate cleavage was detected in the DMSO-treated sample as well. This could be explained by the following: first, the substrate used is not completely specific to cathepsin D. Most aspartate proteases, such as pepsin, proteinase A and gelatinase, have substrate specificities that are nearly identical to that of cathepsin D [69, 110]. They prefer to cleave dipeptide bonds between hydrophobic residues, particularly at Phe-Phe sequences [111]. Therefore, the presence of other cytosolic aspartate proteases that are capable of cleaving the cathepsin D substrate could lead to non-specific cleavage of the fluorogenic substrate, causing high

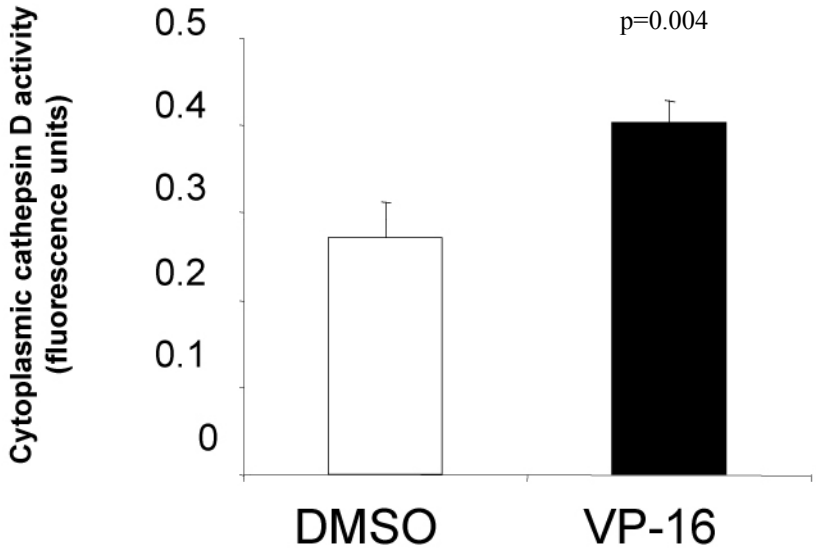


Figure 3.6. Cathepsin D activity in the cytosol increases following VP-16 treatment. U937 cells (20 x 10^6 cells) were treated with 2 μM VP-16 or 0.1% DMSO for 18 hours and cytosolic fractions were prepared. Cytosol was assayed for cathepsin D activity by measuring cleavage of the cathepsin D fluorogenic substrate (MoCAc-Gly-Lys-Pro-Ile-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH2). Cathepsin D activity in the cytosol increased following 2 μM VP-16 treatment. Assays were performed in triplicate and error bars indicate standard deviations. Statistical significance was determined using the VassarStats website (http://faculty.vassar.edu/lowry/VassarStats.html) by comparing cytosolic fractions from U937 cells treated with VP-16 versus cytosolic fractions from U937 cells treated with DMSO. A statistically significant difference ($p<0.05$) in cathepsin D activity was observed between DMSO- and VP-16-treated samples.

background levels. Second, the cathepsin D substrate is extremely photosensitive. Because experimental conditions were such that some degree of light exposure was unavoidable, the high background measured in DMSO-treated extracts may be due to nonspecific substrate degradation. Nonetheless, this result, taken together with the immunoblot depicting the time kinetics of cathepsin D release, shows that cathepsin D does translocate from the lysosomes to the cytosol during the course of VP-16 chemotherapy treatment.

3.2.5. CATHEPSIN B AND LYSOSOMAL MARKERS ARE NOT DETECTED IN THE CYTOSOL

Because chemotherapy drugs cause damage to the cell, it was necessary to establish that the release of cathepsin D from lysosomes was not due to a generalized destruction of the lysosome that results in an integration of the lysosomal contents with cytosol. Three different controls were used as markers of lysosomal integrity: cathepsin B, lysosomal membrane-associated protein (LAMP-1), and β-hexosaminidase.

Studies of the cysteine protease cathepsin B have shown that it is released from the lysosome and is important during cell death resulting from various stimuli including TNF-α, microtubule stabilizing agents, serum deprivation, and bile-salts [67, 69, 72, 112]. To date, a role for cathepsin B in chemotherapy-induced apoptosis has not been examined. Lysosomal and cytosolic fractions obtained from U937 cells were analyzed via immunoblot for release of cathepsin B into the cytosol following treatment with 2 μM VP-16 for 18 hours. As shown in Figure 3.7.A, after 18 hours, cathepsin B was detected in the lysosomal fraction, but was not detected in the cytosol. These results suggest that

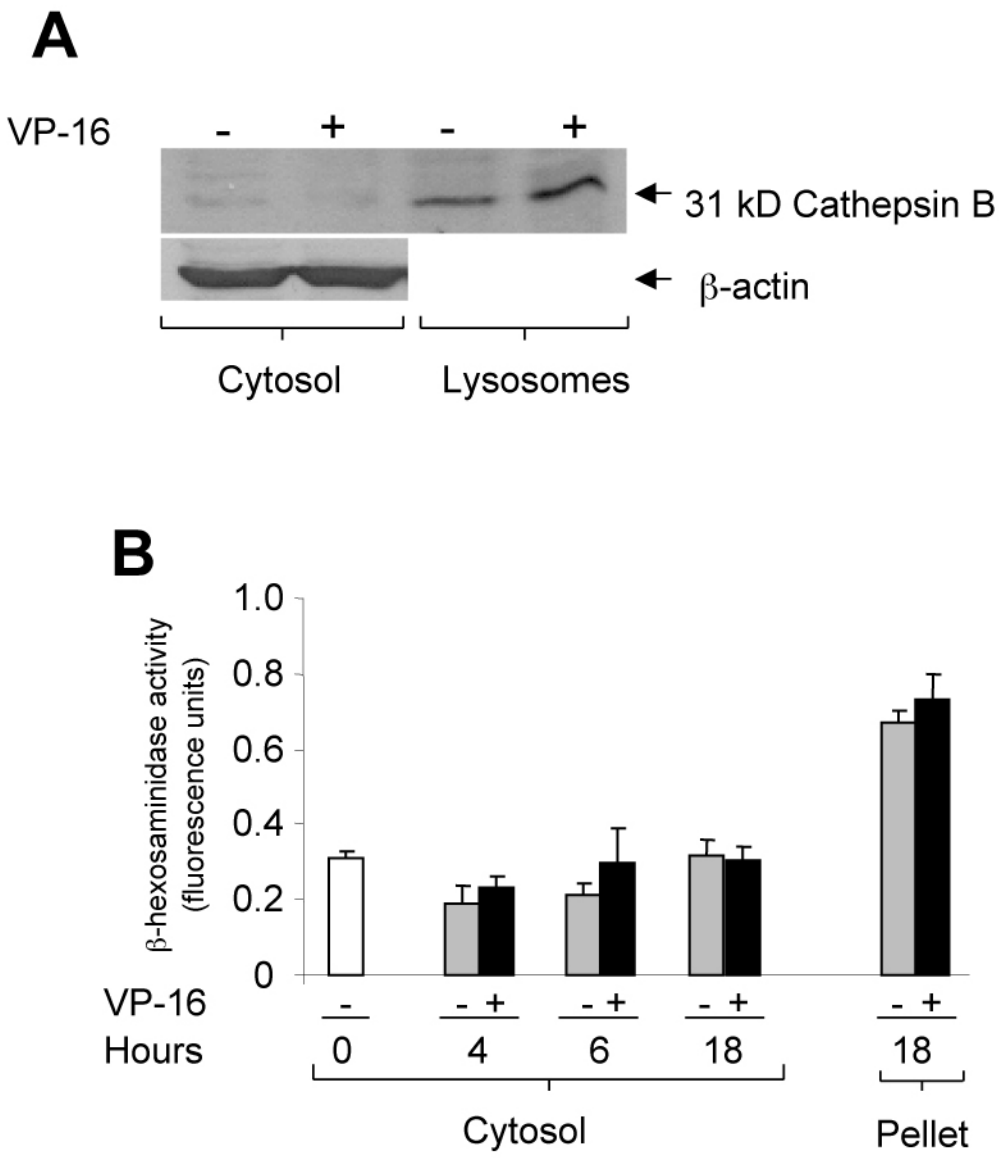

A
VP-16
-
+
-
+
31 kD Cathepsin B
β-actin
Cytosol
Lysosomes
B
β-hexosaminidase activity
(fluorescence units)
1.0
0.8
0.6
0.4
0.2
0
VP-16
Hours
0
4
6
18
18
Cytosol
Pellet
C
VP-16
LAMP-1
Cytosol
Lysosomes

Figure 3.7. Selective release of cathepsin D following VP-16 treatment. Cytosolic and lysosomal fractions from U937 cells treated with 2 μM VP-16 (+) or 0.1% DMSO (-) were immunoblotted for cathepsin B and LAMP-1, or assayed for β-hexosaminidase activity. *A,* An immunoblot of cathepsin B shows that it was not released into the cytosol following VP-16 treatment. *B,* The activity of β-hexosaminidase was accessed by measuring cleavage of the fluorogenic substrate 4-methylumberriferyl-N-acetyl-β-D-glucosaminide dehydrate. β-hexosaminidase was not released into the cytosol following VP-16 treatment. Error bars represent standard deviations. *C,* Immunoblotting with LAMP-1 shows that it was not released into the cytosol following VP-16 treatment.

cathepsin B remains in the lysosomes following chemotherapy treatment and that the pattern of cathepsins released during apoptosis may be either cell type- or stimulus-specific.

β-hexosaminidase is a resident lysosomal protein that functions normally to degrade terminal N-acetyl hexosamines within the cell. It is not released from intact lysosomes, and therefore, is a common control for distribution of lysosomal contents [113, 114]. To examine lysosomal integrity, β-hexosaminidase activity in cytosolic and lysosomal fractions was determined by measuring cleavage of the substrate, 4-methylumberriferyl-N-acetyl-β-D-glucosaminide dehydrate, following treatment of U937 cells with 2 μM VP-16. As shown in Figure 3.7.B, in untreated cells, less β-hexosaminidase activity was detected in the cytosol compared to lysosomes. Some non-specific degradation of the fluorogenic substrate may have occurred, as shown by the fact that there was modest β-hexosaminidase activity detected in the cytosol of untreated cells. More importantly, upon treatment with VP-16, there was no increase in cytosolic β-hexosaminidase activity even after 18 hours. No difference in cytosolic β-hexosaminidase activity was noted between untreated, DMSO-, and VP-16- treated samples, confirming the integrity of the lysosomes following drug treatment.

The lysosomal membrane-associated protein (LAMP-1) is a heavily glycosylated protein that is bound to the luminal surface of lysosomal membranes, where it functions to protect the membrane from attack by lysosomal enzymes [115]. One would expect to find LAMP-1 in the cytosolic fraction only if membranes were compromised or fragmented during apoptosis. Therefore, it is commonly used as a marker of intact lysosomes [116]. The Western blot in Figure 3.7.C shows that LAMP-1 was not detected

in the cytosol following VP-16 treatment. This data provided additional confirmation that lysosomal membranes remained intact during the experimental time course of chemotherapeutic insult. Altogether, these results show that the integrity of lysosomes is not compromised during VP-16-induced apoptosis and that the observed release of cathepsin D into the cytosol (Figure 3.5) was not the result of a general leakage of lysosomal contents.

3.2.6. KINETICS OF CASPASE-3 ACTIVATION AND CYTOCHROME C RELEASE: BOTH OCCUR LATER THAN CATHEPSIN D RELOCALIZATION

One of the hallmark features of apoptosis is the activation of the caspase proteases. Caspases are critical for cell death induced by chemotherapeutic drugs. Apoptotic processes are complex, and although caspase-independent cell death does occur, most cell death pathways involve caspase activation [7, 117, 118]. It was imperative, therefore, to assess the kinetics of cathepsin D release relative to the kinetics of cytochrome c release and caspase activation. Analysis of cytosolic fractions of VP-16-treated U937 cells for cytochrome c release revealed that cytochrome c was first detected in the cytosol roughly 8 hours after VP-16 treatment (Figure 3.8.A). Additionally, U937 cells were treated with VP-16 for varying lengths of time, and whole cells lysates were analyzed for the activation of caspase-3. Active caspase-3 fragments (17 and 12 kD) were first detected approximately 8 hours after VP-16 treatment, presumably shortly after cytochrome c release (Figure 3.8.B). Cathepsin D was first detected in the cytosol 4 hours following VP-16 treatment (Figure 3.5), well before the release of cytochrome c or

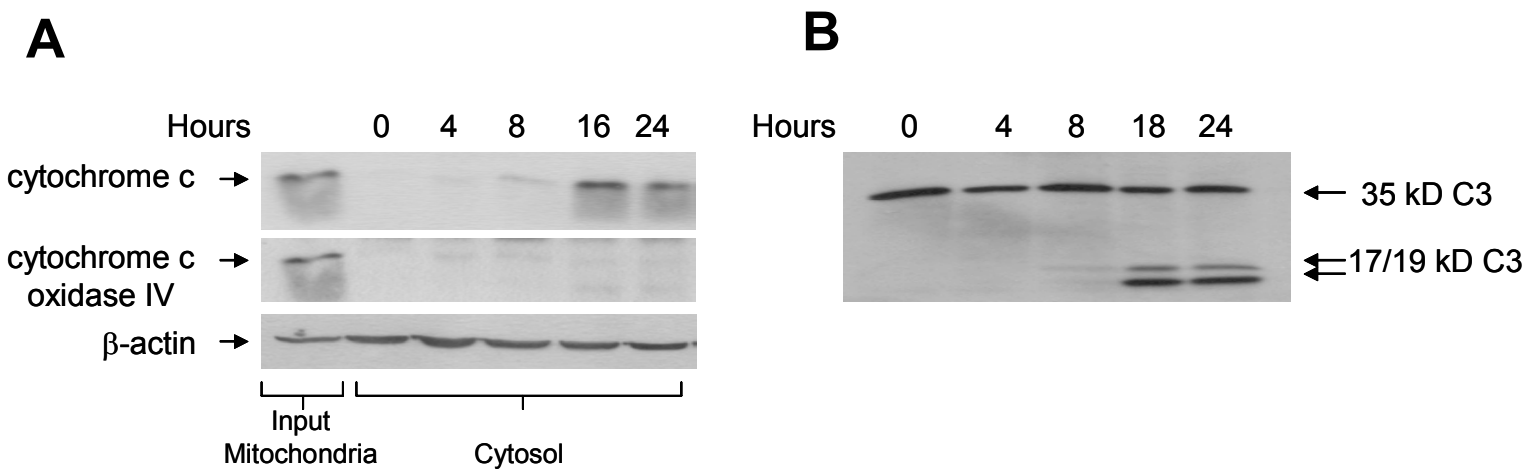


Figure 3.8. Cathepsin D release into the cytosol occurs earlier than cytochrome c release or caspase-3 activation. Wild-type U937 cells were treated with 2 μM VP-16 for varying times. *A,* Cytosolic and mitochondrial extracts were prepared from 20 x 10^6 cells and proteins (25 μg/lane) were electrophoresed on a 13% SDS-PAGE gel. Immunoblotting with anti-cytochrome c shows that cytochrome c was first detected in the cytosol approximately 8 hours following VP-16 treatment. *B,* Whole cell lysates were prepared from 5 x 10^6 cells at varying times and proteins (25 μg/lane) were electrophoresed on a 10% SDS-PAGE gel and immunoblotted for caspase-3. Active caspase-3 fragments were first detected in the cytosol roughly 8 hours following VP-16 treatment.

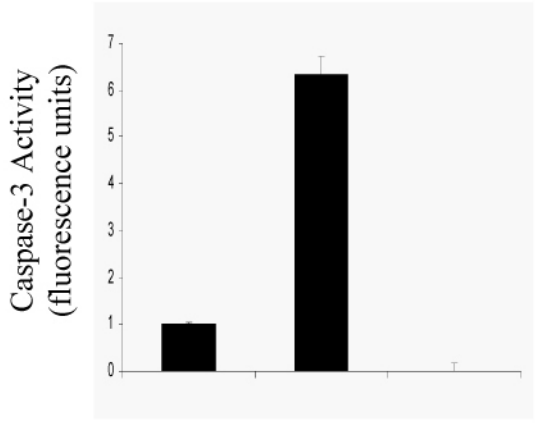
the activation of caspase-3. This data supports the hypothesis that cathepsin D may act upstream of caspase activation in VP-16-treated U937 cells.

3.2.7. CATHEPSIN D RELOCALIZATION IS NOT INHIBITED BY CASPASE INHIBITOR OR CATHEPSIN D INHIBITOR

The appearance of cathepsin D in the cytosol of U937 cells occurs roughly 4 hours after VP-16 treatment. On the other hand, caspase-3 activation was not detected until 8 hours post-drug treatment. To determine whether release of cathepsin D was dependent on the activation of caspases, U937 cells were pretreated with a general cysteine caspase inhibitor, z-VAD-FMK, for 1 hour at 37 °C prior to VP-16 treatment. A caspase activity assay in Figure 3.9.A shows that 50 μM z-VAD-FMK was sufficient to completely inhibit caspase-3 activity in VP-16-treated cells. As shown in Figure 3.9.B, cathepsin D release into the cytosol following VP-16 treatment was not prevented by caspase inhibitor. Therefore, the cytosolic relocalization of cathepsin D was not dependent on caspase activation in VP-16-treated cells.

