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MORTE CELLULARE PROGRAMMATA E NEURODEGENERAZIONE: RUOLO DEL COMPARTIMENTO AUTOFAGICO-LISOSOMICO

PROGRAMMED CELL DEATH AND NEURODEGENERATION: ROLE OF THE AUTOPHAGIC-LYSOSOMAL COMPARTMENT

Relatore:
Prof. Ciro Isidoro

Coordinatore:
Prof. Umberto Dianzani

Candidata:
Roberta Castino

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PREFAZIONE

In questa tesi è riportato il lavoro svolto dalla candidata durante i quattro anni del corso di Dottorato in Patologia Molecolare. Il lavoro è stato svolto in parte nel laboratorio del Prof. Isidoro nel Dipartimento di Scienze Mediche e in parte in collaborazione con il Prof. Murphy, all'Università di Bristol.
Questa tesi è stata scritta quasi interamente in italiano, trattandosi di un Dottorato di Ricerca italiano; alcune parti introduttive alla sessione "Risultati" sono state scritte in inglese, la lingua universalmente riconosciuta in ambito scientifico.

1. LA MORTE CELLULARE

Sulla base delle modalità d'innesco e di esecuzione della morte cellulare, che si riflettono in caratteristiche modificazioni biochimiche e morfologiche, si distinguono tre tipologie di morte cellulare: la morte cellulare programmata di tipo I (apoptosi), la morte cellulare programmata di tipo II (autofagica) e la necrosi (tipo III). La tipologia della morte cellulare dipende evidentemente dall'attività dell'organulo cellulare primariamente coinvolto e dalla via di segnalazione di innesco. Tutti gli organuli possono, in risposta ad uno stress lesivo irreversibile, generare un segnale di innesco della morte cellulare (Ferri e Kroemer, 2001).

Fisiologicamente la morte cellulare programmata riveste un ruolo importante nell'organogenesi e nel continuo rimodellamento cui sono soggetti gli organi nel corso della vita. (Glucksmann, 1965; Saunders, 1966; Kerr et al., 1974).

Nel caso il danno sia provocato dall'accumulo di una proteina difettosa (ad es. con errori conformazionali che ne compromettono il trasporto o la degradazione) il segnale viene generato nel compartimento di accumulo (ad es. il reticolo endoplasmatico o il complesso di Golgi); nel caso l'insulto lesivo provenga dall'esterno (farmaco citotossico, citochina, etc.) il segnale puo' partire dalla plasmamembrana, dal mitocondrio, dal nucleo o dai lisosomi, a seconda di dove è situato il bersaglio dell'azione tossica. Se l'insulto lesivo è quantitativamente elevato rispetto ai tempi di risposta tale da non consentire l'attuazione del programma di morte la cellula soccombe per necrosi.

1.1. LA MORTE CELLULARE PROGRAMMATA

La morte cellulare programmata (PCD -programmed cell death-) è un tipo di morte cellulare che richiede energia, la trascrizione di geni specifici e l'attivazione di proteine. E' caratterizzata da un susseguirsi di eventi regolati che conducono al "suicidio" della cellula e può consistere in un'evidente processo di condensazione cellulare (apoptosi) e/o in una marcata autofagia. Le principali caratteristiche biochimico-morfologiche che contraddistinguono la PCD I e II sono riassunte nella tabella 1.

Apoptosi (morte programmata di tipo I)	Gli organelli sono mantenuti intatti. Si osserva la frammentazione del nucleo, con condensazione della cromatina e taglio del DNA a partire dall'estremità 3'. Si ha l'attivazione delle caspasi. La fosfatidilserina viene traslocata sul lato esterno della membrana plasmatica. Riduzione del volume cellulare per perdita di acqua Formazione di corpi apoptotici
Morte autofagica/lisosomica (morte programmata di tipo II)	E' caratterizzata dalla formazione di vacuoli autofagici. Le proteasi attivate nelle fasi iniziali sono le catepsine lisosomiche. L'attivazione delle caspasi avviene nelle fasi tardive, come anche la frammentazione del DNA. La fosfatidilserina viene traslocata sul lato esterno della membrana plasmatica. Riduzione del volume cellulare per intensa degradazione macromolecolare.

Tab.1 Caratteri morfologici e biochimici peculiari della PCD di tipo I e II

1.1.1 L’APOPTOSI

La morte apoptotica è caratterizzata da eventi molecolari, biochimici e morfologici finementi regolati. E’un processo importante sia nella morfogenesi che nella regolazione dell’omeostasi tissutale nell’adulto.
Nell’uomo i processi apoptotici sono coinvolti in:
-sviluppo embrionale (Jacobson et al 1997)
-sviluppo del sistema nervoso centrale (Naruse e Keino 1995)
-mantenimento dell’omeostasi cellulare
-spegnimento delle risposte immunitarie (Cohen e Duke 1992; Behrens e Muller 1996).
Un’alterazione del processo apoptotico può, inoltre, essere alla base di numerose patologie come, per esempio, infezioni virali (Banda et al 1992), tumori (Wrigth et al., 1994) e malattie autoimmuni (Watanable-Fukunaga et al., 1992).

L’apoptosi nello sviluppo embrionale

Durante lo sviluppo di tutti gli animali che sono stati studiati compare la morte cellulare programmata (PCD) (Clarke e Clarke, 1996), ma solo recentemente sono stati scoperti i meccanismi molecolari che ne stanno alla base. Già nella metà del secolo scorso l’apoptosi è stata descritta durante la metamorfosi degli anfibi (Vogt, 1842), ma successivamente altri studi hanno confermato l’importanza della morte cellulare durante lo sviluppo di numerosi tessuti sia nei vertebrati che negli invertebrati (Glucksmann, 1965; Clarke e Clarke, 1996). La morte cellulare programmata ha un ruolo fondamentale nello sviluppo animale. Sono state riportate almeno quattro funzioni della PCD nello sviluppo: 1) rimodellamento di strutture; 2) eliminazione di strutture inutilizzate; 3) controllo del numero cellulare; 4) eliminazione di cellule abnormi, localizzate nel posto errato, non funzionali.

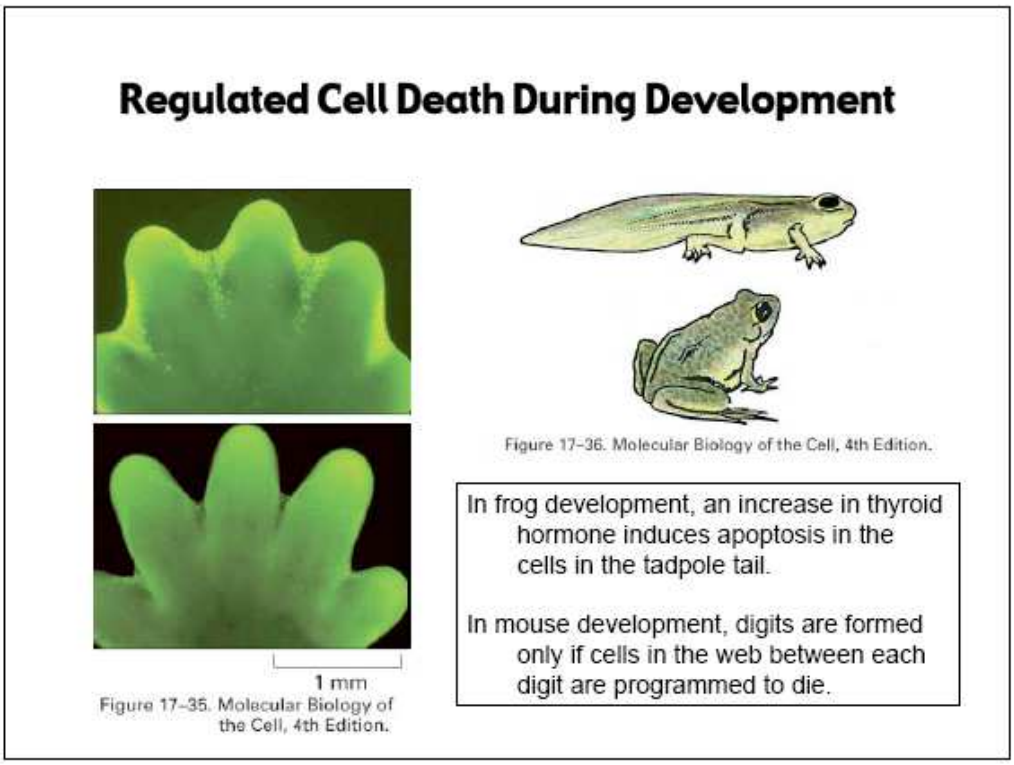

Figure 1. La morte cellulare programmata nello sviluppo.

L'apoptosi nello sviluppo del sistema nervoso centrale

E'stato dimostrato che durante l'ontogenesi degli organismi multicellulari è indispensabile la morte cellulare programmata in periodi temporalmente distinti (Saunders, 1996). Glucksmann (1965) aveva distinto la morte cellulare sulla base della funzione biologica in tre distinte classi: 1) morfogenetica, responsabile dei cambiamenti di forma durante lo sviluppo strutturale; 2) istogenetica, associata al differenziamento di organi e tessuti; e 3) filogenetica, in accordo con la scomparsa delle strutture larvali. La PCD si manifesta con grande intensità durante la neurogenesi (Clarke, 1982; Clarke,1985; Ferrer et al., 1992) così come durante la morfogenesi (Hurle, 1988). Il primo e maggiore evento morfogenetico in cui è coinvolta la PCD nello sviluppo del sistema nervoso centrale (CNS) è la trasformazione della placca neurale nel tubo neurale (Jelinek e Friebora, 1966; Langman et al., 1966; Burnside, 1975; Karfunkel, 1974).

La morte cellulare programmata è un fenomeno comune durante lo sviluppo postnatale del SNC. Sono state evidenziate caratteristiche tipiche della PCD nella corda spinale dei mammiferi (Nurcombe et al., 1981; Bunker, 1982; Lance-Jones, 1982; Harris e McCaig, 1984; Comans et al., 1987), nella retina e nei suoi collegamenti (Arees e Astrom, 1977, Finlay et al., 1982; Cunningham et al.,1982; Dreher et al., 1983; Finlay

e Pallas, 1989). La morte cellulare postnatale è stata anche osservata nell'ippocampo in hamsters e ratti (Janowiski and Finlay, 1983; Ferrer et al., 1990). Nel corso della morfogenesi del cervello un tasso di apoptosi anormale può portare alla formazione di archiencefalo e idroencefalo.

I meccanismi genetici e molecolari dell'apoptosi sono stati studiati e identificati per la prima volta in *Caenorabditis elegans*, un piccolo nematode che vive nel suolo. In questo verme, sono stati individuati geni indispensabili per il processo apoptotico quali ced-3 e ced-4 (Chinnayan et al 1997; Wu et al 1997).

Ced-3 codifica per una proteasi (CED-3) coinvolta nell'inattivazione di proteine necessarie per la sopravvivenza cellulare (es. enzimi coinvolti nella riparazione del DNA) e nell'attivazione di proteine necessarie, invece, per la morte (es. endonucleasi che degradano il DNA).

A differenza della necrosi, nell'apoptosi si osserva perdita di acqua con riduzione del volume e condensazione. Nelle prime fasi, l'organizzazione strutturale della cellula è mantenuta mentre a livello nucleare si osserva la disgregazione del nucleolo, il taglio della lamina, la condensazione e infine, il taglio della cromatina in frammenti. Frammenti di materiale nucleare raggiungono in seguito la membrana plasmatica, dove vengono circondati da evaginazioni della membrana stessa che conferiscono alla cellula un aspetto a bolle (blebbing). Queste "bolle" si staccano dal corpo cellulare portando con sé parte del citoplasma e del materiale nucleare, dando origine ai corpi apoptotici. Questi ultimi possono essere fagocitati da cellule adiacenti dello stesso tipo (omofagia) o da macrofagi (eterofagia) e successivamente degradati all'interno dei lisosomi (Amenta e Baccino 1989, Dèmoz et al 2002). I corpi apoptotici che sfuggono la fagocitosi vanno incontro a lisi spontanea. Questo processo è simile a ciò che si verifica nelle ultime fasi della necrosi e per questo viene definito "necrosi secondaria" (Wyllie et al 1980), anche se a differenza della necrosi presenta la condensazione della cromatina.

1.1.2 REGOLAZIONE DEL PROCESSO APOPTOTICO

L'apoptosi è innescata da una serie di stimoli extracellulari e intracellulari (es. le radiazioni ionizzanti, lo stress ossidativo, l'esposizione a sostanze cancerogene, le infezioni virali, i danni al DNA, la mancanza di fattori di crescita e di citochine).

Sono state descritte tre vie d'innesco dell'apoptosi:

- via estrinseca che dipende dall'attivazione di recettori di morte
- via intrinseca che coinvolge direttamente i mitocondri
- via endosomica che coinvolge le catepsine.

Nel primo caso (**via estrinseca**) l'apoptosi viene innescata dal legame di specifiche molecole-segnale (FasL e TNF) con i propri recettori posti sulla membrana plasmatica (Death Receptor). A questo segue l'oligomerizzazione del recettore, il reclutamento delle procaspasi 8 e 10 che possiedono un dominio DED/DD (Death Effector Domain) e che legano la porzione citoplasmatica dei Death Receptor tramite molecole adattatrici come FADD. Si forma così un complesso proteico detto DISC (Death-Inducing Signaling Complex) in grado di attivare le caspasi esecutrici 3, 6, 7 (Kisckel et al., 2001; Nagata e Golstein 1995; Chinnaiyan et al 1995; Fig.1) Nella fase finale questa via si interseca con quella intrinseca.

Le caspasi sono cisteino-proteasi, residenti nel citoplasma, che vengono sintetizzate come pro-enzimi e poi attivate per scissione proteolitica. Una volta attivate esse attivano per proteolisi parziale i precursori di altre caspasi innescando una cascata proteolitica (Earnshaw et al 1999). L'azione delle caspasi nel processo apoptotico può essere indiretta, attraverso la traduzione del segnale, oppure diretta, intervenendo sulla scissione proteolitica di substrati citoplasmatici e nucleari (PARP, laminine, actina, ecc.) (Porter et al., 1997).

Una seconda via che conduce alla morte cellulare programmata è la via mitocondriale o **intrinseca**. In questo meccanismo sono coinvolte proteine codificate dai geni della famiglia Bcl-2 di cui fanno parte sia proteine pro-apoptotiche (Bax, Bid e Bcl-X_S) sia anti-apoptotiche (Bcl-2, Bcl-X_L e Bag-1). Bcl-2 associa con la membrana mitocondriale esterna e con le membrane del reticolo endoplasmatico e del nucleo e ne garantisce la loro integrità (Cory e Adams, 2002). L'azione di pro-soppravivenza delle proteine della famiglia di Bcl-2 si esplica prevenendo il rilascio del citocromo c e quindi l'attivazione di caspasi 9. Le proteine della famiglia di Bcl-2, probabilmente regolano l'attivazione anche di altre caspasi, indipendentemente dal danno alle membrane mitocondriali (Cory e Adams, 2002). Bcl-2 ha quattro domini funzionali (BH) altamente conservati e sette domini ad alfa-elica che ne garantiscono la struttura. I domini BH1, BH2, e BH3 sono stati ritrovati anche in alcuni membri pro-apoptotici della famiglia di Bcl-2 e studi hanno dimostrato che questi domini sono necessari per l'eterodimerizzazione di Bcl2-Bax (Oltvai et al., 1993; Takayama et al., 1994; Kelekar e Thompson, 1998; Schendel et al., 1997; Schlesinger et al., 1998). Recentemente è stata

dimostrata *in vivo* l'eterodimerizzazione di Bcl-2 e Bax (Mahajan et al., 1998), ritenuta importante poiché in questo modo Bcl-2 è in grado di bloccare la potente azione di Bax come induttore di morte (Mahajan et al., 1998). Studi condotti da May e collaboratori (1994) hanno dimostrato che inteleuchina-3 induce la fosforilazione di Bcl-2 e che questa modificazione è strettamente correlata con la sopravvivenza cellulare.

Bcl-2, in seguito a specifici segnali (mancanza di fattori di crescita, stress ossidativo, ecc.) altera la permeabilità della membrana mitocondriale determinando l'apertura o la chiusura dei pori di transizione; viene alterata la catena di trasporto degli elettroni, si generano specie reattive dell'ossigeno e, infine, viene rilasciato il citocromo c nel citoplasma. Il citocromo c lega e attiva Apaf-1 (apoptotic protease activating factor) che a sua volta innesca la via caspasica che termina con l'attivazione della caspasi 3 (Zou H. et al 1997). La via mitocondriale può essere innescata anche da p53. Un danno al DNA, dovuto ad esempio a radiazioni ionizzanti, può attivare p53, il quale blocca il ciclo cellulare nella fase G1 favorendo la riparazione del DNA. Se la riparazione è produttiva il ciclo cellulare può riprendere e la cellula sopravvive; nel caso invece in cui il danno sia troppo esteso ed irreparabile p53 può attivare l'apoptosi attraverso il blocco dei geni anti-apoptotici come Bcl-2 (Yu e Zhang 2005).

Nel sistema nervoso, è stato riportato che i membri della famiglia di Bcl-2 sono coinvolti nella sopravvivenza e nella morte di cellule neuronali (Merry and Korsmeyer, 1997). Bcl-2, i*n vitro,* previene la morte, indotta da siero-privazione, di motoneuroni (Sato et al., 1998) e, *in vivo,* quella indotta da assotomia (Dubois-Dauphin et al., 1994; Farlie et al., 1995). Kostic e collaboratori (1997) hanno riportato la correlazione tra Bcl-2 e morte cellulare in un modello di topi affetti da sclerosi laterale amiotrofica.

