ABSTRACT

Title of thesis: IDENTIFICATION OF AUTOPHAGIC CELL DEATH AND IMPLICATIONS FOR THERAPY

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Autophagy is an evolutionarily conserved mechanism of bulk protein and organelle degradation that requires the *ATG* class of genes. Although autophagy has been frequently observed in dying cells in several species, a causative role for autophagy in cell death has not been demonstrated. We show that inhibition of caspase-8 in mouse L929 fibroblast cells causes cell death with the morphology of autophagy. Autophagic cell death in L929 cells is dependent on *ATG* genes and involves the receptor interacting protein *(RIP)* and the activation of the MAP kinase kinase 7*(MKK7)* - Jun N-terminal kinase *(JNK)* - *cJUN* pathway. We also show that autophagy occurs in many primary human tumors including cancer of the breast, lung and pancreas. Our findings validate autophagic cell death and might explain the role of autophagy in development, viral infections, neurodegenerative diseases and cancer.

IDENTIFICATION OF AUTOPHAGIC CELL DEATH

AND IMPLICATIONS FOR THERAPY

by

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Dedicated to my parents, my wife and my brother.

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Chapter 1

Introduction

Cell death

In 1951, Glucksmann postulated the seemingly paradoxical concept of significant cell death accompanying cell proliferation during development of multicellular organisms (1). The ability to balance timely and controlled cell death with cell multiplication is also vital during adult life, for example to suppress tumor growth (2).

Types of cell death

The three major morphological types that are common in dying cells are apoptosis, autophagy and necrosis (3). Apoptosis is characterized by cytoplasmic shrinkage, nuclear condensation and chromatin fragmentation, preservation of membrane integrity late into the process, early exposure of phosphatidylserine on the cell surface, and blebbing of the plasma membrane (4). Internucleosomal fragmentation of DNA has generally been considered the hallmark of apoptosis (5). The execution machinery of apoptosis consists of a special class of proteases called caspases (6). Autophagic cell death, on the other hand, is characterized by formation of vacuoles that transport organelles and cytoplasmic content to lysosomes for degradation (7). Cytoplasm is condensed with tightly packed organelles and with evidence of mitochondrial condensation. Nuclear features such as fragmentation are not characteristic. Cells dying by autophagy tend to be in clusters and phagocytes are not primarily responsible for clearance of the dead cells (8, 9). Necrotic death shows cell swelling, loss of membrane

integrity and release of cytoplasmic content to the exterior (10). Nuclear changes are variable and inconsistent (3).

Thirty years of cell death research has uncovered an astounding array of cell death mechanisms, signaling pathways and dying cell morphologies which could occur in various permutations and combinations. For example, caspase activation had been considered to be typical of apoptosis (11). However, caspases are active in autophagic cells in programmed cell death of *Drosophila* salivary glands (12). Caspase 8 activity and phosphatidylserine surface exposure, seen most commonly in apoptosis, have also been noted in necrotic dying cells (10, 13). Furthermore, nuclear condensation has been observed in several instances of non-apoptotic death, albeit with different patterns of condensation (14, 15). An important inference is that there is no single or exclusive linear sequence of events to bring about death in a cell. Death receptors are thought of as one of two pathways to activate caspases and apoptosis. Recent work suggests that death receptors can activate apoptotic and non-apoptotic pathways of cell death in the same cell type in response to the same death stimulus (13, chapter 2). Caspases, long considered to be synonymous with death proteases, are neither necessary nor sufficient to cause cell death (16, 17). Other proteases like cathepsins can cause cell death independent of caspases (18). To illustrate the evolving concepts in cell death and possible relationships between autophagic and apoptotic cell death (Fig. 1), it is worthwhile to consider specific models of cell death.

A) Both autophagy and apoptosis occur and are responsible for PCD.

B) Autophagy necessary for apoptosis but not sufficient for cell death.

C) Autophagy and apoptosis as alternative routes of death.

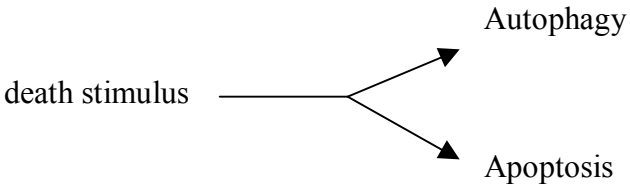

Fig.1. Possible relationships between autophagy and apoptosis in dying cells. Different models of dying cells reveal different relationships between autophagy and apoptosis. A) A single death stimulus triggers death with features of both autophagy and apoptosis. B) Autophagy is necessary to trigger apoptosis but not sufficient by itself for death. C) Either autophagic death or apoptotic death ensues after a death stimulus in a mutually exclusive manner.

The salivary glands of *Drosophila* pupae undergo massive programmed cell death (PCD) beginning at 12 hours after puparium formation in response to a pulse of ecdysone steroid (19). Dying salivary gland cells develop large vacuoles in their cytoplasm (20). Smaller vacuoles with enclosed organelles are observed two hours later and by 16 hours after puparium formation, salivary gland autolysis is complete. Microarray analyses of dying salivary gland cells revealed upregulation of several classes of genes including the *Drosophila* homologues of yeast *ATG* genes that are responsible for starvation induced autophagy (21). Suprisingly, there is a requirement for caspase activity for ecdysone regulated programmed cell death to occur in salivary gland cells and several features of apoptosis are present (22). Inhibition of caspases by over-expression of the viral caspase inhibitor p35 prevents salivary gland PCD (20). Moreover, caspase processing of cytoskeletal elements and nuclear fragmentation are observed in these dying cells (23). Thus, both autophagy and apoptosis genes contribute to *Drosophila* salivary gland PCD (Fig. 1 A).

An interesting model to study PCD in the context of development is the 3-D MCF10A mammary epithelial cell culture model which closely parallels normal physiology of mammary glands including lumen formation in the mammary acini (24, 25). Over-expression of anti-apoptotic Bcl2 family members merely delays and does not prevent cell death during cavity formation (26). Morphology of the dying cells reveals autophagic vacuoles (26). Cell death accompanied by autophagy is also seen in primary mouse mammary epithelial cultures and in pre-pubertal mouse mammary glands (27, 28). Recent reports suggest Tumor necrosis factor related apoptosis inducing ligand (TRAIL) induced apoptosis via death receptor activation in the above mentioned 3-D MCF10A

model requires both autophagy and apoptosis for complete cell death (29) (Fig. 1 A). Inhibition of either process results in partial lumen formation.

Autophagy has been postulated to be necessary for initial steps of the apoptotic program in a leukemic cell line CCRF-CEMVbl in response to TNF-alpha (30). CCRF-CEMVbl cells exhibit autophagic vacuoles before apoptotic features such as nuclear fragmentation appear. Also, inhibition of the early stages of autophagy by the Phospahaditylinositol-3 kinase (PI3K) inhibitor 3-methyladenine (3-MA) prevented TNF-alpha induced death. Surprisingly, enhancing autophagy in these cells did not enhance apoptotic death in terms of extent or time. Thus, autophagy seems to be necessary but not sufficient for cell death (Fig. 1 B).

Stimulation of death receptors by FasL, TNF or TRAIL can all induce caspase independent cell death resembling necrosis in activated T lymphocytes and this mechanism of cell death requires the serine threonine kinase Receptor Interacting Protein (RIP) (13). Furthermore, certain features observed in these dying cells such as membrane blebbing have been shown to be dependent on cathepsin B (18). Therefore, even classical mediators of apoptosis such as the death receptors could function in alternative forms of cell death. It would therefore seem that death signals ensure cell death by activating one of two (or more) alternate pathways (Fig 1 C).

From the lines of thought touched upon in the preceding discussion, it is clear that artificial classifications of cell death into stand alone entities are no longer valid. Cell death is probably best viewed as various overlapping and intertwining combinations of the same components at the sensing (death receptors, steroid receptors), mediating (e.g. MAP kinase kinase pathway, DAPkinase) and executing stages (e.g. caspases, cathepsins,

calpains, reactive oxygen species) with the ultimate end result of death. We shall next consider in detail the two major mechanistic processes, namely apoptosis and autophagy, to elaborate on their morphology and molecular machinery.

Autophagy

Autophagy is an evolutionarily conserved mechanism of protein and organelle degradation that has been observed in organisms that are as different as yeast and humans (31). Autophagy is the process by which cytoplasmic structures are sequestered into vacuoles and then transported to lysosomes predominantly for degradation (7). Although frequently observed in dying cells as discussed above (32, 33), autophagy was first observed as part of a hepatocellular response to lack of nutrients. Formation of autophagic vacuoles was described in rat liver tissue following amino acid deprivation and glucagon stimulation (34, 35). Autophagy is a general term that encompasses an ever-increasing list of related yet sufficiently distinct processes (36).

A) Macroautophagy: It involves formation of a double or multi membrane cytoplasmic vesicle-like structure that sequesters cytoplasmic proteins or organelles. In a series of steps detailed below, this structure is transported to the lysosome. The outer membrane of the structure fuses with the lysosomal limiting membrane, releasing the contents into the lysosome where they are degraded by the action of lysosomal hydrolytic enzymes.

B) Microautophagy: The distinctive feature of this process is the outpouching of a portion of the lysosomal membrane to engulf an organelle. Unlike in

macroautophagy, there is no sequestering double membrane formed in the cytoplasm.

C) Cytoplasm-to-vacuole targeting pathway (cvt): In *S.cerevisiae*, the biosynthetic physiological function of delivering the enzymes aminopeptidase I and alpha-mannosidase to the yeast vacuole is carried out by the autophagy-like Cvt pathway. Several of the gene products required for the process are shared with the macroautophagy pathway.

D) Pexophagy and mitophagy: The selective sequestration or engulfment of peroxisomes and mitochondria respectively and delivery to lysosomes.

The best characterized type and that which is associated with dying cells is macroautophagy. Henceforth, the term 'autophagy' will refer to macroautophagy unless indicated otherwise.

Stages in the formation of Autophagic vacuoles

While there is no precise delineation of the stages in the process or their terminology, the following sequence, based on studies in *S.cerevisiae*, is widely accepted (7).

1. Induction: Autophagy inducing signals such as a lack of nutrients stimulate formation of a crescent shaped cisterna called the 'phagophore'. The origin of the cisterna and its membranes is unknown although an endoplasmic reticulum origin is considered most probable (37).
2. Formation: Elongation and complete encircling to form the double membrane bound 'autophagosome' or 'autophagic vacuole'.