To assess the importance of cathepsin D activity in its release, U937 cells were pretreated with the cathepsin D inhibitor pepstatin A for 1 hour prior to VP-16 treatment. 100 μM pepstatin A was sufficient to completely abrogate cathepsin D activity (data not shown). Figure 3.10 shows that cathepsin D relocalization was not dependent on its own activity. In fact, release of cathepsin D is slightly enhanced by pretreatment with pepstatin A, a reproducible yet puzzling phenomenon which will be addressed in the discussion. Nonetheless, these results illustrate that cathepsin D relocalization to the cytosol in VP-

A



VP-16 (2 μM)	-	+	+
z-VAD (50 μM)	-	-	+

B

z-VAD (50 μM)	-	-	+
VP-16 (2 μM)	-	+	+

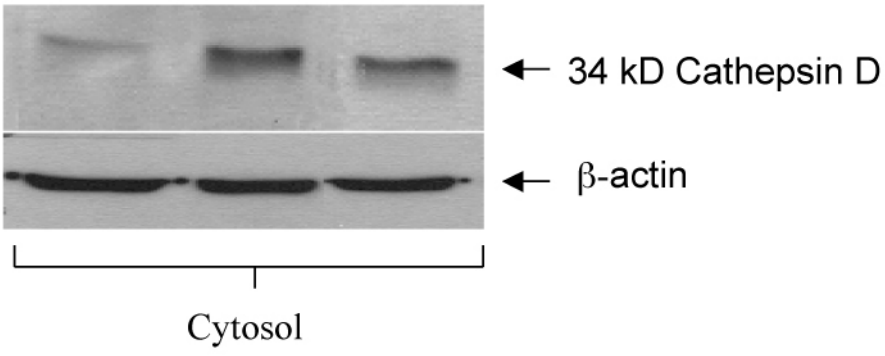


Figure 3.9. Release of cathepsin D into the cytosol is not dependent on caspase activation. Wild-type U937 cells were treated with 50 μM z-VAD-FMK for 1 hour prior to 2 μM VP-16 treatment for 18 hours. Cytosolic fractions were prepared from 20 x 10^6 cells. *A,* Proteins (20 μg/sample) from cytosolic fractions were assessed for caspase-3 activity by measuring cleavage of the fluorogenic substrate Ac-DEVD-AFC. Caspase-3 activity increased substantially following VP-16 treatment, but was completely abolished by z-VAD-FMK. Assays were performed in triplicate and error bars represent standard deviations. *B,* Proteins (25 μg/lane) from cytosolic fractions were electrophoresed on a 10% SDS-PAGE gel and subjected to immunoblotting with anti-cathepsin D antibody. Release of cathepsin D into the cytosol was not inhibited by the pancaspase inhibitor z-VAD-FMK.

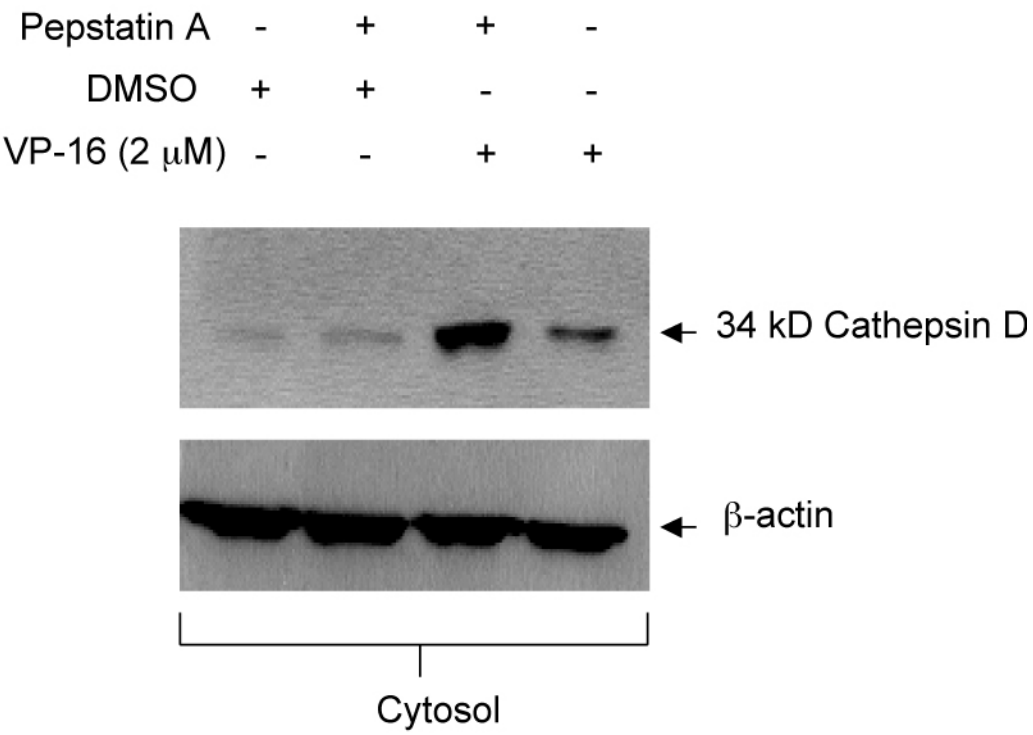


Figure 3.10. Release of cathepsin D into the cytosol is not dependent on cathepsin D activity. Wild-type U937 cells were treated with 100 μM pepstatin A prior to 2 μM VP-16 treatment for 18 hours. Cytosolic fractions were prepared from 20 x 10^6 cells. Proteins (25 μg/sample) were electrophoresed on a 10% SDS-PAGE gel and subjected to immunoblotting with anti-cathepsin D antibody. The Western blot shows that release of cathepsin D into the cytosol was not prevented by pepstatin A.

16-treated cells was not dependent on its own activity or on the activation of caspase proteases.

3.3. DISCUSSION

The results in this chapter show that following treatment with the chemotherapeutic agent VP-16, cathepsin D is relocalized from the lysosomes to the cytosol. By pinpointing the cytoplasm as the location of cathepsin D during apoptosis, these data provide supportive evidence for the hypothesis that cathepsin D plays an important role in chemotherapy-induced apoptosis. The conundrum associated with these findings is that cathepsin D is normally located within an acidic compartment with a pH of 5. Proteolytic enzymes contain an active binding pocket, into which "matching" substrates must fit correctly within the binding pocket in order for cleavage to occur. Alterations in pH can cause significant modifications in secondary structure when the protonation states of ionizable residues change, and amino acids reconfigure to form the most thermodynamically stable structure [119]. The result of these conformational changes can be a protein with a significantly altered binding pocket, often rendering the enzyme inactive. Because cathepsin D is most active at acidic pH, it was necessary to ascertain whether activity was retained at higher pH. While the pH of the cytosol in a normal cell is close to neutral, the cytosolic pH of an apoptotic cell is slightly more acidic, sometimes as low as 6.3 [102, 120]. It was found that cathepsin D retained the ability to cleave caspase-8 up to a pH of 7.0. At a pH of 4.5, cathepsin D completely degraded caspase-8 so that no fragments were discernible by Western blot. However, at pH 6.5, cathepsin D cleaved caspase-8 to 44/47 kD fragments. While these fragments are

not the typical "active" fragments of caspase-8, the ability of cathepsin D to produce them shows that while cathepsin D enzymatic activity is lessened at a higher pH, it is not abolished.

The reduced activity of cathepsin D at higher pH might be advantageous to sustaining the apoptotic process. While destruction of an anti-apoptotic factor during apoptosis might not pose a problem, if fully-activated cathepsin D were to cleave a pro-apoptotic protein, the protein might simply be destroyed rather than activated.

Though a substantial amount of cathepsin D is released into the cytosol following VP-16 treatment, a large amount of cathepsin D remains within the lysosomes. Discerning a difference between the levels of lysosomal cathepsin D in DMSO- and VP-16-treated cells was difficult via Western blot. The release of only a small portion of the total cathepsin D pool still may have very significant consequences for the cell. The cathepsin D protein is expressed in very high levels in virtually all cell types [82]. Therefore, even if only a small percentage of total cathepsin D is released into the cytosol, it still may represent a substantial amount of protein. Additionally, only a small amount of enzyme is required to initiate an enzymatic cascade. For instance, only a fraction of the total amount of mitochondrial cytochrome c must be released from mitochondria to induce apoptosis [121-125]. If the function of cathepsin D is to cleave an apoptotic regulatory factor to initiate a downstream process, only a small amount of cathepsin D would be necessary to initiate enzymatic progression.

It is also possible that the appearance of cathepsin D in the cytosol following VP-16 treatment results from increased expression of a cytosolic form of cathepsin D that lacks lysosomal targeting signals. While this possibility may be unlikely as the cathepsin

D protein contains several lysosomal targeting signals, use of the protein translation inhibitor cycloheximide in relocalization experiments would verify whether cathepsin D appearance in the cytosol is directly due to relocalization of cathepsin D from the lysosomes.

In addition to investigating localization of cathepsin D during chemotherapy-induced apoptosis, the localization of other key lysosomal proteins was also examined. β-hexosaminidase is a resident lysosomal protease that functions in the lysosomes to degrade terminal N-acetyl hexosamines within the cell [113]. Because β-hexosaminidase is not released from intact lysosomes, it is commonly used as a marker of lysosomal integrity [126-128]. Cleavage of a small amount of β-hexosaminidase substrate was detected in the untreated cytosolic fraction, probably due to nonspecific cleavage of the fluorogenic substrate. Importantly, β-hexosaminidase activity in the cytosol was significantly less than in the lysosomes. Moreover, cytosolic β-hexosaminidase activity did not increase even after 18 hours of drug treatment, and there were no differences between DMSO- and VP-16- treated samples. The absence of increased β-hexosaminidase in the cytosol following VP-16 treatment suggests that lysosomes remain intact during chemotherapy treatment.

The cytosolic presence of another lysosomal protein, LAMP-1, following VP-16 treatment was also examined. LAMP-1 is a membrane protein which is bound to lysosomal membranes to protect them from attack from the digestive enzymes of the lysosome [115]. The absence of LAMP-1 in the cytosol suggests that lysosomal membranes remain intact during chemotherapy-induced damage. This data, taken in conjunction with the β-hexosaminidase data, strongly suggests that lysosomal integrity is

preserved during VP-16 induced apoptosis and that cathepsin D release is not due to a general rupture of the lysosome and release of lysosomal contents.

If lysosomes do not release all of their contents during chemotherapy-induced apoptosis, then how is cathepsin D released? Bidere *et al.* [99] have shown that during staurosporine-induced apoptosis, lysosomal proteins are released in a size-selective fashion, as preloaded FITC-dextran molecules under 70 kD were released from lysosomes, while larger dextrans were not. However, relocalization of lysosomal proteins following VP-16 treatment may not be size-dependent since the 34 kD cathepsin D protein was released but the 31 kD cathepsin B protein was not. It is possible, however, that if cathepsin B exists in a complex within lysosomes, its apparent molecular weight may appear much higher. To rule out a size-dependency in the release of lysosomal proteins following VP-16 treatment, dextran experiments could be conducted using VP-16 as in the case of staurosporine [99].

Although the mechanism of cathepsin D release remains unclear, and was not investigated in this project, there are several possible mechanisms. A conformational change resulting from enzyme activation might allow cathepsin D to adopt a temporary unfolded structure that could allow it to pass through the lysosomal membrane. Proteolytic processing of the cathepsin D enzyme occurs during activation to the 34 kD fragment. Though active cathepsin D is always present within the lysosome, it is possible that VP-16 treatment leads to slight additional processing that alters the structure of cathepsin D, allowing it to pass through the lysosomal membrane. Alternatively, the heavily glycosylated side chains of cathepsin D contain many potential phosphorylation sites, and modulation of these sites may play a role in release [79]. It is possible that

removal of the cathepsin D phosphorylation sites via cleavage may alter the structure of cathepsin D so that it no longer possesses "recognition signals" that are necessary for sequestration in the lysosomes. To date, however, no one has offered experimental data to delineate a mechanism for specific cathepsin D release from the lysosomes.

Evidence does exist to indicate that cathepsin B is specifically released from the lysosomes following stimulation with TNF-α [70]. However, I did not observe cathepsin B release in response to VP-16 treatment, suggesting that it is not involved in etoposide-induced apoptosis. It is likely that the pattern of proteolytic enzymes released from lysosomes during apoptosis varies depending on both the apoptotic stimuli and on the cell type.

Although VP-16-induced caspase activation occurred at a later time point than release of cathepsin D into the cytosol, it was important to investigate whether caspase activation impacted cathepsin release. In addition to acting as executioners during apoptosis, some caspases further promote cell death by participating in feedback activation loops. For instance, though caspase-3 is activated downstream of caspase-9 during apoptosis, caspase-3 can in turn cleave caspase-9 to further promote amplification of the caspase cascade [129, 130]. In one study, cytochrome c release initiated by etoposide was found to occur in two stages: an early, modest release of cytochrome c promoted activation of caspase-9 and caspase-3, while late cytochrome c release resulted in a drastic change in mitochondrial membrane potential and substantial loss of mitochondrial cytochrome c. Interestingly, the late stage cytochrome c release was inhibited by the caspase inhibitor z-VAD, suggesting that a feedback loop causes caspases to induce further mitochondrial dysfunction and late stage cytochrome c release

[17]. One of the potential roles of cathepsin D in chemotherapy-induced apoptosis may be to enhance caspase activation either by direct cleavage or by stimulation of upstream apoptotic factors that lead to caspase activation. To investigate whether the release of cathepsin D from lysosomes was dependent on caspase feedback activation, cells were treated with the pancaspase inhibitor z-VAD-FMK prior to treatment with VP-16. The data in this chapter showed that caspase inhibition did not prevent cathepsin D relocalization to the cytosol. Cathepsin D release from lysosomes, therefore, is not dependent on caspase activation.

To investigate whether cathepsin D relocalization was dependent on its own activity, cells were treated with 100 μM pepstatin A, a dose sufficient to inhibit cathepsin D activity in the cell, prior to VP-16 treatment. Pepstatin A contains an unusual statyl residue (4-amino-3-hydroxy-6-methylheptanoic acid) that is necessary for irreversible binding of the inhibitor to the active enzyme [131, 132]. The data in this chapter suggests that pepstatin A does not prevent release of cathepsin D from the lysosomes; in fact, pepstatin A modestly enhanced cathepsin D release. This is a puzzling phenomenon that has not been noted in other studies. It was suggested earlier in this discussion that cleavage of cathepsin D might temporarily alter the enzyme's structure so that it can pass easily out of the lysosomes. It is possible that, if pepstatin A binds to active cathepsin D, it might "freeze" this structure so that it remains in an advantageous conformation for release. Further studies are necessary to determine the importance of pepstatin A on the cytosolic relocalization of cathepsin D following chemotherapy treatment.

3.4. CONCLUSION

In conclusion, the data presented in this chapter have shown that cathepsin D was not present in the cytosol of untreated U937 cells. Cathepsin D was released from the lysosomes about four hours after VP-16 treatment, prior to release of cytochrome c or activation of caspase-3. Cytosolic accumulation of the lysosomal proteins β-hexosaminidase, LAMP-1, and cathepsin B was not observed, suggesting that the release of cathepsin D is the result of a selective process. Cathepsin D release was not dependent on caspase activity, nor was it dependent on its own activity. These results provide support for the hypothesis that cathepsin D is important in chemotherapy-induced apoptosis by placing cathepsin D at the cellular location of important apoptosis signaling events.

4. DOWNREGULATION OF CATHEPSIN D INHIBITS CHEMOTHERAPY-INDUCED CELL DEATH

4.1. INTRODUCTION

I have hypothesized that cathepsin D is important in chemotherapy-induced apoptosis. To directly test this hypothesis, I chose to utilize RNA interference technology to downregulate expression of the cathepsin D protein in U937 and HeLa cells to determine whether cathepsin D downregulation provides protection against chemotherapy-induced cell death.

4.1.1. COMMON METHODS USED TO DOWNREGULATE PROTEIN EXPRESSION

There are advantages and disadvantages to common methodologies for downregulating the expression or activities of proteins. Before the conception of gene-targeting methods, the primary means of enzyme inhibition was use of a pharmacologic inhibitor. In fact, pharmacologic inhibitors such as DEVD, z-VAD, and IETD are still commonly used to inhibit caspase proteases [27, 133, 134]. Using pharmacologic inhibitors is advantageous in several ways. Many are cell permeable, and by requiring only a short incubation with cells to confer their inhibitory effects, they are easy to use. The mechanism of most protease inhibitors is to bind the active site of the enzyme, thus preventing substrate entry and binding [135]. However, because many enzymes have analogous substrate specificities, pharmacologic inhibitors are often not specific for one enzyme and can even inhibit an entire family of enzymes. Many of the studies that have investigated the importance of cathepsin D in apoptosis have employed the

pharmacologic inhibitor pepstatin A. For example, Deiss *et al.* [88] utilized pepstatin A to examine the importance of cathepsin D in TNF-α-, IFN-γ-, and Fas/APO-1 ligand-induced apoptosis. Similarly, Bidere *et al.* [99] found that staurosporine-induced cell death was delayed by pepstatin A. The serious problem with using pepstatin A to study the effects of cathepsin D is that pepstatin A inhibits several aspartic proteases, including renin, pepsin, and proteinase A [131, 132]. Thus, the promiscuity of pepstatin A precludes drawing definitive conclusions about the specific role of cathepsin D.

Technology developed in the 1980s allowed researchers to target enzymes at the level of gene expression by introducing an antisense molecule complementary to the mRNA of the gene of interest [136]. Once introduced into the cell, the antisense molecule binds to the complementary mRNA sequence to form a duplex, and thus prevents translation of the gene product. Antisense-mediated protein downregulation can be inefficient, due partly to the stoichiometry of the methodology [140]. One molecule of antisense cDNA is required to silence one mRNA target. Therefore, large numbers of antisense molecules are required to elicit efficient silencing.