I neuroni cerebellari indotti a morire per privazione di potassio extracellulare sono stati utilizzati come modello di studio di morte cellulare neuronale e Harris e Jonhson (2001) hanno dimostrato che la via proptotica coinvolta è la via mitocondriale e che dipende dall'attivazione di Bax (Harris and Johnson, 2001; Linseman et al., 2002; Linseman et al., 2004). Bim è una proteina pro-apoptotica della famiglia di Bcl-2 che svolge un ruolo fondamentale nell'apoptosi di cellule neuronali (Putcha et al., 2001; Whitfield et al., 2001; Putcha et al., 2002). Si pensa che proteine unicamente con il dominio BH3, come Bim, promuovano l'apoptosi per via mitocondriale interagendo e neutralizzando membri anti-apoptotici della famiglia di Bcl-2, come Bcl-2 stesso e Bcl-xL (Strasser et al. 2000; Bouillet e Strasser 2002). Utilizzando la tecnica del doppio ibrido (two-hybrid assay) alcuni ricercatori hanno dimostrato che Bim interagisce

direttamente con diverse proteine della famiglia di Bcl-2 e inibisce la funzione di sopravvivenza di queste proteine (Hsu et al., 1998; Wilson-Annan et al., 2003). In ultimo, proteine antiapoptotiche della famiglia di Bcl-2 promuovono la sopravvivenza cellulare sequestrando e inibendo la funzione di Bax e Bak, che permeabilizzano la membrana mitocondriale esterna e determinano il rilascio di citocromo c (Wei et al., 2001; Degenhardt et al., 2002)

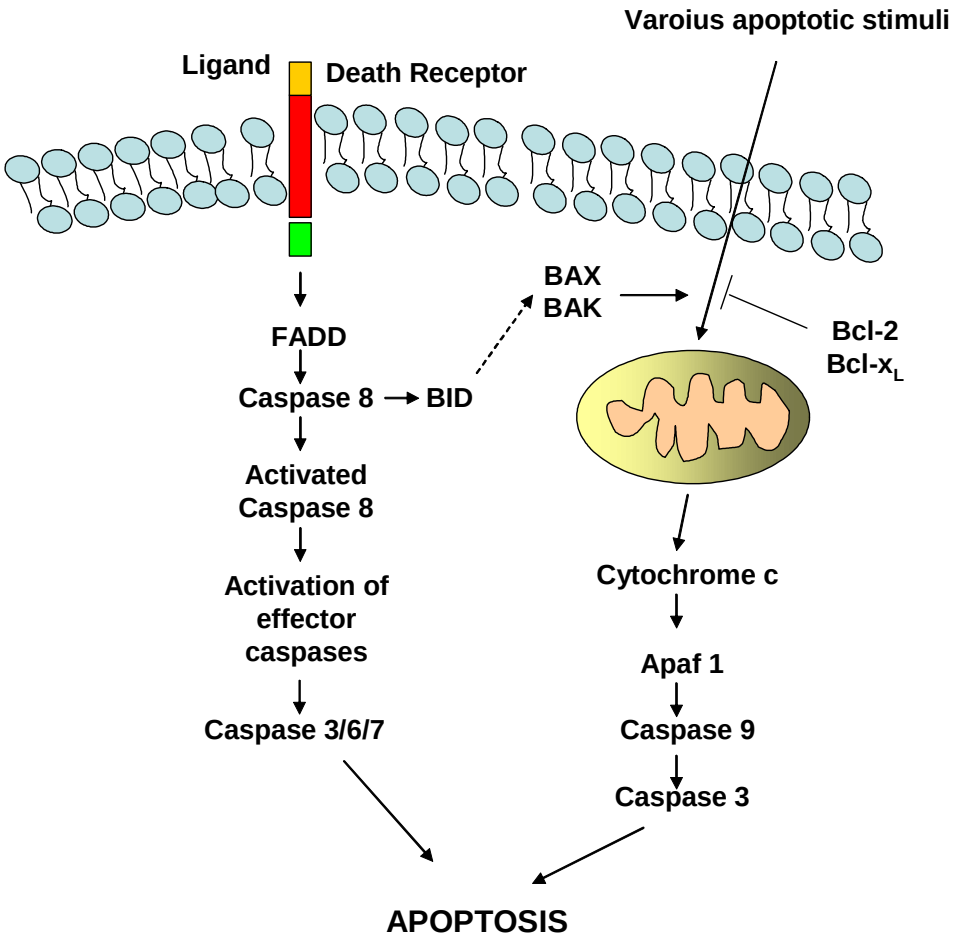

Figura 2. V**ia intrinseca ed estrinseca dell'apoptosi**

La terza modalità di attivazione dell'apoptosi (**via endosomica-lisosomica**) è di scoperta molto più recente rispetto alle due precedenti. Inizialmente i lisosomi venivano associati esclusivamente alla necrosi cellulare. Diversi studi hanno messo in evidenza come il rilascio di enzimi idrolitici lisosomici, in seguito ad un danno cellulare acuto,

rappresenti l'evento finale che determina la lisi delle membrane cellulari e la morte per necrosi delle cellule (Zahrebelski et al., 1995). L'attivazione degli enzimi lisosomici non è però esclusiva della necrosi, infatti molti agenti lisosomotropici intervengono anche nell'induzione dell'apoptosi (Kessel et al., 2000; Ollinger, 2000). In generale la rottura dei lisosomi e il conseguente rilascio di enzimi idrolitici nel citosol potrebbe indurre sia un danno reversibile ma anche la necrosi e l'apoptosi della cellula (Olejnicka et al., 1999; Li et al., 2000; Brunk e Svensson, 1999).

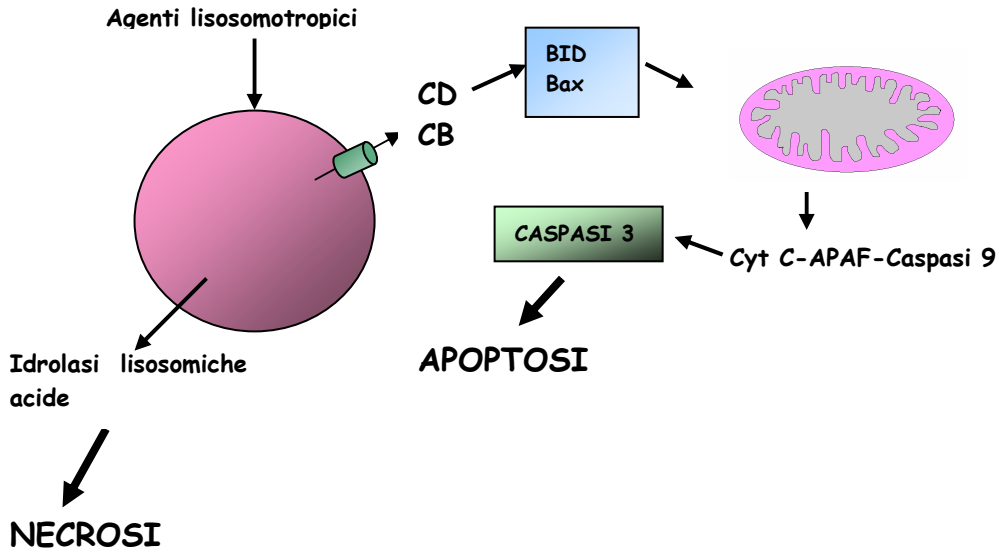

Fig. 3 Via endosomica-lisosomica dell'apoptosi

Diversi enzimi proteolitici sono implicati nella regolazione dell'apoptosi. Le catepsine lisosomiche sono anch'esse implicate nell'attivazione delle caspasi e di conseguenza dell'apoptosi (Ollinger, 2000; Roberg et al., 1999). Le catepsine sono proteasi che, in condizioni fisiologiche, sono localizzate all'interno dei compartimenti endosomici-lisosomici. In risposta a determinati segnali, dai lisosomi esse sono rilasciate nel citoplasma dove avviano trasduzione del segnale di morte. Studi su fibroblasti e cardiomiociti in coltura mostrano che la destabilizzazione dei lisosomi (misurata come rilascio di catepsina D) e l'alterazione del potenziale di membrana dei mitocondri precedono il rilascio del Citocromo c (Ollinger 2000; Roberg et al., 1999). Le proteasi lisosomiche, catepsina B, D ed L agiscono come mediatori dell'apoptosi in molte linee cellulari (Guicciardi et al., 2000; Foghsgaard et al., 2001; Kagedal et al., 2001; Ollinger, 2000; Roberg et al., 1999; Katunuma et al., 2001; Ishisaka et al., 1998,

Demoz et al., 2002). In uno studio recente, Stoka e collaboratori hanno dimostrato che Bcl-2 veniva attivato mediante idrolisi in presenza di estratti lisosomici, suggerendo il possibile coinvolgimento delle proteasi lisosomiche nell'apoptosi (Stoka et al., 2001). La proteasi responsabile del taglio idrolitico di Bcl-2 non è ancora stata identificata, ma le catepsine lisosomiche sono tra i possibili candidati. Katunuma e collaboratori e Ishisaka e collaboratori hanno mostrato che la caspasi-3 è attivata dalla catepsina lisosomica L (Katunuma et al., 2001; Ishisaka et al., 1998, 1999). Guicciardi e collaboratori hanno riportato il coinvolgimento di un'altra catepsina lisosomica, la catepsina B, nell'apoptosi (Guicciardi et al., 2000). In epatociti trattati con TNF-alfa, la caspasi 8 induce il rilascio di catepsina B che, a sua volta, media il rilascio di citocromo c dai mitocondri. La catepsina B, in realtà, agisce come regolatore negativo dell'apoptosi (Shibata et al., 1998; Isahara et al., 1999). L'iperespressione di catepsina B in cellule PC12 riduce la sensibilità di queste cellule all'apoptosi. Nel caso di catepsina D, Deiss e collaboratori furono i primi a riportare che questa proteasi avesse un ruolo come mediatore dell'apoptosi (Deiss et al., 1996). Utilizzando l'RNA antisenso per catepsina D o l'inibitore specifico pepstatina A, hanno riportato l'inibizione dell'apoptosi indotta da IFN gamma o Fas in cellule Hela, così come l'apoptosi indotta da TNF-alfa in cellule U937. Il coinvolgimento della catepsina D nel'apoptosi di fibroblasti indotta da TNF-alfa è stato dimostrato anche da Isidoro e collaboratori (Demoz et al., 2002). Roberg e collaboratori (2002) hanno dimostrato che in seguito allo stress ossidativo, la catepsina D trasloca dai lisosomi al citosol. Questa traslocazione precede il rilascio del citocromo c e l'attivazione delle caspasi, suggerendo un ruolo di catepsina D a monte degli eventi mitocondriali. In accordo con ciò, l'inibitore di catepsina D, pepstatina A, previene il rilascio del citocromo c, l'attivazione delle caspasi e la morte cellulare (Kagedal et al., 2001; Roberg et al., 1999; Roberg, 2001).

1.1.3 LA MORTE AUTOFAGICA

La morte autofagica è un fenomeno filogeneticamente molto antico che potrebbe essersi sviluppato prima dell'apoptosi. La metamorfosi degli insetti puo' essere considerata una condizione estrema di rimodellamento tissutale in cui la morte autofagica ha un ruolo preminente (Beaulaton e Lockshin, 1977). Nei vertebrati la morte autofagica sembra importante durante la morfogenesi di alcuni organi (formazione della

cavità intestinale, regressione delle ghiandole sessuali, etc.) (Beaulaton e Lockshin, 1982; Schweichel e Marker, 1973). Dal punto di vista fisiopatologico la morte autofagica pare associata anche alla morte neuronale (Anglade et al., 1997) che si osserva in seguito all'esposizione a sostanze citotossiche (Rez et al., 1991). Le osservazioni morfologiche confermano la teoria secondo la quale la morte autofagica avrebbe caratteristiche diverse rispetto a quelle dell'apoptosi. L'evento principale della morte autofagica consiste nella degradazione delle componenti cellulari; il numero dei mitocondri diminuisce ma quelli presenti rimangono intatti e mantengono livelli di ATP necessari per completare il processo di autodigestione. La maggior parte del citoplasma viene degradato attraverso il processo autofagico prima che il nucleo incominci a collassare. Attraverso il sistema autofagico vengono rimossi organelli e membrane danneggiate con lo scopo di mantenere l'omeostasi cellulare. Questo meccanismo potenzialmente ha un effetto di "salvaguardia" della cellula analogo all'apoptosi indotta da p53 in seguito ad una danno al DNA (Castino et al., 2003). In analogia con la definizione di "p53 guardiano del genoma" l'autofagia è stata definita "guardiano del proteoma". L'apoptosi e la morte autofagica possono agire più o meno contemporaneamente all'interno di uno stesso tessuto.

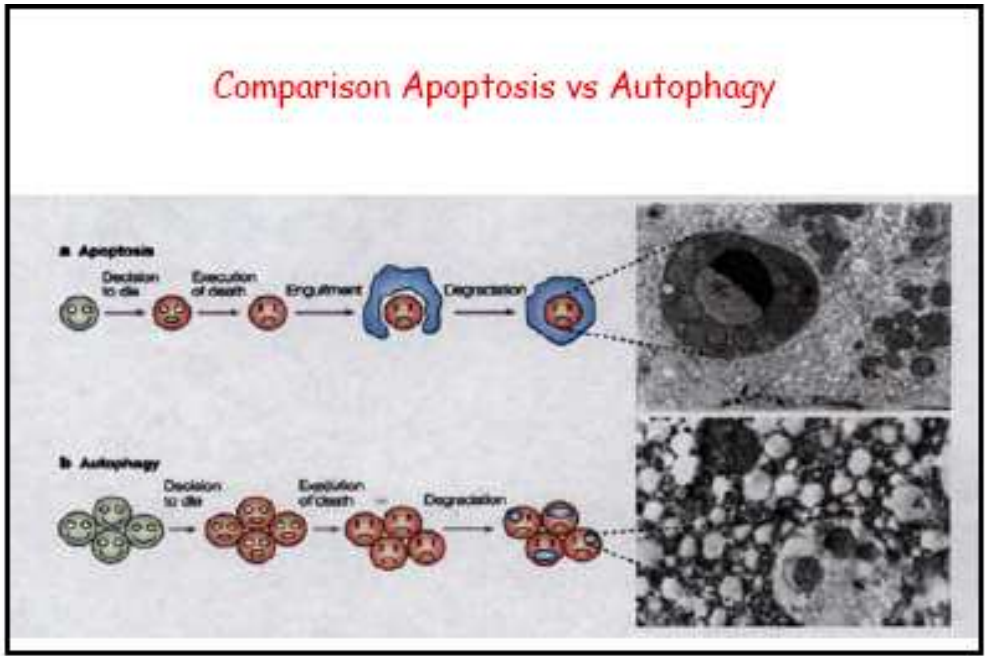

Figura 4. Confronto tra l'apoptosi e la morte autofagica.

2. LA MORTE CELLULARE PROGRAMMATA NEL SNC

Nel corso dello sviluppo del SNC si osserva un'elevata mortalità di neuroni immaturi. Come tutte le cellule, anche quelle neuronali richiedono di apporti trofici per la loro sopravvivenza. Fino a pochi anni fa si riteneva che i neuroni morissero unicamente per mancanza di nutrienti ("starvation") e di fattori trofici. Nel 1988, usando un modello in vitro di neuroni simpatetici, Johnson e collaboratori hanno riportato che l'inibizione della sintesi proteica inibiva la morte cellulare di neuroni simpatetici indotta dalla privazione di fattore di crescita nervoso (nerve growth factor NGF). L'identificazione in *Caenorhabditis elegans* dei geni *ced-3*, *ced-4* e *ced-9* coivolti nella morte cellulare programmata, e dei rispettivi omologhi in mammifero ha permesso di analizzare i meccanismi della morte cellulare neuronale a livello molecolare. Fu poi dimostrato che nei vertebrati, la morte cellulare neuronale indotta da privazione di fattori trofici richiede la partecipazione di cisteino proteasi, che più tardi furono denominate caspasi e che rappresentano l'omologo in mammifero del gene CED-3 in *C. elegans*.

L'apoptosi nei mammiferi è regolata da proteine della famiglia di Bcl-2, dalla proteina Apaf-1 e dalle proteine della famiglia delle caspasi. Le suddette proteine sono, rispettivamente, gli omologhi delle proteine in *C. elegans* CED-9, CED-4, e CED-3. I neuroni condividono con gli altri tipi cellulari le caratteristiche peculiari della morte cellulare programmata. Le proteine della famiglia di Bcl-2 hanno un ruolo cruciale nella trasduzione del segnale di morte apoptotica. A questa famiglia appartengono sia proteine anti-apoptotiche che pro-apoptotiche che contengono uno o piu' domini di omologia con Bcl-2 (domini BH). I principali membri anti-apoptotici della famiglia di Bcl-2 sono Bcl-2 e Bcl-x_L. Garcia e collaboratori (2004) hanno riportato che Bcl-2 può garantire la sopravvivenza di neuroni simpatetici in assenza di NGF, e ciò dimostra che l'iperespressione di Bcl-2 può sopperire al segnale di morte indotto dall'assenza di fattori trofici. Inoltre, è stato dimostrato che topi transgenici che esprimono Bcl-2 nel loro sistema nervoso, durante lo sviluppo sono protetti dalla morte cellulare neuronale e da danni neuronali quali occlusione dell'arteria cerebrale o assotomia del nervo faciale (Dubois-Dauphin et al., 1994; Sagot et al., 1995). Questi dati suggeriscono che la soppressione dell'apoptosi potrebbe proteggere da vari stress quali la privazione di fattori trofici o stimoli patologici.

L'espressione di Bcl-2 è elevata nel sistema nervoso centrale durante lo sviluppo ed è regolata negativamente dopo la nascita, mentre la sua espressione nel sistema nervoso periferico rimane costante durante tutto il corso della vita (Martinou et al., 1995). Sebbene lo sviluppo del sistema nervoso di topi knockout per Bcl-2 sia normale, si osserva una progressiva perdita di neuroni motori, sensoriali e simpatetici dopo la nascita (Veis et al., 1993; Michaelidis et al., 1996), suggerendo che Bcl-2 abbia un ruolo cruciale per il mantenimento della sopravvivenza neuronale. Bcl-x_L è espressa in cervelli in via di sviluppo; a differenza di Bcl-2, Bcl-x_L continua ad aumentare durante la vita adulta (Gonzalez-Garcia et al., 1995).

Bcl-2 e Bcl-x_L agiscono inibendo membri pro-apoptotici della famiglia di Bcl-2 attraverso l'eterodimerizzazione (Merry e Korsmeyer, 1997). Bax è un membro pro-apoptotico della famiglia di Bcl-2 che è ampiamente espresso nel sistema nervoso (Deckwerth et al., 1996). In topi difettivi per Bax, i gangli cervicali superiori e i nuclei faciali presentano un aumentato numero di neuroni. Inoltre, i neuroni simpatetici neonatali e i neuroni motori faciali dei topi difettivi per Bax sono più resistenti alla morte cellulare indotta da privazione di NGF. Quindi, l'attivazione di Bax potrebbe eseere un evento cruciale nella morte neuronale indotta sia da privazione di fattori trofici sia da danni esterni.

2.1 APAF-1 E LE CASPASI NELLA MORTE DI CELLULE NEURONALI

Apaf-1 è l'omologo in mammifero della proteina CED-4 prodotta in *C. elegans* e trasmette segnali di morte apoptotica che vanno ad attivare le caspasi. Apaf-1 insieme con la caspasi-9 forma un complesso con il citocromo c rilasciato dai mitocondri al fine di attivare la pro-caspasi-9 (Zou et al., 1997). La caspasi-9 attivata, a sua volta, attiva la caspasi-3. Topi deficienti per Apaf-1 muoiono durante lo sviluppo embrionale, esibendo una ridotta apoptosi nel cervello ed una ingrandita area proliferativi paraventricolare (Cecconi et al., 1998). Così, Apaf-1 è indispensabile nell'apoptosi di cellule progenitrici delle cellule nervose.

La capacità degli inibitori delle caspasi di bloccare la morte cellulare neuronale indotta da privazione di fattori trofici e da altre condizioni di stress citotossico, ha fornito indiscutibili evidenze del ruolo delle caspasi nella morte cellulare nel sistema nervoso (Cryns et al., 1997). Considerando che nelle cellule di mammifero ci sono almeno 14 caspasi diverse, i ricercatori si sono impegnati a capire il differente ruolo delle caspasi

nella morte cellulare neuronale. Come gli altri tipi cellulari, i neuroni possono esprimere diverse caspasi simultaneamente. Analisi biochimiche e genetiche effettuatte su topi mutanti per una o l'altra caspasi hanno permesso di capire che queste proteasi sono organizzate in vie parallele e alcune volte anche incrociate che sono specializzate a rispondere a determinati stimoli. Le caspasi sono sintetizzate come proenzima enzimaticamente inattivo composto da un pro-dominio all'ammino terminale, una subunità minore e una subunità maggiore. Queste proteasi possono essere classificate in base a sequenze caratteristiche del dominio pro. Le caspasi che contengono il cosiddetto "dominio effettore di morte", come la caspasi 8 e la caspasi 10, sono attivate mediante l'interazione con i domini intracellulari dei recettori di morte come il CD95 (Apo-1/Fas) e il recettore per fattore di necrosi tumorale (TNF). Le caspasi che contengono i domini di attivazione (CARDs), come le caspasi 1,2,4,5,9,11, e 12 sono molto probabilmente attivate mediante un complesso intracellulare di attivazione composto dal complesso citocromo c/Apaf-1/caspasi 9 (Li et al., 1997). Mentre le caspasi che contengono corti domini "pro" , come per esempio la caspasi 3, possono essere attivate dalla maggior parte delle vie caspasiche, dati recenti hanno dimostrato che alcune caspasi, come la 11 e la 12, sono attivate solo in particolari condizioni patologiche (Kang et al., 2000; Nakagawa et al., 2000). Queste ricerche sono particolarmente interessanti perché sulla base di cio' si potrebbero inibire le vie di attivazione associate a stati patologici senza interferire sull'apoptosi coinvolta nello sviluppo e nell'omeostasi cellulare.