3. Docking and fusion: Fusion of the autophagosome with a lysosome, whereupon the inner membrane and its enclosed cytoplasmic contents (called the ‘autophagic body’) are released into the lumen of the lysosome.
4. Breakdown and recycling: Lysosomal enzymes degrade the contents of the autophagic body.

A **B**

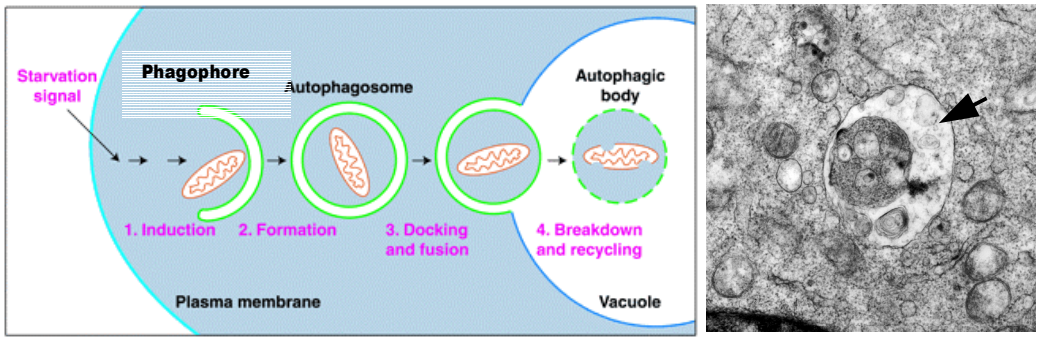

Fig.2. Stages of Autophagy in yeast. A. Autophagy commences with induction of a 'phagophore' that encircles and sequesters organelles and cytoplasm to form the 'autopaghosome' which then docks and fuses with a lysosome, and releases its contents into the lysosomal interior as the 'autophagic body'. Lysosomal hydrolases and proteases ensure degradation of the autophagic body (modified from 7). B. Transmission Electron Microscope view of an autophagic vacuole (arrow) in a L929 mouse fibroblast cell. Magnification is 16000.

Molecular pathways and regulation of autophagy

Genetic screens for defects in the formation of autophagic vacuoles and protein degradation in *S.cerevisiae* resulted in a better understanding of autophagy at the molecular level and the identification of a class of genes called *ATG/APG/AUT/CVT* in *S.cerevisiae* (38, 39, 40). Yeast lacking *ATG genes* show defects in autophagy in response to starvation. Homologues for twelve yeast *ATG* genes and their proteins have been identified in other species including humans (Table 1). Several of the *ATG* genes are postulated to participate in a pair of ubiquitin-like conjugation systems (41, 42).

It is not surprising, given the manifold functions of autophagy, that the upstream pathways are diverse and range from nutrient regulation, cell proliferation, cell size regulation, development and cell death (43).The prominent regulating molecules in mammalian cells are Class I and Class III PI3K, Akt-PTEN-TSC1/2-Rheb-mTOR circuit, the Insulin Receptor and downstream targets. Briefly, Class I PI3K, Akt, Rheb and mTOR components inhibit autophagy while Class III PI3K, PTEN and TSC1 and 2, and the mTOR inhibitor rapamycin positively regulate autophagy. It is interesting that several of these molecules (for example, PI3K) also regulate apoptosis, proliferation, growth, protein translation and cell survival. Indeed, autophagy seems to be one node in a complex network of sensors, mediators and effectors that enable cells to respond to diverse challenges.

S. cerevisiae	***C. elegans***	***D. melanogaster***	***H. sapiens***
Atg1	NP_507869	CG10967	XP_008514
Atg2	ns	CG1241	NP_060506
Aut1/Atg3	NP_500024	CG6877	NP_071933
Aut2/Atg4	NP_502208	CG6194	NP_116241
Atg5	ns	CG1643	NP_004840
Atg6/Vps30	T29537	CG5429	NP_003757
Atg7	NP_502064	CG5489	NP_006386
Aut7/Atg8	NP_495277	CG1534	NP_009216
Atg9	NP_503178	CG3615	BAB15246
Atg10	ns	ns	ns
Atg12	NP_498228	CG10861	NP_004698
Atg13	ns	ns	ns
Atg14	ns	ns	ns
Atg16	ns	ns	ns
Atg17	ns	ns	ns
Aut10	T26730	LD38705p	AAH07596
Aut4	ns	ns	ns
Cvt17	ns	ns	ns

Table 1. Presumed Atg proteins in *S.cerevisiae ,C.elegans, D.melanogaster* and *Homo sapiens* (from 7). Many of the Atg proteins are conserved across several species, from yeast to humans.

Apoptosis

Apoptosis is characterized by exposure of phosphatidylserine on the cell surface, preservation of membrane integrity until late in the process when the apoptotic cell becomes permeable to vital dyes such as DAPI, nuclear condensation and fragmentation, and engulfment by scavenger phagocytes (44). Stimuli that induce apoptosis include growth factor or hormone withdrawal, cell lineage, abnormally persistent cells during development, toxic chemicals, drugs and radiation. Apoptotic stimuli trigger activation cascades of a special class of proteases called caspases (45). There are two distinct pathways of caspase activation, namely the death receptor mediated pathway and the mitochondrial pathway (11).

The Death Receptor family, that includes Fas, TNFR1, and TRAIL-R1, is located on the plasma membrane (fig.3). Death receptors cluster together when bound to their respective ligands (46, 47) and recruit adaptor molecules such as Fas associated death domain protein (FADD) (48). Specialized domains of the adaptor molecules in turn recruit procaspase molecules via homotypic interactions (49). The signaling complex thus formed, with its constituent death receptors, adaptor molecules and procaspases, is called the Death Inducing Signaling Complex (DISC) (50). Procaspase molecules in the DISC process each other through proteolysis utilizing their feeble enzymatic activity, the so called 'Induced Proximity' model (51), resulting in active caspases. The intrinsic or mitochondrial pathway requires APAF , cytochrome c and ADP released from mitochondria to recruit procaspase 9 in the formation of the 'apoptosome' (52), which is analogous to the DISC in that it contains active caspases.

Caspases are cysteine containing aspartate specific proteases (45). They are classified into initiator caspases and effector caspases (25). The subset of caspases called initiator caspases (including caspase 8, caspase 9, caspase 10, caspase 2) are activated in response to a death inducing stimulus at the DISC or at the apoptosome. Initiator caspases in turn process effector caspases. The effector caspases (including caspase 3, caspase 6, caspase 7) are responsible for the morphological hallmarks of apoptosis through their protease activity on a variety of substrates. For example, caspase-3 degrades the Inhibitor of Caspase Activated DNase (ICAD), thereby releasing the DNase (CAD) from inhibition. DNase action results in the characteristic ladder of DNA fragments detected in apoptotic cells (53).

It should be mentioned that death receptors in spite of their name do not invariably cause cell death. For example, death receptors, adaptors like FADD and caspases activation has been shown to induce NF-KB expression and modulate immune and pro-survival responses (54, 55, 56). The polar outcomes of death receptor signaling are probably determined by the receptor type and domains, the identity of the adaptor molecules and their interacting proteins and the molecular milieu downstream.

The focus of my thesis work has been to investigate autophagic cell death in mammalian cells and its relationship to apoptosis, to explore the regulatory pathways of autophagic death and to identify autophagy in primary human tumors. Non apoptotic cell death mechanisms such as autophagic cell death have wide ranging therapeutic implications in the context of human diseases.

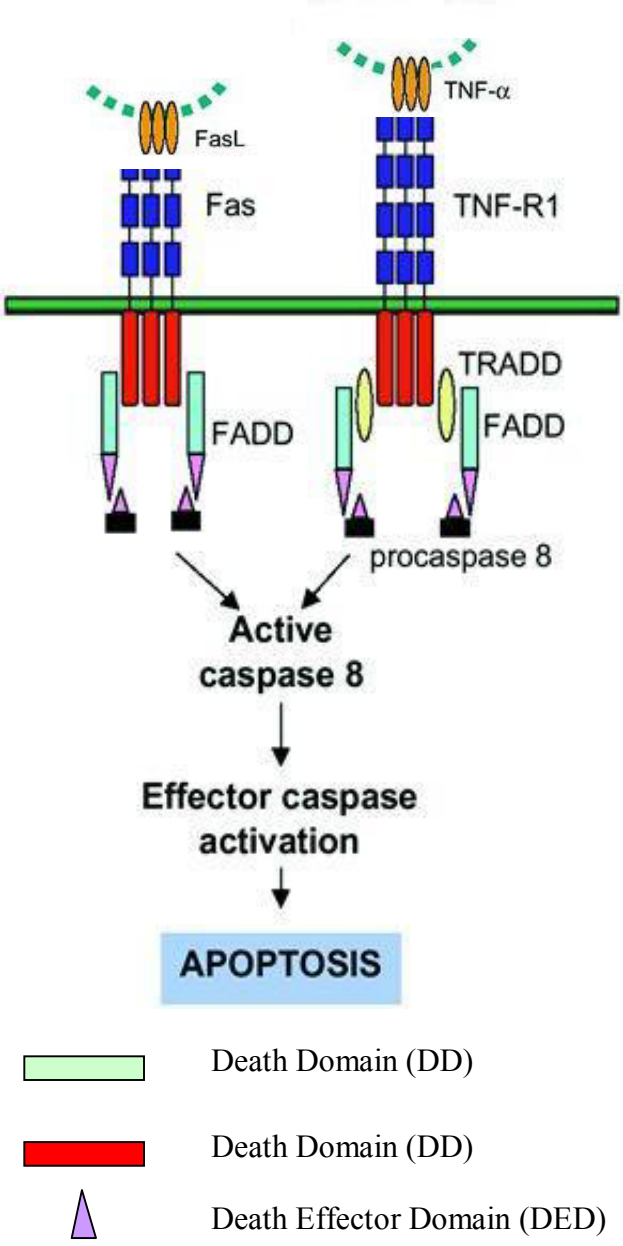

Fig. 3. Death Inducing Signaling Complex (DISC). Binding of the ligand (FasL or TNF-α) to the trimeric death receptor(TNF-R, Fas) triggers formation of the Death Inducing Signaling Complex (DISC) by recruitment of adaptor molecules (FADD, TRADD) via homotypic interactions. The DISC activates the initiator caspases caspase 8 and caspase 10. Active caspase 8 and 10 process and activate effector caspases like caspase 3. [TNF, tumor necrosis factor; TNF-R1, TNF receptor type 1; TRADD, TNFR-associated death domain protein; FasL, Fas ligand; FADD, FAS-associated death-domain protein.]