4.1.2. RNA INTERFERENCE

Direct cellular introduction of small interfering RNAs (siRNAs) has overcome many of the hurdles associated with the inefficiency of antisense RNAs and the cytotoxicity associated with long double-stranded RNA molecules. siRNAs are small double-stranded RNAs that, like antisense and long dsRNA, target and degrade mRNA homologous to the siRNA sequence [137, 138]. RNA interference (RNAi) was first observed inadvertently by plant biologists who were attempting to enhance the petal color

of petunias by introducing a promoter sequence for chalcone synthase, a pigment-inducing gene. Instead of generating brighter flowers, the plants produced flowers completely devoid of color [139, 140]. Though these results puzzled scientists at the time, the discovery that direct injection of a double-stranded RNA into the nematode *C. elegans* posttranscriptionally silenced the genes homologous in sequence to the injected dsRNA enlightened researchers to the world of RNA interference [141, 142].

There are two commonly accepted methods for producing siRNAs in cells. In the first method, short (typically 21-nucleotide) siRNA duplexes with 2-nucleotide 3'-overhangs are synthesized and transfected into cells. The duplexes then directly activate the cellular RNAi system for silencing of homologous mRNA targets. The advantage of this method is that siRNA-mediated protein downregulation occurs within 1-2 days of transfection. The disadvantage of direct siRNA delivery is that protein downregulation is transient (5-6 days) because the siRNAs are degraded quickly. Additionally, because cells must be transfected prior to each experiment, the degree of protein downregulation may change due to variable transfection efficiency. In the second method, a plasmid or virus that codes for an oligonucleotide homologous to a sequence of interest and a selectable marker such as hygromycin B, puromycin, or neomycin is transfected into cells. Vectors used for siRNA production typically contain an RNA polymerase III promoter that is capable of producing large numbers of the siRNAs. The transfected cells are cultured in the presence of the appropriate selection media, and presumably only cells that stably express the transfected plasmid will grow. These cells are capable of continually producing the appropriate siRNAs to elicit specific protein downregulation. While siRNA clones may take weeks to grow via this method, once clones are

established, their levels of protein downregulation presumably remain stable. For the experiments described in this chapter, I chose to establish stable siRNA clones that downregulate cathepsin D expression in both HeLa cells and U937 cells.

There are distinct advantages to using siRNAs to silence cathepsin D. First, because of their small size (typically not more than 21-23 nucleotides in length), siRNAs do not activate the mammalian dsRNA-inducible interferon system that is normally initiated in response to cellular introduction of long double-stranded RNA [137, 138]. This interferon response can lead to alteration of cellular phenotype or cytotoxicity by causing nonspecific gene suppression. Second, siRNA-mediated gene suppression can be highly efficient [140]. In this chapter, downregulation of cathepsin D using siRNA was measured to be at least 95% via Western blotting and cathepsin D activity assays. Third, it is possible to search for and use nucleotide sequences that are homologous only to the gene of interest. Therefore, the specificity of downregulation using siRNAs can be better than the specificity of enzyme inhibition using a pharmacologic inhibitor such as pepstatin A.

Figure 4.1 outlines a schematic model for the molecular mechanism of RNA interference. Double-stranded RNAs are introduced to the cell and are cleaved by the Dicer endonuclease to 21-nucleotide duplexes [138, 143]. In the cell, these duplexes recruit and activate the RNA-induced silencing complex (RISC), a multiprotein endonuclease complex that is responsible for specific degradation of target mRNA [144]. Though the mechanism by which siRNA constrains RISC is not clearly understood, it is known that siRNAs shuttle RISC to homologous mRNAs, where the antisense strand

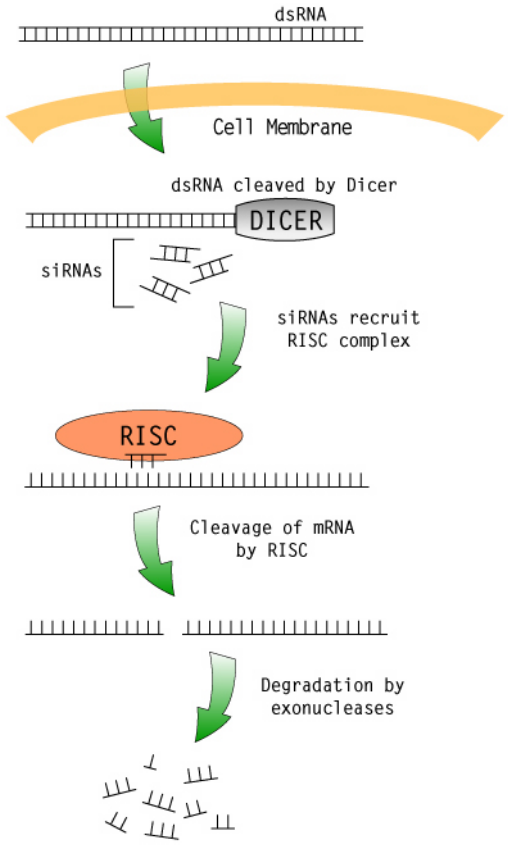


Figure 4.1. Schematic diagram of the siRNA-induced silencing mechanism. Double-stranded RNA enters the cell and is cleaved by the endonuclease Dicer to 21-25 nucleotide small interfering RNAs (siRNAs) with 2-nt 3' overhangs. These small dsRNAs recruit and associate with the endonuclease complex RISC. By a process that is poorly understood, the siRNAs unwind and guide RISC to complementary mRNAs. A base-pairing mechanism between the mRNA and siRNA promotes activated RISC to cleave and degrade target mRNAs.

of the siRNA duplex unwinds and base-pairs with complementary mRNAs [138, 140, 144]. Activated RISC cleaves and degrades sequence-specific mRNA, resulting in gene silencing.

Ideal siRNA nucleotide sequences have several characteristics. Most are 21-23 nucleotides in length, with 2 nucleotide "overhangs" at the 3' ends [137]. The overhangs are typically a TT sequence, which acts as an efficient cleavage recognition sequence for Dicer, as a recognition sequence for RISC, and also as a feature that renders the siRNA itself more resistant to further degradation by nucleases [138, 145]. Appropriate target sequences in the gene exist in the open reading frame of the mRNA, at least 50-100 nucleotides downstream of the start codon [145]. Most importantly, it is critical that the target sequences are only found in the gene of interest so that silencing is specific.

4.2. RESULTS

4.2.1. DESIGN AND PREPARATION OF CATHEPSIN D siRNA OLIGONUCLEOTIDES

To downregulate the cathepsin D protein, target sequences at varying locations in the cathepsin D mRNA were chosen using criteria from the Ambion siRNA target finder (Figure 4.2.A) [146]. Five oligonucleotides were designed to target the cathepsin D mRNA (Figure 4.2.B). For each siRNA sequence, two 62-63-nt oligonucleotides were constructed with a 9-nt "loop" separating a 21-nt sense sequence and its complementary sequence, a 5-6 nucleotide poly(T) sequence at the 3' end, and *Bam HI* and *Hind III*

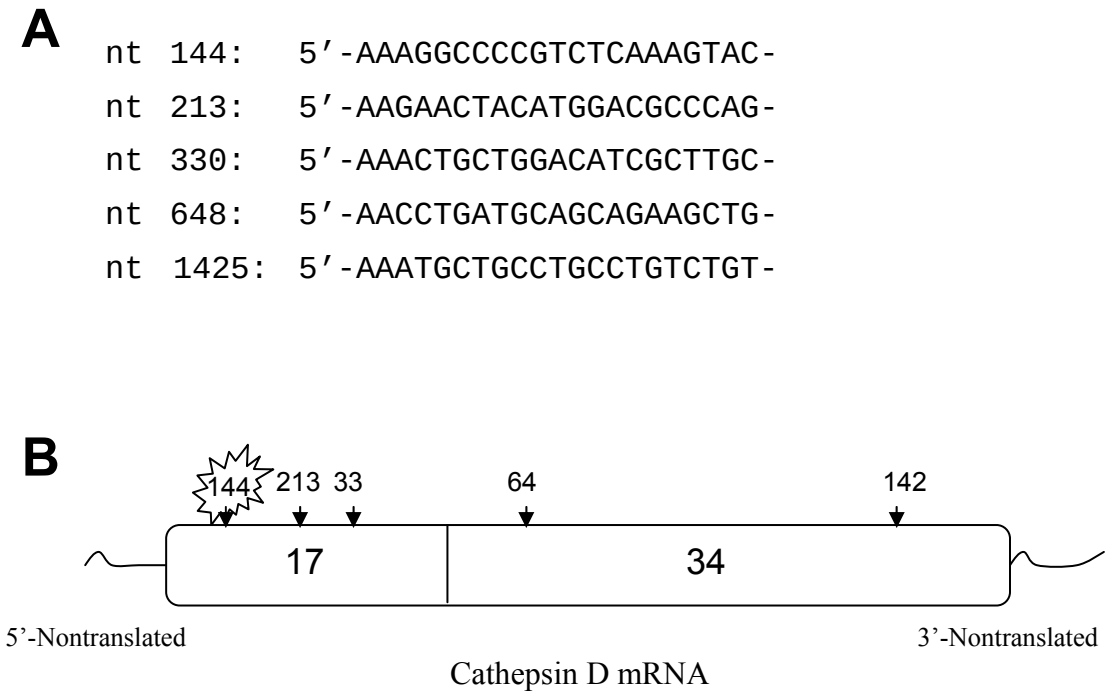


Figure 4.2. Nucleotide sequences selected as silencing targets in the cathepsin D mRNA. *A,* The five 21-nucleotide cathepsin D siRNA sequences. The 144 siRNA sequence targeting codons 50-55 in human cathepsin D mRNA was the only sequence to evoke efficient downregulation of cathepsin D protein. *B,* A schematic representation of the five sites targeted for silencing in the cathepsin D mRNA is shown.

restriction sites at the ends. (Figure 4.3). Typically, to produce stable siRNA molecules in cells, expression vectors with dual promoters are used to express the two strands of the siRNA. However, this project utilized hairpin structure siRNAs because only one vector is necessary for expression of the entire siRNA oligonucleotide and folding into the double-stranded structure is more efficient [147]. In our case, the oligonucleotide sequences were designed to generate *Bam HI* or *Hind III* restriction sites at the 5' and 3' ends, respectively, of the annealed insert fragments to facilitate insertion into the silencing vector. The oligonucleotides were ligated and annealed into the p*Silencer* 2.1-U6 hygro vector, which contains a hygromycin B resistance gene for selection in cells. Hygromycin B is an aminoglycoside antibiotic that inhibits protein synthesis by interfering with translocation of ribosomes [148]. The hygromycin resistance gene initiates phosphorylation of hygromycin, thus inactivating it and conferring antibiotic resistance. The p*Silencer* 2.1-U6 vector also utilizes a U6 RNA polymerase III promoter, frequently used in siRNA production because it is a very strong promoter capable of driving expression of large amounts of siRNAs (www.ambion.com). Figure 4.4 depicts the p*Silencer* 2.1-U6 hygro vector map.

4.2.2. CATHEPSIN D IS DOWNREGULATED IN U937 AND HELA CELLS BY siRNA

Five oligonucleotides corresponding to unique sequences in the cathepsin D mRNA were subcloned into p*Silencer* vector 2.1-U6, then transfected into HeLa and U937 cells using the GenePORTER2 transfection reagent (see Materials and Methods). Stably transfected cells that grew in the presence of hygromycin B were expanded in cell

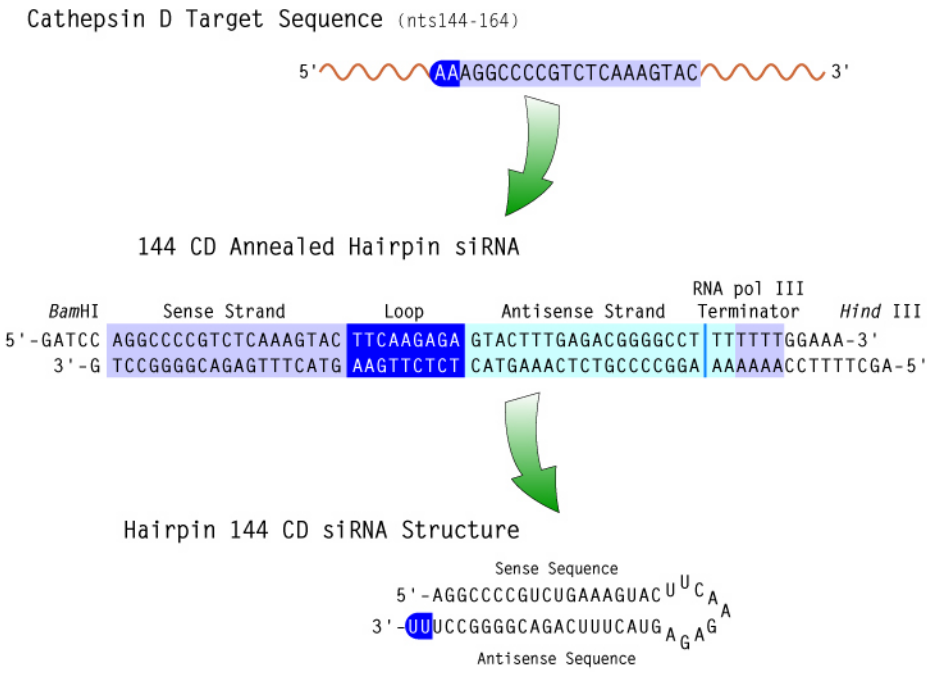


Figure 4.3. Design of the 144 CD siRNA oligonucleotide hairpin loop. Oligos are designed by connecting a 21-nucleotide siRNA sequence to the complementary sequence with a 9-nucleotide "loop" sequence. *Bam HI* and *Hind III* restriction sites are added to the ends for ligation into the vector. When transcribed, the siRNA structure forms a double-stranded structure that is capable of recruiting the RISC complex *in vivo*.

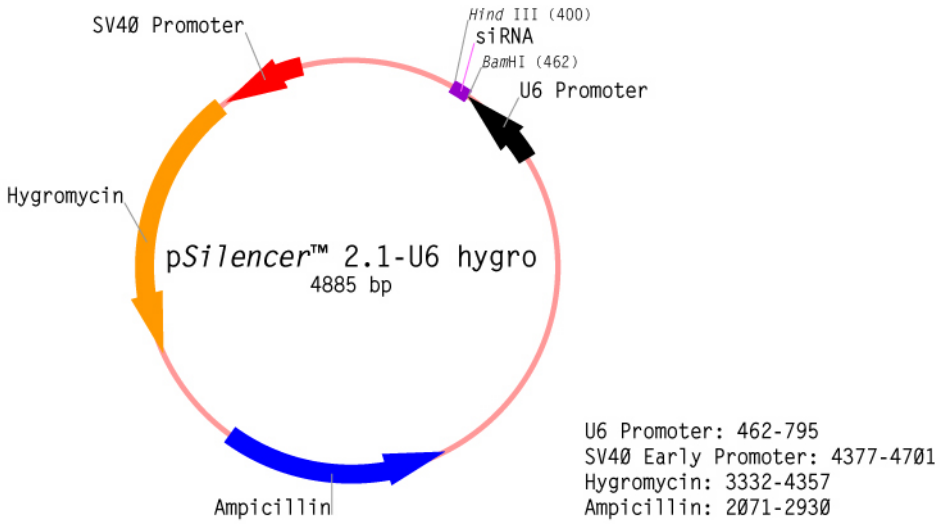


Figure 4.4. Vector map of p*Silencer* 2.1-U6 hygro. siRNA oligonucleotides were ligated into p*Silencer* between the *Bam HI* and *Hind III* restriction sites. Following transfection of U937 and HeLa cells, cell lines harboring the siRNA constructs were selected in media containing 250 μM hygromycin B.

culture, and independent clonal lines were isolated by limiting dilution (U937) or colony cloning (HeLa). Additionally, a p*Silencer* 2.1 U-6 construct containing a 21-nucleotide siRNA sequence with no homology to any known gene was transfected into HeLa and U937 cells as a control. Of the five transfections, three (containing siRNA sequences directed towards nucleotides-144, -248, and -1425) produced viable clones. These clones were expanded in hygromycin B-containing media and whole cell lysates from the clones were examined for the presence of cathepsin D. Cathepsin D downregulation was successfully achieved in the clones containing a sequence directed towards nucleotides 144 -164 of cathepsin D mRNA (Figure 4.5). In Figure 4.5.A, Western blotting was used to compare cathepsin D protein expression in wild-type HeLa cells, HeLa cells expressing the control siRNA sequence (control siRNA/HeLa), and HeLa cells expressing the 144 cathepsin D (CD) siRNA (144 CD siRNA/HeLa). While cathepsin D expression in cells harboring the control siRNA was unaffected, cathepsin D expression in cells containing the 144 CD siRNA was downregulated by greater than 95% (densitometric scanning, not shown). Control immunoblots for caspase-8, caspase-3, β-actin (Figure 4.5.A), PARP, and XIAP (data not shown) verified that expression of these proteins was unaffected and that downregulation of cathepsin D was specific. In support of this data, a cathepsin D enzymatic activity assay of whole cell lysates showed that cleavage of the cathepsin D fluorogenic substrate MoCAc-Gly-Lys-Pro-Ile-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2 was inhibited in two different HeLa cell clones expressing the 144 CD siRNA (Figure 4.5.B).