Le due caspasi principalmente coinvolte nella morte apoptotica di cellule neuronali sono la caspasi 3 e la caspasi 9. Sia topi difettivi di caspasi 3 (Kuida et al., 1996) che topi difettivi di caspasi 9 (Kuida et al., 1998) mostrano alterazioni anche gravi nella morte cellulare durante lo sviluppo neuronale. I topi difettivi di Apaf-1, o di caspasi 3 o 9 mostrano numerosi difetti nella morte neuronale e ciò suggerisce che queste vie di segnalazione sono importanti nella regolazione della morte cellulare durante lo sviluppo del cervello.

2.2 NEUROTROFINE: UN CASO DI VITA O DI MORTE

Il ruolo di speciali proteine, le **neurotrofine**, nello sviluppo dei neuroni e del cervello è stato sottolineato da tempo, cioè dai primi studi di Rita Levi Montalcini sul Fattore di accrescimento nervoso (NGF, nerve growth factor). E' stato infatti scoperto che non solo le neurotrofine entrano in azione nelle fasi precoci dello sviluppo per

guidare la formazione delle reti nervose, ma continuano ad agire per tutta la vita come fattori di protezione delle cellule nervose da fattori di stress che potrebbero comprometterne il funzionamento fino alla morte stessa dei neuroni. In particolare si è visto che, in risposta a lesioni del cervello, nel neurone si scatena una precisa serie di eventi intracellulari mirate, da un lato, a eliminare il più rapidamente possibile i neuroni compromessi, e dall'altro a ristabilire le connessioni perdute a causa della lesione da parte dei neuroni superstiti. A questo scopo, numerosi geni codificanti neurotrofine e proteine del citoscheletro si attivano in tempi più o meno successivi alla lesione, in modo abbastanza selettivo a seconda dell'area cerebrale interessata. Non solo: secondo alcuni studiosi, le neurotrofine esercitebbero un ruolo facilitatore sull'attività sinaptica, che sembra esprimersi maggiormente in certe fasi dello sviluppo e in certe condizioni di attivazione delle sinapsi. Parimenti, al malfunzionamento delle neurotrofine è stato attribuito un ruolo importante nel mediare i deficit neuropsichiatrici riscontrabili in malattie neurodegenerative come il morbo di Alzheimer (Abbott e Nelson, 2000; Pennypacker et al., 2000; Schinder e Polo, 2000). Come sopra menzionato, la sopravvivenza dei neuroni immaturi durante lo sviluppo dipende dalla disponibilità di fattori neurotrofici. Le neurotrofine generalmente attivano e legano i recettori Trk (TrkA, TrkB e TrkC) che sono recettori di superficie con attività tirosina chinasica intrinseca e in grado di autofosforilarsi (Barbacid, 1995); Dopo il legame del NGF con TrkA, per esempio, il recettore fosforila diversi residui di tirosina nella propria coda citoplasmatica. Queste fosfotirosine, a loro volta, funzionano come legame di attracco per altre molecole come la fosfolipasi Cγ, il fosfoinositide 3 chinasi (PI3K) (Fruman et al., 1998) e proteine adattatrici come Shc. L'ipotesi delle neurotrofine si basa sul fatto che le neurotrofine agiscano come segnale di sopravvivenza per inibire il programma di morte. Comunque, l'interazione delle neurotrofine con il loro recettore p75NTR, in certe condizioni, puo' indurre morte cellulare, suggerendo che le neurotrofine possano agire anche da ligandi di morte in certe situazioni. Il recettore p75 (p75NTR) è un membro della famiglia del recettore per il TNF che può legare tutte le neurotrofine (Dechant and Barde, 1997). Il suo dominio intracellulare contiene una regione con alta omologia con il "dominio di morte" che media le interazioni proteina-proteina ed è presente in altri membri della famiglia del recettore del TNF. Inizialmente, si pensava che p75NTR cooperasse con Trk per modulare la risposta alle neurotrofine. In realtà p75NTR potrebbe avere un ulteriore ruolo nel controllo della morte cellulare nel sistema nervoso. Barde e collaboratori hanno riportato che l'uso di anticorpi che inibiscano il

legame di NGF con p75NTR, inibiscono la morte di cellule della retina di polli che esprimono p75NTR ma non TrkA (Frade et al., 1996), indicando che l'interazione di NGF con p75NTR promuove la morte cellulare in questo sistema.

2.3 VIA DI TRASDUZIONE DI PI3K-AKT

Un ruolo chiave della via di traduzione del segnale di PI3K nella sopravvivenza neuronale fu proposto inizialmente in seguito all'osservazione che gli inibitori della PI3K bloccano gli effetti di sopravvivenza del NGF (Yao and Cooper, 1995). Gli enzimi PI3K sono normalmente presenti nel citosol e possono essere attivati direttamente mediante il reclutamento del recettore Trk attivato, o indirettamente attraverso Ras attivato. Gli enzimi PI3K attivi catalizzano la formazione del lipide 3' fosfoinositide fosforilato, che regola la localizzazione e l'attività di un componente cruciale nella sopravvivenza cellulare, la serina/treonina chinasi Akt (Philpott et al., 1997).

Akt ha tre isoforme cellulari, delle quali c-Akt3/Rac-PKγ è la specie maggiormente espressa nei neuroni (Datta et al., 1999). Oltre al dominio chinasico posto in posizione centrale, Akt contiene un dominio di omologia alla plecstrina posto all'ammino terminale, che media la sua interazione con proteine e fosfolipidi. Dopo il suo legame con i lipidi, Akt viene traslocato dal citoplasma sulla superficie interna della plasmamembrana, e ciò porta Akt molto vicina alle molecole attivatrici. Le chinasi che fosforilano e attivano Akt, le chinasi 3-fosfoinositolo dipendenti, sono esse stesse regolate da fosfolipidi. Possiamo, perciò, dire che i prodotti lipidici generati dagli enzimi PI3K controllano l'attività di Akt regolando la sua localizzazione e la sua attivazione.

La proteina Akt attivata controlla la sopravvivenza dei neuroni in assenza di fattori trofici, al contrario, una forma dominante negativa di Akt inibisce la sopravvivenza neuronale persino in presenza di fattori di sopravvivenza (Datta et al., 1999). I risultati finora riportati avvalorano l'ipotesi che Akt abbia un ruolo essenziale nella sopravvivenza neuronale. Akt agisce su numerose proteine chiave per mantenere le cellule in vita, compresi regolatori dell'apoptosi e fattori di trascrizione. Uno dei bersagli di Akt è, per esempio, Bad, un membro pro-apoptotico della famiglia di Bcl-2, che nella sua forma non fosforilata può legare Bcl-x_L e quindi bloccare la sopravvivenza cellulare. L'attivazione di Akt induce la fosforilazione di Bad e promuove la sua

interazione con la proteina chaperone 14-3-3, che sequestra Bad nel citoplasma e inibisce la sua azione pro-apoptotica (Datta et al., 1997). E' stato dimostrato che Akt agisce, direttamente o indirettamente, su tre famiglie di fattori di trascrizione. Akt agisce sulla proteina CREB e su NF-kB, entrambe coinvolte nella regolazione della sopravvivenza cellulare, ma puo' anche regolare negativamente i segnali di morte (Brunet et al., 1999). Akt è, dunque, una chinasi ampiamente coinvolta nella sopravvivenza di cellule nervose. E' probabile che, nel prossimo futuro, verranno identificati altri substrati di queste chinasi.

Le neurotrofine non sono le uniche sostanze a controllare la sopravvivenza neuronale: la stimolazione elettrica e la depolarizzazione ad alte concentrazioni di KCl inibiscono la morte neuronale (Koike et al., 1989). Studi più recenti indicano che la depolarizzazione della membrana attiva anche le vie di sopravvivenza neuronale. Non è noto se le vie di sopravvivenza neuronale che vengono attivate in questi casi siano le stesse che vengono attivate dalle neurotrofine (Mao et al., 1999; Vaillant et al., 1999).

Sebbene sia chiaro che le neurotrofine e la depolarizzazione delle membrane attivano la via di trasduzione del segnale che inibisce l'apoptosi, non è del tutto chiaro che cosa inneschi l'attivazione dell'apoptosi in assenza dei segnali di sopravvivenza. E' possibile che le neurotrofine inibiscano semplicemente un segnale basale di morte cellulare programmata.

La rimozione di NGF determina una diminuzione nell'attività della MAP p38 chinasi e della PI3K, a cui segue una serie di cambiamenti metabolici inclusa l'aumentata produzione di specie reattive dell'ossigeno, un diminuito "uptake" del glucosio e diminuita sintesi proteica. In alcune cellule, la rimozione dell'NGF determina un lento ed evidente aumento dell'attività della chinasi JNK e della p38 MAP chinasi (Xia et al., 1995); in altre cellule, c-Jun, uno dei substrati di JNK, è indotto e fosforilato (Estus et al., 1994; Ham et al., 1995). L'attivazione di JNK stesso può essere necessaria, anche se non sufficiente, a indurre l'apoptosi neuronale.

Come in altri tipi cellulari, il rilascio del citocromo c dai mitocondri induce l'attivazione delle caspasi nei neuroni simpatetici. L'aggiunta di un inibitore di pan-caspasi, ma non del NGF, protegge i suddetti neuroni anche dopo che è avvenuto il danno alle membrane mitocondriali e il rilascio del citocromo c (Deshmukh and Johnson, 1998). Questi neuroni, in pratica, non sono destinati a morire fino a quando le caspasi non vengono attivate. Ciò significa che il cosiddetto "punto di non ritorno" è a livello dell'attivazione delle caspasi o a valle e suggerisce che che l'inibizione dell'attività

delle caspasi potrebbe essere sufficiente per bloccare la morte neuronale in alcune condizioni patologiche.

4.4 APOPTOSI PATOLOGICA NEL CERVELLO ADULTO

L'apoptosi fisiologica che si osserva nello sviluppo del cervello e l'apoptosi patologica nel cervello adulto hanno in comune simili meccanismi molecolari di azione. Ci sono, comunque, alcune differenze chiave nei meccanismi di regolazione dell'apoptosi. Mentre l'apporto di nutrienti ha un ruolo chiave nell'apoptosi durante lo sviluppo, ci sono poche evidenze dell'implicazione di fattori trofici come meccanismo primario di morte cellulare nelle malattie neurodegenerative nell'adulto. Piuttosto, gli insulti tossici che risultano da danni di tipo biochimico o genetico potrebbero regolare le malattie neurodegenerative cooperando con la morte cellulare programmata, per esempio attraverso la formazione di specie radicali dell'ossigeno o l'attivazione delle caspasi. Un punto chiave nelle malattie neurodegenerative nell'adulto è la tossicità determinata da abnormi strutture proteiche e aggregati che si accumulano nelle cellule. Ciò è stato evidenziato in malattie neurodegenerative come la malattia di Alzheimer, il morbo di Parkinson, la malattia di Huntington e la sclerosi laterale amiotrofica.

2.5 MORTE CELLULARE INDOTTA DA ISCHEMIA

La morte cellulare indotta dal danno ischemico, tradizionalmente, è stata descritta come necrosi. Negli ultimi decenni, caratteristiche morfologiche e biochimiche dell'apoptosi sono state in realtà documentate in modelli sperimentali animali di danno ischemico cerebrale (Charriaut-Marlangue et al., 1996).

Durante l'ischemia i mitocondri sembrano avere un ruolo fondamentale nella trasmissione del segnale di morte per attivare le caspasi. Ci sono evidenze dell'attivazione della caspasi 3, mediata dalla caspasi 11, una caspasi che viene indotta in modo significativo in seguito a danno ischemico (Kang et al., 2000). Inoltre, inibitori delle caspasi diminuiscono notevolmente il danno ischemico sui neuroni. Sebbene ci sia una forte evidenza di apoptosi nel danno cerebrale ischemico, non tutte le cellule muoiono di apoptosi. Accanto alle cellule con caratteristiche tipiche dell'apoptosi, ci sono cellule che appaiono ingrandite e altamente vacuolizzate (Marti net al., 1998); è

probabile che la morte di un numero significativo di neuroni, nell'ischemia cerebrale , avvenga attraverso un meccanismo non caspasi-dipendente.

2.6 MORTE CELLULARE NEURONALE NELLA MALATTIA DI ALZHEIMER

La malattia di Alzheimer (AD) è una delle più comuni malattie neurodegenerative. E' una malattia neurodegenerativa insidiosa e progressiva caratterizzata da demenza correlata all'età, declino cognitivo, e accumulo di depositi beta-amiloidi nel cervello. Colpisce l'11% della popolazione sopra i 65 anni di età e il 50% sopra i 50 anni (Vickers et al., 2000). Nel cervello del paziente affetto da AD si riscontrano caratteristiche placche neuritiche senili e accumuli di neurofibrille. Le placche senili sono depositi extracellulari di peptide beta-amiloide mentre le placche neurofibrillari sono aggregati citoplasmatici composti principalmente dalla proteina tau (una proteina associata ai microtubuli nel cervello). Questi depositi abnormi di proteina beta-amiloide, e di tau sono la causa principale delle perdita di cognizione e di memoria e della demenza caratteristica dei pazienti affetti da malattia di Alzheimer.

Circa una quindicina di anni fa, i ricercatori hanno dimostrato una base genetica alla predisposizione alla malattia. Geneticamente, la malattia di Alzheimer è una malattia complessa ed eterogenea ed è strettamente correlata all'età. Esistono due forme della malattia: una forma familiare rara, precoce e altamente penetrante (EOFAD) e una piu' tardiva (LOAD). EOFAD rappresenta solo una piccola parte di tutti i casi (meno del 5%) e tipicamente si manifesta in pazienti con meno di 65 anni. Nella forma familiare della malattia di Alzheimer sono state documentate più di 160 mutazioni a carico di tre geni: il gene che codifica il precursore della proteina beta-amiloide (APP) sul cromosoma 21 (Goate et al., 1991); il gene che codifica la presenilina 1 (PSEN 1) sul cromosoma 14 (Sherrington et al., 1995) e il gene che codifica la presenilina 2 (PSEN 2) sul cromosoma 1 (Rogaev et al., 1995; Levy-Lahad et al., 1995). Il gene APP codifica una proteina di membrana integrale che è soggetta a taglio proteolitico da parte degli enzimi alpha e beta-secretasi. Il taglio proteolitico di APP determina la formazione di una proteina di 100-120 kDa e di una di 10-20 kDa. Un'altra mutazione associata alla malattia si ritrova nel geni della presenilina PSN1 e PSN2. Numerosi studi su questi proteine sono stati condotti in Drosophila, in particolare sulla proteina (Martin-Morris e White, 1990). Nella Drosophila in cui viene silenziato il gene APP1, i neuroni sono vitali e non mostrano grossi difetti morfologici a dimostrazione del fatto che APP1 non

è indispensabile per la vita, e tuttavia gli insetti “transgenici” presentano difetti nella fototassi. Questi difetti possono essere in parte eliminati se viene espresso un gene APP umano nella Drosophila (Fossgreen et al., 1998) suggerendo che il gene APP non differisce molto nelle due specie.

LOAD, d’altro canto, colpisce prevalentemente persone con più di 65 anni e rapresenta la stragrande maggioranza dei casi di AD.

Nel corso della malattia di Alzheimer si verifica una progressiva perdita cellulare. E’ difficile stabilire il contributo dell’apoptosi in questa perdita cellulare poiché il processo patologico dell’AD è cronico e progressivo. Alcuni neuroni mostrano caratteristiche morfologiche tipiche dell’apoptosi, ma molti neuroni mostrano caratteristiche diverse suggerendo quindi che l’apoptosi non sia l’unico meccanismo implicato nella degenerazione della malattia di Alzheimer. LeBlanc (2005) ha dimostrato che la morte cellulare che si osserva nell’Alzheimer è apoptosis. Il ricercatore ipotizza che l’attivazione della caspasi 6 effettrice nella malattia di Alzheimer sia responsabile della rottura del citoscheletro dei neuriti con conseguente blocco del trasporto di proteine e organelle determinando le caratteristiche cliniche e patologiche della malattia.

La macroautofagia è considerata da molti ricercatori la chiave per la sopravvivenza cellulare e per la longevità. In uno studio recente, Yu e collaboratori hanno dimostrato che la macroautofagia neuronale nella malattia di Alzheimer è indotta precocemente e prima della deposizione delle placche di beta-amiloide. Successivamente, gli autofagosomi e le vescicole autofagiche tardive si localizzano all’interno dei dendriti e determinano un’alterata maturazione dei vacuoli nei lisosomi. L’analisi morfologica identifica vacuoli autofagici nel cervello come principale riserva di beta amiloide. Vacuoli purificati contengono il precursore della beta amiloide, presenilina 1, e attività gamma secretasica presenilina-dipendente. Inibendo o inducendo l’autofagia in cellule neuronali e non, modulando con rapamicina si osservano cambiamenti nello stato di proliferazione dei vacuoli autofagici.

2.7 MORTE CELLULARE NEURONALE NELLA MALATTIA DI PARKINSON

La malattia di Parkinson (PD) è caratterizzata da progressiva perdita di neuroni dopaminergici nella “substantia nigra” e inclusioni citoplasmatiche formate da aggregati proteici insolubili (corpi di Lewy). Ciò porta ad una progressiva perdita delle

funzionalità motorie, inclusi tremore, bradichinesia, e rigidità. Colpisce approssimativamente un milione di persone negli Stati Uniti (Olanow e Tatton, 1999). Una piccola parte dei casi di PD è stata correlata a mutazioni in geni specifici inclusi la alpha-sinucleina, parkin e ubiquitina C-terminal idrolisi L1.

2.8 LA MALATTIA DI HUNGTINTON E LA MORTE CELLULARE

La Corea di Huntington è una malattia neurodegenerativa, ereditabile, ad esordio tardivo, causata da una mutazione a livello del gene codificante per l'huntingtina. Tale gene presenta, a livello della regione codificante, una espansione della tripletta CAG che si traduce in una sequenza poliglutamminica a livello della proteina. La proteina, mutata e troncata nella regione N-terminale, forma degli aggregati intranucleari che unitamente alla eccitotossicità, allo stress ossidativo e alla deplezione di energia provocano la morte dei neuroni dello striato. In laboratorio, in seguito alla scoperta del gene responsabile della malattia, è stato possibile produrre un modello di topi transegnici che presentassero la malattia (Mangiarini et al., 1996). In questi topi sono stati studiati e dimostrati i meccanismi apoptotici di morte cellulare (Reddy et al., 1998; Ferrante et al., 2000). Uno dei primissimi eventi che subentrano nella malattia è la iper-regolazione della caspasi 1 (Ona et al., 1999). In seguito, con l'avanzare della malattia, vengono iper-espressa la caspasi 3, le caspasi 8 e 9 e il citocromo c viene rilasciato dai compartimenti mitocondriali (Kieckle et al., 2002; Sanchez et al., 1999).
Nelle aree celebrali colpite da degenerazione neuronale, dei pazienti affetti da Corea di Huntington, si osservano livelli elevati sia di radicali liberi che di altri prodotti tipici del danneggiamento ossidativo: malondialdeide 8-idrossideossiguanosina, 3-nitrotirosina e eme-ossigenasi (Browne et al., 1999).

3. LA MACROAUTOFAGIA

A seconda del modo in cui il substrato viene trasportato nel lume lisosomiale, nelle cellule di mammifero sono state descritte tre principali forme di autofagia: la macroautofagia, la microautofagia e l'autofagia mediata da chaperone (fig.5) (Seglen e

Bohley, 1992; Kim e Klionsky, 2000; Cuervo and Dice, 2000). Sono state descritte differenze sulla natura del substrato, il tipo cellulare, e le condizioni cellulari.