Chapter Two

Regulation of an Atg7-Beclin 1 Program of Autophagic Cell Death by Caspase-8

Section 2.1: Abstract

Caspases play a central role in apoptosis, a well-studied pathway of programmed cell death. Other programs of death potentially involving necrosis and autophagy may exist, but their relationship to apoptosis and mechanisms of regulation remain unclear. We define a new molecular pathway in which inhibition of caspase-8 activity induces cell death with the morphology of autophagy. Cell death and autophagy involve the activation of the receptor-interacting protein (RIP, a serine-threonine kinase), MAP kinase kinase 7 (MKK7), Jun N-terminal kinase (JNK) and c-JUN, and are dependent on the genes *ATG7* and *BECLIN-1*. Therapies involving caspase inhibitors, presently in development for a range of clinical conditions, may arrest apoptosis but may have the unanticipated effect of promoting autophagic cell death.

Section 2.2: Introduction

Apoptosis is a well-studied pathway of programmed cell death conserved from *C.elegans* to humans (57). Caspases, a family of cysteine containing aspartate specific proteases, produce the morphological changes associated with apoptotic death (58, 59). Non-apoptotic forms of cell elimination include those with features of necrosis and autophagy (3, 13, 60, 61). Necrosis can result when cell metabolism and integrity are compromised by a non-physiological insult. Recently, evidence has emerged that death receptors and

RIP can induce caspase-independent cell death that appears necrotic (13, 61). Autophagy promotes a cell survival response to nutritional starvation involving membrane-bound vacuoles that target organelles and proteins to the lysosome for degradation (7, 62). Two pathways containing ubiquitin-like genes that are highly conserved from yeast to humans, function in autophagy (*ATG* genes). Certain examples of cell death have autophagic features, but a role for *ATG* genes in cell death has not been established (31). Here we show that autophagy is associated with death of some mammalian cells, that this death depends on the expression of genes that are homologous to yeast *ATG* genes, and that this mechanism of cell death is negatively regulated by caspase 8.

Section 2.3: Materials and Methods

Tissue Culture

The mouse L929 cell line was obtained from the American Type Culture Collection (Rockville, MD). L929 cells were cultured in Dulbecco's modified Eagle's medium with 4.5 g/L glucose. Media were supplemented with 2 mM L-glutamine, 1% penicillin/streptomycin solution, and 10% fetal bovine serum (FBS).

Preparation of siRNA

Non-specific RNAi oligoribonucleotides and RNAi oligoribonucleotides corresponding to the following cDNA sequences were purchased from Dharmacon (Boulder, CO).

CAGTTTGGCACAATCAATA for *BECLIN 1*.

GTTTGTAGCCTCAAGTGTT for mouse *ATG*7.

CCACTAGTCTGACTGATGA for RIP.

TGAGATACTCGAGGTGGAT for MKK7.

CATTCGATCTCATTCAGTA for c-Jun.

GATCGAGGATTATGAAAGA for caspase-8.

Transfection of siRNA

0.5 nmol RNAi were transfected by Amaxa nucleofectionTM (Gaithersburg, MD). Cells were then cultured in growth medium for 96 hrs before further analysis.

Cell death analysis

Cell viability was determined after treatments by staining with propidium iodide (2 μg/mL) and flow cytometric analysis on a FACScan. Percent cell death was quantitated as previously described (63). [Note: The above experiments were done by Yu, L]

Electron microscopy analyses

Cells were fixed in 3% glutaraldehyde in 0.1 M MOPS buffer (pH 7.0) for at least 16 hours at 4^{o}C, post-fixed in 1% osmium tetroxide for 1 hour, embedded in Spurr's resin, sectioned, double stained with Uranyl acetate and Lead citrate, and analyzed using a Zeiss EM 10 transmission electron microscope. For each treatment or control group, at least 100 cells from randomly chosen transmission electron microscopy fields were analyzed for quantification of morphological features. Cells with $\geq$ 10 vacuoles were scored as autophagy positive. Statistical analysis of zVAD and DMSO treated groups were done with Statview 5.0.1 program.

Section 2.4: Results

zVAD, a caspase inhibitor, induces cell death in L929 cells.

In mouse L929 fibroblastic cells, tumor necrosis factor (TNF), oxidants, ceramide, and radiation can induce caspase-independent death (64). However, benzyloxycarbonyl-

Valyl-Alanyl-Aspartic-acid (O-methyl)- fluoromethylketone (zVAD), a pan-caspase inhibitor with broad specificity, induced the death of L929 cells (Fig. 4). Death began 12 hours after zVAD treatment and was complete by 40 hours (Fig.5). The dead cells appeared to be round, detached (Fig. 4 b), and had a convoluted plasma membrane permeable to vital dyes (Fig. 4 d); this differed from apoptosis in which nuclei are condensed and membrane integrity is preserved.

Transmission electron microscopy (TEM) revealed intact mitochondria and endoplasmic reticulum, condensed osmophilic cytoplasm, and numerous large cytoplasmic inclusions that were membrane-bound vacuoles characteristic of autophagy (Fig. 6). A time course revealed that vacuolated cells accumulated prior to cell death (Fig. 5 and 7). Similar results were obtained in human U937 monocytoid cells and mouse RAW 264.7 macrophage cells (65).

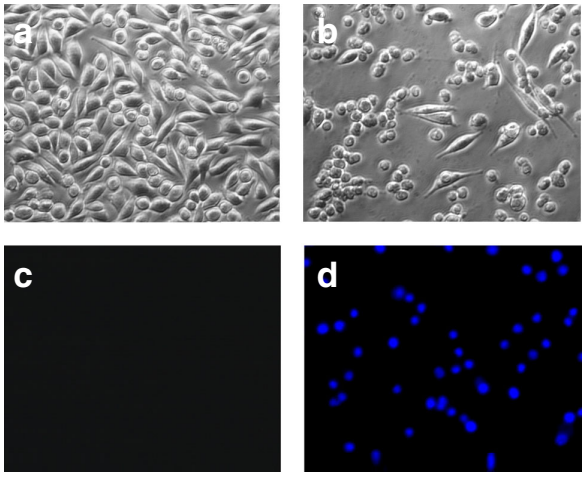

Fig.4. zVAD induces cell death in L929 cells. L929 cells were treated with 1ul DMSO vehicle (panels a and c) or 20 uM zVAD (panels b and d) for 24 hours and then examined by light microscopy (panels a and b) or DAPI-staining and fluorescent microscopy (panels c and d). Magnification is 200X. [Note: This work was done by Yu, L]

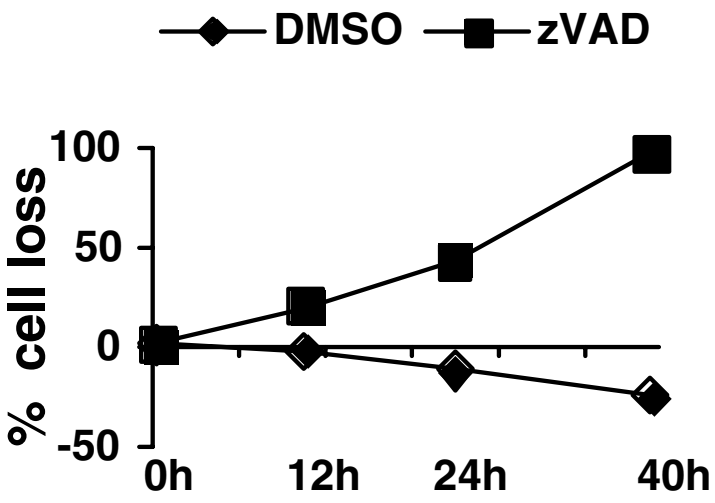

Fig.5. Time course of zVAD induced cell death in L929 in hours (h).Cells were harvested, stained with propidium iodide, and cell loss was quantified by flow cytometry. [Note: This work was done by Yu, L]

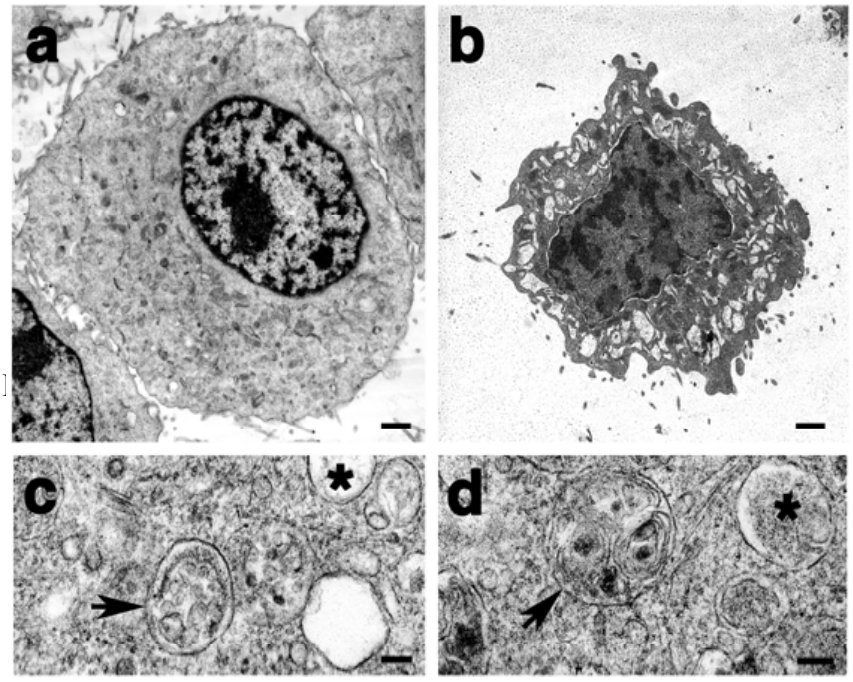

Fig.6. TEM analysis of L929 cells treated with zVAD. L929 cells were treated for 12 hours with vehicle (a) or zVAD (b-d). For (c, d), early membrane-bound vacuoles (black arrowheads) and later vacuoles (asterisks) are shown. Scale bars in (a, b) are 1 um and in (c, d) are 0.1 um.