In U937 cells, immunoblotting reveals that cells harboring the 144 CD siRNA (144 CD siRNA/U937) also exhibited greater than 95% downregulation of cathepsin D

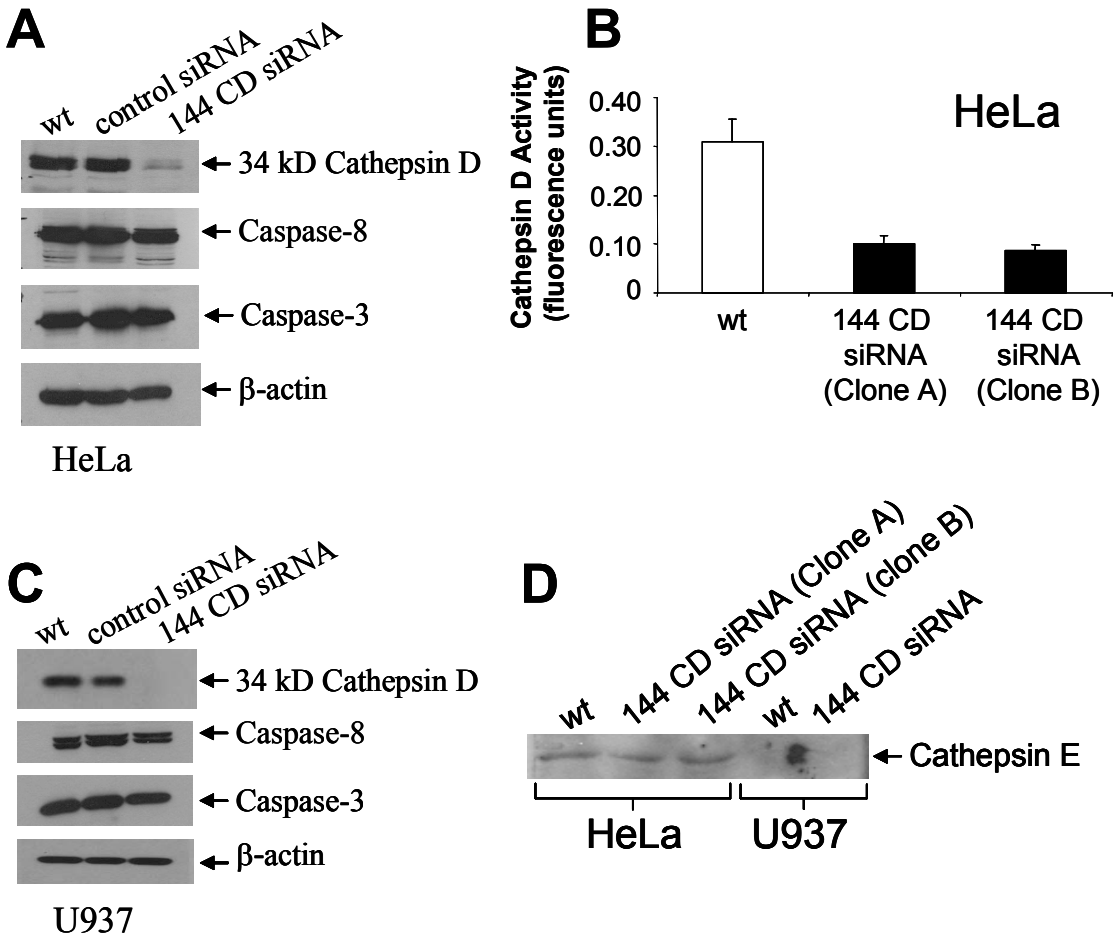

A
wt
control siRNA
144 CD siRNA
34 kD Cathepsin D
Caspase-8
Caspase-3
β-actin
HeLa
B
Cathepsin D Activity (fluorescence units)
0.40
0.30
0.20
0.10
0
HeLa
wt
144 CD siRNA (Clone A)
144 CD siRNA (Clone B)
C
wt
control siRNA
144 CD siRNA
34 kD Cathepsin D
Caspase-8
Caspase-3
β-actin
U937
D
wt
144 CD siRNA (Clone A)
144 CD siRNA (clone B)
wt
144 CD siRNA
Cathepsin E
HeLa
U937

Figure 4.5. siRNA-mediated specific downregulation of cathepsin D in U937 and HeLa cells. *A,* Cathepsin D protein expression is downregulated in clonal HeLa cells transfected with p*Silencer* plasmid containing the cathepsin D (CD) 144 siRNA sequence (see Materials and Methods). The control lane depicts a 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 siRNA/HeLa cells, and 144 CD siRNA/HeLa cells were subjected to immunoblotting with cathepsin D, caspase-8, caspase-3, or β-actin antibody. The data show specific downregulation of cathepsin D protein in the 144 CD siRNA/HeLa cells. *B,* Cathepsin D enzymatic activity was assayed in whole cell extracts (5 μg per sample) of wild-type HeLa cells and two clonal HeLa cell lines expressing the 144 CD siRNA (clones A and B). Enzymatic activity was measured as described in Materials and Methods using the fluorogenic substrate MoCAc-Gly-Lys-Pro-Ile-Phe-Phe-Arg-Leu-Lys(Dnp)-D-Arg-NH_2. The depicted values represent the mean of triplicate assays and error bars represent standard deviations. *C*, Cathepsin D expression is also downregulated in clonal U937 cells transfected with p*Silencer* plasmid containing the 144 CD siRNA sequence. Whole cell extracts from wild-type U937 cells, control siRNA/U937 cells, and 144 CD siRNA /U937 cells were subjected to immunoblotting with cathepsin D, caspase-8, caspase-3 or β-actin antibody. *D,* Whole cell extracts from two clonal HeLa cell lines (clones A and B) and one clonal U937 cell line expressing the 144 CD siRNA were subjected to immunoblot analysis for the aspartic cathepsin protease, cathepsin E. Cathepsin E was not downregulated by the 144 CD siRNA in HeLa cells. Expression of cathepsin E was not detected in parental, wild-type U937 cells.

protein expression as compared to wild-type U937 cells and U937 cells containing the control siRNA sequence (Figure 4.5.C and densitometric scanning). Immunoblots for caspase-8, caspase-3, and β-actin showed that, as with HeLa cells, the 144 CD siRNA specifically downregulated the cathepsin D protein in U937 cells. Expression of cathepsin E, the closest related enzyme and the only other known aspartate cathepsin, was unaffected in two different HeLa clones expressing 144 CD siRNA (Figure 4.5.D). Cathepsin E was not detected in wild-type U937 cells or in 144 CD siRNA/U937 cells (Figure 4.5.D)

To ensure that downregulation of cathepsin D did not alter the metabolic phenotype of these cells, healthy U937 cells were plated at a density of 1 x 10^5 cells/ml and cell growth was assessed under normal cell culture conditions for 4 days. Figure 4.6 demonstrates that the growth kinetics of the 144 CD siRNA/U937 cell were identical to that of wild-type U937 cells. Similar results were seen in HeLa cells (data not shown). These results confirm that the use of RNAi is an efficient method to downregulate cathepsin D expression in both suspension leukemic cells (U937) and adherent epithelial cells (HeLa).

4.2.3. DOWNREGULATION OF CATHEPSIN D BY siRNA INHIBITS CELL DEATH INDUCED BY VARIOUS APOPTOTIC STIMULI

Cathepsin D has been shown to play a role in apoptosis induced by death ligands such as TNF-α [88, 149], by oxidizing agents such as naphthazarin [90-93], by the protein kinase C inhibitor staurosporine [98, 99], and by sphingosine, a potent inducer of ceramide production [51, 97]. If cathepsin D is important for apoptosis induced by TNF-

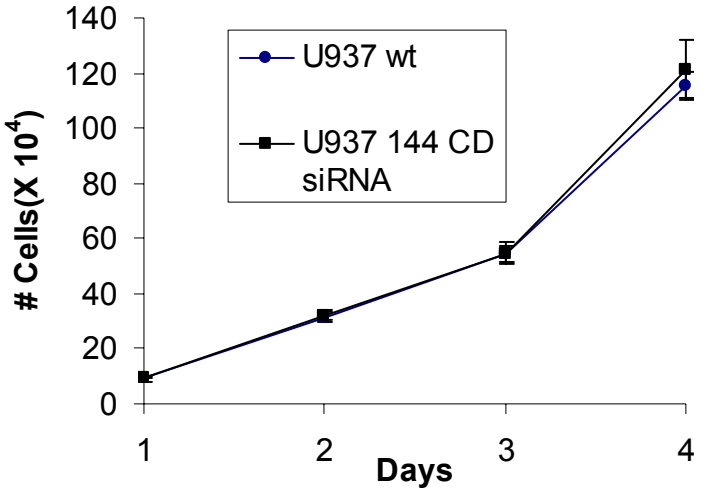
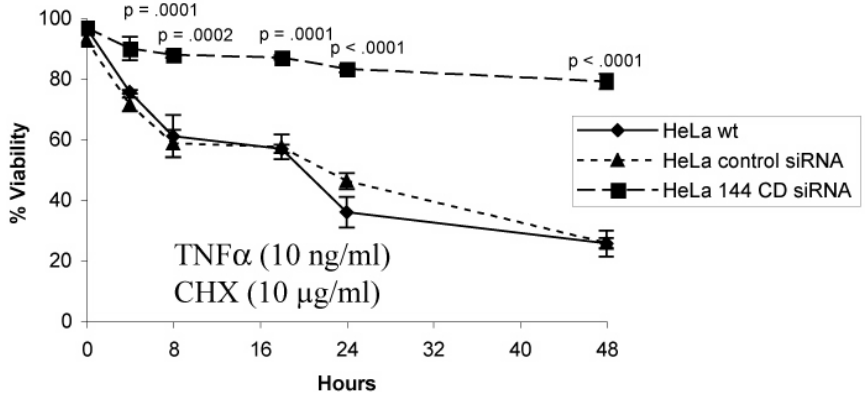


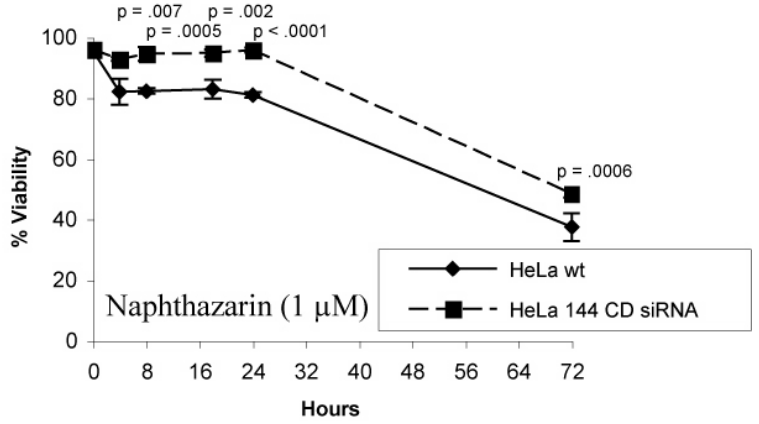
Figure 4.6. Growth kinetics of wild-type U937 and 144 CD siRNA/U937 cells. Wild-type U937 cells and 144 CD siRNA/U937 cells were plated at a density of 1 x 10^5 cells/ml and cultured under normal conditions (see Materials and Methods). At the indicated time points, aliquots of cells were removed for cell counting. The growth curves shows that the growth of the 144 CD siRNA/U937 clone is identical to that of wild-type U937 cells.

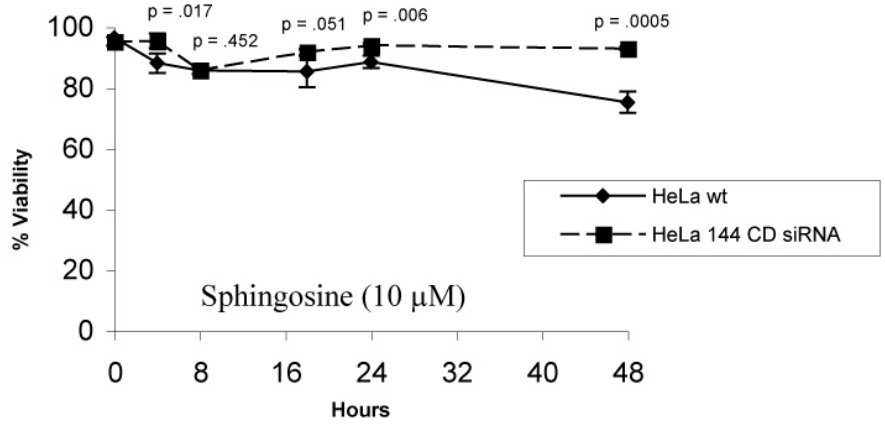
α, naphthazarin, staurosporine, or sphingosine, then I predicted that cathepsin D downregulation would inhibit cell death following these treatments. To test this hypothesis, the kinetics of cell death were assessed via trypan blue exclusion assays following treatment with apoptotic stimuli in wild-type cells, cells expressing the control siRNA, and cells expressing the 144 CD siRNA. As described in Materials and Methods, cells were treated with apoptotic stimuli and at various time points, aliquots of cells were removed for analysis by trypan blue dye exclusion. As shown in Figure 4.7, downregulation of cathepsin D did impact cell death following several different treatments in both HeLa and U937 cells. HeLa cells were treated with 10 ng/ml TNF-α in combination with 10 μg/ml cycloheximide. After 48 hours of TNF-α treatment, 30% of wild-type HeLa cells and control siRNA/HeLa cells remained viable (Figure 4.7.A). Strikingly, roughly 90% of 144 CD siRNA/HeLa cells remained viable after 48 hours of TNF-α treatment. Likewise, downregulation of cathepsin D in HeLa cells protected against cell death induced by 1 μM napthazarin and 10 μM sphingosine (Figure 4.7.A). By contrast, downregulation of cathepsin D expression in HeLa cells did not protect against cell death induced by the PKC inhibitor staurosporine (data not shown).

Downregulation of cathepsin D in U937 cells resulted in protection against cell death induced by a variety of stimuli as well. Figure 4.7.B shows that cell death was significantly inhibited in 144 CD siRNA/U937 cells following treatment with 10 ng/ml TNF−α + 10 μg/ml cycloheximide, 2 μg/ml TRAIL, 1 μM staurosporine, or 4 μM naphthazarin. These results confirm that the siRNA clones are functional and that, in our

A


p = .0001
p = .0002
p = .0001
p < .0001
p < .0001
HeLa wt
HeLa control siRNA
HeLa 144 CD siRNA
TNFα (10 ng/ml)
CHX (10 μg/ml)
% Viability
Hours


p = .007
p = .002
p = .0005
p < .0001
p = .0006
HeLa wt
HeLa 144 CD siRNA
Naphthazarin (1 μM)
% Viability
Hours


p = .017
p = .452
p = .051
p = .006
p = .0005
HeLa wt
HeLa 144 CD siRNA
Sphingosine (10 μM)
% Viability
Hours

B

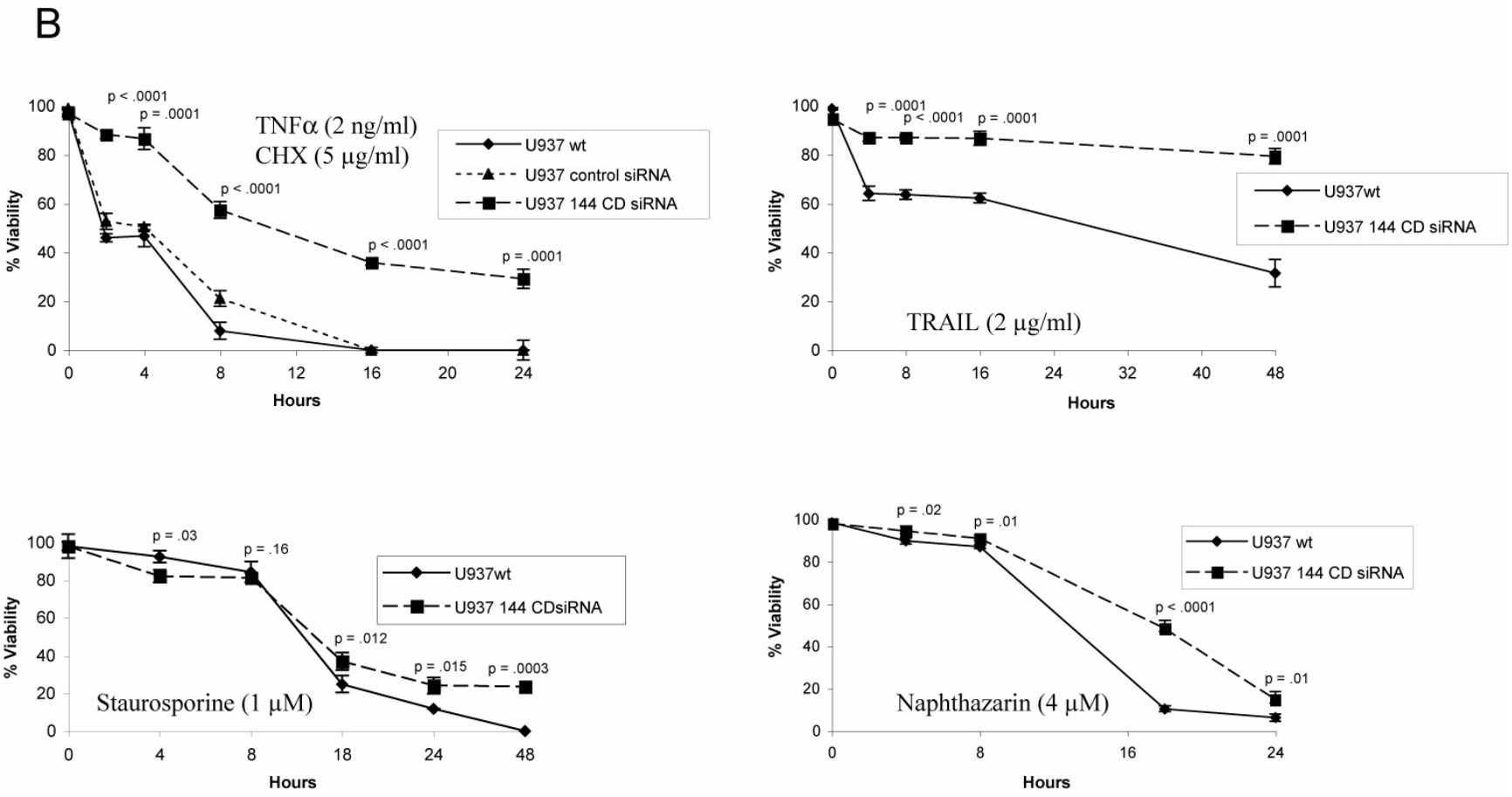

TNFα (2 ng/ml)
CHX (5 µg/ml)
U937 wt
U937 control siRNA
U937 144 CD siRNA
% Viability
Hours
p < .0001
p = .0001
p < .0001
p < .0001
p = .0001
TRAIL (2 µg/ml)
U937wt
U937 144 CD siRNA
p = .0001
p < .0001
p = .0001
p = .0001
Staurosporine (1 µM)
U937wt
U937 144 CDsiRNA
p = .03
p = .16
p = .012
p = .015
p = .0003
Naphthazarin (4 µM)
U937 wt
U937 144 CD siRNA
p = .02
p = .01
p < .0001
p = .01