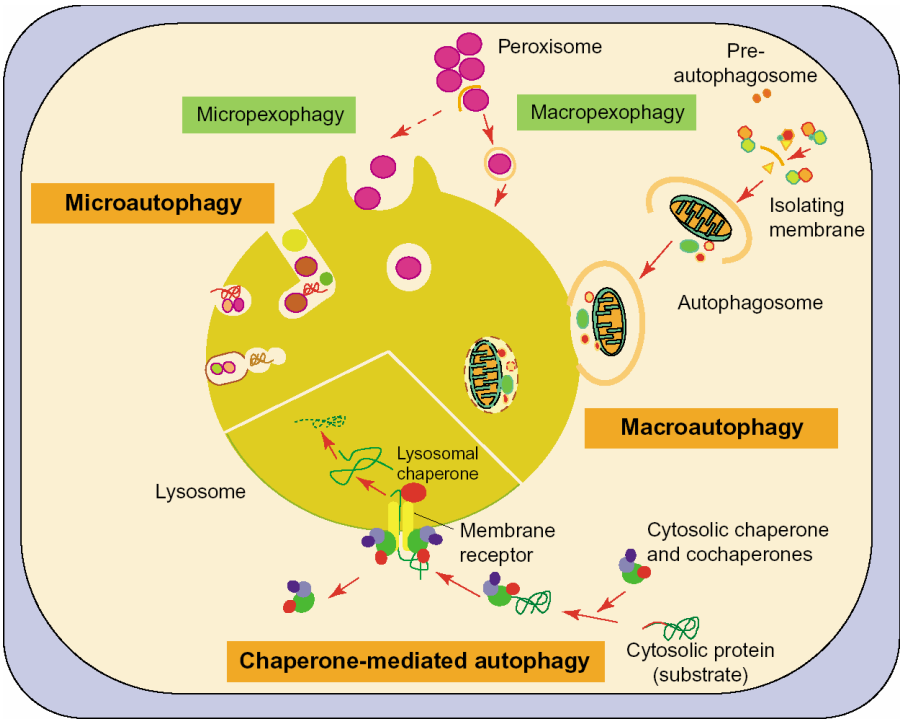

Figura 5. Tipi di autofagia nelle cellule di mammifero. Esistono tre principali forme di autofagia: la macroautofagia, la microautofagia, e l'autofagia mediata da chaperone.I substrati internalizzati possono essere di diversa natura: organuli citosolici e/o singole proteine. Come esempio di autofagia selettiva di organelli, nel diagramma sono mostrati i perossisomi che sono trasportati nei lisosomi attraverso un meccanismo di macroautofagia selettiva detta macropexofagia. L'autofagia mediata da chaperone è mostrata ad ingrandimento maggiore per evidenziare le proteine che partecipano all'internalizzazione delle proteine substrato.

La macroautofagia è un processo dinamico altamente regolato in cui le membrane cellulari subiscono un drammatico cambiamento morfologico. Regola finemente l'equilibrio tra la formazione e la degradazione di proteine e organelli garantendo la normale crescita e lo sviluppo cellulare. Permette il turnover di costituenti cellulari durante lo sviluppo ed in particolari condizioni di stress cellulare, come per esempio in situazioni di "affamamento", quando cioè la cellula scarseggia di nutrienti e di fattori trofici. Le proteine citosoliche vengono degradate principalmente attraverso due vie: una lisosomica e una non lisosomica. La via non lisosomica prevede

prevalentemente la degradazione selettiva di proteine cellulari in condizioni metaboliche basali. Avviene mediante il coinvolgimento dell'ubiquitina e la degradazione idrolitica attraverso il proteasoma. Per contro, il processo che coinvolge i lisosomi porta alla degradazione di proteine extracellulari (introdotte per pinocitosi o endocitosi) e la degradazione all'interno di vescicole di proteine intracellulari e organelli, in condizioni di stress (macroautofagia che d'ora in poi, per comodità, chiameremo semplicemente autofagia). Ricordiamo che esistono anche due processi di degradazione lisosomica di specifiche proteine citosoliche detti microautofagia e autofagia mediata da chaperone.

L'autofagia è il meccanismo attraverso cui proteine stabili a vita lunga vengono degradate, ed è il solo meccanismo per mezzo del quale interi organuli come mitocondri e perossisomi sono riciclati. Inizialmente, l'autofagia era stata descritta come la risposta della cellula alla privazione di siero e fattori trofici (affamamento). Una delle principali funzioni dell'autofagia è infatti il recupero di substrati (aminoacidi, zuccheri, lipidi) dalla degradazione macromolecolare necessari per la sopravvivenza della cellula quando i nutrienti scarseggiano. In ragione di ciò, l'autofagia è stimolata dalla privazione di aminoacidi (Mortimore and Poso, 1986) ed è controllata dagli ormoni; il glucagone promuove, mentre l'insulina inibisce, l'autofagia (Blommaart et al., 1995). Analogamente, un eccesso di aminoacidi riduce i livelli di autofagia. La cellula è particolarmente sensibile alle concentrazioni citoplasmatiche di asparagina, glutammina, leucina, istidina, fenilalanina e triptofano (Grinde e Seglen, 1981; Seglen et al., 1980). L'autofagia, negli eucarioti, è altamente regolata da numerosi fattori incluse le purine (Kovacs et al., 1981), i fattori di crescita (Xue et al., 1999), il siero (Amenta, 1978), agonisti adrenergici (Seglen et al., 1990) e secondi messaggeri (Codogno et al., 1997).

Nell'autofagia, porzioni di citoplasma sono sequestrate all'interno di vescicole, dotate di una doppia membrana, conosciute con il nome di vacuoli autofagici o autofagosomi. Il processo autofagico può essere suddiviso in almeno quattro fasi dette: I) induzione; II) formazione dell'autofagosoma; III) fusione dell'autofagosoma con il lisosoma; IV) degradazione del corpo autofagico (Fig 6).

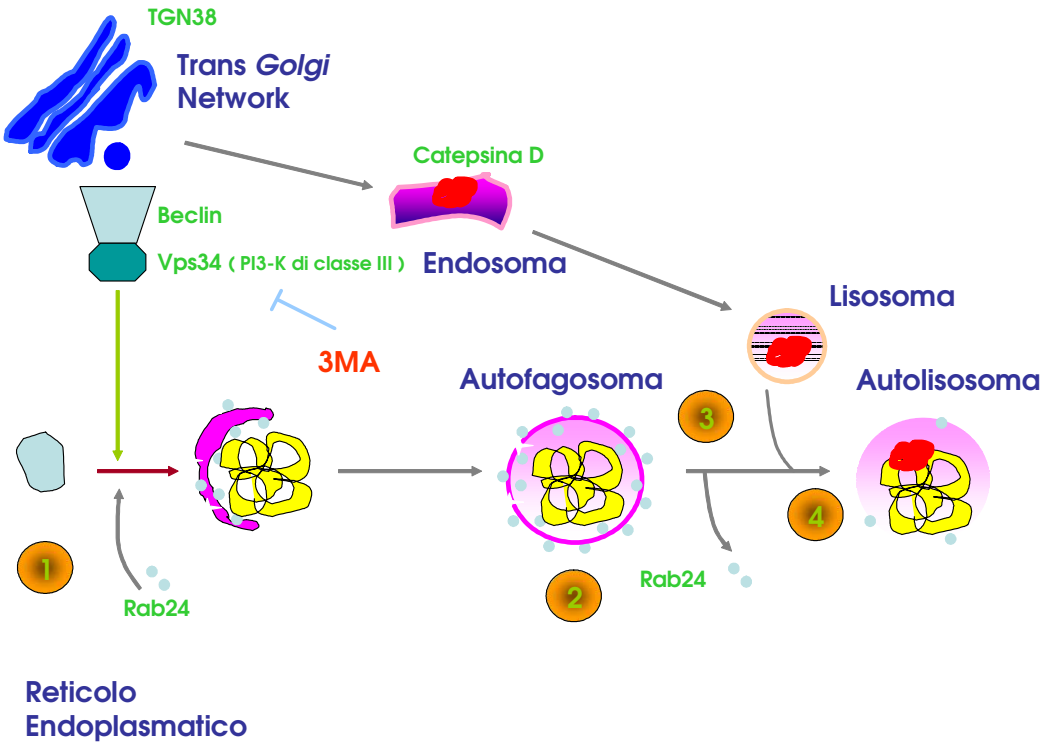

Figura 6. L'autofagia è un processo multifasico.

La vescicola autofagica pare abbia origine da un'estroflessione del reticolo endoplasmatico liscio (Dunn, 1990), ma non è escluso che le membrane del vacuolo possano originare anche dal TGN (trans Golgi network) (Stromhaug et al., 1998). Questo processo è altamente regolato ed è sotto il controllo di GTPasi, fosfatidilinositolo chinasi e varie fosfatasi tra cui la protein fosfatasi PP2A. Nelle cellule animali la fusione dell'autofagosoma con il lisosoma dipende dalla presenza dei microtubuli e dal mantenimento delle proprietà di acidificazione. Dopo la fusione dell'autofagosoma con il lisosoma la membrana esterna dell'autofagosoma viene incorporata nella membrana del lisosoma stesso. Il contenuto della vescicola viene riversato nel lume del lisosoma in cui idrolisi di diverse classi (lipasi, glicosidasi, proteasi) intervengono nella degradazione. I meccanismi molecolari che regolano la formazione della vescicola autofagica sono stati studiati per la prima volta in *Saccaromyces cerevisiae* (Klionsky et al 2003) e i geni coinvolti in questo processo sono stati designati con il nome Apg acronimo di Autophagy (inizialmente Atg da

Autophagy) . Molte delle proteine della famiglia Apg isolate in lievito presentano il loro omologo nelle cellule di mammifero.

3.1 REGOLAZIONE DEL PROCESSO AUTOFAGICO

Il processo autofagico è regolato a diversi livelli. Come si è detto in precedenza l'autofagia non-specifica è inibita in condizioni di abbondanza di nutrienti ed è indotta in condizioni di stress e privazione di fattori trofici. Nelle cellule animali la fosforilazione della proteina S6 ribosomale è fortemente correlata con l'inibizione dell'autofagia (Blommaart et al., 1995). L'attività della p70s6 chinasi è regolata dalla chinasi mTOR (Thomas e Hall, 1997, Brown et al., 1995). TOR (target of rapamycicn) è una chinasi che inibisce la via autofagica, e che a sua volta, è inibita dall'antibiotico rapamicina. L'inibizione della fosforilazione di p70S6 (e forse altri substrati non noti), come risultato dell'inattivazione di mTOR in seguito al trattamento con rapamicina, induce l'autofagia anche in condizioni di abbondanza di nutrienti. Come nelle cellule animali, l'autofagia può essere indotta in lievito mediante l'inibizione rapamicina-dipendente di Tor2 (Noda e Oshumi, 1998). Nei lieviti Tor2 fosforila Tap42 e questo induce l'interazione della Tap42 con la fosfatasi 2A (PP2A). Questa interazione inibisce l'attività della PP2A importante per l'induzione dell'autofagia (Beck e Hall, 1999). L'inibizione di Tor2 determina invece l'attivazione di PP2A e l'induzione dell'autofagia. Nelle cellule animali l'inibizione di PP2A da parte dell'acido ocadaico ha un forte effetto inibitorio sull'autofagia (Holen et al., 1993). Il cambiamento della localizzazione e/o dell'attività di PP2A potrebbe essere dunque un meccanismo di controllo dell'autofagia. Quando mTOR è inattiva essa non esercita alcuna inibizione sulla PP2A, che può allora defosforilare Apg13. La defosforilazione di Apg13, l'attivazione di Vps34 (omologo della PI3-K di classe III dei mammiferi) e l'interazione di Vps34 con Apg6 determinano l'attivazione dell'autofagia e innescano la formazione della vescicola autofagica. Nei lieviti, il complesso della PI3-K di classe III è costituito da Apg6, Apg14, Vps15 (serin-chinasi miristilata) e da Vps34. Apg14 funge da adattatore per l'interazione Vps15-Vps34 dove Vps15 è la chinasi che regola l'attività del Vps34 (Stack et al., 1995). L'associazione Apg14-Apg34, invece, conferisce la specificità del complesso (Kihara et al., 2001).

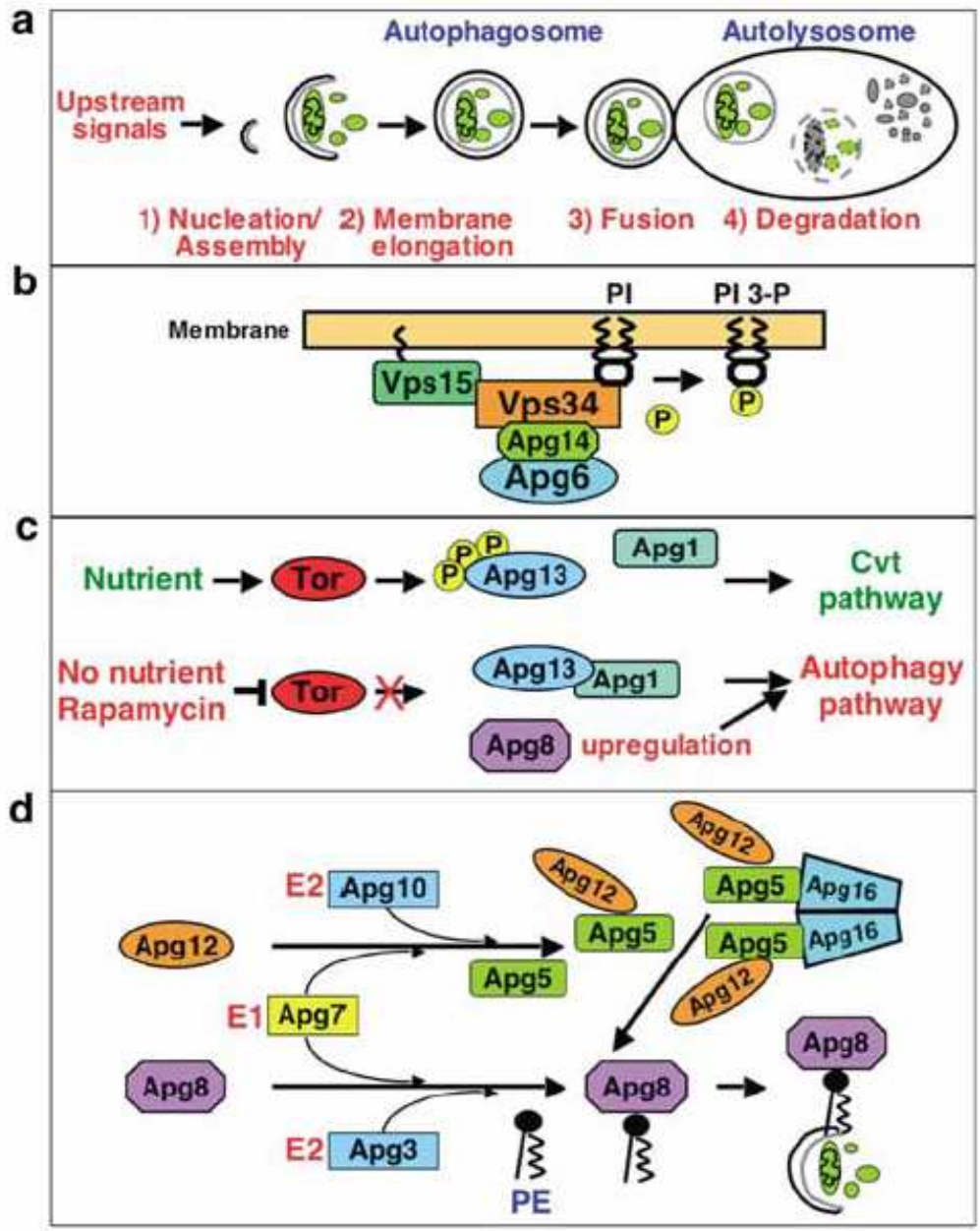

Fig. 7 Proteine coinvolte nella regolazione del processo autofagico

In studi recenti si è visto come la PI3-Kinasi (PI3-K) di classe III e la PI3-K di classe I agiscano in direzioni opposte nell'attivazione dell'autofagia (Petiot et al., 2000). La PI3-K di classe III ha un'azione attivante l'autofagia ed è inibita dalla 3-Metiladenina (3-MA), wortmannina e LY294002 (Kim e Kliosky, 2000; Petiot et al., 2000). L'attivazione della PI3-K di classe III determina l'accumulo di fosfatidil-inositoli-fosfato PIP2 e PIP1, due messaggeri lipidici che segnalano l'innesco dell'autofagocitosi (Petiot et al., 2000). Beclin 1, l'omologo in mammifero di Vps30p/Apg 6p, forma complessi con la PI3-K di classe 3 favorendone l'attivazione e

conseguentemente promuovendo l'avvio del sequestro autofagico (Kihara et al., 2001). L'overespressione di Beclin-1 in cellule MCF-7 induce un aumento del numero delle vescicole autofagiche. La 3-MA è in grado di prevenire l'autofagia indotta da Beclin-1 in cellule MCF-7 (Liang et al., 1999). L'incremento invece della PI3-K di classe I inibisce la macroautofagia e favorisce la sintesi proteica, la proliferazione cellulare e la sopravvivenza cellulare (Kim e Kliosky, 2000; Petiot et al., 2000). Ad esempio, la stimolazione da parte di fattori di crescita della PI3-K I determina l'attivazione PKB/Akt e di conseguenza l'attivazione di mTOR, che promuove la sintesi delle proteine e inibisce la degradazione proteica autofagica (Dennis et al., 1999). In condizioni di stress PTEN (Phosphatase and Tensin homolog deleted on chromosome Ten), una lipide-proteasi, interviene riducendo i livelli di PIP-3 (fosfatidilinositolo 3,4,5-trisfosfato), che si riflette in una diminuzione dell'attività di Akt e di conseguenza di mTOR (Petiot et al., 2000; Arico et al., 2001). PTEN è una proteina con duplice funzione, protein-lipido fosfatasi che risulta mutata in molti tipi di cancro (Di Cristofano e Pandolfi, 2000; Li et al., 1997; Myers et al., 1997). E' stato dimostrato che PTEN defosforila al 3' del fosfatidilinositolo prodotto dalla PI3-K di classe I (Maehama e Dixon, 1998) e di conseguenza regola negativamente la via di PI3-K/PKB (Wu et al., 1998). PTEN è espresso in cellule di colon carcinoma HT-29 e regola negativamente la via di segnalazione PI3-K/AKB dipendente da IL-13 (Arico et al., 2001).