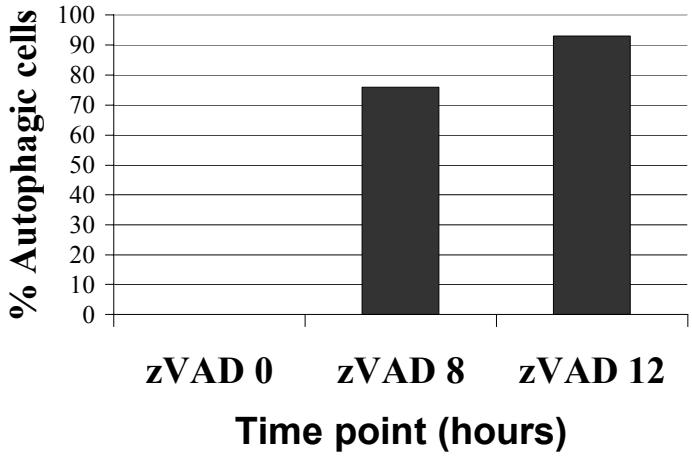

Fig.7. Autophagy precedes cell death and increases temporally in zVAD treated L929 cells. L929 cells were treated with 20uM zVAD and were harvested at different time points as indicated and analyzed for TEM for autophagic structures. % Autophagic cells refers to cells with >10 autophagic vacuoles/cell as a fraction of all cells.

Inhibition of Autophagy prevents zVAD induced cell death.

The association of autophagic vacuoles with cell death has been observed in developing animals, but it has not been clear if it was a process to rescue or condemn the cell (8). *Drosophila* cells manifesting autophagy and death have increased *ATG* gene transcripts (21, 66), but there is no known requirement for *ATG* genes in any model of cell death. We sought evidence that autophagy was required for cell death by treating L929 cells with two inhibitors of autophagy, 3-methyladenine (3-MA) and Wortmannin (7, 30). Both inhibitors prevented zVAD-induced cell death (Fig.8). However, these inhibitors are general phosphatidylinositol-3 kinase (PI-3 kinase) inhibitors and could simultaneously affect autophagy and other cellular processes.

We tested whether *ATG* genes were required for cell death. *ATG7 (HsGSA7 / mAPG7)* is an important autophagy gene encoding a protein resembling an El-type ubiquitin-activating enzyme that is used in both ubiquitin-like pathways required to form autophagic vacuoles in yeast (67, 68). We reduced expression of *ATG7* by RNAi and found that zVAD-induced cell death was almost completely inhibited (Fig. 9 A). Another *ATG* gene, *BECLIN-1*, the mouse homolog of yeast *ATG6*, encodes a Bcl-2-interacting, candidate tumor suppressor and antiviral protein (69, 70). Molecular alterations in beclin 1 are common in human cancers and *BECLIN-1* gene knockouts in mice cause a dramatic increase in epithelial and hematopoietic malignancies (71, 72). Reduction of the Beclin-1 protein by RNAi also decreased zVAD-induced death (Fig. 9 A). TEM analyses of cells with reduced Atg7 or Beclin-1 protein levels showed a parallel inhibition of autophagic vacuole formation associated with reduced cell death (Fig. 9 B) Thus, *ATG7* and *BECLIN-1* are required for autophagic cell death triggered by zVAD.

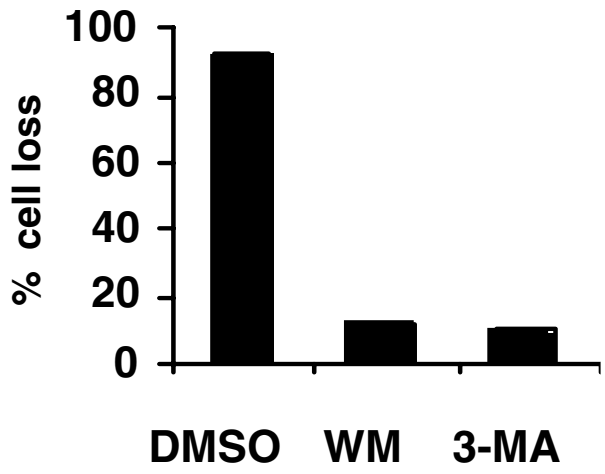

Fig.8. Inhibitors of autophagy prevent zVAD induced cell death. L929 cells were pretreated with 1 ug/ml Wortmannin (WM) or 10 mM 3-methyladenine (3-MA) for 1 hour, then with 20 uM zVAD for 36 hrs, after which cell loss was quantitated. [Note: This work was done by Yu, L]

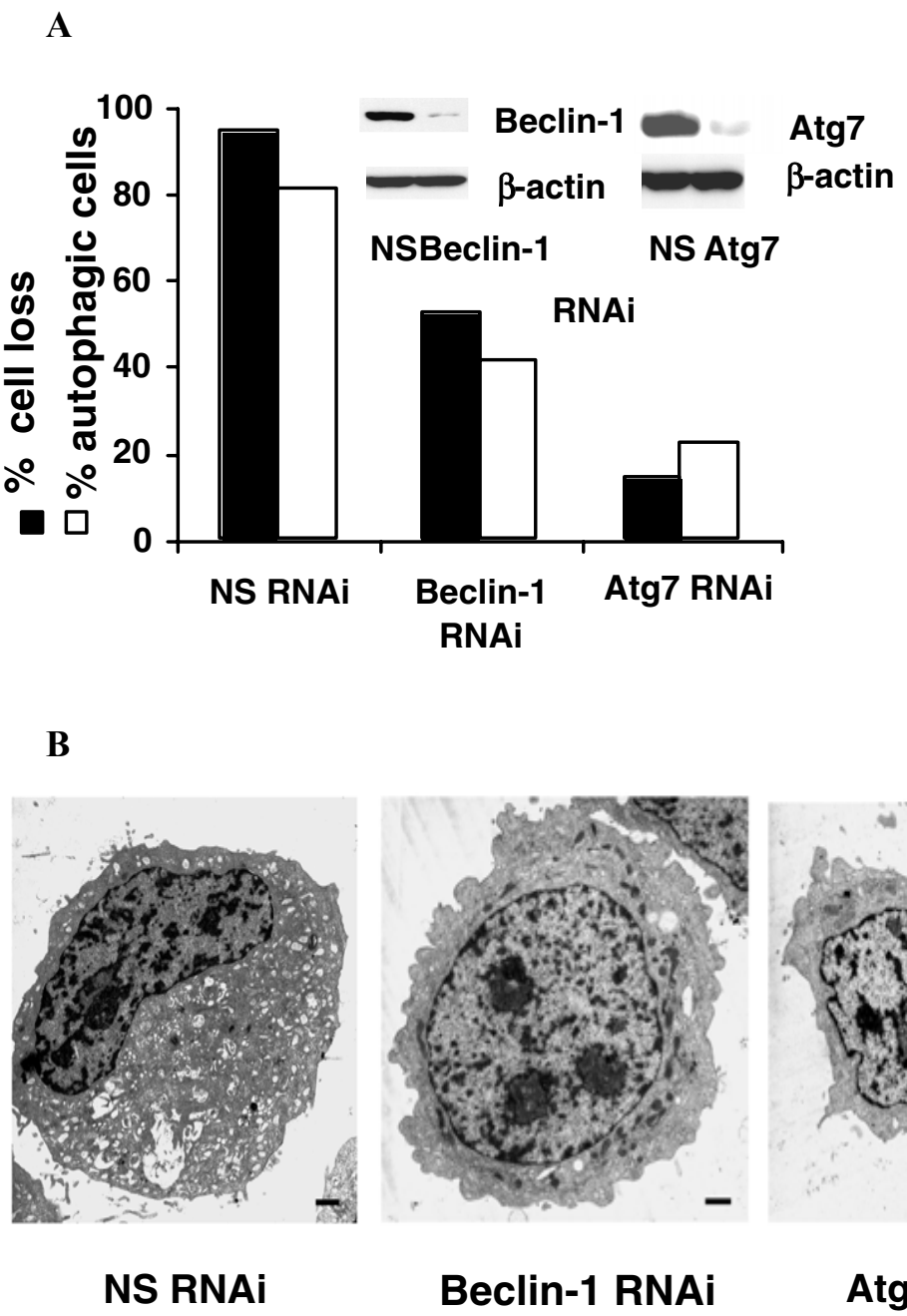

Fig.9. zVAD induced cell death and autophagy requires Atg7 and Beclin-1. A) Cells were treated with zVAD or vehicle for 36 hour, 96 hours after transfection with RNAi or non-specific oligonucleotides. The % cell loss (solid bars) and the fraction of cells with autophagy features (>10 autophagic vacuoles/cell) based on TEM (open bars) were quantitated. All quantitative data represent at least 3 experiments. The steady state levels of the corresponding proteins are shown by Western blot (inset). B) TEM of L929 cells transfected with *ATG* RNAi and treated with zVAD. Representative TEM of L929 cells transfected RNAi as indicated or non-specific oligonucleotides. After 96 hours, these cells were treated with zVAD for 36 hours. Scale bars are 1 um.

RNAi of RIP prevents zVAD induced cell death.

Death receptors can elicit nonapoptotic death through the 'receptor-interacting protein' (RIP), a death-domain-containing, serine-threonine kinase (13, 61). We therefore reduced RIP expression by RNAi and observed decreased autophagy and decreased cell death (Fig. 10 A and B).