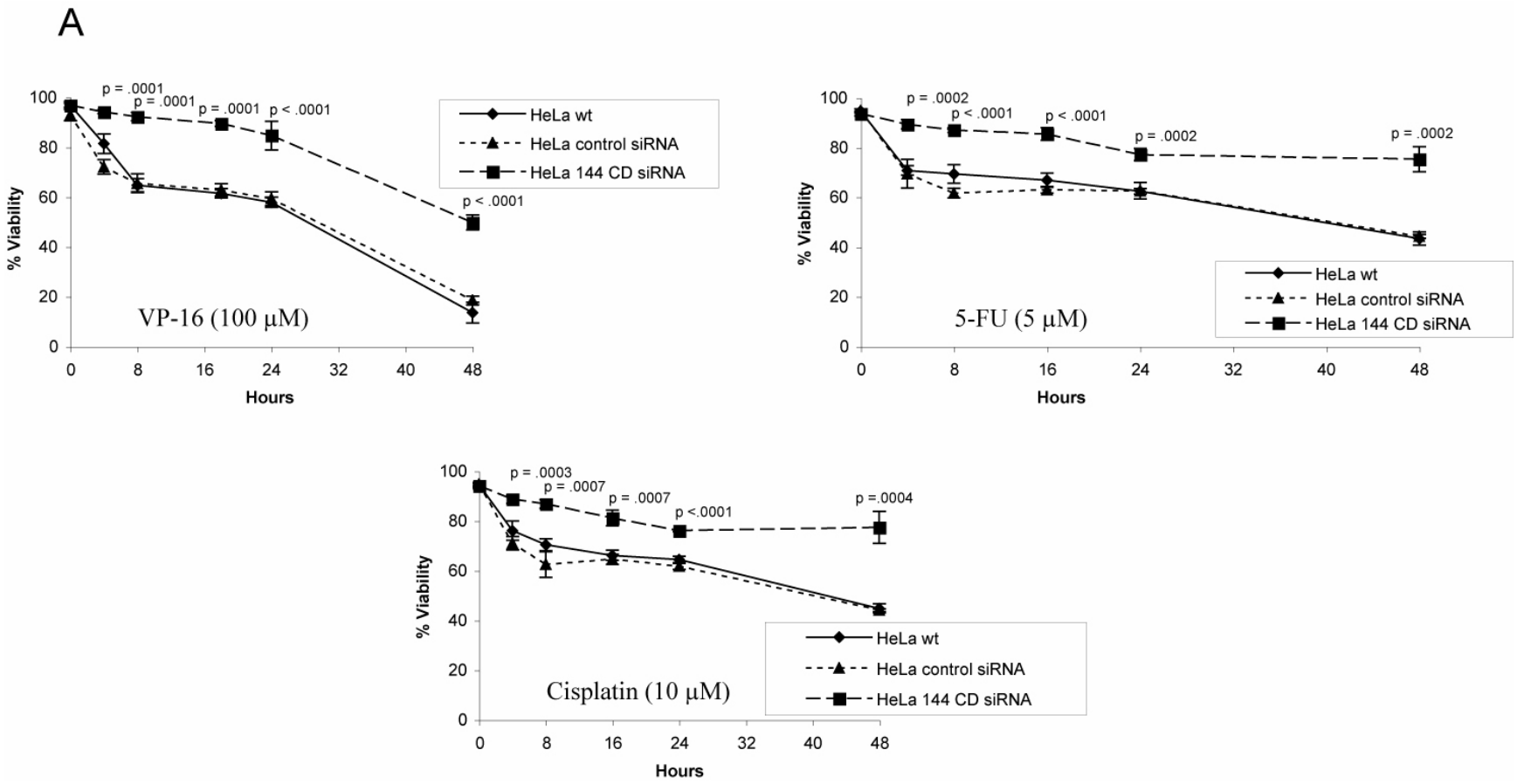
Figure 4.7. Death ligand-, oxidative stress-, and ceramide-induced cell death is inhibited by siRNA-mediated downregulation of cathepsin D. *A,* HeLa cells were exposed for varying lengths of time to 10 ng/ml TNF-α + 10 μg/ml cycloheximide, 1 μM napthazarin, or 10 μM sphingosine. At the indicated timepoints, 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. Error bars represent standard deviations. Control, untreated cultures always maintained greater than 97% viability out to 48 hours. The statistical significance of differences seen at each time point was determined using the VassarStats website (http://faculty.vassar.edu/lowry/VassarStats.html) and Microsoft Excel Data Analysis tools by comparing cells expressing 144 CD siRNA versus cells expressing control siRNA (or wild-type cells in the case of naphthazarin and sphingosine). As depicted, statistically significant differences (p-values < 0.05 using a directional t-test for independent samples) were observed with all drugs. *B*, U937 cells were treated with 2 ng/ml TNF-α + 5 μg/ml cycloheximide, 2 μg/ml TRAIL, 1 μM staurosporine, or 4 μM naphthazarin for varying lengths of time and cell viabilities determined as in Panel A. Statistical significance was determined as described in Panel A.

cell systems, cathepsin D plays an important role in apoptosis induced by TNF-α, TRAIL, oxidative stress (naphthazarin), and ceramide (sphingosine).

4.2.4. DOWNREGULATION OF CATHEPSIN D BY siRNA INHIBITS CHEMOTHERAPY-INDUCED APOPTOSIS

In this section, the effects of cathepsin D downregulation on chemotherapy-induced apoptosis were assessed. If cathepsin D is important for chemotherapy-induced cell death, as my hypothesis states, then it is expected that cells that do not express the cathepsin D protein would exhibit significant protection against chemotherapy drugs. Figure 4.8 illustrates the effects of multiple chemotherapy drugs on cell viability in wild-type cells, cells expressing control siRNA, and cells expressing 144 CD siRNA. 144 CD siRNA/HeLa cells were found to be significantly protected against cell death induced by 100 μM VP-16 over 48 hours of treatment (Figure 4.8.A). 144 CD siRNA/HeLa cells were also protected against cell death by the chemotherapy drugs 5-fluorouracil and cisplatin, particularly late in apoptosis. To verify that the protection observed in these cells was not unique to this clone of transfected cells, a second HeLa cell clone harboring CD siRNA (144 B) was tested and found to exhibit similar responses to all three chemotherapy drugs (Figure 4.9). U937 cells expressing the 144 CD siRNA also were significantly protected against 2 μM VP-16-induced cell death throughout the course of the 48-hour treatment (Figure 4.8.C). An inherent, marked resistance of wild-type U937 cells to 5-fluorouracil and cisplatin precluded inclusion of studies with these drugs in U937 cells. A comprehensive table of results from the trypan viability experiments in HeLa and U937 cells is shown in Figure 4.10. The first column in the table indicates the type of stimulus used to induce cell death. The second column indicates whether the

A


p = .0001
p = .0001
p = .0001
p < .0001
p < .0001
HeLa wt
HeLa control siRNA
HeLa 144 CD siRNA
% Viability
VP-16 (100 μM)
Hours
p = .0002
p < .0001
p < .0001
p = .0002
p = .0002
5-FU (5 μM)
p = .0003
p = .0007
p = .0007
p <.0001
p =.0004
Cisplatin (10 μM)

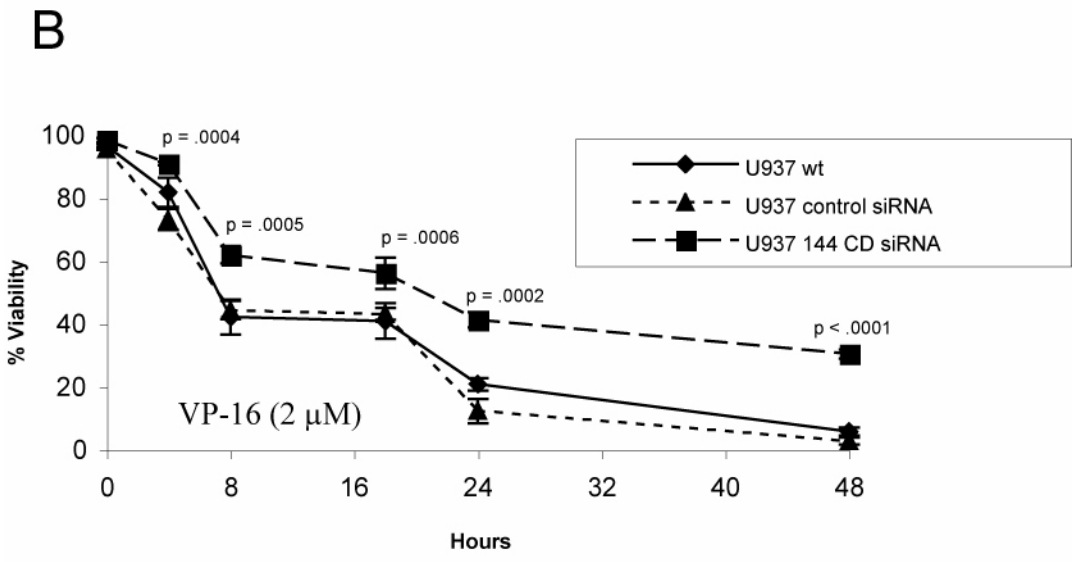


Figure 4.8. Chemotherapy-induced cell death is inhibited by siRNA-mediated downregulation of cathepsin D. *A,* HeLa cells were exposed for varying lengths of time to 100 μM VP-16, 5 μM 5-fluorouracil, or 10 μM cisplatin. At the indicated timepoints, aliquots of cells were removed and assayed for viability by trypan blue exclusion. For each data point, quadruplicate counts were performed using a minimum of 100 cells per count. Error bars represent standard deviations. Control, untreated cultures always maintained greater than 97% viability out to 48 hours. *B*, U937 cells were treated with 2 μM VP-16 for varying lengths of time and cell viabilities determined as in Panel A. Statistical significance was determined as described in Figure 4.7.

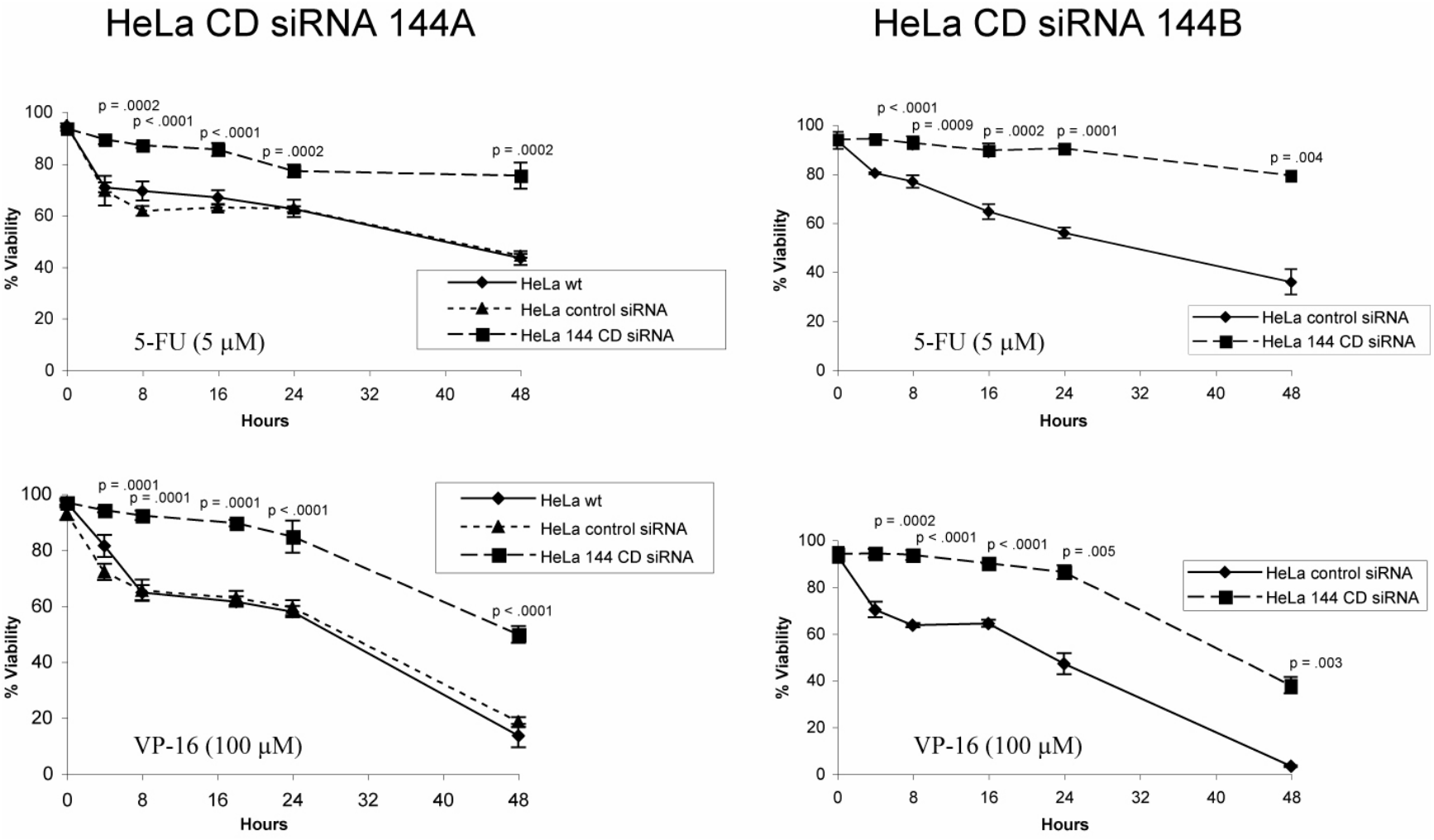

HeLa CD siRNA 144A
HeLa CD siRNA 144B
% Viability
Hours
5-FU (5 μM)
VP-16 (100 μM)
HeLa wt
HeLa control siRNA
HeLa 144 CD siRNA
p = .0002
p < .0001
p < .0001
p = .0002
p = .0002
p < .0001
p = .0009
p = .0002
p = .0001
p = .004
p = .0001
p = .0001
p = .0001
p < .0001
p < .0001
p = .0002
p < .0001
p < .0001
p = .005
p = .003

Figure 4.9. Two different 144 CD siRNA/HeLa cell clones (A and B) protect against 5-fluorouracil- and VP-16-induced cell death. Two different transfected clones of HeLa cells harboring the 144 CD siRNA were exposed for varying lengths of time to 5 μM 5-FU or 100 μM VP-16. At the indicated timepoints, 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. Error bars represent standard deviations. Control, untreated cultures always maintained greater than 97% viability out to 48 hours. Statistical significance was determined as described in Figure 4.7. As shown, both 144 CD siRNA/HeLa clones exhibited significant protection from 5-FU- and VP-16-induced cell death.

HeLa:

	Drug kills wild-type cells?	Protection afforded by 144 CD siRNA
TRAIL	N	Not Tested
TNF-α	Y	++
Staurosporine	Y	-
VP-16	Y	++
Cisplatin	Y	++
5'-Fluorouracil	Y	++
Sphingosine	Y	+
Naphthazarin	Y	+

U937:

TRAIL	Y	++
TNF-α	Y	++
Staurosporine	Y	+
VP-16	Y	++
Cisplatin	N	Not Tested
5'-Fluorouracil	N	Not Tested
Sphingosine	N	Not Tested
Naphthazarin	Y	++

Figure 4.10. Summary of cathepsin D siRNA protection against cell death induced by a variety of stimuli.

stimulus was capable of causing apoptosis in wild-type cells. The third column indicates the relative degree of protection conferred by the 144 CD/siRNA. A minus sign indicates that the cathepsin D siRNA offered no protection against apoptosis. One plus sign indicates that cell death was inhibited between 15 and 30% by cathepsin D downregulation (% difference = control siRNA – 144 CD siRNA), while two plus signs indicate that inhibition of cell death was greater than 30%. These results demonstrate that downregulation of cathepsin D protein expression in both HeLa cells and U937 cells confers significant protection against apoptosis induced by chemotherapy drugs. Together these findings provide strong support for my hypothesis that cathepsin D is important for chemotherapy-induced cell death.

4.3. DISCUSSION

There are several advantages associated with using stable siRNA clones to downregulate the cathepsin D protein compared to antisense-mediated downregulation or pharmacologic inhibition. The only maintenance required to sustain the stable CD siRNA clones was to culture them in media containing hygromycin B. With a stable clone, cathepsin D downregulation does not vary throughout the course of experiments, whereas the efficiency of inhibition by a pharmacologic inhibitor or transient transfection may vary throughout the experimental time course and from experiment to experiment. Also, the siRNA oligonucleotides were found to downregulate cathepsin D extremely efficiently (>95%), more so than downregulation expected with an antisense RNA approach.

Nonetheless, problems can arise when using RNA interference strategies. Recently, reports have suggested nonspecific downregulation of other genes during the RNAi process, presumably due to siRNA duplex binding nonspecifically to sequences which are not 100% homologous to the targeted sequence [150]. Therefore, great care must be taken to ensure that critical proteins in the pathway of interest (in our case, apoptosis) are not also inadvertently silenced. We addressed this issue by ensuring that expression of key apoptotic proteins (caspase-3, caspase-8), as well as the aspartic protease, cathepsin E, was not altered in the silencing process. Additionally, care was taken to ensure that the growth properties of the siRNA clones were similar to that of the wild-type cells.