Dopo l'induzione dell'autofagia ha inizio la formazione della vescicola autofagica con il sequestro delle componenti citoplasmatiche. Il processo di sequestro implica un sistema di coniugazione proteica in cui gioca un ruolo fondamentale l'interazione di due dimeri Apg12-Apg5. L'interazione covalente di Apg12 con un'Apg5 richiede l'azione dell'Apg7 (proteina coinvolta nell'attivazione dell'ubiquitina) e dell'Apg10 (enzima che funge da coniugatore di proteine). Un'altra proteina, Apg16, si lega a Apg5 e dimerizza le coppie Apg12-Apg5. Il legame covalente tra l'Apg5 e l'Apg12 prevede l'attivazione della glicina 187, posta all'estremità carbossi-terminale dell'Apg12, attraverso l'interazione prima con l'Atg7 e poi con l'Atg10. Solo dopo l'attivazione si ha il legame covalente tra la glicina187 e la lisina 130 dell'Apg5 (Mizushima et al., 1998). Forme mutanti della proteina Apg5 prive del residuo di lisina130 non formano il complesso Apg5–Apg12–Apg16 ma rimangono associati alla membrana da cui dovrebbe originare il vacuolo autofagico. L'Apg5 potrebbe esso stesso possedere un dominio in grado di dirigere il complesso al reticolo (Mizushima et al., 2001). Importante per la formazione della vescicola è anche

l'aggiunta di un fosfatidiletanolammina lipide all'estremità carbossi terminale di una proteina associata ai microtubuli, LC3, di nuova sintesi. LC3 è la proteina omologa all'Apg8/Aut7 di *S. cerevisiae*. L'aggiunta della fosfatidiletanolammina è preceduta dal taglio, in posizione C-terminale, dell'LC3, da parte della cistein-proteasi Apg4 generando una forma solubile nota come LC3-I ed esponendo una glicina al carbossi terminale, indispensabile per le reazioni successive (Kabeya et al., 2000; Kabeya et al., 2004). La forma LC3-I, a sua volta, è modificato in una forma associata alle membrane, LC3-II, da parte di omologhi di Apg7 e Apg3 e si localizza sull'autofagosoma e sull'autolisosoma. Nelle cellule che esprimono EGFP-LC3 l'induzione dell'autofagia, attraverso l'affamamento, promuove una ricollocazione della proteina nelle vescicole autofagiche di nuova formazione (Mizushima et al., 2004). Così, la quantità di LC3-II in cellule di mammifero è considerato un buon marcatore della formazione di autofagosomi (Kabeya et al., 2000). Successivamente, l'autofagosoma fonde con il lisosoma per degradare il materiale intrappolato. E' probabile che LC3-II venga rapidamente degradato all'intermo del lume dell'autofagolisosoma. Purtroppo, poco è noto sulla degradazione di LC3-II nell'autolisosoma durante l'autofagia indotta da affamamento. Studi morfologici indicano che, in culture primarie di epatociti, solo pochi autofagosomi e autolisosomi si osservano in condizioni di affamamento senza l'utilizzo di inibitori di idrolasi lisosomiche (Pfeifer, 1977; Ueno e Kominami, 1991), probabilmente perché la fusione con il lisosoma e la degradazione sono processi molto veloci durante l'affamamento. Gli autolisosomi sono facilmente isolati in ratti in condizioni di affamamento e trattati con leupeptina ma non in ratti non trattati.

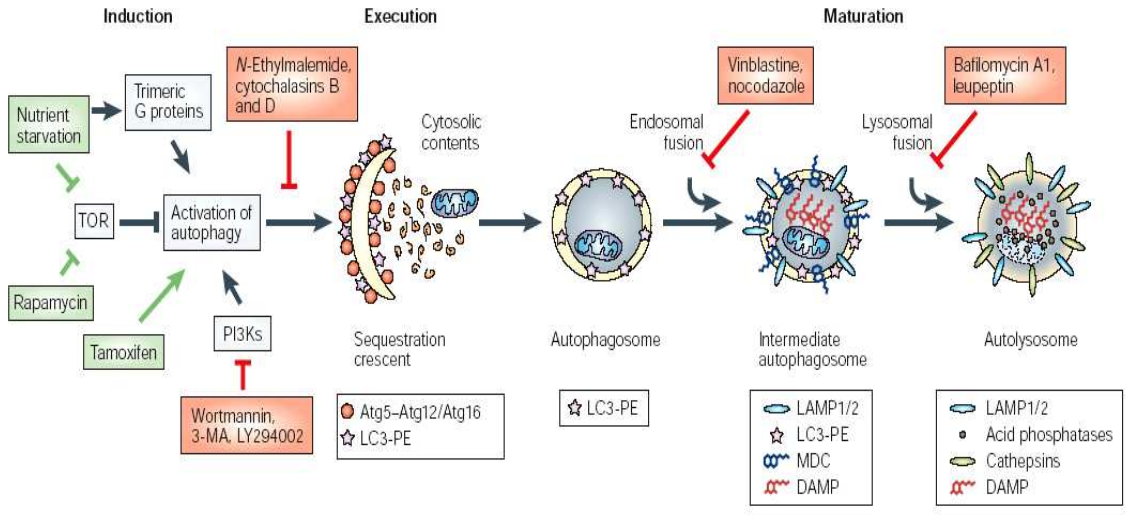

Figura 8. Attivazione e formazione della vescicola autofagica: regolatori e inibitori

Le membrane lisosomiche contengono diverse proteine N-glicosilate, incluse LAMP-1 e LAMP-2. LAMP-2 è stato descritto come un recettoreper la degradazione autofagica, selettiva nei lisosomi (Cuervo e Dice, 1996;1998). Dopo la formazione, l'autofagosoma si fonde con il lisosoma e acquisisce proteine di membrana lisosomica, come LAMP-1 e LAMP-2, pompe protoniche vacuolari e idrolasi acide. Il recettore per il mannosio-6-fosfato (MPR) viene usato come marcatore dei compartimenti prelisosomici, l'amina acidotropica (3-(2,4-dinitroanilino)-3'-amino-N-methyl-dipropylamine (DAMP), come marcatore dei compartimenti prelisosomici, lisosomici e autofagolisosomici.

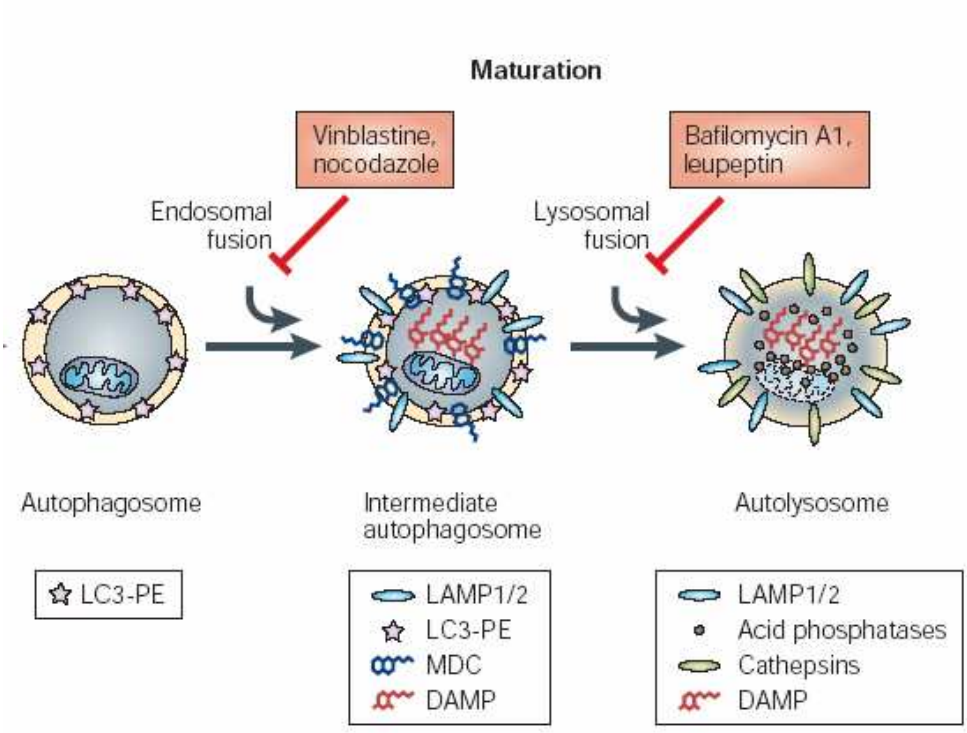

Figura 9. Fusione e maturazione della vescicola autofagica

Il legame tra autofagia e morte cellulare può essere evidenziata utilizzando 3-Metiladenina (3-MA). La 3-MA blocca la formazione del vacuolo autofagico attraverso l'inibizione della PI3-K di classe III. La 3-MA inibisce sia la formazione dell'autofagosoma che la morte autofagica (Seglen e Gordon, 1982; Jia et al., 1997). La morte autofagica indotta dal TNF-α non è però inibita da asparagina che inibisce la fusione dell'autofagosoma con il lisosoma (Jia et al., 1997). La condensazione e la frammentazione del nucleo nella morte autofagica non è associata al rilascio di enzimi citoplasmatici e positività alla colorazione con blu tripano. Questo indica che la morte autofagica, come quella apoptotica, almeno nei primi stadi non è associata alla perdita

dell'integrità di membrana. Studi recenti evidenziano una differente organizzazione del citoscheletro durante la morte autofagica rispetto al processo apoptotico. Nell'apoptosi si ha la depolimerizzazione e il taglio dell'actina, delle citocheratine, delle lamine e di altre proteine del citoscheletro (Bursh et al., 2000). Nella morte autofagica invece il citoscheletro viene ridistribuito ma preservato anche in quelle cellule che presentano la condensazione e la frammentazione del nucleo (Bursh et al., 2000). Durante la morte autofagica, a differenza del processo apoptotico, non c'è attivazione delle caspasi (Kitanaka e Kuchino, 1999; Borner e Monney, 1999; Quignon et al., 1998).

L'autofagia e l'apoptosi sono due eventi distinti e indipendenti?

Diversi studi mettono in evidenza come in alcune situazioni la morte autofagica e quella apoptotica potrebbero essere tra loro interconnesse, anche se i meccanismi non sono ancora perfettamente chiariti. Nella malattia di Parkinson, studi ultrastrutturali hanno dimostrato che i neuroni della substantia nigra di pazienti affetti mostrano sia segni di autofagia che caratteristiche proprie del processo autofagico (Anglade et al., 1997). Comunque, è stato dimostrato che l'espressione di alfa-sinucleine mutate, una condizione frequente in alcune forme di malattia di Parkinson familiare, induce la morte cellulare autofagica senza attivazione delle caspasi, dovuta ad alterazioni del sistema di degradazione proteico ubiquitina dipendente (Stefanis et al., 2001). Ciò suggerisce che non necessariamente in tutte le circostanze si abbia interconnessione tra apoptosi e autofagia in questa malattia. In particolari contesti cellulari e sotto l'influenza di particolari stimoli l'autofagia potrebbe essere indispensabile per il successivo innesco del processo apoptotico mentre in altre situazioni potrebbe avere un'azione antagonista sull'apoptosi. In altri casi invece i due sistemi si manifesterebbero indipendentemente l'uno dall'altro. In alcuni sistemi sperimentali l'apoptosi è sempre preceduta dall'autofagia. Infatti, in questa situazione l'utilizzo di inibitori dell'autofagia come la 3-MA inibisce l'apoptosi mentre gli inibitori delle caspasi non inibiscono l'autofagia. Un esempio di questo tipo è l'apoptosi indotta dal TNF-α in linee cellulari di linfoblasti (Jia et al., 1997). In questo caso, comunque, l'autofagia è essenziale ma non sufficiente nel determinare l'apoptosi; il TNF-α oltre che l'autofagia stimola il rilascio di segnali di morte indipendenti dall'autofagia (Jia et al., 1997). In altre situazioni l'autofagia può antagonizzare l'apoptosi, e l'inibizione dell'autofagia potrebbe incrementare la sensibilità delle cellule agli stimoli pro-apoptotici. Un esempio ben caratterizzato è

l'apoptosi indotta dal sulfide sulindaco in cellule HT-29 di colon carcinoma (Bauvy et al., 2001). In questo modello la morte apoptotica è accelerata in cellule HT-29 in cui si riduce l'attività autofagica facendo iperesprimere una GTPasi mutata nella subunità Gαi3; analogamente, il trattamento delle cellule non trasfettate in presenza di inibitori dell'autofagia aumenta l'apoptosi. Nelle cellule trasfettate con la GTPasi mutata il rilascio di citocromo c dai mitocondri è accelerato, suggerendo agli autori che l'autofagia potrebbe ritardare l'apoptosi attraverso il sequestro di fattori mitocondriali che promuovono l'apoptosi. Apoptosi e autofagia potrebbero anche agire in maniera mutuamente esclusiva. L'inibizione dell'autofagia in questo caso potrebbe indirizzare i segnali di morte nell'induzione dell'apoptosi. Per esempio varie linee cellulari di glioma maligno esibiscono, in seguito all'esposizione con triossido di arsenico, una morte di tipo autofagica (Kanzawa et al., 2003). L'inibizione dell'autofagia con bafilomicinaA (un inibitore dell'H-ATPasi, inibisce l'acidificazione delle vescicole autofagiche) determina invece la morte delle cellule per apoptosi (Kanzawa et al., 2003).

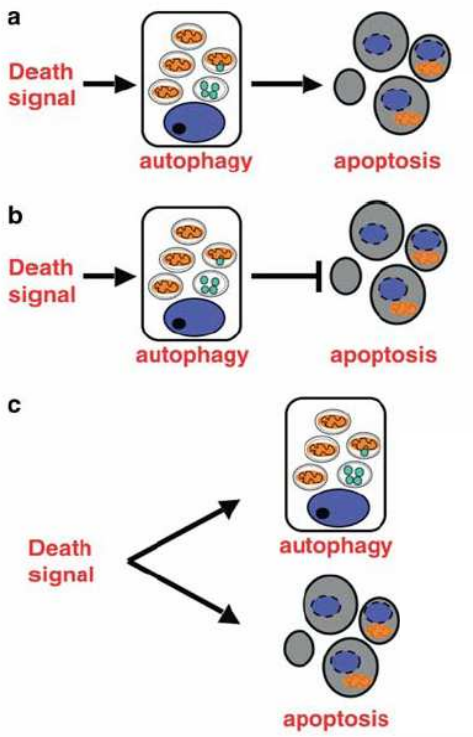

Figura 10. Relazione tra morte apoptotica e autofagica

La relazione tra i due tipi di morte cellulare potrebbe essere in parte spiegata dal fatto che esistono delle interazioni fisiche tra proteine coinvolte nell'autofagia e proteine coinvolte nell'apoptosi. Tra queste troviamo Beclin-1 che da una parte regola l'autofagia e dall'altra interagisce con Bcl-2, una proteina anti-apoptotica (Liang et al., 1998). Analisi mutazionali rilevano che la regione di Beclin compresa tra l'aa 88 e l'aa 150 è sufficiente per mediare l'interazione con Bcl-2. La stessa regione media anche l'interazione con bcl-xl, membro della famiglia di bcl-2, che inibisce l'apoptosi (Boise et al., 1993). Curiosamente la sequenza codificante per questa regione di Beclin è deleta in alcuni cloni cDNA di cervello umano non adulto (Liang et al., 1998). Ciò succerisce che a livello cerebrale esistono almeno due forme di Beclin, una contenente il sito d'interazione con Bcl-2 e una senza questo dominio.

3.1.1 LA REGOLAZIONE FARMACOLOGICA DEL PROCESSO AUTOFAGICO

Nelle cellule *in vitro* l'aggiunta di siero (5-10%), ricco di fattori di crescita, al terreno inibisce il catabolismo proteico. Il glucagone può stimolare la proteolisi negli epatociti con stimolazione della via autofagica-lisosomica (Hopgood et al., 1980). Anche altre sostanze che stimolano la produzione di AMP ciclico, come il dibutiril-AMPc e l'adrenalina, possono avere effetti simili (Morata et al., 1982) provocando l'attivazione della proteina chinasi A (PKA) (Holen et al., 1996). I glucocorticoidi favoriscono l'incremento della proteolisi nel fegato, risposta che si somma a quella del glucagone (Morata et al., 1982). Nel muscolo la prostaglandina E2 induce la sintesi e l'accumulo di catepsine cellulari e conseguentemente aumenta la proteolisi (Rodemann et al., 1982). Le sostanze acidotropiche che accumulano nei lisosomi possono alterarne le proprietà fisico-chimiche in due modi: elevando il pH del lume (neutralizzazione) o causando la destabilizzazione della membrana (cui contribuisce l'accumulo di materiale non degradato conseguente al blocco delle idrolasi acide). L'ammonio e altri composti analoghi (a concentrazioni millimolari) possono virtualmente inibire la degradazione proteica lisosomica associata a privazione di aminoacidi (Stevens et al., 1975). Queste sostanze, nella loro forma dissociata (neutra) si comportano come basi (es. NH_3) e attraversano agevolmente la membrana e accumulano nei compartimenti acidi. Nei lisosomi queste sostanze vengono protonate sottraendo H^+ e quindi alcalinizzando il pH interno. Anche se più protoni sono traslocati all'interno di questi organelli, il pH rimane

elevato per la continua protonazione delle basi. Come risultato finale si ha l'accumulo dell'ammonio (e analoghi) che non può attraversare le membrane essendo carico positivamente con conseguente destabilizzazione lisosomica. La clorochina si comporta anch'essa come una base lisosomotropica e inibisce le proteasi, le fosfolipasi, la sintesi degli steroidi, la sintesi del DNA e la proteolisi (Bertini e Bari, 1970). Le ammine sono utilizzate a concentrazioni tra 5mM e 20 mM. La pepstatina, la chimostatina, la leupeptina, l'antipaina e gli epossi-succinil-peptidi E-64, Ep-459 e Ep-475 sono tutti potenti inibitori delle proteasi lisosomiche. Un singolo inibitore, come la leupeptina a 0.3 mM, può inibire più dell'80% della proteolisi (le ammine esercitano un effetto maggiore). Il vanadato inibisce le ATPasi, le fosfato-transferasi e, usato alla concentrazione di 10 mM, la proteolisi lisosomica con effetto diretto sugli enzimi lisosomici (Van Dyke et al.,1984). La vinblastina (che distrugge i microtubuli) e la colchicina provocano accumulo degli autofagosomi bloccando la loro fusione con i lisosomi. La 3-metil-adenina (3-MA) alla concentrazione di 10 mM blocca completamente e specificatamente la via autofagica-lisosomica di degradazione negli epatociti e in altri tipi cellulari; non ha effetti sulla sintesi proteica e sui livelli di ATP (Ege et al., 1984).

LY294002 e la wortmannina, in quanto inibitori specifici di kinasi della famiglia della fosfatidilinositolo 3-chinasi (PI3K) (Vlahos et al., 1994), sopprimono completamente l'autofagia (Seglen e Gordon, 1982; Blommaart et al. 1997).

La 3-MA ed anche altri inibitori delle proteine serina/treonina fosfatasi, come l'acido okadaico e la caliculina A, sopprimono l'autofagia. L'attività inibente l'autofagia dell'acido okadaico (7 nM) si esplica attraverso l'inibizione della PP2A, (Cohen et al., 1990), la cui attività fosfatasica è essenziale per il mantenimento del sequestro autofagico (Holen et al., 1993).

4. L' AUTOFAGIA E SISTEMA NERVOSO

In seguito ad assotomia di neuroni vitali, si osserva un notevolo aumento di autofagia, con formazione di enormi vescicole di vario tipo che sono state descritte come autofagosomi. La risposta autofagica è rapida; per esempio, gangli superiori assotomizzati in ratti adulti, mostrano aumentata autofagia nel corpo cellulare dopo solo 2 ore. Le vescicole che si formano contengono mitocondri, e i classici substrati

dell'autofagia derivanti dal Golgi o dal reticolo endoplasmatico. Inoltre, molte di queste vescicole contengono precursori della sintesi dei neurotrasmettitori.

4.1 ALTERAZIONI LISOSOMICHE E MACROAUTOFAGIA NELL'INVECCHIAMENTO NEURONALE

La proteolisi lisosomica sembra essere implicata nell'invecchiamento di molti tipi cellulari e tissutali (Bahr e Bendiske, 2002, Lynch e Bi, 2003). Tra gli aspetti da prendere in considerazione vi è la stabilità dei lisosomi, il danneggiamento dei sistemi di trasporto delle macromolecole ai lisosomi e l'alterazione dell'espressione di idrolasi lisosomiche. Alcuni studi hanno dimostrato che durante il normale invecchiamento neuronale si osserva un notevole incremento dell'espressione di catepsina D e catepsina E (Bahr e Bendiske, 2002; Dunlop et al, 2002; Lynch e Bi, 2003). Data l'importanza dei lisosomi nella degradazione dei complessi macromolecolari, i deficit funzionali di questi organelli possono determinare un rapido aumento dell'ossidazione proteica e della formazione di aggregati. Studi "in vitro" hanno evidenziato il ruolo della proteolisi lisosomica nella regolazione del turn-over dell' α-sinucleina. L'α-sinucleina è una proteina di 140 aminoacidi associata a diverse malattie neurodegenerative. L'alterazione del processo lisosomico ne può favorire l'accumulo a livello neuronale (Gomez-Santos et al., 2003; Stefanis et al., 2001) con conseguenze sul mantenimento dell'omeostasi cellulare, sulle vie di traduzione del segnale e sulla regolazione trascrizionale (Lundvig et al., 2005).