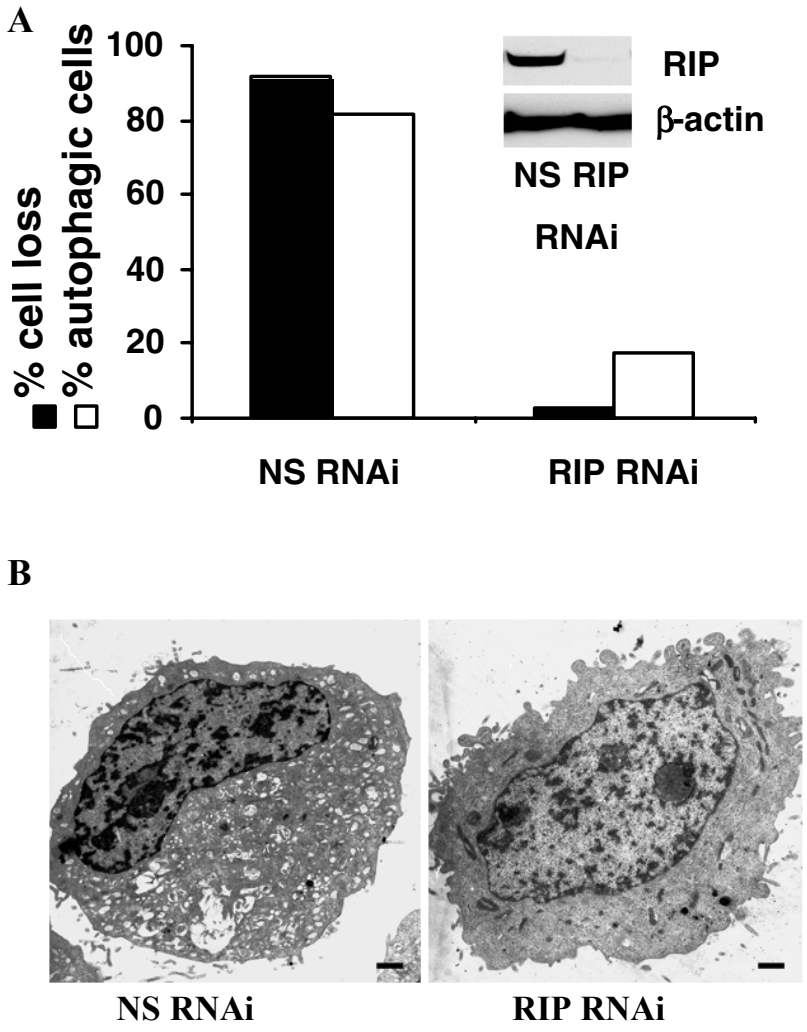

Fig.10. zVAD induced cell death and autophagy require RIP. A) L929 cells were treated with zVAD or vehicle 96 hours after transfection with RIP RNAi or non-specific oligonucleotides and the % cell loss (solid bars) and the fraction of cells with autophagy features based on TEM (open bars) was quantitated. The steady state level of RIP is shown by Western blot (inset). B) TEM of L929 cells transfected with RIP RNAi and treated with zVAD. L929 cells were transfected with non specific oligonucleotides or RIP RNAi. After 96 hours, these cells were treated with zVAD for 36 hours. Scale bars in both are 1 um.

JNK Inhibitor II, RNAi of MKK7 and c-Jun prevent zVAD induced Cell Death. zVAD activated c-Jun N-terminal kinase (JNK) which is also activated by RIP in response to cytokines (73). Moreover, a JNK inhibitor, but not inhibitors against p38 or Erk, blocked zVAD-induced cell death, further indicating a specific role for JNK (Fig. 11). The protein synthesis inhibitor cycloheximide (CHX) blocked cell death, indicating that protein synthesis was required (Fig. 11).

RNAi silencing of the JNK-activating kinase MAP kinase kinase 7 (MKK7) also completely prevented cell death and formation of autophagic vacuoles (Fig. 12 A and B). RNAi suppression of the transcription factor c-Jun reduced but did not eliminate the c-Jun protein and inhibited autophagy and cell death by 45 to 50% (Fig. 12 A). Thus, a signal pathway involving RIP, MKK7, JNK, and c-Jun appears to activate autophagy and cell death. The involvement of c-Jun and new protein synthesis implies transcription of target genes may also be required.

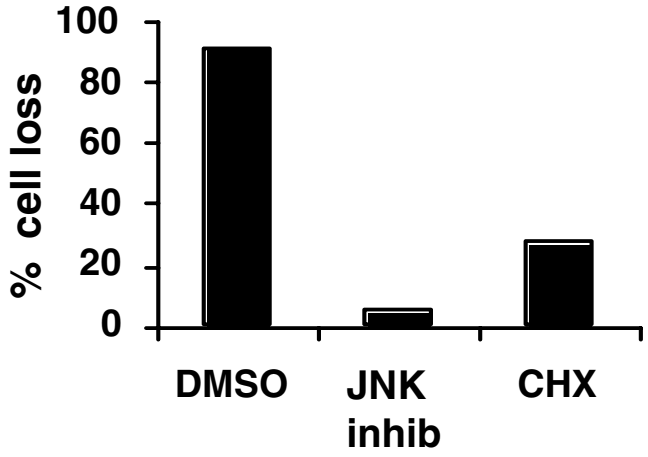

Fig.11. JNK inhibitor and Cycloheximide prevent zVAD induced cell death. zVAD-induced cell death in cells treated with control, JNK inhibitor II (1ug/ml), or cycloheximide (CHX) (2 ug/ml). [Note: This work was done by Yu,L]

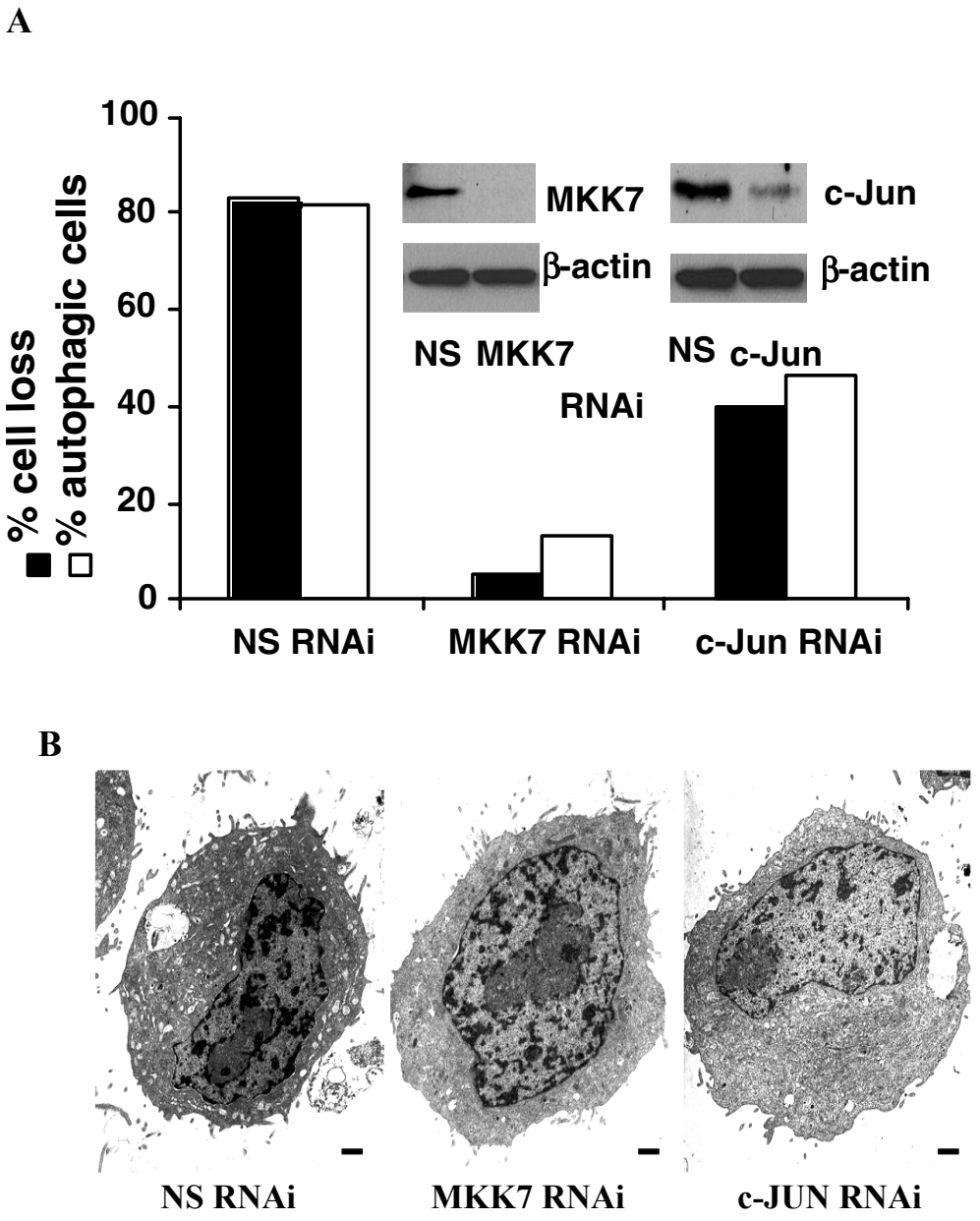

Fig.12. zVAD induced Cell Death and Autophagy require the JNK Pathway. A) Cells were treated with zVAD or vehicle 96 hours after transfection with MKK7 RNAi, c-Jun RNAi, or non-specific oligoribonucleotides. The % cell loss (solid bars) and the fraction of cells with autophagy features (>10 autophagic vacuoles/cell) on TEM (open bars) were quantitated. The steady state level of the corresponding proteins is shown by Western blot (inset). Data represent at least 3 experiments. B) Representative TEM images.

Caspase-8 RNAi induces Cell Death.

Finally, we addressed how zVAD induced autophagic cell death. Active caspase-8 functions in lymphocyte receptor signaling pathways that do not cause cell death (74). We therefore used RNAi to progressively reduce caspase-8 expression over time and found that cell death was correspondingly increased (Fig. 13 A). Cells in which caspase-8 was reduced showed features of autophagy (Fig. 13 B). Because zVAD is a potent inhibitor of caspase-8, it likely exerted its death effect through inhibition of caspase-8.