The results of this chapter are summarized in Figure 4.10. The data support the hypothesis that cathepsin D is important not only in death ligand- and oxidative stress-induced apoptosis, but also in chemotherapy-induced apoptosis. Though protection against cell death was conferred with most treatments in both HeLa and U937 cells harboring the 144 CD siRNA, the degree of protection varied. For instance, 144 CD siRNA/HeLa cells were not protected against staurosporine–induced cell death and 144 CD siRNA/U937 cells were protected only to a small degree. Bidere *et al*. [99] have reported that inhibition of cathepsin D using pepstatin A in T lymphocytes markedly protected against staurosporine-induced cell death.. Thus, the importance of cathepsin D in cell death may vary depending on both the cell type and the stimulus used. However, for the chemotherapy drugs I studied (VP-16, cisplatin, and 5-fluorouracil), drug-induced apoptosis was always inhibited by the 144 CD siRNA. As VP-16 efficiently induces

death in both U937 cells and HeLa cells, it was utilized to further investigate the mechanism of cathepsin D-induced cell death (Chapter 5).

4.4. CONCLUSION

The results from this chapter demonstrate that the cathepsin D protein can be efficiently and specifically downregulated using RNA interference. Cells exhibiting downregulation of cathepsin D were protected against a variety of apoptotic stimuli, including death receptor activation, oxidative stress, ceramide, and inhibition of PKC. Importantly, downregulation of cathepsin D protected cells against chemotherapy-induced cell death, supporting the hypothesis that cathepsin D is important in this cell death pathway.

5. THE ROLE OF CATHEPSIN D IN CYTOCHROME C RELEASE AND CASPASE ACTIVATION FOLLOWING CHEMOTHERAPY TREATMENT

5.1. INTRODUCTION

The data in this project have thus far established that, following chemotherapeutic stimuli such as VP-16, lysosomal cathepsin D is released into the cytosol prior to cytochrome c release and caspase activation. The importance of cathepsin D in chemotherapy-induced apoptosis has also been demonstrated by showing that siRNA-induced downregulation of cathepsin D in HeLa and U937 cells confers protection against cell death induced by chemotherapy drugs. The goal of experiments described in this chapter was to explore potential mechanistic roles for cathepsin D during chemotherapy-induced apoptosis. VP-16 was used in these studies because it efficiently induces cell death in both HeLa and U937 cells and because HeLa and U937 cells harboring cathepsin D 144 siRNA were significantly protected against VP-16-induced cell death.

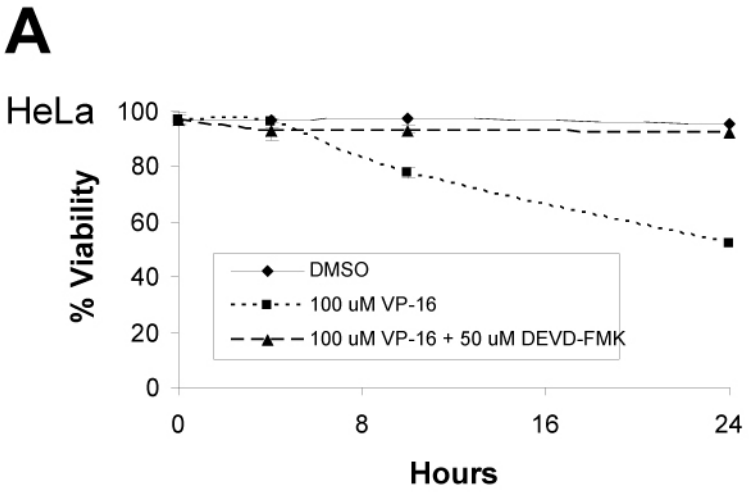
VP-16 induces DNA damage to cells by promoting production of irreversible DNA-binding derivatives from oxidation reduction reactions [105, 106] and by inhibiting topoisomerase II [151]. As discussed previously, DNA damage initiates the intrinsic apoptotic pathway, promoting release of mitochondrial apoptogenic proteins such as cytochrome c and SMAC, formation of the apoptosome, and activation of caspase-9 [8, 22].

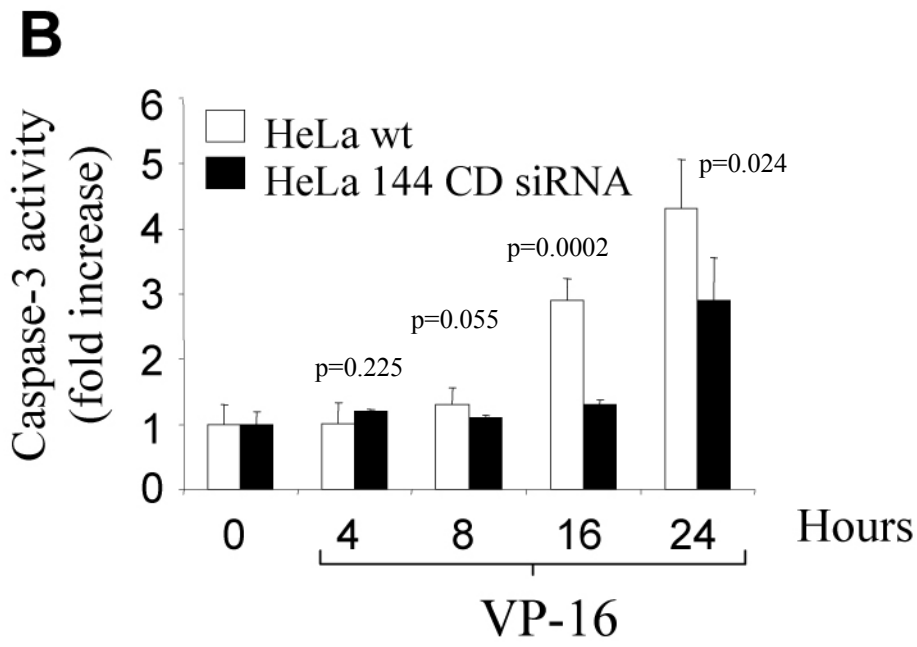
In preliminary studies, I found that 2 μM VP-16 treatment of U937 cells or 100 μM VP-16 treatment of HeLa cells efficiently induced 80-90% cell death over a 48-hour period. This chapter provides demonstration that cathepsin D downregulation impacts well-characterized apoptotic processes during chemotherapy-induced cell death, including mitochondrial release of cytochrome c and the activation of caspase proteases. Furthermore, evidence is provided that cathepsin D enzyme acts via a cytosolic factor to provoke cytochrome c release in a cell-free system. In this chapter, I propose a model for the mechanistic role of cathepsin D in chemotherapy-induced apoptosis.

5.2. RESULTS

5.2.1. DOWNREGULATION OF CATHEPSIN D INHIBITS CASPASE-3 ACTIVATION

The activation of caspase-3 is a critical step in the apoptotic execution of chemotherapy-treated U937 cells [26, 152, 153]. Moreover, inhibition of caspase-3 with the pharmacologic inhibitor DEVD-FMK almost completely attenuated VP-16-induced cell death in wild-type HeLa cells (Figure 5.1.A). In the relocalization studies described in Chapter 3, cathepsin D was shown to be released into the cytosol of U937 cells approximately 4 hours after treatment with 2 μM VP-16 (Figure 3.5). VP-16-induced activation of caspase-3 was first observed 8 hours following treatment, as detected by immunoblotting (Figure 3.8). It is possible then, that cathepsin D may act to either directly or indirectly activate caspase-3 in chemotherapy-induced apoptosis. To determine whether cathepsin D is important for VP-16-induced caspase-3 activation,


A
HeLa
% Viability
100
80
60
40
20
0
0
8
16
24
Hours
DMSO
100 uM VP-16
100 uM VP-16 + 50 uM DEVD-FMK


B
Caspase-3 activity
(fold increase)
6
5
4
3
2
1
0
HeLa wt
HeLa 144 CD siRNA
p=0.225
p=0.055
p=0.0002
p=0.024
0
4
8
16
24
Hours
VP-16

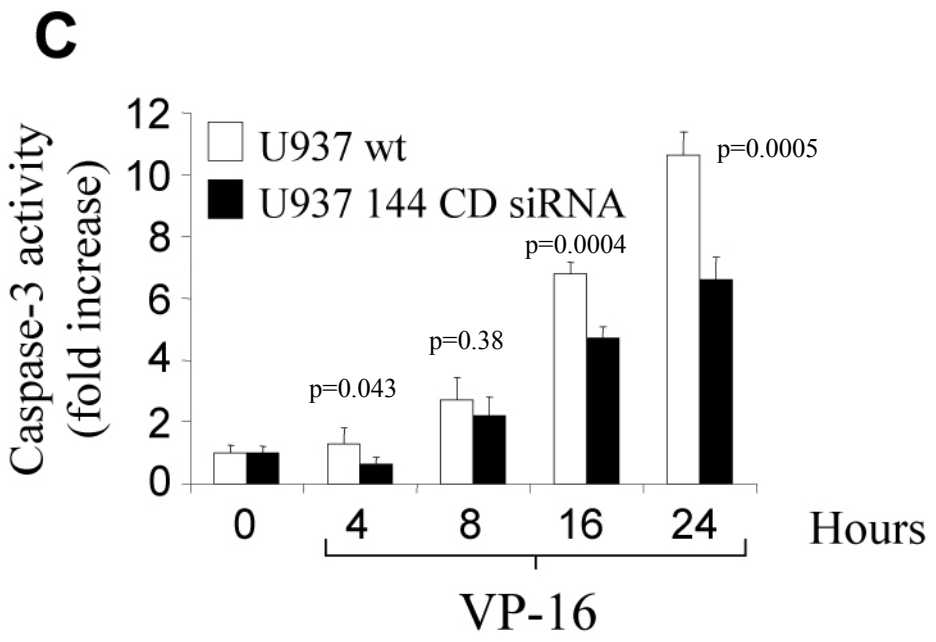


Figure 5.1. Downregulation of cathepsin D inhibits caspase-3 activation. *A,* Wild-type HeLa cells were pretreated in the presence or absence of 50 μM DEVD-FMK for 1 hour prior to treatment with 100 μM VP-16. Cells were harvested at varying time points, and trypan blue exclusion assays were performed to assess cell viability. DEVD-FMK prevented VP-16-induced cell death. *B,* Wild-type HeLa cells and 144 CD siRNA/HeLa cells were treated with 100 μM VP-16 for varying times. Whole cell lysates were prepared and assessed for caspase-3 activity by measuring cleavage of the caspase-3 fluorogenic substrate Ac-DEVD-AFC. Caspase-3 activation was inhibited in HeLa cells that express the 144 CD siRNA. *C,* Wild-type U937 cells and 144 CD siRNA/U937 cells were treated with 2 μM VP-16 for varying times. Whole cell lysates were prepared and caspase-3 activity was measured by cleavage of the caspase-3 substrate. Caspase-3 activation was inhibited in U937 cells that express 144 CD siRNA. Error bars represent the standard deviations of triplicate wells.

wild-type U937 cells and 144 CD siRNA/U937 cells were treated with 2 μM VP-16 for varying lengths of time. Similarly, wild-type HeLa cells and 144 CD siRNA/HeLa cells were treated with 100 μM VP-16. Whole cell lysates were then prepared and caspase-3 enzymatic activity in the lysates was assessed by incubating the lysates with the caspase-3 fluorogenic substrate Ac-DEVD-AFC and measuring cleavage of the substrate by spectrofluorometry. Similar to what was seen in Chapter 3, caspase-3 activation was first detected in wild-type HeLa and U937 cells around 8 hours after VP-16 treatment and levels continued to increase up to 24 hours. Caspase-3 activation in cells expressing the control siRNA was similar to corresponding wild-type HeLa or U937 cells (data not shown). Importantly, caspase-3 activation was significantly reduced in 144 CD siRNA/HeLa cells and 144 CD siRNA/U937 cells (Figure 5.1.B,C). These data suggested that cathepsin D is important for caspase-3 activation during VP-16-induced cell death.

To test whether cathepsin D has the potential to directly activate caspase-3, whole cell lysates were prepared from U937 cells and incubated with either 2 μg cathepsin D or 0.2 μg caspase-3 in the presence or absence of 50 μM z-VAD for one hour at 37 °C. Caspase-3 activity in the lysates was measured by cleavage of the fluorogenic caspase substrate Ac-DEVD-AFC. Addition of recombinant caspase-3 to lysates resulted in marked cleavage of the caspase-3 substrate, which was completely inhibited by z-VAD-FMK (Figure 5.2). However, no increase in caspase-3 activity was noted in cathepsin D-treated lysates. The fact that cathepsin D failed to induce rapid activation of caspase-3 suggests that cathepsin D indirectly promotes caspase-3 activation during chemotherapy-induced apoptosis or requires some cofactor that is inactivated in extracts.

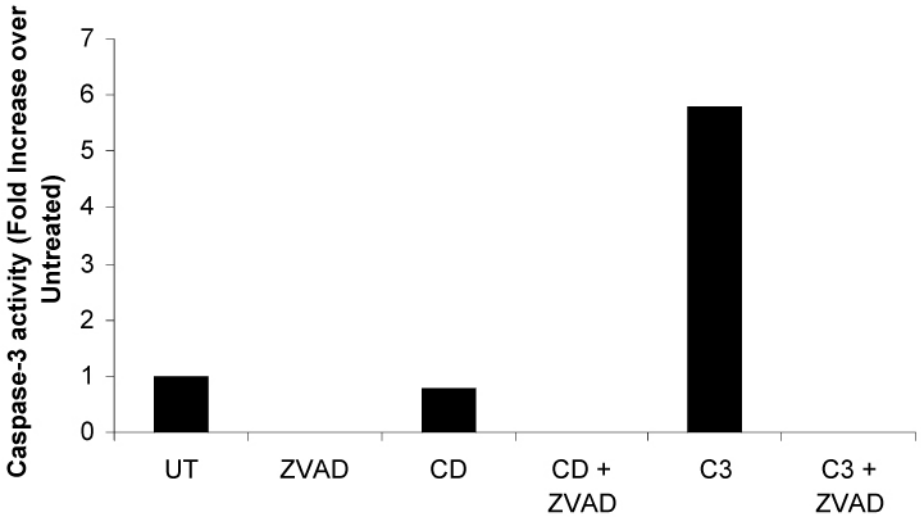


Figure 5.2. Cathepsin D does not directly activate caspase-3. Whole cell lysates were prepared from 5 x 10^5 wild-type U937 cells. Cell lysates were treated with 2 μg cathepsin D or 0.2 μg caspase-3 at 37 °C for one hour in the presence or absence of the pancaspase inhibitor z-VAD-FMK (50 μM). Caspase-3 activity was assessed by measuring cleavage of the caspase-3 fluorogenic substrate Ac-DEVD-AFC. Caspase-3 activity was completely inhibited by z-VAD-FMK. Cathepsin D does not induce rapid caspase-3 activation in U937 cell lysates.

5.2.2. DOWNREGULATION OF CATHEPSIN D INHIBITS CYTOCHROME C RELEASE

In addition to performing experiments to assess the impact of cathepsin D downregulation on caspase-3 activation, we investigated the upstream apoptotic process of cytochrome c release. As discussed earlier, release of cytochrome c from the mitochondria results in formation of the apoptosome complex, activation of caspase-9, and subsequent activation of caspase-3. To investigate the importance of cathepsin D in cytochrome c release during chemotherapy treatment, wild-type U937 cells and 144 CD siRNA/U937 cells were treated with 2 μM VP-16 for varying lengths of time. Cytosolic fractions were then prepared as described in Materials and Methods, and aliquots of the cytosolic proteins (25 μg/lane) were electrophoresed on a 13% SDS-PAGE gel. The Western blot for cytochrome c in Figure 5.3 illustrates the kinetics of cytosolic cytochrome c release in both wild-type U937 cells and 144 CD siRNA/U937 cells. Cytochrome c was first detected in the cytosol approximately 8 hours after VP-16 treatment in wild-type cells, but was not detected in 144 CD siRNA/U937 cells until 16 hours following drug treatment. Even after 16 or 24 hours, the degree of cytochrome c release was reduced in cells expressing the 144 CD siRNA compared to cells expressing the control siRNA. Immunoblotting for cytochrome c oxidase IV showed that cytosolic fractions were not contaminated with mitochondria, nor was there general mitochondrial rupture or leakage. Therefore, the observed cytochrome c release was due to VP-16-induced activation of the intrinsic apoptosis pathway. Together, these data illustrate that

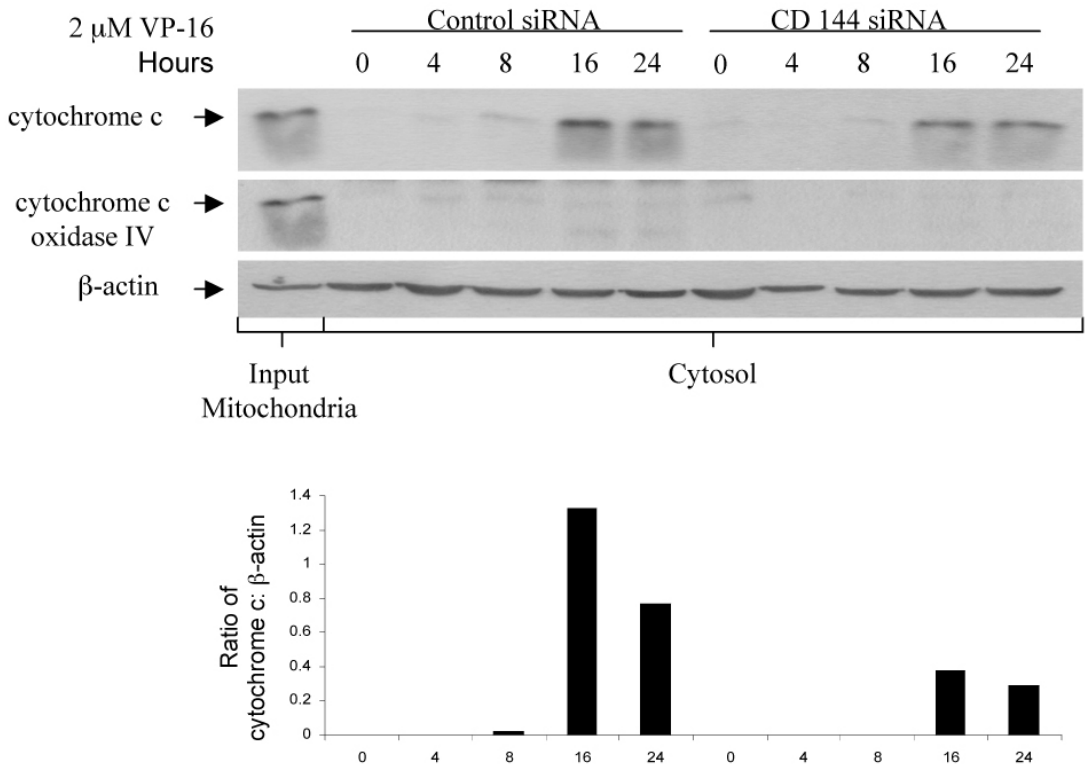


Figure 5.3. Cathepsin D downregulation inhibits cytochrome c release. Control siRNA/U937 cells or 144 CD siRNA/U937 cells were treated for varying lengths of time with 2 μM VP-16. At the indicated times, cytosolic fractions were prepared from 20 x 10^6 cells. Cytosol was immunoblotted for the appearance of cytochrome c. Cytochrome c was first detected in the cytosol at 8 hours, and markedly increased by 16 hours following VP-16 treatment. Cytochrome c release was inhibited in 144 CD siRNA/U937 cells up to 24 hours after VP-16 treatment. Densitometry of the immunoblot was analyzed as the ratio of cytochrome c to β-actin. Immunoblotting with cytochrome c oxidase IV was used to verify mitochondrial integrity. The above immunoblot is representative of repeated experiments with similar results.

cathepsin D is important for the release of cytochrome c into the cytosol following VP-16 treatment in U937 cells.