Numerosi studi hanno dimostrato che l'inibizione dell'attività lisosomica può inoltre favorire l'accumulo di lipofuscine (Terman and Brunk, 1998; Szweda et al., 2003) e dunque esaltare l'effetto che lo stress ossidativo ha nell'indurre l'accumulo di lipofuscine (Sundelin et al., 2001). Molti studi, inoltre, mettono in evidenza l'associazione tra un'eccessiva attivazione della macroautofagia e l'insorgenza di alcune patologie del SNC quali la corea di Huntington, la sindrome d'Alzheimer e il morbo di Parkinson (Bahr e Bendiske, 2002; Larsen e Sulzer, 2002).

4.2 AUTOFAGIA E NEURODEGENERAZIONE

Nella maggior parte delle malattie neurodegenerative, compresa la sclerosi laterale amiotrofica, il morbo di Parkinson, la malattia di Alzheimer e la malattia di Huntington o le malattie prioniche, la causa pare riconducibile alla formazione di aggregati di proteine che presentano una conformazionate anomala. Numerosi studi hanno dimostrato il coinvolgimento sia del sistema dell'ubiquitina-proteasoma sia del sistema endosomico-lisosomico nella degradazione di questi aggregati (Ardley et al., 2005; Artal-Sanz e Tavernarakis, 2005; Cuervo, 2005; Inoki et al., 2005; Jeyakumar et al., 2005; Libersiki et al., 2004; Ravikumar et al., 2002; Ravikumar et al., 2004; Ravikumar e Rubinstein, 2004; Shintani and Klionski, 2004). In generale, sembrerebbe che il sistema dell'ubiquitina agisca prevalentemente nel degradare proteine solubili, mentre l'autofagia è maggiormente coivolta nella degradazione di aggregati proteici (Cuervo, 2005; Shintani and Klionski, 2004). Per esempio, la prima manifestazione a livello cellulare del morbo di Parkinson pare essere la formazione di inclusioni (i corpi di Lewi) che contengono alpha-sinucleina come proteina principale. In colture cellulari, l'alpha-sinucleina mutata, ma non la forma wild-type, accumula in vacuoli autofagici e parallelamente il sistema ubiquitina proteasoma è alterato (Stefanis et al., 2001). E' interessante notare che in cervelli di pazienti malati di Parkinson sono state riscontrate mutazioni nel sistema dell'ubiquitinazione; l'alterazione del sistema potrebbe essere una delle cause dell'accumulo di aggregati proteici che non vengono rimossi (Ardley et al., 2005). Inoltre, studi effettuati utilizzando cellule SH-SY5Y hanno dimostrato che l'inibizione prolungata del sistema ubiquitina-proteasoma (utilizzando l'inibitore MG115 alla concentrazione di 100nM) puo' causare una eccessiva attivazione del sistema endosomico-lisosomico (Ardley et al., 2005; Ding et al., 2003). D'altro canto, nella malattia di Huntington non sembra essere coinvolto il sistema ubiquitina-proteasoma in quanto quest'ultimo non riesce a digerire le sequenze di poliglutammina che si formano (Venkatraman et al., 2003). Piuttosto,in questa patologia, l'autofagia sembra essere la via maggiormente implicata. In modelli cellulari che riproducono la malattia di Huntington, l'inibizione della proteina mTOR e di conseguenza l'induzione dell'autofagia l'accumulo di poliglutamine e la morte cellulare. L'inibizione dell'autofagia ha gli effetti opposti (Ravikumar et al., 2004; Venkatraman et al., 2004). Inoltre, i cervelli di pazienti con malatti di Alzheimer presentano un'aumentata espressione del compartimento endosomico-lisosomico, come viene mostrato da studi

morfologici (Cataldo et al., 1996). In generale, in numerose malattie neurodegenerative si riscontra un malfunzionamento dell'apparato endosomico-lisosomico (Cataldo et al., 1996; Ravikumar et al., 2004).

Alterazioni del compartimento endosomico-lisosomico sono state correlate alla morte neuronale in molte malattie neurodegenerative, così come in patologie nervose trasmissibili (malattie prioniche) (Larsen e Sulzer, 2002; Nixon et al., 2000; Liberski et al., 2002). La morfologia del compartimento endosomico-lisosomico è alterato in numerose malattie neurodegenerative come malattia di Alzheimer (Nixon et al., 2000), malattia di Huntington (Kegel et al., 2000) e morbo di Parkinson (Stefanis et al., 2001). Anche i neurotrasmettitori possono attivare una via di segnalazione che si interseca con quella che regola l'autofagia. La dopamina, ad esempio, può elevare i livelli basali di autofagia e quindi avere un ruolo in molte malattie neurodegenerative (Petersen et al., 2001; Gomez-Santos et al., 2003). Per contro, non è stato finora riportato un ruolo attivo protettivo dell'autofagia nella neurodegenerazione. L'attivazione dell'autofagia potrebbe facilitare lo smaltimento di aggregati proteici intracellulari, caratteristica fondamentale delle malattie neurodegenerative (Larsen e Sulzer, 2002; Jellinger e Stadelmann, 2000). In numerose malattie neurodegenerative si riscontra sia la presenza di aggregati proteici intracellulari e sia alterazioni dell'attività del sistema proteolitico. Sebbene la proteina mutata sia diversa in ognuna di queste malattie, la sequenza di eventi che porta all'accumulo di aggregati proteici è generalmente la stessa. In primo luogo (I), la conformazione anomala della proteina in questione espone siti idrofobici che sono normalmente nascosti; (II) la cellula risponde a questa proteina anomala attivando il sistema delle chaperones (per promuore il ritorno alla conformazione proteica normale) e attivando le proteasi citosoliche (per rimuovere la proteina danneggiata); (III) nelle fasi iniziali della malattia, talora, le chaperone e le proteasi riescono a riportare le cellula alla normalità. Quando i livelli di proteina alterata aumentano le proteine alterate sono intrappolate in aggregati e il processo diventa irreversibile (Michalik e Van Broeckhoven, 2003). Le proteine diventano così resistenti alla degradazione da parte di proteasi citosoliche e l'unica via possibile è l'attivazione dell'autofagia per la degradazione di aggregati neoformati (Webb et al., 2003; Ravikumar et al., 2002). Se la deposizione di aggregati aumenta e il sistema autofagico non riesce a degradare il materiale neoformato, l'attivazione dell'autofagia potrebbe portare alla morte cellulare programmata di tipo II (autofagica).

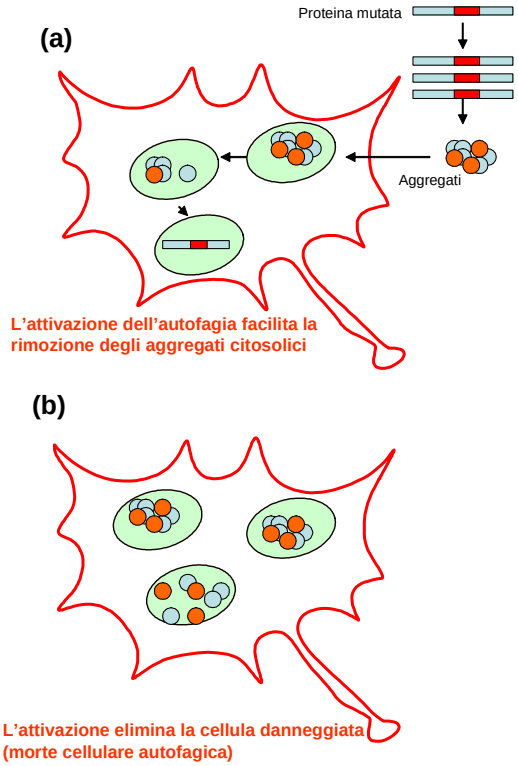

Figura 11. **L'autofagia nella progressione delle malattie neurodegenerative.** L'accumulo citosolico di proteine malconformazionate è comune a molte malattie neurodegenerative. Queste proteine mutate espongono regioni idrofobiche (rosso), che promuovono l'associazione con altre molecole anche di origine diversa. Successivamente, vengono reclutate altre molecole fino a formare aggregati di grosse dimensioni. (a) L' attivazione dell'autofagia nelle prime fasi della malattia potrebbe degradare gli aggregati, prevenendo o almeno rallentando l'accumulo. (b) Durante la progressione della malattia, i materiali indigeriti accumulano all'interno di vescicole autofagiche, l'autofagia è attivata, gli aggregati sono sequestrati all'interno le vescicole autofagiche e il materiale degradato.

In questo modo si cerca di prevenire o rallentare la formazione degli aggregati proteici facilitandone la degradazione. DiFiglia e collaboratori hanno recentemente dimostrato che nella malattia di Huntington la degradazione autofagica di piccoli frammenti della proteina mutata previene la formazione di aggregati di grosse dimensioni (Qin et al., 2003); l'attivazione dell'autofagia nelle prime fasi della neurodegenerazione potrebbe quindi avere un potenziale ruolo terapeutico.

Un secondo caso in cui si osserva un ruolo protettivo dell'autofagia nei neuroni è rappresentato dal danno assonale (Matthews e Raisman, 1972). Quando gli assoni sono disconnessi dal loro corpo cellulare, l'autofagia è attivata (Matthews e Raisman, 1972), facilitando la rimozione di organuli danneggiati e di vescicole secretorie che non possono più essere trasportate lungo i terminali sinaptici. L'autofagia contribuisce,

anche, alla rigenerazione assonale procurando all'area danneggiata, i componenti energetici (attraverso la degradazione di macromolecole intracellulari nei loro componenti essenziali) e membrane (attraverso la fusione diretta di vescicole autofagiche con la plasmamembrana) (Matthews e Raisman, 1972).

4.3 LA PROTEOLISI LISOSOMICA E L'EPILESSIA MIOCLONICA

L'epilessia mioclonica progressiva di Unverricht-Lundborg (EPM1) è una malattia neurodegenerativa ereditaria recessiva che si manifesta in età pediatrica ed è caratterizzata da stimolo mioclonico, atassia e lento declino nelle funzioni cognitive. L'EPM1 è causata da mutazioni funzionali nel gene della cistatina B (CSTB), un inibitore di cisteino protesi della famiglia delle catepsine lisosomiche (Pennacchio et al., 1996; Pennacchio et al., 1998; Alakurtti et al., 2005; Houseweart et al., 2003). Gli studi suddetti suggeriscono un ruolo diretto della proteolisi lisosomica nella patogenesi dell' EPM1, come osservato per altre malattie neurodegenerative. Sono stati mostrati ridotti livelli di acido 5-idrossilinoleiacetico (5-HIAA) nel fluido cerebrospinale di pazienti affetti da EMP1, indicando un alterato metabolismo della serotonina in questi pazienti (Leino et al., 980; Airaksinen et al., 1982.). In accordo con i dati finori riportati, la somministrazione di L-triptofano, un precursore della serotonina, da solo o in combinazione con carbidopa (l'inibitore della DOPA decarbossilasi periferica), determina un miglioramento nel movimento e nelle condizioni generali del paziente affetto da EPM1 (Koskiniemi et al., 1980; Leino et al., 1981; Pranzatelli et al., 1995). Dati preliminari di D'Amato e collaboratori (Arbatova et al., 2005) indicano un coinvolgimento del 3-idrossichinureina, un derivato del metabolismo del triptofano che è tossico per le cellule nervose nella patogenesi dell' EPM1. E' noto che l'enzima che metabolisma il triptofano, "la triptofano ossigenasi", viene degradato nei lisosomi (Kopitz et al., 1990), e che lo stesso triptofano è un inibitore dell'autofagia (Seglen et al., 1980). Il legame molecolare tra il metabolismo del triptofano e la funzionalità del compartimento lisosomico che porta alla morte cellulare di cellule nervose, non è noto.

4.4 IL COMPARTIMENTO ENDOSOMICO LISOSOMICO E LE MALATTIE CEROIDI LIPOFUSCINOSI

Le malattie ceroidi lipofuscinosi (NCL, malattia neurodegenerativa di Batten) comprendono un gruppo di otto o più malattie ereditarie da accumulo lisosomico. Hanno una frequenza di 1 su 12500 nati. Sono caratterizzate da cecità, demenza e accumulo di pigmenti autofluorescenti nelle cellule neuronali e in altri tipi cellulari (Goebel, 1995). Sono state classificate sulla base dell'età di comparsa dei sintomi, delle caratteristiche fenotipiche cliniche e ultrastrutturali del materiale accumulato. Puo' trattarsi di depositi granulari osmiofilici e corpi curvilinei.

Sulla base della comparsa dei sintomi, dei geni coinvolti e della differenziazione ultrastrutturale del materiale accumulato, le malattie ceroidi lipofuscinosi vengono classificate in quattro gruppi: a) forma infantile (INCL), forma tardo infantile (LINCL), forma giovanile (JNCL) e forma adulta (ANCL) (Goebel, 1995; Santavuori, 1974). Finora, non è stata trovata una terapia efficace per la cura di questa patologia. La diagnosi si basa sull'esame al microscopio elettronico del materiale accumulato, su analisi genetiche o enzimatiche. L'analisi genetica ha permesso di identificare due geni implicati nella patologia codificanti due noti enzimi lisosomici: la proteina tioesterasi palmitoil e la proteinasi lisosomica insensibile alla pepstatina. Il primo è responsabile della forma INCL e il secondo della forma LINCL.

Ceroido lipofuscinosi neuronale infantile (INCL)

L'INCL è causata da mutazioni del gene palmitoil tioesterasi 1 (PPT1) posto sul cromosoma 1p32 (Vesa et al., 1995). L'enzima PPT1 è stato, inizialmente, identificato come una lipasi citosolica (Camp e Hofmamm, 1993), ma, studi successivi hanno confermato la sua localizzazione nei compartimenti lisosomici (Hellsten et al., 1996; Verkruyse e Hofmann, 1996). Altri ricercatori, utilizzando la metodica del frazionamento subcellulare, hanno evidenziato la presenza del PPT1 anche in altri compartimenti cellulari e nel citosol. E' stato riportao che l'enzima ha attività enzimatica a diversi range di pH, pH 4-5 (Cho e Dawson, 1998); pH 7 (Waliany et al., 1994) e ciò ha fatto presupporre un'azione da parte di questo enzima anche in compartimenti non lisosomici. Studi piu' recenti hanno poi dimostrato che il diverso pH di azione corrisponde ad una diversa specificità di substrato (Cho et al., 1999).

Non è chiaramente noto il meccanismo per cui una mancanza dell'enzima PPT nella INCL porterebbe alla morte neuronale precoce. Il materiale accumulato nella INCL è

molto simile ai pigmenti invecchiati che normalmente vengono accumulati nel cervello in età molto avanzata (Goebel, 1995). E' stato riportato che l'iperespressione di PPT1 protegge le cellule nervose dalla morte apoptotica e il trattamento con inibitori del PPT1 aumenta l'apoptosi indotta farmacologicamente (Cho et al., 2000; Cho e Dawson, 2000). Verosimilmente, e' possibile che la mancanza di PPT1, descritta nella INCL, comprometta la traduzione del segnale di sopravvivenza cellulare e resulti, quindi, in una morte cellulare precoce.

Ceroido lipofuscinosi neuronale tardo-infantile (LINCL)

La causa della LINCL fu ritrovata nel gene codificante la proteinasi lisosomiale insensibile alla pepstatina (LPIP) (Sleat et al., 1997). Finora, sono state riportate 25 mutazioni in questo gene (Sleat et al., 1999). I corpi cellulari curvilinei che vengono accumulati nella LINCL sono formati, prevalentemente, da proteine e parzialmente da materiale lipidico. Studi sulle sequenze aminoacidiche e immunocitochimici hanno mostrato che si tratta di accumuli formati da un'unica proteina, la subunità c della ATP sintetasi mitocondriale (Palmer et al., 1989). Per caratterizzare la proteasi responsabile della degradazione della subunità c, Ezaki e collaboratori (1996), hanno dimostrato che il trattamento di cellule normali con gli inibitori pepstatina ed E64 determinava l'accumulo della subunità c. Per contro, essi mostrarono nessuna significativa differenza tra le classi di aspartico proteasi quali catepsina B, L, D o H (Dawson e Glaser, 1988; Ezaki et al., 1996; Vines e Warburton, 1999). Dawson e Glaser (1988) hanno anche dimostrato che l'attività di catepsina B era diminuita in fibroblasti LINCL invecchiati. Per esempio, il 4-idrossinonenale, che accumula nei tessuti NCL, era in grado di inibire catepsina B. Sebbene l'inibizione della degradazione all'interno dei lisosomi, della subunità c da parte di pepstatina (Ezaki et al., 1996) non sia supportata dal fatto che la proteina CLN2 è insensibile alla pepstatina (Sleat et al., 1997), è stato osservato che gli estratti lisosomici normali, se depleti della proteina CLN2, mostrano una ridotta capacità a degradare la subunità c comparati alla frazione di controllo (Ezaki et al., 1999).

Ceroido lipofuscinosi neuronale giovnile (JNCL)

Il gene difettivo in questa forma di lipofuscinosi giovanile è localizzato sul cromosoma 16p12,48. Il gene codifica per una proteina di 438 aminoacidi del peso molecolare di 48kDa. Contiene diverse sequenze aminoacidiche idrofobiche e cio' è

compatibile con la sua struttura transmembrana e puo' avere una funzione nel trasporto e, nella fusione degli organuli, nel controllo del pH o nella localizzazione degli organuli stessi. Le proteine lisosomiche di nuova sintesi subiscono modificazioni posttraduzionali con aggiunta di un gruppo mannosio-6-fosfato che è riconosciuto da un recettore specifico per il trasporto nei compartimenti endosomici-lisosomici. Il pH acido dei lisosomici garantisce la dissociazione ligando-recettore e la rimozione del mannosio-6-fosfato. Nei cervelli dei pazienti JNCL, i livelli delle glicoproteine sono significativamente elevati se confrontati con i cervelli di controllo (Sleat et al., 1998).

I lisosomi degradano proteine trasportate in questi compartimenti attraverso l'autofagia e attraverso la via di degradazione associata a proteine di membrana (LAMPs) (Cuervo e Dice, 1996). Non sono note mutazioni a carico delle proteine LAMP, nella malattia di Batten. Cellule CHO trasfettate con LAMP2 mostrano un'aumentata proteolisi (Cuervo e Dice, 1996). In uno studio recente, è stata riportata una severa perdita di cellule neuronali nell'area corticale e nell'ippocampo e accumulo di materiale proteico di pecore con una forma congenita di NCL ovina (Tyynela et al., 1997). Nel cervello di queste pecore non si riscontrava attività enzimatica di catepsina D, mentre altri enzimi lisosomici erano normali. Cio' supporta l'idea che nelle malattie ceroido lipofuscinosi la causa della neurodegenerazione sia un'alterata degradazione proteica da parte di catepsina D.