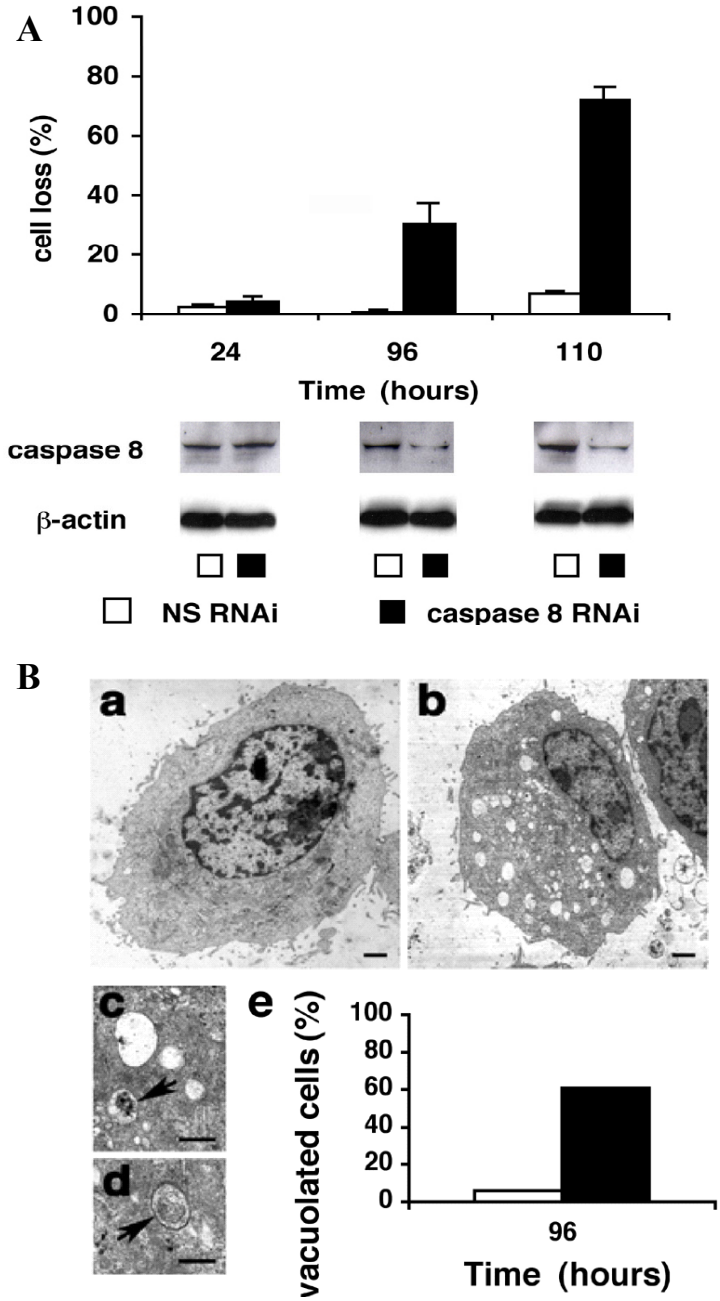

Fig.13. Downregulation of caspase 8 induces cell death. A) Time course of viability of L929 cells transfected with either nonspecific (NS) (open symbol) or caspase-8-specific (solid symbol) RNAi at 24, 96, 110 hrs after transfection. Panels below show the abundance of caspase-8 protein by Western blot [done by Yu,L] B) Representative TEM pictures and quantification of the cells treated with either nonspecific or caspase-8-specific RNAi s. Cells were harvested at 96 hrs after RNAi transfection. (a) NS control cell, (b-d) caspase-8 RNAi at different magnifications. Scale bars in a and b, 1 uM, c and d, 0.5 uM. Arrowheads indicate double membrane autophagic vacuoles. (e) The fraction of cells with autophagic features based on TEM was quantified (for NS vs. Caspase-8, $p<0.0001$, Mann-Whitney U test). NS control cell, open bar, caspase-8 RNAi , solid bar.

Extent of autophagy correlates with cell death.

As mentioned above, zVAD treated L929 cells had significantly more autophagy compared to control DMSO treated cells as measured by both morphometric analysis and number of autophagic vacuoles compared (for DMSO control vs. 8 hour or 12 hour zVAD treated, $p<0.0001$, Mann-Whitney U test). Inhibition of *ATG7* or *BECLIN-1* by RNAi prevented formation of autophagic structures as determined by TEM (for NS vs. *ATG7* or *BECLIN-1*, $P<0.000.1$, Mann-Whitney U test). Similarly, inhibition of *RIP, MKK7* and *c-JUN* by RNAi before zVAD treatment decreased the number of autophagic vacuoles significantly in all three cases (for NS vs. RIP or MKK7 or c-Jun, $p<0.0001$, Mann-Whitney U test). Finally, *CASPASE-8* RNAi mimicked zVAD treatment with respect to induction of autophagy (for NS vs. *CASPASE 8* RNAi, $p<0.0001$, Mann-Whitney U test).

Section 2.5: Discussion

We have shown that two key autophagy genes, *ATG7* and *BECLIN-1*, are necessary for an autophagic death pathway in mammalian cells. This may explain other forms of non-apoptotic death (29). To our knowledge, this report is the first direct evidence for an autophagy dependent process of cell death and one that can be blocked by inhibiting the *ATG* class of genes. The conservation of mammalian autophagic death genes in yeast suggests that this process might have arisen early in eukaryotic evolution. *BECLIN-1* gene knockouts cause an unexplained increase in spontaneous tumors (71, 72), and it is possible that Beclin 1 may act as a tumor suppressor by causing autophagic cell death.

We have shown a role for Caspase-8 in the regulation of autophagy. The suppression of autophagic death by Caspase-8 in mammalian cells indicates caspases can regulate both apoptotic and non-apoptotic cell death. We favor the idea that there is a low constitutive level of caspase-8 activation that carries out cellular regulatory processes (74). Interestingly, this regulatory role is specific for Caspase-8 alone and inhibition of other caspases does not affect autophagy (unpublished Yu, L et al).

Because viral pathogens have caspase inhibitors, the autophagic pathway could be poised to counter viral infection as a "fail-safe" mechanism. The existence of two distinct pathways, one activated by Caspase-8 and the other supressed by Caspase-8, might act to ensure cell obliteration when cell death is of paramount importance as during normal embryogenesis. Lastly, caspase inhibitors are currently being developed as therapeutic agents for neurodegenerative diseases characterized by abnormal cell death (75,76). Our findings indicate that caspase inhibition could have the untoward effect of exacerbating cell death and disease severity by activating the autophagic death pathway.

Chapter Three

Autophagy in Human tumors. Cell Survival or Death?

Section 3.1: Introduction

The development of tumors involves multiple genomic changes that result in abnormal neoplastic cells and necessary alterations in the surrounding support tissue. Similar to the dynamics of a developing tissue or organism, tumors can be viewed as amalgamations of multiple cell types of epithelial, stromal, angiogenic and connective tissue origin that are intricately linked by their interactions (2). Tumor growth involves two essential deviations from the normal state including the induction of proliferative stimuli, such as c-Myc and E2F, and simultaneous suppression of potentially compensatory cell death (77). It is well recognized that apoptosis is impaired in many cancers by mutations in genes such as p53 (2, 78), but it remains to be determined if non-apoptotic cell death mechanisms are also impaired in neoplastic cells. While compelling evidence indicates that aberrations in cell proliferation and death are the critical determinants of neoplastic growth, recent discoveries suggest that less studied mechanisms may contribute to tumor growth control.

Autophagy is an evolutionarily conserved mechanism of protein and organelle degradation that has been observed in organisms that are as different as yeast and humans. Autophagy involves the sequestration of cytoplasmic structures into vacuoles that are transported to lysosomes for degradation (7). Recent studies of autophagy suggest that this mechanism of proteolysis may function in the regulation of cell survival

and death (79 and chapter 2). There are at least three ways in which autophagy might enhance cancer cell survival. Autophagy may serve to optimize nutrient utilization in rapidly growing cancer cells when faced with hypoxic or metabolic stress similar to the starvation response observed in normal cells (80). Alternatively, autophagy might aid in degradation of organelles such as depolarized mitochondria that activate death pathways (81). Autophagy might also prevent cells from accumulating free radical induced damage to lethal levels by removing organelles that are sources or targets of such damage.

While the role of autophagy in cell survival during nutrient deprivation is well characterized, less is known about the possible role of this form of proteolysis in cell death even though autophagy occurs in dying cells of diverse organisms (8, 31). Therefore, it is important to consider the possibility that autophagy may play an important role in some forms of programmed cell death (chapter 2). While autophagy might commence as an adaptive response that sacrifices mass for homeostasis and enhances survival, cell death may ensue if the process is carried beyond a threshold. Thus, autophagy may suppress tumor growth by causing cell death, limiting cell size, or otherwise maintaining a low mutation rate, and decreasing the likelihood of aberrant growth. We initiated a morphological survey for autophagic structures in several different primary human tumors on account of the paucity of evidence for autophagy in cancer.

Section 3.2: Materials and Methods

Tissues were all obtained from surgical resection specimens, immediately fixed in 3% glutaraldehyde, processed, and embedded in Embed 812. Semi-thin sections were cut, stained with Toluidine-Blue, and examined using light microscopy to verify the presence

and preservation of viable neoplastic tissue. Thin-sections were stained with uranyl acetate-lead citrate and examined using a Zeiss EM 10 transmission electron microscope.

Section 3.3: Results

Autophagic structures were observed in neoplastic cells, and displayed the morphological features of double and multi-lamellate membrane bound vacuoles enclosing cytoplasmic content and organelles (Fig.14). These autophagic vacuoles were typically in the vicinity of the nucleus and were frequently adjacent to swirls of endoplasmic reticulum devoid of ribosomes. The nuclei of these cells lacked apoptotic features such as fragmentation and chromatin margination. Of the 12 tumors studied, 7 had evidence of autophagy including ganglioneuroma, infiltrating ductal carcinoma of the breast, adenocarcinoma of the lung, pancreatic adenocarcinoma, and pancreatic islet cell tumor. Taking into account the small sample size for some of the tumor types examined, it seems reasonable to expect that autophagy occurs in many tumors.

Table 2. Survey of human tumors for autophagic structures.