5.2.3. CATHEPSIN D INDUCES RELEASE OF CYTOCHROME C FROM ISOLATED MITOCHONDRIA

In the preceding section, I have demonstrated that cathepsin D is important for the timely release of cytochrome c and activation of caspases following chemotherapy treatment. To determine whether purified cathepsin D can initiate direct release of cytochrome c, isolated mitochondria were treated with purified cathepsin D in a cell-free system. Mitochondrial fractions were isolated from 20 x 10^6 untreated wild-type U937 cells using the fractionation protocol described in Materials and Methods. Mitochondria (50 μg) were then treated with 2 μg of cathepsin D for 1 hour at 37 °C, followed by centrifugation at 14,000 rpm for 30 minutes at 4 °C to pellet the mitochondria. Supernatants, which contained proteins released from the mitochondria, were centrifuged twice more at 14,000 rpm for 30 minutes at 4 °C. The purified supernatants were electrophoresed on a 13% SDS-PAGE gel and immunoblotted for cytochrome c. As shown in Figure 5.4, cathepsin D enzyme provoked a modest release of cytochrome c into the supernatant, an action that was inhibited by 100 μM pepstatin A.

Though cathepsin D alone did induce some cytochrome c release, the amount of protein released was not markedly different than control. It was questionable whether this level of cytochrome c release could initiate a substantial apoptotic response or whether some degree of cytosolic contamination might falsely enhance cathepsin D-

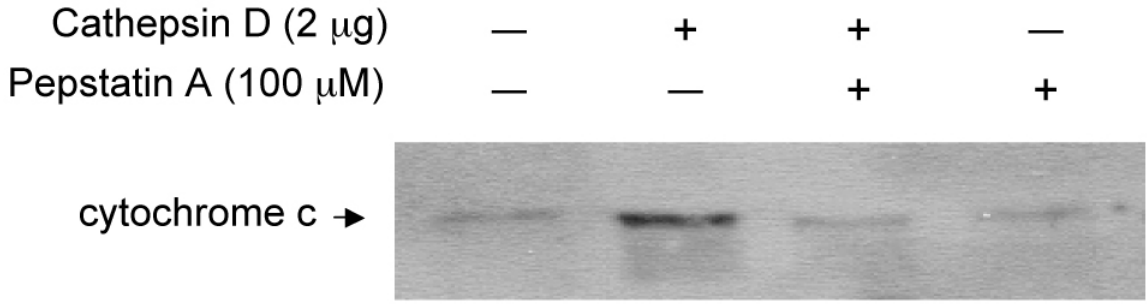


Figure 5.4. Cathepsin D induces release of cytochrome c from isolated mitochondria. Mitochondria were isolated from wild-type U937 cells as described in Materials and Methods. Mitochondria (50 μg) were then treated with 2 μg purified cathepsin D enzyme for 1 hour at 37 °C in the presence or absence of 100 μM pepstatin A. Supernatants were isolated by centrifugation and immunoblotted for cytochrome c. Cathepsin D elicits release of cytochrome c from mitochondria that is completely inhibited by pepstatin A.

induced cytochrome c release. To examine whether cytosolic proteins might enhance cathepsin D-mediated cytochrome c release, mitochondrial and cytosolic extracts were prepared from untreated wild-type U937 cells. Mitochondria (50 μg) were then incubated with 2 μg cathepsin D in the presence or absence of 10 μg cytosolic extract. Following incubation, supernatants were purified, electrophoresed on a 13% SDS-PAGE gel, and immunoblotted for cytochrome c. As shown in Figure 5.4, cathepsin D alone was again found to provoke only a modest release of cytochrome c from mitochondria. However, mitochondria that were incubated with cathepsin D in the presence of cytosolic extracts exhibited robust release of cytochrome c (> 25-fold) relative to mitochondria incubated with cathepsin D alone, as shown by densitometric scanning of the immunoblot (Figure 5.5). This release of cytochrome c was completely inhibited by 100 μM pepstatin A, demonstrating that the release was due to cathepsin D enzymatic activity. These results suggest that cathepsin D cleaves a cytosolic factor, which then mediates the effects of cathepsin D on cytochrome c release.

5.2.4. DOWNREGULATION OF CATHEPSIN D INHIBITS SMAC/DIABLO RELEASE

If cathepsin D plays a role in cytochrome c release during chemotherapy-induced apoptosis, it also may play a role in the release of other apoptogenic mitochondrial proteins as well. The proapoptotic factor, SMAC/Diablo, is known to be released from mitochondria during chemotherapy-induced apoptosis, where it enhances caspase activity by binding and inactivating caspase inhibitory proteins in the Inhibitor of Apoptosis protein family (IAPs), particularly XIAP [154, 155]. To determine whether cathepsin D

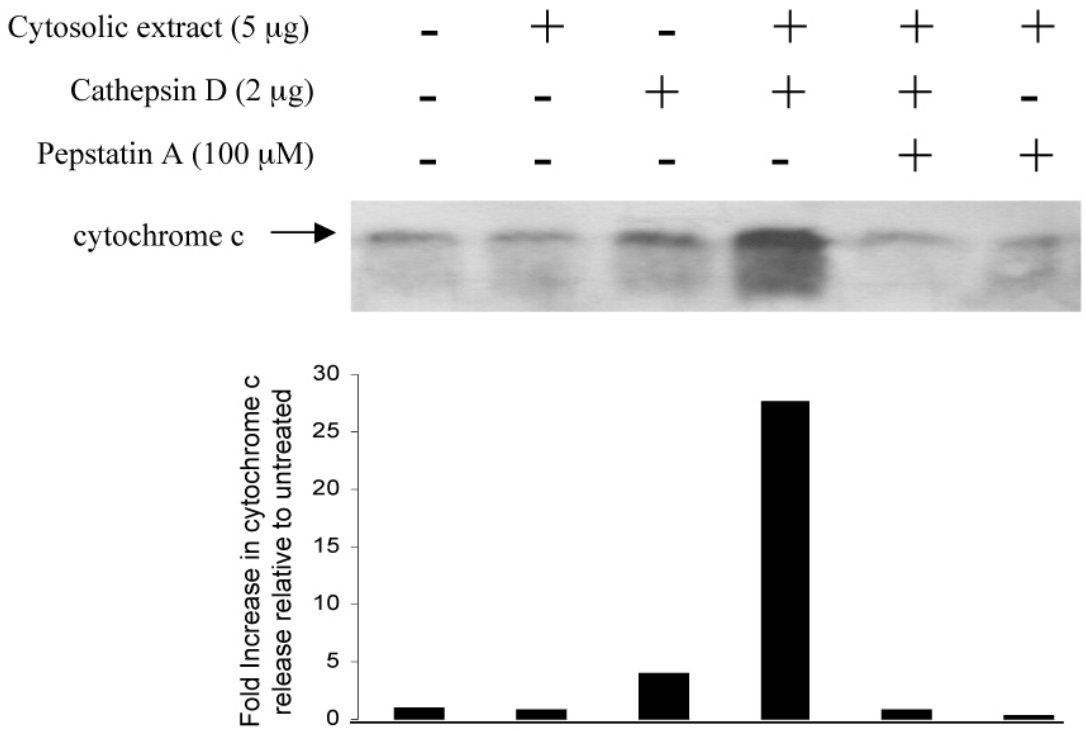


Figure 5.5. Cytochrome c release stimulated by cathepsin D is enhanced by the presence of cytosolic extracts. Mitochondrial and cytosolic fractions were isolated from wild-type U937 cells as described in Materials and Methods. Mitochondria (50 μg) were then incubated for 1 hour at 37 ºC with 10 μg of cytosol (Lane 2), 2 μg cathepsin D alone (Lane 3), or cytosol preincubated with cathepsin D for 1 minute (Lane 4). Supernatants were isolated by centrifugation and probed for the presence of cytochrome c. Although cathepsin D alone induced a small amount of cytochrome c release, incubation of cathepsin D with cytosol robustly enhanced cytochrome c release. The release of cytochrome c was completely inhibited by 100 μM pepstatin A. Densitometry represents the fold increase of cytochrome c release relative to untreated mitochondria.

impacts the cytosolic release of SMAC during chemotherapy treatment, wild-type U937 cells and 144 CD siRNA/U937 cells were treated with 2 μM VP-16, and cytosolic fractions were prepared at varying time points. Cytosolic proteins (25 μg/lane) were then electrophoresed on a 13% SDS-PAGE gel, and subjected to immunoblotting with anti-SMAC. Figure 5.6 shows that the release of SMAC into the cytosol in U937 cells expressing control siRNA was first detected at 16 hours. By contrast, no release of SMAC was detected in 144 CD siRNA/U937 cells, even after 24 hours of VP-16 treatment. These data suggest that cathepsin D can enhance the activation of caspases during chemotherapy treatment by stimulating not only release of cytochrome c, but also release of SMAC from mitochondria, thus perhaps relieving caspases from IAP inhibition.

5.2.5. CATHEPSIN D-INDUCED CYTOCHROME C RELEASE IS NOT DEPENDENT ON CASPASES

The data thus far have determined that cathepsin D acts on a cytosolic factor to promote cytochrome c release. Though cathepsin D does not activate caspase-3 through direct cleavage (Figure 5.2), it may promote caspase activation by prompting cytochrome c and SMAC release. Although caspase activation typically occurs downstream of cytochrome c release, it has been reported that the release of cytochrome c may occur in 2 stages [17, 130]. Following an initial release, caspase-3 becomes activated. Activated caspase-3 may then act in a positive feedback amplification loop to promote additional cytochrome c release [17, 130]. To investigate whether caspases are necessary for cathepsin D-induced cytochrome c release, mitochondrial and cytosolic fractions were

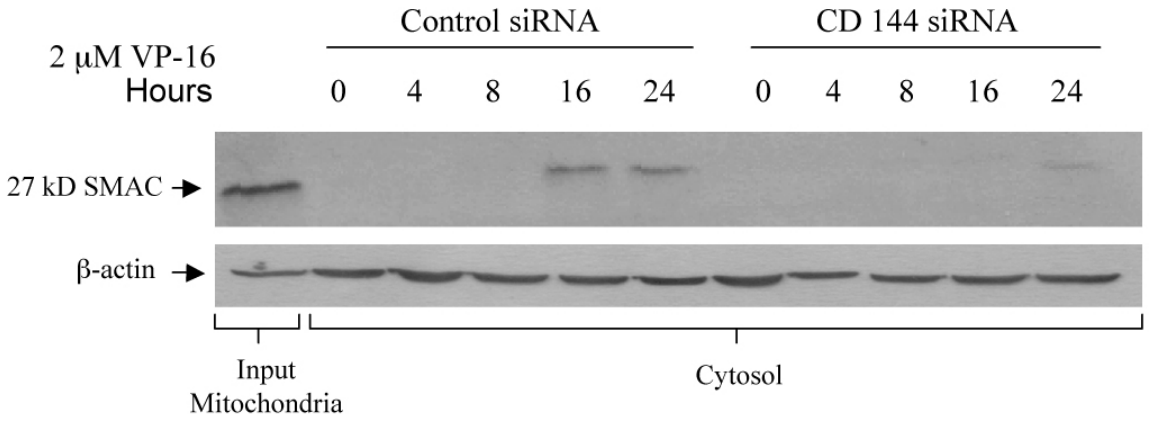


Figure 5.6. Cathepsin D siRNA inhibits SMAC/Diablo release. Control siRNA/U937 cells and 144 CD siRNA/U937 cells were treated with 2 μM VP-16 for varying lengths of time and cytosolic extracts were prepared as described in Materials and Methods. Cytosolic proteins (25 μg/lane) were immunblotted to detect release of cytochrome c. SMAC was released from control siRNA/U937 cells 16 hours after VP-16 treatment, but release was inhibited dramatically in 144 CD siRNA/U937 cells.

isolated from wild-type U937 cells. Mitochondria (50 μg) and cytosolic extracts (10 μg) were then preincubated with caspase-3 inhibitor (50 μM z-VAD-FMK) or cathepsin D inhibitor (100 μM pepstatin A) for 5 minutes prior to addition of 2 μg cathepsin D enzyme. In the presence of cytosol, cathepsin D promoted substantial release of cytochrome c from mitochondria (Figure 5.7). Cathepsin D-mediated cytochrome c release was completely inhibited by pepstatin A, but was unaffected by the caspase inhibitor. Therefore, caspases do not appear to mediate the effects of cathepsin D on cytochrome c release.

The effects of cysteine cathepsin inhibitors on cathepsin D-mediated cytochrome c release were also examined. Isolated mitochondria and cytosolic extracts were pretreated with cathepsin B inhibitor (50 μM z-FA-FMK) or cathepsin L inhibitor (50 μM z-FF-FMK) for 5 minutes prior to addition of 2 μg cathepsin D (Figure 5.7). As expected, cathepsin B inhibition had no effect on cathepsin D-induced cytochrome c release. Unexpectedly, inhibition of cathepsin L resulted in a 50% reduction in cathepsin D-induced cytochrome c release. This data opens up the possibility that cathepsin L may act as an intermediary factor in cathepsin D-induced cytochrome c release. This issue will be addressed further in the discussion.

5.3. DISCUSSION

The data in this chapter have shown that caspase activation and cytochrome c release, as well as SMAC release, are inhibited by siRNA-mediated downregulation of cathepsin D during chemotherapy-induced apoptosis. I propose a model in which

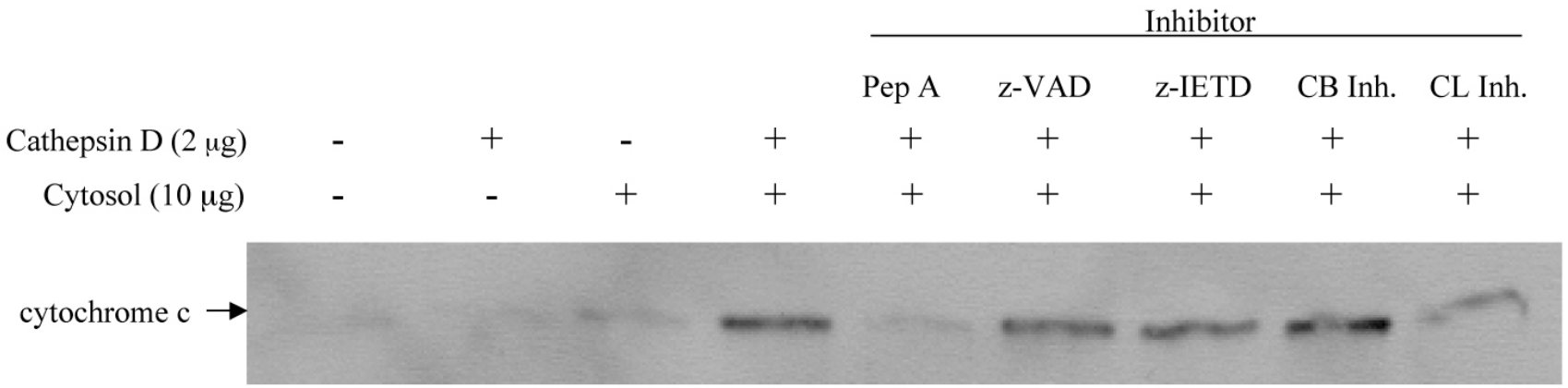


Figure 5.7. Impact of pharmacologic enzyme inhibitors on cathepsin D-induced cytochrome c release. Mitochondria and cytosol were isolated from untreated wild-type U937 cells and treated with 2 μg cathepsin D in the presence of 100 μM pepstatin A, 50 μM z-VAD-FMK (pancaspase inhibitor), 50 μM z-IETD-FMK (caspase-8 inhibitor), 50 μM cathepsin B inhibitor, or 50 μM cathepsin L inhibitor. Following incubation for 1 hour at 37 °C, reactions were centrifuged and the supernatants were subjected to immunoblotting to detect release of cytochrome c. Cathepsin D-induced cytochrome c release was inhibited by pepstatin A, but not by caspase inhibitors z-VAD or z-IETD. Cathepsin B inhibitor (50 μM z-FA) had no impact on cathepsin D-induced cytochrome c release. By contrast, cathepsin L inhibitor (50 μM z-FF) inhibited cytochrome c release by almost 50% (densitometric scanning, not shown).

chemotherapy drugs initiate release of cathepsin D into the cytosol. I hypothesize that in the cytosol, cathepsin D cleaves a cytosolic factor to provoke cytochrome c release and subsequent caspase activation (Figure 5.8). While identification of this factor is beyond the scope of this project, some possibilities will be discussed.