4.4 LE MALATTIE PRIONICHE

Le malattie prioniche sia umane che animali sono un gruppo di malattie strettamente correlate con i disordini neurodegenerative fatali come la scrapie ovina, BSE (bovine spongiform encephalopathy) e la CJD (Creutzfeltd-Jacob disesae), kuru e GSS (Gerstmann-Straussler-Scheinker sindrome) e FFI (fatal familial insomnia) (Collins, 2000). Le malattie prioniche umane hanno tre caratteristiche comuni: sono ereditarie, acquisite in seguito all'esposizione con materiale infettato, o acquisite sporadicamente. Sono caratterizzate dall'accumulo di una proteina endogena (PrP) con una conformazione errata (Jackson e Collinge, 2001). L'evento principale della malattia è la conversione della proteina prionica normale PrPc, in una forma non degradabile, PrPsc (Prusiner, 1998). La proteina PrP è modificata attraverso la formazione di legami disolfuro tra i residui 179 e 214 (Stahl e Prusiner, 1991), la

glicosilazione sull'Asp181 e sull'Asp197 (Rudd et al., 2001), e la successiva rimozione di 22 aminoacidi al carbossi-teminale, l'aggiunta di un gruppo GPI (glicosilfosfatidilinositolo) al carbossiterminale (Stahl et al., 1987). La proteina PrP cellulare è ancorata alla plasmamembrana attraverso il gruppo GPI ed è localizzata alle vescicole lipidiche ricche di colesterolo (Vey et al., 1996). La coda GPI puo' venire idrolizzata determinando il rilascio della proteina dalla superficie cellulare (Caughey e Raymond, 1991). Numerosi studi hanno cercato di chiarire i meccanismi molecolari della neurodegenerazione causata dall'infezione con agenti prionici ma purtroppo rimangono tuttora sconosciuti. Rimane, tuttora, non noto il ruolo delle modificazioni post-traduzionali della PrP. E' stato, recentemente, osservato un ruolo fondamentale della coda di GPI nello sviluppo della malattia (Chesebro et al., 2005). Le cellule che mancano della forma PrPc ancorata a GPI appaiono immuni agli effetti citotossici della PrPsc. Lewis e collaboratori (2006) hanno utilizzato catepsina D per rimuovere una breve sequenza al C-terminale dalla PrPsc associata all'infezione prionica.

5. STRESS OSSIDATIVO, MORTE CELLULARE E AUTOFAGIA

5.1 STRESS OSSIDATIVO E DANNO NEURONALE

Tra i molti fattori che causano danno neuronale possiamo certamente includere lo stress ossidativo. L'elevato utilizzo di ossigeno e l'elevata attività mitocondriale dei neuroni rende, infatti, queste cellule particolarmente sensibili allo stress ossidativo mediato dai radicali liberi dell'ossigeno (ROS). Lo stress ossidativo è considerato uno dei principali mediatori del declino delle funzioni cellulari che si osserva progressivamente durante l'invecchiamento.

Alcuni studi, negli ultimi decenni, hanno dimostrato un ruolo diretto dello stress ossidativo nelle alterazioni fisiologiche che si osservano in vari organi e tessuti, tra cui il sistema nervoso centrale (SNC) (Beckman and Ames, 1998). Lo stress ossidativo determina, infatti, l'aumento, all'interno di una cellula o di un tessuto, dei livelli di proteine, lipidi e acidi nucleici ossidati. In seguito all'esposizione con specie radicaliche dell'ossigeno (ROS) le proteine possono subire numerose modificazioni post-trascrizionali tra cui: ossidazione degli aminoacidi, racemizzazione e deaminazione dei residui di acido aspartico e/o di asparagina, e ossidazione dei gruppi sulfidrilici

(Agarwal and Sohal, 1994; Davies, 1987; Sohal et al., 2002; Stadtman e Levine, 2000). L'ossidazione proteica può causare l'inattivazione di alcuni enzimi, con un effetto che si ripercuote a livello di diverse funzioni cellulari incluse: la sintesi proteica, la produzione di energia, l'organizzazione citoscheletrica e la trasmissione di segnali intracellulari (Agarwal and Sohal, 1994; Squier, 2001; Standtman and Levine, 2000). Oltre a questi effetti, le modificazioni ossidative delle proteine potrebbero contribuire alla formazione di aggregati intracellulari insolubili dannosi per la cellula (Agarwal and Sohal, 1994; Squier, 2001; Standtman and Levine, 2000). La formazione di questi aggregati è favorita dal fatto che l'ossidazione delle proteine compromette il naturale ripiegamento terziario e aumenta l'idrofobicità delle proteine stesse. Queste alterazioni favoriscono le interazioni, non specifiche, proteina-proteina che a loro volta promuovono la formazione di aggregrati proteici (Agarwal and Sohal, 1994; Squier, 2001; Standtman and Levine, 2000). Gli aggregati proteici possono influire sull'omeostasi cellulare sia alterando il traffico vescicolare (Butterfield e Kanski, 2001) sia inducendo il rilascio di segnali di stress all'interno della cellula (Bednarsky e Lynch, 1996; Cuervo e Dice, 2000; Davies, 1987).

Nei neuroni la presenza di aggregati potrebbe essere una delle cause della perdita della funzionalità neuronale che si osserva a livello del SNC durante l'invecchiamento e le malattie neurodegenerative. Un esempio tipico è rappresentato dall'accumulo intracellulare di lipofuscine nei neuroni durante l'invecchiamento (Yin e Yuan, 1995; Yin, 1996; Nilsson e Yin, 1997). Le lipofuscine sono aggregati composti principalmente da proteine, lipidi e alcune tracce di carboidrati e metalli (Brunk e Terman, 2002; Szweda et al., 2003) che spesso contengono anche un pigmento chiamato ceroide (Brunk e Terman, 2002; Szweda et al., 2003). Il complesso lipofuscine-ceroide non è degradabile, per la presenza di legami crociati. E' probabile che la formazione delle lipofuscine abbia inizio a livello dei lisosomi dove la degradazione di macromolecole ricche di Fe (citocromi mitocondriali, ferritina, etc.) (Brunk e Terman, 2002) favorisce il rilascio di Fe e la sua possibile interazione con perossido d'idrogeno (H_2O_2). L'interazione Fe e H_2O_2 attraverso la reazione di Fenton porta alla formazione di specie radicaliche attive che sono responsabili dell'ossidazione di lipidi e proteine all'interno della cellula.

Nel metabolismo la riduzione dell'ossigeno molecolare avviene per trasferimento ogni volta di un solo elettrone, perciò può essere accompagnata dalla produzione di radicali liberi intermedi (O^-_2, H_2O_2, OH^-). La produzione enzimatica e

non enzimatica dei ROS può avvenire sia fisiologicamente che in corso di particolari situazioni fisiopatologiche (iperossigenazione di un tessuto, riperfusione, processi infiammatori, esposizione a radiazioni ionizzanti). In condizioni fisiologiche la produzione di radicali liberi è contrastata dalla presenza di sistemi difensivi. In queste situazioni l'anione superossido è convertito per opera della superossido dismutasi a perossido d'idrogeno. Il perossido d'idrogeno è, a sua volta, ridotto ad H_2O dalla glutatione perossidasi o a O_2 e H_2O dalla catalasi. La neutralizzazione del perossido d'idrogeno è importante perché l'interazione del perossido d'idrogeno con metalli di transizione come il Fe e il Cu porta alla formazione di radicali idrossilici particolarmente reattivi (reazione di Haber-Weiss).
Lo stress ossidativo, dunque, compare quando vi è uno squilibrio tra la produzione di radicali liberi e la capacità della cellula di difendersi contro questi. Lo stress ossidativo incorre, quindi, quando la formazione di radicali aumenta e/o quando vengono a meno i sistemi di riparo.

I ROS possono indurre alterazioni a livello dei lipidi, delle proteine e degli acidi nucleici. Il danno ossidativo ai lipidi esita nella perossidazione lipidica che determina la progressiva perdita della fluidità di membrana, la riduzione del potenziale di membrana e l'incremento della permeabilità a ioni come il Ca^{2+}. Le modificazioni a livello degli amino gruppi operato dai ROS può indurre l'inattivazione delle proteine (Davies, 1987). I ROS possono determinare alterazioni anche a livello del DNA e dell'RNA (Brawn e Fridovich 1981). La superossido dismutasi, le catalasi, la glutatione perossidasi, la vitamina E e altri antiossidanti riducono ma non prevengono i danni macromolecolari indotti dai ROS (rottura della doppia elica del DNA, formazione di legami crociati proteina-DNA e proteine-proteine, frammentazione proteica e decomposizione dei lipidi con formazione di idroperossidi, endoperossidi ciclici e aldeidi).

5.2 SRESS OSSIDATIVO E INVECCHIAMENTO NEURONALE

Tra gli organi e i tessuti, il sistema nervoso centrale è il più vulnerabile allo stress ossidativo. Questo può essere spiegato tenendo conto di alcune caratteristiche del SNC che contribuiscono a incrementare i livelli dei ROS intracellulari: l'elevato metabolismo, l'alto contenuto di metalli reattivi e l'azione pro-ossidante di molti neurotrasmettitori. Inoltre, nel SNC si ha un'elevata concentrazione di lipidi, soprattutto acidi grassi polinsaturi, che in seguito all'esposizione ai ROS possono

andare incontro a perossidazione lipidica generando prodotti di perossidazione lipidica cosiddetti “LPPs”. Una volta formati, gli LPPs, possono rapidamente interagire con le proteine provocandone l’ossidazione (Butterfield e Kanski, 2001). Si tenga poi presente che nell’invecchiamento la capacità antiossidante, a livello del SNC, diminuisce. L’aumento dell’ossidazione proteica, nell’invecchiamento, varia a seconda del tipo cellulare (Grune et al., 2004). Le cellule post-mitotiche presentano, ad esempio, livelli più alti di proteine ossidate rispetto alle cellule mitotiche. Questo potrebbe essere dovuto al fatto che le cellule mitotiche, durante ogni processo di divisione, sintetizzano nuove macromolecole. Come risultato le macromolecole intracellulari delle cellule mitotiche saranno esposte per un periodo limitato ai ROS. Alcune aree del sistema nervoso centrale sono costituite, quasi esclusivamente, da cellule post-mitotiche e ciò rende queste zone particolarmente vulnerabili allo stress ossidativo. Secondo alcuni studi, con l’invecchiamento, il 40% delle proteine totali risulta ossidato (Stadtman e Berlett, 1997; Stadtman e Levine, 2000). Gli elevati livelli di proteine ossidate potrebbe essere dunque una delle cause principali del declino osservato a livello neuronale, durante l’invecchiamento. La presenza di aggregati proteici, infatti, sembra interferire con la capacità dei neuroni di formare e mantenere le sinapsi e con il mantenimento dell’integrità del citoscheletro e delle normali vie di trasmissione dei segnali intracellulari.

Un altro aspetto importante dell’invecchiamento neuronale è l’accumulo di lipofuscine. Alcuni studi hanno dimostrato che circa il 75% dei neuroni, in persone anziane, presentano un accumulo di lipofuscine (Brunk e Terman, 2002; Szweda et al., 2003). Recentemente, è stato dimostrato che l’accumulo di lipofuscine-ceroide potrebbe direttamente inibire l’attività del proteosoma (Giasson et al., 2000) e interferire con la capacità del lisosoma di degradare i mitocondri (Brunk e Terman, 2002). Le lipofuscine, quindi, potrebbero promuovere la produzione dei ROS favorita dall’accumulo di mitocondri danneggiati e favorire l’accumulo di altri aggregati cellulari (Tau, α-sinucleina). Oltre ai ROS anche l’ossido nitrico (NO), se presente ad alte concentrazioni, può avere un effetto neurotossico. L’NO è una piccola molecola bioattiva, generata dall’attivazione dell’enzima NO-sintetasi, che a livello del sistema nervoso gioca un ruolo importante nel rilascio dei neurotrasmettitori, nello sviluppo neuronale, nella plasticità sinaptica e nella regolazione dell’espressione genica (Dawson e Dawson, 1998). Un eccessiva produzione di NO, come conseguenza dell’induzione della NO-sintetasi indotta nelle cellule della microglia una volta attivate, pare associata

alla neurodegenerazione (Liu et al., 2002). L'NO ad alte concentrazioni può interagire con l'anione superossido formando perossinitriti, molecole altamente reattive che possono danneggiare proteine, DNA e RNA (Vaananen et al., 2005; Cover et al., 2005); e puo' stimolare un eccessivo rilascio di glutammato (Brown e Bal-Price, 2003).

5.3 MORTE NEURONALE E H_2O_2

Sebbene il perossido d'idrogeno non sia particolarmente reattivo nei confronti delle componenti cellulari esso è in grado d'indurre la morte cellulare di neuroni corticali (Whittemore et al., 1994). La tossicità dell' H_2O_2 potrebbe dipendere dalla formazione di radicali idrossilici reattivi. Il perossido d'idrogeno potrebbe indurre la morte apoptotica dei neuroni attraverso il reclutamento di molecole pro-apoptotiche (Wei et al., 2000) come le caspasi. Il perossido d'idrogeno esogeno può incrementare l'accumulo intracellulare di H_2O_2 e indurre l'attivazione delle MAP-kinasi (Ruffels et al., 2004). Le MAP-K rappresentano un gruppo eterogeneo di proteine coinvolte in diversi vie di trasduzione del segnale: ERK regola la crescita e il differenziamento cellulare mentre Jun-N-terminal-kinasi (JNK) e la p38 MAP-kinasi intervengono principalmente nella morte cellulare. A basse concentrazioni l'H_2O_2 induce la proliferazione cellulare attraverso l'attivazione di ERK e della via di PI3-K/Akt mentre a più alte concentrazioni sembra determinare un'arresto del ciclo cellulare e successiva morte per l'attivazione di JNK e della p38MAP-K (Sang-Oh Yoon et al., 2002).

Il selenio, un antiossidante, blocca l'apoptosi indotta dall' H_2O_2 favorendo l'attivazione di Akt e inibendo l'attivazione dei geni di morte (Sang-Oh Yoon et al., 2002).

Il perossido d'idrogeno, inoltre, può ossidare e modulare direttamente canali, pompe e scambiatori del Ca^{2+} . L'ossidazione delle pompa Ca-ATPasi, ad esempio, ne inibisce la funzione con un aumento dei livelli citosolici di Ca^{2+} (Rohn et al., 1993), a questo può seguire l'innesco del processo apoptotico (Poern-Ares 1998).

Il perossido d'idrogeno media la morte cellulare in diverse linee cellulari neuronali (Ishihara et al., 2000; Jiang et al., 2001; Wei et al., 2000).

L' H_2O_2 induce, modificazioni nucleari (ingrossamento dei pori nucleari, frammentazione, addensamento della cromatina) tipiche del processo apoptotico e rigonfiamento di organelli quali mitocondri e reticolo endoplasmatico (marcatori della necrosi) (Lim et al., 2002). Questi dati suggeriscono che le cellule neuronali trattate con perossido d'idrogeno potrebbero presentare sia caratteri tipici dell'apoptosi che della

necrosi. L'insorgenza della morte apoptotica o necrotica dipende sia dal tempo che dalla concentrazione dei ROS (Manev et al., 1995; Cole e Pere-Polo, 2002).

Recentemente alcuni studi hanno mostrato che lo stress ossidativo indotto da H_2O_2 causa la depolimerizzazione della membrana mitocondriale e la formazione di pori a cui segue la traslocazione del citocromo c dai mitocondri al citoplasma (Takeyama et al., 2002).

Sia la caspasi 3 che la caspasi 8 sembrano essere attivate nella morte cellulare indotta dal perossido (Zhuang et al., 2000; Kirkland e Franklin, 2002).

L'inibizione della caspasi 3 ma non della caspasi 6 sembra essere in grado d'inibire la morte apoptotica, in cellule di feocromocitoma PC12, indotta dal perossido d'idrogeno (Yamakawa et al., 2000).

Gli effetti intracellulari dell' H_2O_2 esogeno sono simili a quelli osservati nella morte cellulare indotta da ceramide nelle cellule PC12 (Muriel et al., 2000).

La ceramide stimola l'espressione di fattori di trascrizione, come l'NFkB, che intervengono nell'induzione del processo apoptotico (Muriel et al., 2000).

La pergolide, un agonista della dopamina, utilizzata nel trattamento delle prime fasi del Parkinson (Barone et al., 1999) protegge "*in vitro*" cellule di neuroblastoma dalla morte cellulare indotta dall' H_2O_2 (Uberti et al., 2002).

Anche i flavonoidi, componenti polifenoliche presenti in vari di tipi di frutta e verdura, sembrano proteggere i neuroni contro la morte cellulare indotta dal H_2O_2 (Sam Sik Kang et al., 2004).

INTRODUCTION

NEURONAL CELL DEATH AND NEURODEGENERATION

Neurodegeneration and neuronal cell death underlie the symptoms of many neurological diseases (Mattson, 2000). The best-understood mechanism of neuronal cell death, classically defined as “apoptosis,” is mediated via caspase 9 activation and a mitochondrial route, or directly via caspase 8 to the primary effector, caspase 3 (Yuan et al., 2003; Lockshin e Zakeri, 2002). Programmed cell death is an essential process for proper neural development. Cell death, with its similar regulatory and executory mechanisms, also contributes to the origin or progression of many or even all neurodegenerative diseases. An understanding of the mechanisms that regulate cell death during neural development may provide new targets and tools to prevent neurodegeneration.

Apoptosis represents a highly regulated suicide death program. The term was first introduced to characterize the morphological features of a specific type of programmed cell death (PCD) in mammalian system: cell shrinkage, nuclear condensation, chromatin margination, and formation of apoptotic bodies. There is no presence of an inflammatory response . The pathways of apoptosis have been elucidated in large part in several studies. The apoptotic pathway may differ depending on the cell type and death stimulus, morphological features of apoptosis does not always correlate with its biochemical features, activation of the biochemical features of apoptosis does not always lead to cell death. Caspases are cysteine proteases that mediate apoptotic death in a variety of cellular systems, including neurons. Caspases are activated through extrinsic or intrinsic pathways. The latter is used by most neurons in most situations. In this pathway, release of mitochondrial cytochrome c into the cytoplasm induces formation of the apoptosome, which leads to the activation of caspase 9 and subsequently other caspases. Caspase-dependent apoptosis is now viewed as only one of various possible pathways that a cell may use to undergo cell death.

LYSOSOMES, AUTOPHAGY AND CELL DEATH

Lysosomes were first described in 1955 by De Duve and his collaborators as organelles enriched with acidic hydrolases and potentially harmful for the cell (De Duve, 1983). Lysosomes were considered to be 'suicide bags' which in case of accidental rupture would cause complete dissolution of all cellular constituents. Regarding its physiological role, the lysosome was for long time considered simply as the 'waste-disposal' compartment of the cell (De Duve, 1983). This view has been challenged with the discovery of other related acid organelles and the finding that lysosomes are dynamic organelles that move along the cytoskeleton and interact with other cellular compartments. It is now well documented that in all eukaryotic cells the acid vacuolar compartments take part in various cellular processes such as partial or extensive degradation of various substrates, trafficking and recycling of molecules among internal organelles to and from the exterior of the cell, post-translational maturation of secretory products (so called 'crinophagy') and storage of undigested material. While some of these functions are prominent or exclusive in certain specialised cells (e.g., crinophagy in endocrine cells and phagolysosome formation in phagocytic cells), there is no doubt that the main function of lysosomes in all cells is to degrade internalised material. In cells of the immune system, acid compartments perform additional specialised functions such as the processing and presentation of exogenous antigens (Riese and Chapman, 2000; Pathak and Blum, 2000) and they constitute the secretory granules of cytotoxic lymphocytes (Stinchombe and Griffiths, 1999) and of mastocytes (Dragonetti et al., 2000). Interestingly, the death-inducing protein Fas ligand is sitting on the membrane of cytotoxic granules of lymphocytes (Bossi and Griffiths, 1999). This is an efficient way to store preformed Fas L which can be promptly inserted in the plasma membrane upon degranulation, thus providing a killing signal for Fas expressing target cells, in addition to the well-known action of perforin and granzymes (Bossi and Griffiths, 1999). As expected, the lysosomal apparatus is particularly extended and active in professional immune cells devoted to phagocytosis (Tapper et al., 2002). Further roles of lysosomes or lysosome-related organelles include the resealing of the plasma membrane following calcium-dependent exocytosis (Jaiswal et al., 2002) and the enlargement of the membrane surface (Borgonovo et al., 2002).