Tumor types with evidence of autophagy	Tumor types with NO evidence of autophagy
Ganglioneuroma, mediastinum	Medullary carcinoma, thyroid
Mesothelioma, pleura	Lymphoma, lymph node
Invasisve ductal carcinoma, breast	Meningioma, brain
Adenocarcinoma, lung	Adenocarcinoma, ovary
Adenoma, pituitary gland	Glioma, brain
Adenocarcinoma, pancreas	
Islet-cell tumor, pancreas	

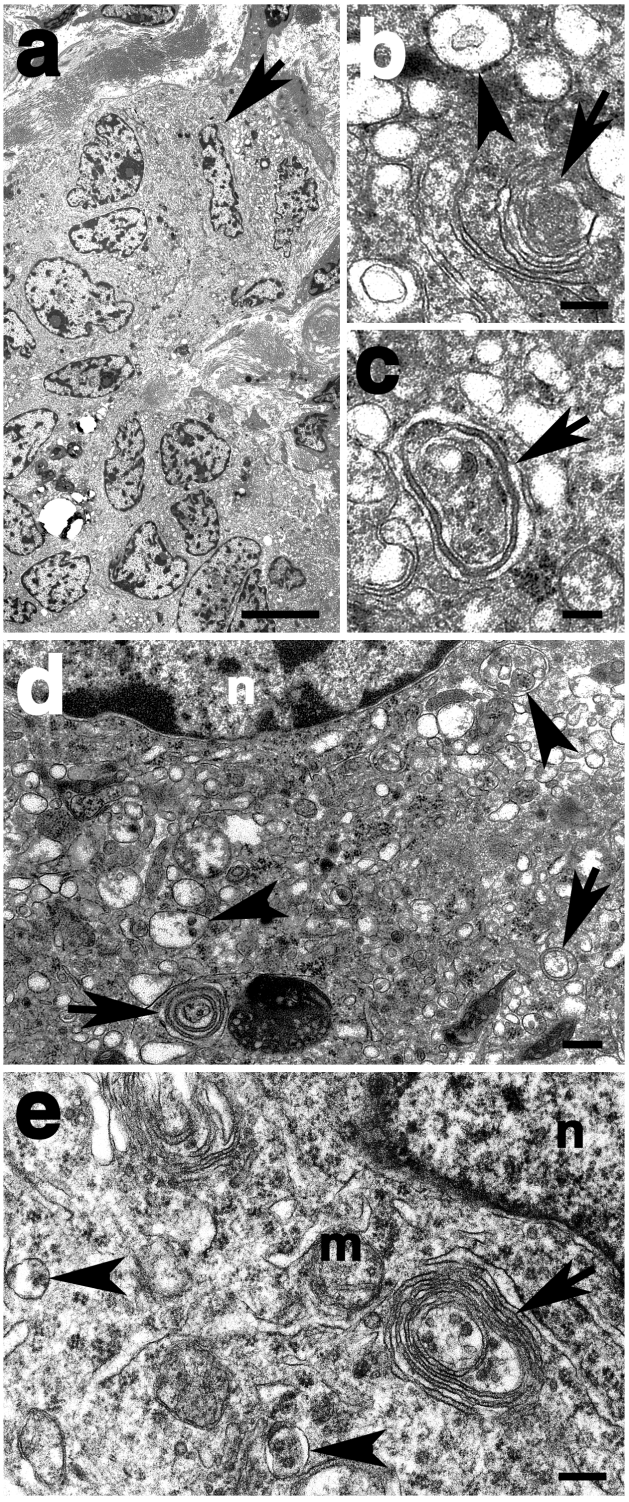

Fig.14. Autophagic structures are present in neoplastic cells of multiple types of primary tumors.

(a) Cells of a pancreatic islet cell tumor that display autophagic features and lack the hallmarks of apoptosis (arrow).

(b, c) Neoplastic pancreatic islet cell tumors cells contain early stage multilamellate (arrows) and single membrane-bound (arrow head) autophagic structures.

(d) Adenocarcinoma of the lung contains several multi-lamellate (arrows) and single membrane-bound (arrow head) autophagic structures, while the nucleus (n) appears normal.

(e) A ganglioneuroma cell with a normal nucleus (n) and mitochodria (m) contains multi-lamellate (arows) and single membrane-bound (arrow head) autophagic structures. Scale bars = 10 μM (a), 0.3 μM (b-e).

Section 3.4: Discussion.

The precise role of autophagy in cancer development, progression and response to therapy is not understood. The recognition of Beclin-1 (Atg6), a gene that functions in autophagy, as a haploinsufficient tumor suppressor raises intriguing possibilities about the importance of autophagy in cancer (70, 71, 72). It is possible that the mechanism of tumor suppression is through promotion of cell death. Autophagy peaks at pre-cancerous stages and diminishes at the malignant stage in some rat tumor models (82), suggesting a tumor suppressor role. It is interesting that autophagy is regulated by some of the same pathways of cell growth control that are altered in tumor formation such as the PI3K system (83). Further, analogs of rapamycin, which stimulate autophagy by inhibiting mTOR have shown promise in models of tumor therapy (84, 85, 86). Rapamycins are thought to stabilize tumor size rather than cause marked regression, possibly indicating control of cell size as the mechanism. In addition, therapeutics such as tamoxifen, an estrogen antagonist in breast tissue, have been shown to potently induce autophagy in MCF7 breast cells (87), suggesting the possibility that autophagy contributes to their anti-neoplastic activity.

A greater understanding of the regulation of autophagy in higher animals would provide better targets for cancer therapies. Although many of the genes that regulate autophagy in yeast appear to be conserved in diverse species (31, 89), several autophagy genes are absent in higher animals suggesting possible differences in the regulation of this form of proteolysis. Autophagy is thought to be present at basal levels in most tissues and is regulated by the pleiotropic mTOR pathway (88). Normal cells, unlike rapidly

growing cancer cells, would be expected to be less sensitive to pro-autophagic stimuli due to minimal metabolic demands and normal activity of regulators such as PI3K and Akt. Therefore, it seems likely that drugs that specifically enhance autophagy would be of value because of their high therapeutic index. Elucidation of the pathway downstream of mTOR would help avoid the pleiotropic effects of this kinase. In tumors with deficient apoptosis and/or up-regulation of the PIK3/Akt pathway, a combinatorial approach that utilizes autophagy modulators in addition to other chemotherapeutic agents might add value to therapy efficacy (43).

Non-apoptotic mechanisms of cell death have been largely overlooked in studies of cancer causation, progression and therapy. Although the variation in cell complexity has been recognized in tumors (2), modest progress has been made in understanding this aspect of cancer biology. It is important for cancer researchers to consider the presence and impact of autophagy and similar less studied processes when interpreting clinical trials and developing drugs for modulation of aberrant cellular pathways.

Chapter Four

Conclusions and Discussion

The possibility of autophagy as a cell death mechanism has been controversial. Notwithstanding the observation of autophagy in dying cells in several species, there has been no direct evidence for autophagic cell death. We have shown that L929 cells undergo autophagic cell death upon treatment with the pan-caspase inhibitor zVAD-fmk (chapter 2). We have also demonstrated that zVAD induced death can be prevented by RNAi inhibition of *ATG* genes. We have presented evidence of the involvement of the MKK7-JNK-cJUN pathway in autophagic cell death. We have also shown that inhibition of caspase-8 by RNAi mimics zVAD treatment, suggesting that zVAD induces death most likely by inhibiting caspase-8. Our work defines a novel regulatory role for caspase-8 in suppressing autophagy. Our work has implicated RIP as a possible switch between apoptotic and autophagic cell death, and as a potential mediator of the induction of autophagy following caspase-8 inhibition. We therefore propose a tentative model to explain the role of caspase-8 in autophagy (Fig. 15).

Our work on autophagy in tumors has demonstrated the presence of autophagic structures in several primary cancer tissues (chapter 3), including cancers of the breast and lung. These two tumors account for more deaths from cancer than any other type. In view of the frequency of resistance to conventional therapy in these cancers and the high mortality rate, a therapeutic approach based on enhancing autophagic cell death would be expected to have a significant impact. Investigation of the role of autophagy in tumors is likely to be a fruitful area of research in the future.

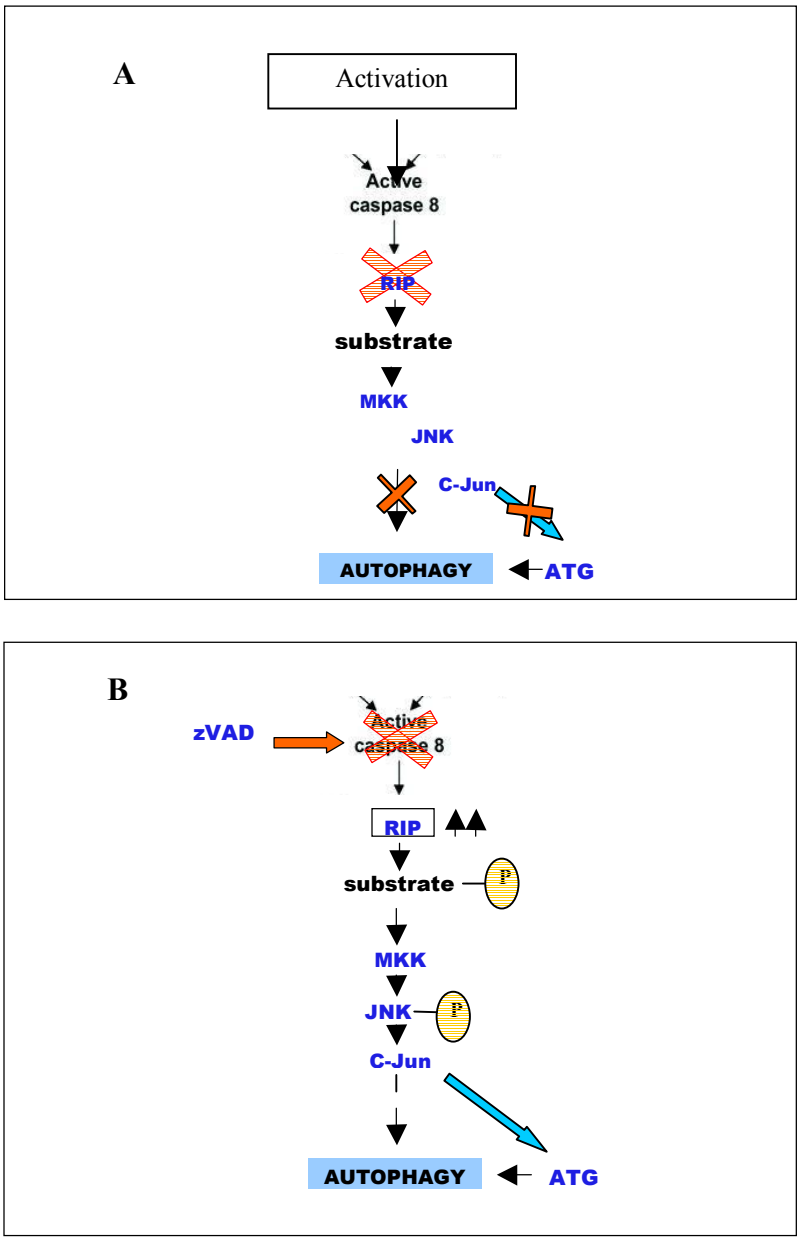

Fig.15. Model for caspase-8 regulation of autophagy in L929 cells. A. Sequestered fraction of caspase 8 is activated at basal states by unknown mechanisms. Active caspase 8 processes RIP, thereby inactivating it. JNK is not phosphorylated and no autophagy ensues. B. When caspase 8 is inhibited, either by zVAD or RNAi, RIP cleavage ceases and RIP is free to induce autophagy. The induction involves the MKK7-JNK-cJUN pathway and requires the *ATG* gene products.