Though cytochrome c release typically precedes caspase-3 activation, it has been shown that only a small amount of cytochrome c is initially released [17, 130]. Figure 5.3 also showed that the initial release of cytochrome c at 8 hours was not substantial. Formation of the apoptosome following cytochrome c release initiates activation of caspase-9, which is followed soon thereafter by activation of caspase-3. Chen *et al.* [17] demonstrated that, following VP-16 treatment, active caspase-3 acts in an amplification loop to induce additional loss of mitochondrial potential and massive release of cytochrome c. It was possible, therefore, that cathepsin D cleavage of caspases may lead to a large-scale release of cytochrome c. In Figure 5.7, however, inhibition of caspase-3 by DEVD-FMK and caspase-8 by IETD-FMK had no effect on cathepsin D-induced cytochrome c release. Additionally, cathepsin D was not able to rapidly activate caspase-3 (Figure 5.2) or caspase-8 (not shown). Therefore, caspases do not appear to be cytosolic intermediates of cathepsin D-induced cytochrome c release.

The effects of cysteine cathepsin inhibitors on cathepsin D-induced cytochrome c release from isolated mitochondria were also assessed. In previous experiments, I had shown that cathepsin B remained in the lysosomes during the course of VP-16 treatment (Figure 3.7). As expected, inhibition of cathepsin B had no impact on cathepsin D-induced cytochrome release (Figure 5.7). Cathepsin B has been implicated in apoptosis

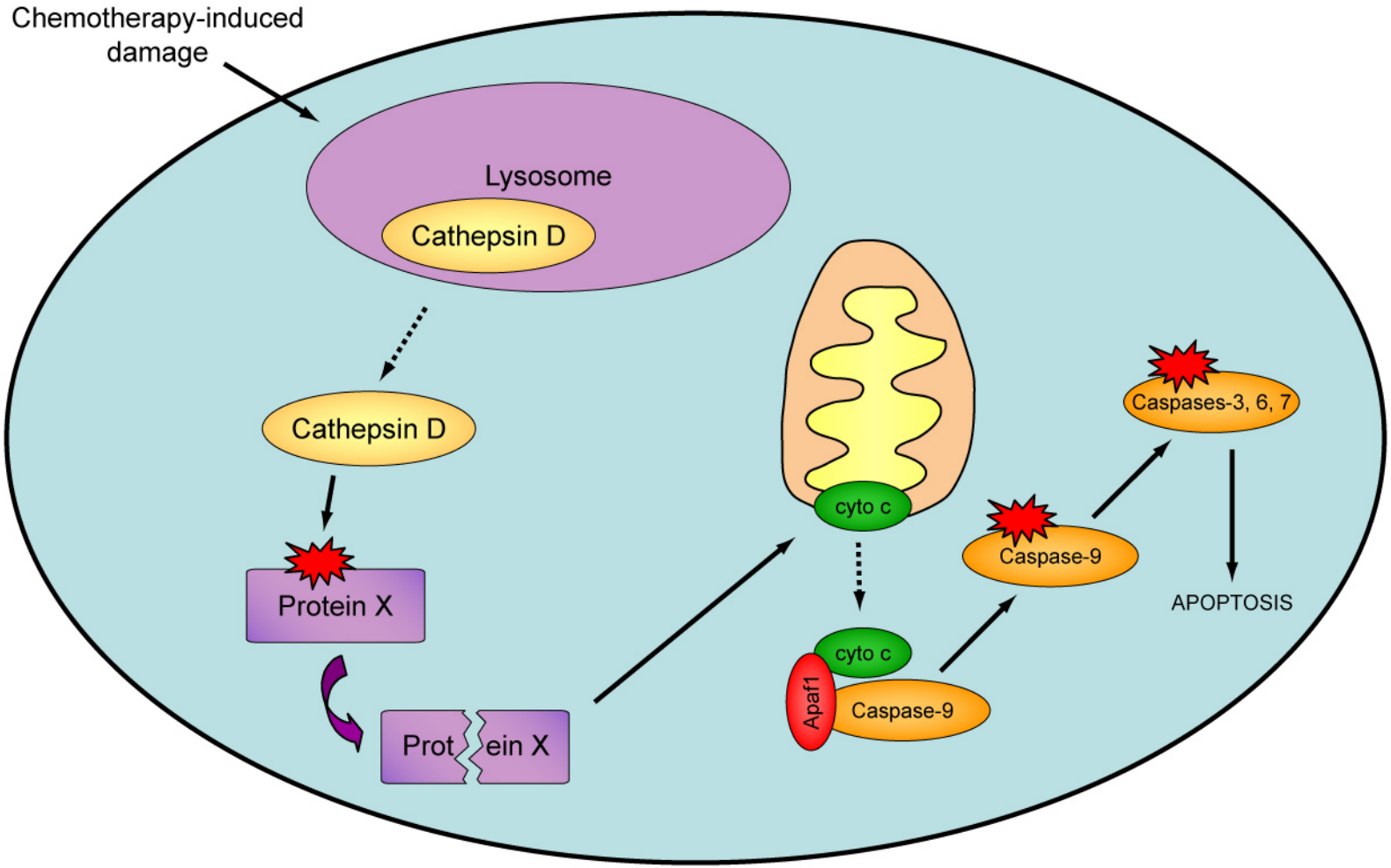


Figure 5.8. Potential mechanism of cathepsin D action. denotes cleavage or activation. See text for details.

induced by the death ligand TNF-α and by microtubule-stabilizing agents, but there is no evidence to suggest that cathepsin B is important for chemotherapy-induced apoptosis [64, 67-69]. Investigations of cathepsin L in apoptosis are contradictory. Levicar *et al.* [156] found that cathepsin L upregulation was associated with reduced apoptosis. Additionally, antisense-mediated downregulation of cathepsin L sensitized cells to apoptosis by reducing expression of the antiapoptotic protein Bcl-2, suggesting that cathepsin L might have an antiapoptotic effect. In another study, cathepsin L-deficient A549 cells displayed increased sensitivity to apoptosis [157]. In contrast, Ishisaka *et al.* [77] found that caspase-3 cleavage in cytosolic extracts by a crude lysosomal fraction was inhibited by two specific cathepsin L inhibitors, suggesting that cathepsin L might contribute to the apoptotic process. In this project, cathepsin D-induced cytochrome c release from isolated mitochondria was reduced by about 50% with the cathepsin L inhibitor z-FF-FMK (50 μM) (Figure 5.7). If cathepsin L is released from lysosomes following VP-16 treatment, it could potentially act as an intermediary for cathepsin D-induced cytochrome c release. The non-specificity of cathepsin L antibodies precluded study of the localization of cathepsin L following chemotherapy drug treatment in my project, but others have shown that it is released from the lysosomes during oxidative stress- and supraoptimal activation-induced apoptosis [60, 98, 158]. If cathepsin D is released following disruption of lysosomal targeting motifs, as suggested in Chapter 3, it would not be surprising to note mutual relocalization of cathepsin L and cathepsin D, as they both contain similar targeting motifs [79]. Inhibition of cathepsin L prevented cathepsin D-mediated cytochrome c release; therefore, it is possible that cathepsin D

cleaves cathepsin L following cytosolic relocalization of both enzymes and the cleaved cathepsin L serves to mediate cytochrome c release.

Another possible mediator of cathepsin D action is the proapoptotic Bcl-2 family member Bid. Bid resides in the cytoplasm as an inactive 26 kD protein. Following specific apoptotic stimuli, Bid is cleaved to a 15 kD truncated form (tBid), and this truncated form translocates to the mitochondria to provoke cytochrome c release [117, 159, 160]. Bid is normally activated by the death-receptor pathway in response to stimuli such as TNF-α or Fas ligand, but it can be activated by some drugs as well [161]. Thus, it can act as a "bridge" between the death-receptor mediated cell death pathway and the intrinsic mitochondrial pathway [162]. Reports on the role of lysosomal proteases in Bid cleavage are incongruous. A recent study demonstrated that disruption of lysosomes in HeLa cells, resulting in translocation of lysosomal proteases to the cytosol, initiated the cleavage of Bid [163]. Likewise, extracts of purified lysosomes were found to process Bid to an active fragment *in vitro* [57, 62]. Additionally, mitochondria that were incubated with Bid that had been treated with lysosomal extracts released a significant amount of cytochrome c. By contrast, mitochondria did not release cytochrome c in response to Bid-deficient cytosolic extract that had been incubated with lysosomal extract. [57]. Cirman *et al*. [163] reported that cathepsins B, H, L, and S can cleave Bid *in vitro* and that incubation of Bid with these cathepsins initiated rapid cytochrome c release from isolated mitochondria. However, Reiners *et al.* [62] and Mandic *et al.* [164] found that pharmacologic inhibition of cathepsins B, D, and L failed to suppress Bid cleavage following lysosomal photodamage. Another study showed that cystatin B-deficiency-induced apoptosis, which is promoted by cathepsins, was unaffected in Bid-

deficient mice, suggesting that cathepsins can promote apoptosis via a Bid-independent mechanism [165].

Likewise, data examining the importance of Bid in cathepsin D-induced apoptosis is also conflicting. In a recent study, Bid was reported to be cleaved to tBid by purified cathepsin D *in vitro* [149]. Moreover, in another study, Bid was not activated in cathepsin D-deficient fibroblasts during TNF-α-induced apoptosis [149]. However, most reports have demonstrated that cathepsin D cannot directly cleave Bid *in vitro* or *in vivo* [62, 163, 166]. Cathepsin D preferentially cleaves dipeptide bonds between hydrophobic residues, and Bid contains no sequences which are compatible with this stipulation [167]. With this in mind, investigations on the importance of cathepsin D in Bid cleavage are currently ongoing.

Because inadvertent activation of caspases would prove disastrous to healthy cells, regulation of caspase activation is stringent. Several safeguards must be surmounted before caspases are fully activated during the apoptotic process. SMAC/Diablo is a 25 kD protein that is released from the mitochondria during apoptosis and binds to IAP (Inhibitor-of-APoptosis) family members [154, 168]. IAPs, particularly XIAP, inhibit caspase activation by binding to the active pocket of several caspase enzymes, thereby preventing substrate entry [154, 169, 170]. When SMAC/Diablo is released into the cytosol during apoptosis, it binds to XIAP and "peels" it away from caspases, exposing their active site [155]. The importance of cathepsin D for SMAC release into the cytosol following chemotherapy treatment was examined by comparing the kinetics of cytosolic SMAC appearance in wild-type U937 cells and 144 CD siRNA/U937 cells. VP-16-induced release of SMAC/Diablo was inhibited in 144 CD

siRNA/U937 cells. Though purified cathepsin D did not induce direct activation of caspase-3 *in vitro* (Figure 5.2), cathepsin D-downregulation in U937 cells did impact caspase-3 activation in whole cells (Figure 5.1). It is possible, therefore, that in addition to stimulating release of cytochrome c, cathepsin D potentiates caspase-3 activation by augmenting release of SMAC/Diablo from the mitochondria and promoting liberation of caspases from IAP inhibition.

We have not yet identified the mediator responsible for cathepsin D-induced cytochrome c release. Preliminary experiments suggest that Bid cleavage is unaffected in VP-16-treated CD siRNA cells; therefore, other candidates must also be considered. Recent studies have demonstrated that during VP-16-induced apoptosis, the nuclear histone H1.2 is released into the cytoplasm, where it stimulates cytochrome c release in a Bak-dependent/Bid-independent manner [171]. Bak is a 23 kD pro-apoptotic member of the Bcl-2 family that is normally activated by tBid. Bak contributes to apoptosis by oligomerizing and forming pores that contribute to mitochondrial membrane permeabilization [172, 173]. Wei *et al*. [174] have shown that VP-16 induces apoptosis in Bid-deficient mouse embryonic fibroblasts (MEFs), but that Bak is still activated and is absolutely necessary for cell death. In non-apoptotic cells, Bak is sequestered in an inactive form by the antiapoptotic protein Bcl-X_L in the cytosol [175]. Activation of Bcl-X_L occurs via cleavage of the full-length protein into an 18 kD fragment [176]. Therefore, if cathepsin D perhaps cleaves Bcl-X_L protein, causing release of Bak, Bcl-X_L may represent another potential intermediate of cathepsin D-induced cytochrome c release.

An alternate possibility of cathepsin D action during VP-16-induced apoptosis is that a cytosolic factor may initiate supraoptimal activation of the cathepsin D enzyme to induce cytochrome c release. Experiments in which cytosolic extracts are incubated with pepstatin A either before or after addition of the cathepsin D enzyme would determine whether this unknown factor acts upstream or downstream of cathepsin D action in cytochrome c release following VP-16 treatment.

5.4. CONCLUSION

In this chapter, I have demonstrated that cathepsin D mediates cytochrome c release and caspase-3 activation following VP-16 treatment. Additionally, cathepsin D-cleaved cytosolic extract induces cytochrome c release from isolated mitochondria, suggesting that cathepsin D cleavage of a cytosolic factor is likely important for promoting cytochrome c release in cells. Moreover, cathepsin D may enhance the release of proapoptotic SMAC into the cytosol during chemotherapy-induced apoptosis, perhaps further augmenting caspase activation.

5.5. FINAL STATEMENT

Cell lines containing siRNA directed against cathepsin D mRNA were protected from chemotherapy-induced cell death, but cell death was not completely attenuated. Most likely, cathepsin D promotes chemotherapy-induced cell death by accelerating and augmenting various key apoptotic processes. The importance of understanding the mechanism of cathepsin D action in apoptosis should not be underestimated. Current

chemotherapy strategies are plagued by problems with cytotoxicity in normal cells and with acquired resistance, which leads to reduced therapeutic potential. Cytotoxic responses develop when chemotherapy drugs reduce expression or function of a target protein that, while contributing to the neoplastic potential of one cell, is vital to the normal function of another. The development of novel drug targets and targeting strategies is critically important for circumventing the resistance and cytotoxic problems incurred with current drugs and targets. With its ability to significantly enhance chemotherapeutic drug-induced cell death, cathepsin D and the pathway it mediates may represent a novel therapeutic target for combinatorial anti-cancer strategies.

APPENDICES

APPENDIX A

ABBREVIATIONS

AIF……..... Apoptosis-inducing factor

Apaf-1…….. Apoptotic protease activating factor

ATP…….. Adenosine triphosphate

CB…… Cathepsin B

CD……... Cathepsin D

DD……... Death domain

DED……. Death effector domain

DISC…… Death-inducing signaling complex

DMSO……. Dimethyl sulfoxide

DR……... Death receptor

dsRNA……. Double-stranded RNA

DTT……. Dithiothreitol

ECV……. Endosomal carrier vehicles

EDTA…….. Ethylenediaminetetraacetic acid

FADD…….. Fas-associated protein with death domain

IFN-γ…… Interferon-γ

LAMP…….. Lysosome-associated membrane protein

M6P……. Mannose-6-phosphate

MEF……. Mouse embryonic fibroblast

MSA…… Microtubule stabilizing agent

PARP……... Poly-ADP-ribose polymerase

PBS…….. Phosphate-buffered saline

PCD……. Programmed cell death

PKC……. Protein kinase C

PMSF……... Phenyl methane sulphonyl fluoride

RISC……. RNA-induced signaling complex

RNAi……. RNA interference

SDS-PAGE……... Sodium dodecyl sulfate polyacrylamide gel electrophoresis

siRNA……... Small-interfering RNA

TBST…… Tris-buffered saline + Tween-20

TNF-α…….. Tumor necrosis factor-α

TNFR…… Tumor necrosis factor receptor

TRADD…… TNF receptor-associated protein with death domain

TRAIL…….. TNF-related apoptosis-inducing ligand

WT……... Wild-type

XIAP…… X-linked inhibitor of apoptosis protein

APPENDIX B

ANTIBODIES

Antibody name (anti-)	Type	Primary Antibody Dilution	Secondary Antibody Dilution	Company
β-actin	Mouse	1:5000	1:5000	Sigma
Caspase-3	Rabbit	1:1000	1:4000	Cell Signaling
Caspase-8	Mouse	1:1000	1:4000	Cell Signaling
Cathepsin B	Rabbit	1:250	1:4000	Calbiochem
Cathepsin D	Rabbit	1:1000	1:4000	Calbiochem
Cathepsin E	Goat	1:1000	1:4000	Santa Cruz
Cytochrome c	Mouse	1:1000	1:4000	BD Pharmingen
Cytochrome c oxidase IV	Mouse	1:1000	1:4000	Molecular Probes
LAMP	Mouse	1:1000	1:4000	RDI
PARP	Mouse	1:1000	1:4000	BD Pharmingen
SMAC	Goat	1:1000	1:4000	R&D Systems
XIAP	Mouse	1:1000	1:4000	BD Transduction

APPENDIX C

ENZYME SUBSTRATES

Enzyme Name	Enzyme Substrate
β-hexosaminidase	4-methylumberriferyl-N-acetyl-β-D-glucosaminide
Caspase-3	Ac-DEVD-AFC
Cathepsin D	MoCAc-Gly-Lys-Pro-Ile-Phe-Phe-Arg-Leu(Dnp)-D-Arg-NH_2

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