Our model of autophagic death places caspase-8 and RIP at a nodal point in normal cellular processes, apoptosis and autophagy. We favor the idea that some caspase-8 activity is essential for cellular processes. There is evidence that caspases are important for proliferation and maturation functions (90, 91). Also, death receptors and caspases play a role in the immune response (92, 93, 94). These non-death functions might be served by a distinct pool of caspase molecules that are active at basal states. Although caspase-8 is classically activated by the death receptor pathway, recent reports suggest that an alternative cytosolic compartment may exist in which caspase-8 is activated through unknown mechanisms (95, 96). This possibility is enticing as it could also explain why caspase-8 is necessary for normal cell function and imply that cells are programmed to die in its absence or inhibition. Alternatively, autophagic death might be triggered only when death pathways, most likely the death receptor pathway, are activated in response to a death stimulus but with caspase-8 activity inhibited due to viral inhibitors or mutations in apoptosis genes. TNF stimulation is a possible source of the death stimulus as L929 cells synthesize TNF endogenously. TNF addition to L929 cells treated with zVAD greatly accelerated death (data not shown). Survival or death might also depend on the molecular milieu in the cell in the context of caspase 8 activation (for e.g. adaptor molecules like FADD, c-FLIP). Clearly, much remains to be known about caspase-adaptor interactions and their effects.

Caspase-8 homozygous deficient patients are almost entirely normal in development and have normal cell populations except for slightly increased blood leukocyte levels and some defects in lymphocyte activation and immune response (94). We hypothesize that autophagic death, possibly in addition to other non-apoptotic death

mechanisms, is responsible for normal programmed cell death during development in these patients. Another possibility is that caspase 10, which is functional in these patients, could substitute for caspase 8 (97 and see below). L929 mouse cells lack caspase-10, possibly allowing caspase-8 inhibition alone to uncover the autophagic pathway.

Mice deficient in caspase-8 die during embryogenesis with defective myocardial development (98). This observation may point to a role for caspase-8 in cell growth and differentiation, either directly in the myoblast progenitors or in other tissue cells necessary for cell-cell interactions during development. These mice also show fewer blood cell precursors in the bone marrow. An absence of caspase-8 in precursor cells might cause excessive autophagic death or defective differentiation.

Data from the caspase-8 RNAi experiments confirm a specific role for caspase-8 in suppressing autophagy, leading us to believe that inhibition of caspase-8 activity is the mode of action of zVAD. Also, inhibition of caspases 1, 2, 3, 9 and 12 did not enhance cell death in L929 cells (data not shown). However, it is not possible to completely rule out the involvement of another zVAD target. zVAD has been reported to inhibit several kinases and kinases are prominently involved in the known regulatory pathways of autophagy. An interesting parallel is the efficacy of 3-MA and Wortmannin in blocking autophagy by inhibiting PI3K (7). Assay of the activity of kinases presumed to function upstream of autophagy might delineate more precisely the targets of zVAD. Obvious candidates to be studied would include RIP itself, PI3K, PI3K dependent kinase 1 (PDK1), Akt, S6 kinase (pp70S6K), DAP-kinase and DRP. Unlike zVAD, other common caspase inhibitors (e.g. IETD, zFA, LEHD, DEVD, zAAD) surprisingly do not kill L929 cells significantly (data not shown). Our experience with pan-caspase inhibitors has

shown that zVAD causes the most reduction in caspase 8 activity in L929 cells (data not shown). Protein levels and activity levels of caspase 8 in the presence of IETD would illuminate the reasons for our negative results with IETD. Also, fibroblasts from mice lacking caspase-8 show defective Fas induced cell death (98). Our model would seem to require that fibroblasts from caspase 8 deficient mice be sensitive to autophagic killing upon Fas activation. We cannot entirely reconcile this conflicting piece of data as of now. Repeating the experiments in primary cell cultures from mice fibroblasts and other cell types might help in resolving this tricky issue.

We have some evidence suggesting that RIP is a substrate for caspase-8 and that zVAD treatment causes an increase in the amount of full length RIP protein but does not completely prevent RIP cleavage (99 and data not shown). The truncated fraction of RIP presumably acts as a dominant negative inhibitor of RIP activity. We have also analyzed p38MAP kinase and Erk in zVAD-treated L929 cells. Neither showed an appreciable effect in either inducing or preventing autophagic cell death. DAP kinase (death associated protein kinase) and DRP (DAP kinase related protein) are involved in mediating both extrinsic and intrinsic death pathways (100, 101). DAP-kinase and DRP also cause formation of plasma membrane blebs during apoptosis (101). Interestingly, zVAD treated cells manifest similar membrane blebbing (Fig. 6). We propose to investigate the involvement of DAP-kinase and DRP in regulation of the autophagic death pathway. Also, inhibition of autophagy by *ATG* RNAi, while blocking autophagy, did not prevent membrane blebbing, suggesting that DAP and DRP kinases, if involved, are probably upstream of the Atg proteins or activated independently (Fig. 9 B).

We recognize the gaps in our understanding of this mechanism of death, especially with regard to two areas. The target molecule of RIP's kinase activity that presumably mediates the autophagy inducing effect is unknown. Experiments to identify the substrate are complex due to the large set of potential candidate proteins. Secondly, we do not know the mediators that link the MKK7-JNK-cJUN pathway to the Atg pathway. Although, the known transcription factor activity of c-JUN and evidence from Drosophila salivary gland PCD studies suggest transcriptional control, we cannot confirm direct regulation of ATG gene transcription. RT-PCR experiments to quantitate ATG transcripts in zVAD treated L929 cells have been inconclusive in this regard.

We have not analyzed changes in mitochondrial polarization and permeability, the activity of the Bcl2 proteins and changes in effector caspases when L929 cells are treated with zVAD. Caspase-9 inhibition did not cause cell death and therefore, a primary role for the mitochondrial death pathway is unlikely. Given that caspase-8 can cleave Bid, a pro-apoptotic Bcl2 protein, to initiate the mitochondrial or intrinsic pathway (102), studies to investigate Bid cleavage and mitochondrial function in zVAD induced death would be interesting.

The demonstration of autophagic cell death (chapter 2) suggests a causative role in some of the instances in which autophagy is associated with dying cells. Autophagic death could underlie the tumor suppressor effect of Atg6 / Beclin-1 (70, 71, 72). So far, we have evidence of autophagic cell death in two other cell types namely U937 human lymphoblast cells and RAW mouse macrophage cells (data not shown). The universality of autophagic cell death remains under investigation. Autophagic cell death might explain

several observations of non-apoptotic death in development, viral and bacterial infections, neurodegeneration and cancer.

Development

The best documented association of autophagy with cell death has been in development (31). The presence of an alternate cell death route probably ensures removal of redundant cells or impaired cells, a process that is vital for normal organogenesis. Although we have no direct evidence implicating the death receptor or mitochondrial pathway in autophagic cell death, input from classical apoptosis inducing pathways to autophagic death might indeed exist. It is then tempting to speculate that an extrinsic or intrinsic death inducing signal, entrusted with the mission of ensuring proper development, is executed through autophagic death when apoptosis fails. The induction of autophagic death upon inhibition of apoptotic mechanisms (Fig. 1 C) might explain why, for example, Apaf-1 knockout mice which are defective in apoptosis exhibit normal development of digital webs (103).

Infectious diseases

Certain viruses endeavor to prevent killing of their host cells (infected cells) by cytotoxic lymphocytes of the immune system. Different viruses subvert the apoptotic process at different points (104). Some viruses (e.g. Cytomegalovirus / CMV) encode for a protein called 'viral inhibitor of caspase' (vICA) that deactivates caspase 8 (105). We expect CMV infected cells to be extremely susceptible to autophagic death based on our model. On the contrary, several bacteria (e.g. *Brucella abortus, Porphyromonas gingivalis, Legionella pneumophila, Leishmania donovani, Coxiella burnetii*) and some viruses (e.g. Picornavirus) subvert autophagy to facilitate their replication (106, 107, 108). SipB

protein from *Salmonella enterica* induces autophagic vacuoles and causes cell death in host macrophages as part of its pathogenicity, but it is not clear if autophagy directly causes the observed cell death (109). Thus, the role of autophagy in the context of viral and bacterial infections, as in tumors (chapter 3), remains perplexing. Regulatory influences other than caspase-8 are probably modified differently in different situations.

Neurodegenerative diseases and myopathy

Autophagic structures have been identified in Huntington's disease (110), Parkinson's disease (111), Alzheimer's disease (112) and Creutzfeldt-Jakob disease (113). Progressive loss of neurons is characteristic of all these conditions. Autophagic cell death might contribute to neurodegeneration, given the frequency of non-apoptotic cell death observed in neurons (3). The possibility of autophagic cell death also urges caution in developing therapies based on caspase inhibitors to treat neurodegeneration. Certain myopathies such as X-linked Myopathy with Excessive Autophagy (XMEA) and Infantile Autophagic Vacuolar Myopathy or atypical Danon's disease exhibit prominent autophagic structures (114, 115). As noted above, caspase 8 deficient mice are defective in myocardial development. It is possible that autophagic death in the myoblasts or myocytes or in adjacent stromal cells may contribute to the pathology of these myopathies.

Cancer

Inactivation of apoptosis is common in cancer (2, 116), but mutations in caspases have not been identified. One possible reason could be that caspase mutations, especially caspase 8, in the light of our model, may not confer a survival advantage to the cell. Augmentation of autophagic cell death shows promise as a therapeutic approach (chapter

3). Rapamycin, an enhancer of autophagy, is being studied in several clinical trials, either alone or in combination with other modalities. *ATG* genes are potentially exciting targets for induction of autophagy. Anti-Fas antibodies are under evaluation as chemotherapeutic agents. While they are intended to promote apoptosis, they might be expected to enhance autophagic death when apoptosis is impaired as is the case in most tumors.

The mechanism of autophagic death and the evidence for autophagy in primary tumors suggest that autophagy functions as a barrier to tumor development. Future work to elucidate the complex control of autophagy and autophagic cell death could uncover specific targets for therapy in infectious diseases, neurodegenerative disorders and cancer.

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