The acid vacuolar system is composed of membrane-sealed organelles distributed throughout the cytoplasm and connected via carrier vesicles with the exterior

of the cell as well as with the endoplasmic reticulum, the Golgi complex and secretory vesicles. At least three distinct stages can be recognised: early endosomes, with an intralumenal pH of 6.0-6.5, late endosomes, with a more acidic intralumenal pH (the value is about 5.0) and lysosomes with the most acid compartment (the pH is below 4.5). Early and late endosomal compartments are pleiomorphic and constitute a dynamic network of tubular and vesicular elements, while lysosomes are circular organelles of about 0.5 μm diameter. The early endosome represents the first stage to which plasma membrane-derived endocytic vesicles deliver their content (Gu and Gruenberg, 1999). Both early and late endosomes receive from the Trans Golgi Network (TGN) newly-synthesised lysosomal acid hydrolases via mannose-6-phosphate receptor (MPR)-mediated or MPR-independent transport (Kornfeld and Mellman, 1989). In these organelles receptors are sorted and recycled back to the plasma membrane or the TGN after acid-dependent dissociation of their ligands (Kornfeld and Mellman, 1989; Mellman, 1996). Final transport from late endosome to lysosome may occur by means of carrier vesicles (though no evidence for such a pathway has been provided as yet) or by transient and limited fusion between the two organelles (the so called 'kiss and run' hypothesis) or via fusion of the two organelles with formation of a transient hybrid organelle from which lysosome and (a smaller) endosome are re-formed (Luzio et al., 2000). Membrane traffic within the endocytic and exocytic pathways is regulated by several transducers, such as Protein and Lipid Kinases (Cardone et al., 1994; Chiarpotto et al., 1999; Brown et al., 1995), mono- and heterotrimeric-Gproteins (Riederer et al., 1994; Feng et al., 1995; Nuoffer and Balch, 1994), calcium ions (Togo et al., 1999; Pryor et al., 2000; Dragonetti et al., 2000), and it is specified by interaction of paired proteins on vesicle and target organelles (the so called 'SNARE hypothesis') (Rothman, 1994; Hay and Scheller 1997). Early endosomes, late endosomes and lysosomes can be discriminated on the basis of the relative presence or absence of integral or membrane-associated proteins, such as for instance EEA1, LAMP1, LAMP2, MPR300, MPR46 and members of the rab family (Karlsson and Carlsson, 1998; Tikkanen et al., 1997; Meresse et al., 1995; Plitz and Pfeffer, 2001; Novick and Zerial, 1997). These organelles also differ in their hydrolase content and, most probably in their functions. For instance, while in endosomes cathepsins mainly effect a limited proteolysis, which eventually results in activation of inactive precursors, in lysosomes these proteases accomplish extensive degradation of the proteins they contain (Berg et al., 1995).

Cellular homeostasis implies that in quiescent cells synthetic and catabolic rates are balanced to insure control of cell mass. Although in principle this holds for all classes of biomolecules present in the cell, it is particularly evident for lipids and proteins, as these classes of molecules are by far the most abundant ones. Moreover, turnover of lipids and proteins occurs constantly in the cell for renewal of membranes and enzymes. Normal cells show the ability to modulate the rates of macromolecular synthesis and degradation in response to stimuli and environmental conditions that influence cell growth; however this ability is lost in transformed and tumour-derived cells (Lockwood et al., 1982; Lockwood and Minassian, 1982; Knecht et al., 1984; Tessitore et al., 1987; 1988). Lysosomes, the organelles with the highest concentration of proteases and other hydrolytic enzymes inside the cell (De Duve, 1983), are primarily involved in the maintenance of cellular homeostasis by accomplishing the degradation of autologous material (Mitchener et al., 1976; Knecht et al., 1984). Lysosome-mediated protein degradation is reduced in carcinogenesis (Kisen et al., 1993) and in tumours (Bradley, 1977; Tessitore et al., 1988). Moreover, the expression and activity of lysosomal cathepsin D were found to be down-regulated in proliferating normal and in transformed cells (Lockwood and Shier, 1977; Isidoro et al., 1995). Conversely, up-regulation of the expression and activity of lysosomal cathepsins B, D and L was reported in association with enterocytic-like differentiation of human colon carcinoma cells (De Stefanis et al., 1997; Isidoro et al., 1997). Substrates to be degraded can be delivered to lysosomes by means of three pathways: microautophagy, in which small pieces of cytoplasm are directly entrapped by invagination of the lysosomal membrane (Dunn, 1994), an hsc73 chaperone-mediated mechanism, which exploits the lysosomal membrane protein LAMP2a as receptor to translocate cytosolic proteins bearing the KFERQ pentapeptide motif (Cuervo and Dice, 1998; Cuervo et al., 2003), and macroautophagy, by which entire organelles along with a portion of cytosol are disposed of (Kim and Klionsky, 2000). In all eukaryotic cells, basal autophagy (in its three forms) ensures the physiological turnover of old cytoplasmic structures, thus contributing to the homeostatic equilibrium between protein synthesis and organelle biogenesis versus protein degradation and organelle disposal (Dunn, 1994). Macroautophagy is certainly the major process for degradation of cellular constituents, its rate being enhanced under stress circumstances that produce organelle damage (Lemasters et al., 1998) or under extreme nutrients restriction, in order to recycle biomolecules for the synthesis of essential constituents (Kopitz et al., 1990; Munafo and

Colombo, 2001). The latter are examples in which autophagy elicits a protective action in favour of cell survival. In other cases, extensive autophagic degradation involving large portions of the cell is induced as part of programmed cell death, the so called 'type II' cell death (Bursch, 2001). The autophagic process starts with the entrapment of a portion of cytoplasm that may include an entire organelle by a double-membrane vesicle, generally derived from the smooth endoplasmic reticulum (Dunn, 1990; Bohley and Seglen, 1992), and probably also from the TGN (Kihara et al., 2001), to form the autophagic vacuole. The subsequent steps include docking and fusion of the autophagic vacuole with the lysosome and breakdown of the autophagic body. The autophagic process is tightly regulated at various levels (Klionski and Emr, 2000; Kim and Klionsky, 2000) and is under the control of the αi3 subunit of the heterotrimeric G protein (Ogier-Denis et al., 1995; 1996), various protein phosphatases (Holen et al., 1992), and class I and class III phosphoinositide 3-kinases (PI 3-K) (Petiot et al., 2000).

How are cell growth (increment in cellular mass) and cell proliferation (increment in cell number) coordinated ? It is conceivable that in quiescent cells the rates of synthesis and degradation are in equilibrium, while in growing and proliferating cells the rate of synthesis is likely to override that of degradation to achieve net accumulation of macromolecules (Baccino et al., 1984; Knecht et al., 1984). How is the autophagic degradative pathway down regulated in this circumstance ? One mechanism relies on the phosphorylation of the ribosomal protein S6, that functions as a signal for protein synthesis (Chou and Blenis, 1995) as well as an inhibitory signal of autophagy (Blommaart et al., 1995). The kinase cascade leading to S6 phosphorylation starts with the activation of class I PI 3-K by stimulated growth factor receptors and proceeds via PKB/AKT and mTOR (the mammalian homologue of the yeast Target of Rapamycin) (Chung et al., 1992). Therefore, under environmental conditions favourable to cell growth, TOR promotes protein synthesis and inhibits autophagic protein degradation, while under unfavourable conditions TOR is inactive and allows the upregulation of autophagy (Dennis et al., 1999). In this scheme, TOR functions as a sensor that permits the cell to adjust mass accumulation to a level appropriate to nutrient availability (Schmeizie and Hall, 2000). A crucial messenger in this pathway is the phosphoinositide 3,4,5, triphosphate (PiP3), the principal (and first) product of class I PI 3-K (Petiot et al., 2000). The level of PiP3, and consequently that of inhibition of autophagy, is physiologically lowered by the lipid phosphatase called PTEN (phosphatase and tensin homolog deleted on chromosome ten), which removes one

phosphate group to generate PiP2. Therefore, PTEN acts as a positive regulator of basal autophagy (Petiot et al., 2000). Another important positive regulator of autophagy is the Bcl 2-interacting protein Beclin 1 (Liang et al., 1999), the human homologue of the protein Vps30p/apg6p involved in autophagy and vacuole protein sorting in yeast. Intriguingly, Beclin 1 has been shown to also interact with the class III PI 3-K required for the formation of the autophagic vacuole in mammalian cells (Kihara et al., 2001). The finding that PTEN and Beclin 1 act as oncosuppressors that are mutated in as many as 50% of human carcinomas, further illustrates the strict link between autophagy and cancer (Di Cristofano and Pandolfi, 2000; Aita et al., 1999). PTEN plays a central role in regulating the signalling responsible for growth, adhesion and migration. Altered levels of PTEN expression have also been implicated in tumour-associated angiogenesis (Wen et al., 2001; Huang and Kontos, 2002). Re-expression of PTEN in PTEN-deficient tumour cells restores the susceptibility to cell death (Simpson and Parsons, 2001) and suppresses tumour cell invasion across basement membranes (Tamura et al., 1999). Similarly, it has been shown that reconstitution of high level of Beclin 1 expression in breast cancer cells results in induction of autophagy and enhanced proteolysis that are associated with inhibition of cell proliferation and reduced tumourigenesis in *nude* mice (Liang et al., 1999). These observations show that there is a link between the down-regulation of the autophagic-lysosomal system and the acquisition of a more aggressive phenotype in tumours.

Autophagic cell death is associated with up-regulation of the autophagic pathway that leads to uncontrolled digestion of self structures by lysosomal hydrolases, mainly cathepsins (Schwartz et al., 1993; Bursch et al., 2000). Endocytosis and delivery to endosomes of TNF-TNFR1 complex provide a functional link with the activation of acidic sphingomyelinase that leads to ceramide production (Wiegmann et al., 1994; Monney et al., 1998) and of lysosomal cathepsin D (Deiss et al., 1996; Démoz et al., 2002), both associated with TNFα-induced apoptosis. Also cathepsin B has been shown to mediate the death signal of TNFα (Guicciardi et al., 2000; Foghsgaard et al., 2001). In both studies, cathepsin B was shown to translocate from lysosomes to the cytosol upon treatment with TNFα. In L929 fibroblasts apoptosis by this cytokine was shown to depend on the activity of cathepsin D, although no cytosolic release of the lysosomal protease could be demonstrated by immunofuorescence (Dèmoz et al., 2002). In cells undergoing apoptosis, leakage of lysosomal cathepsins, if it occurs, must be finely controlled since there is no disruption of the lysosomal membrane, as shown by the fact

that lysosomes are still intact and able to retain the pH gradient (Foghsgaard et al., 2001; Démoz et al., 2002). On the other hand, massive leak of lysosomal hydrolases is expected to induce a necrotic type of cell death, rather than apoptosis. The proposed mechanism that links cathepsins with caspases involves the cytosolic release of cytochrome c from mitochondria via cleavage of Bid (Guicciardi et al., 2000; Stoka et al., 2001). Alternatively, it has been proposed that cathepsin L can activate procaspase 3 with the aid of an as yet unidentified lysosomal membrane-bound protease (Katunuma et al., 2001). However, in a model of lysosomal photodamage-induced apoptosis the processing of Bid was clearly operated by a lysosomal hydrolase, although inhibitors of cathepsins B, D and L were not effective (Reiners et al., 2002). It should be noted also that lysosomotropic agents (Neuzil et al., 1999; Li et al., 2000) and the synthetic retinoid CD437 (Zang et al., 2001) cause leakage of lysosomal cathepsins associated with induction of apoptosis. How this leakage occurs is not known, though it is probably favoured by the formation of free radicals in the lumen of lysosomes via a Fenton-like reaction (Roberg et al., 1999; Brunk and Svensson, 1999; Ollinger, 2000). The lysosomal-autophagic system is also probably involved in apoptosis, perhaps only in the initial steps. The following two observations support this hypothesis: Beclin 1, a positive regulator of autophagy, interacts with the antiapoptotic protein Bcl-2 (Liang et al., 1998) and some of the same signals that trigger apoptosis may also induce autophagy (Xue et al., 1999). In conclusion, in many circumstances, lysosome acts as a central organelle which determine which type of death pathway will dominate in injured cells as a result of the type, intensity and duration of the cytotoxic treatment (Ferri and Kroemer, 2001).

RISULTATI E CONCLUSIONI

1. LISOSOMI E CATEPSINE LISOSOMICHE COME POTENZIALI MARCATORI PER LA MORTE DI CELLULE TUMORALI: UNO STUDIO SPERIMENTALE SUL NEUROBLASTOMA

Nel 1979 Greenbaum e collaboratori hanno riportato che '*in vivo'* la Pepstatina A, un inibitore specifico di catepsina D, rallenta la formazione e l'accumulo di fluido in peritoneo nei topi portatori di tumore ascite (Greenbaum et al., 1979). Più recentemente, Leto e collaboratori hanno dimostrato che le sostanze E-64 and pepstatina A, inibitori rispettivamente di cisteino-proteasi lisosomiche e di catepsina D, riducono e impediscono la formazione di metastasi in topi portatori di tumori mammari e ovarici (Leto et al., 1994). Nel nostro laboratorio abbiamo dimostrato che l'inibizione delle catepsine lisosomiche conduce a morte cellule di neuroblastoma umano "*in vitro*" (Castino et al., 2002). Effetti analoghi degli inibitori delle catepsine furono riportati anche in cellule neuronali e in epatociti primari (Isahara et al., 1999). Queste osservazioni sembrerebbero in contraddizione con il presupposto ruolo delle catepsine come mediatori dell'apoptosi. A questo proposito, è bene notare che la proteolisi mostra un ruolo fondamentale nel turnover proteico così come nell'attivazione di pro-enzimi, pro-ormoni e di fattori di sopravvivenza (Berg et al., 1995). Se queste funzioni vitali sono compromesse le cellule possono andare incontro a morte. Rimane da spiegare, comunque, come l'inibizione delle proteasi lisosomiche possa determinare una morte cellulare apoptotica con coinvolgimento delle caspasi, enzimi che risiedono nel citoplasma (Castino et al., 2002). Una possibile spiegazione è che l'inibizione prolungata della proteolisi lisosomica porti all'accumulo di substrati ossidati che possono causare il "leakage" (letteralmente "gocciolamento") delle membrane lisosomiali. Questo è, probabilmente, anche il meccanismo d'azione degli agenti lisosomotropici che accumulano nei lisosomi e innalzano il pH lisosomico. Tra i farmaci antiblastici, che causano apoptosi inducendo il "leakage" dai lisosomi, ricordiamo l'inibitore C1311 della topoisomerasi II (Burger et al., 1999), il retinoide sintetico CD437 (Zang et al., 2001) e l' α-Tocopheryl succinato (Neuzil et al., 2002). Queste osservazioni rafforzano l'ipotesi del ruolo delle catepsine lisosomiche nello sviluppo di tumori e nella progressione neoplastica e incoraggiano gli studi per ricercare nuovi e più efficaci farmaci chemioterapici. Sia in "*in vivo*" che "*in vitro*" questi inibori causano inevitabilmente effetti sistemici e ciò rimane una limitazione al

loro uso nella terapia. Un obbiettivo della ricerca è quello di trovare un sistema per veicolare questi inibitori limitando gli effetti sistemici.

Il neuroblastoma è una neoplasia del nevrasse a localizzazione extracranica, altamente maligna e con elevata capacità di metastatizzazione. Il neuroblastoma rappresenta l'8-10% di tutte le neoplasie maligne solide della prima infanzia ed è secondo per frequenza ai tumori del sistema nervoso centrale. La neoplasia insorge più frequentemente nel primo e nel secondo anno di vita e comunque nei primi cinque anni di vita nella quasi totalità dei casi. L'incidenza è di 10 casi per anno per milione di bambini di età compresa tra o e 4 anni, e di 4 casi per anno per milione di bambini tra i 6 e i 9 anni.

Il neuroblastoma appartiene ad una famiglia di tumori derivati dalla trasformazione neoplastica di un precursore del sistema nervoso periferico originato da cellule migrate dalla cresta neurale (Heyes et al., 1989). Fra queste diverse neoplasie alcune si presentano con caratteristiche proprie delle cellule primitive del sistema nervoso simpatico e vengono denominate neuroblastomi; altre, a carattere più differenziato, e costituite da cellule affini agli elementi maturi del tessuto nervoso, sono denominate ganglioneuromi (Heys et al., 1989). L'eziologia del neuroblastoma è sconosciuta nella maggior parte dei casi. In alcuni casi la comparsa della neoplasia pare correlare con l'esposizione prenatale a certe sostanze, quali fenobarbital, alcool, coloranti (Kramer et al., 1987).

La chemioterapia è la principale modalità di trattamento del neuroblastoma. Sono stati individuati, a questo riguardo, diversi farmaci efficaci: ciclofosfamide, cisplatino, doxorubicina, vincristina e peptichenio (miscela di sei oligopeptidi sintetici contenenti m-L fenilalanina mostarda) (Keshelava et al., 1997).

Abbiamo analizzato il ruolo della proteolisi endosomica-lisosomica nella sopravvivenza di due linee di neuroblastoma umano (GI-LI-N e LAN-5) che presentano una diversa suscettibilità al trattamento con farmaci citotossici e citochine e hanno un diverso livello di espressione delle catepsine lisosomiche B e D. In questo modello cellulare, l'inibizione delle catepsine B e D con gli inibitori farmacologici E-64 o CA074Me (inibitori di CB) o pepstatina A (inibitore di CD) si sono rivelati citotossici per le due linee. La morte cellulare ha le caratteristiche tipiche dell'apotposi (condensazione e frammentazione della cromatina). Inoltre, l'inibizione delle caspasi protegge le cellule di neuroblastoma dalla morte cellulare indotta dall'inibizione delle

catepsine B e D. I dati riportati indicano, perciò, che l'inibizione prolungata della proteolisi lisosomica è incompatibile con la sopravvivenza cellulare.

ENGLISH VERSION

LYSOSOMES AND LYSOSOMAL CATHEPSINS AS POTENTIAL TARGETS FOR TUMOUR CELL KILLING: AN EXPERIMENTAL STUDY ON NEUROBLASTOMA CELLS

In 1979 Greenbaum and co-workers reported that the 'in vivo' administration of Pepstatin A, an inhibitor of cathepsin D, retarded the formation and the accumulation of ascitic fluid in mice bearing ascites tumours (Greenbaum et al., 1979). More recently, Leto and co-workers have reported on the beneficial effects of the administration of E-64 and pepstatin A, inhibitors of lysosomal cysteine-proteases and of cathepsin D, respectively, with regard to metastasis formation in mice bearing metastatic mammary and ovarian cancers (Leto et al., 1994). In the same line, we found that inhibition of lysosomal cathepsins was detrimental for human neuroblastoma cells, resulting in induction of apoptosis (Castino et al., 2002). Similar effects of cathepsin inhibitors were reported in neuronal cells and in primary hepatocyte cultures by other groups (Isahara et al., 1999). These latter observations seem to contradict the supposed role of cathepsins D and B as mediators of apoptosis, as outlined in previous paragraphs. However, it should be noted that lysosomal proteolysis plays a unique role in protein turnover as well as in the activation of pro-hormones and pro-survival factors (Berg et al., 1995), as revealed by studying the phenotypes of cathepsin knock-out mice (Saftig et al., 1995). If these functions are compromised the cells become frail and eventually die. It remains unexplained, however, how the inhibition of a lysosomal protease can trigger an apoptotic signal that involves cytosolic caspases (Castino et al., 2002). One possibility is that the prolonged inhibition of lysosomal proteolysis leads to the accumulation of oxidized substrates that eventually cause lysosomal leakage. This is probably also the mechanism of action of lysosomotropic agents that accumulate within lysosomes and buffer the intravacuolar pH. Among the known antiblastic drugs that cause apoptotic cell death by inducing primary leakage from lysosomes are the topoisomerase II-inhibitor C1311 (Burger et al., 1999), the synthetic retinoid CD437 (Zang et al., 2001) and the α-Tocopheryl succinate (Neuzil et al., 2002). Altogether these observations emphasize the role of lysosomal cathepsins in tumour development and progression and encourage the search for new and more effective chemotherapeutic drugs that target the lysosomal function. However, the 'in vivo' use of lysosomal cathepsin inhibitors or of lysosomotropic agents has obvious limitations because of unavoidable systemic toxic effects. A major goal of research in cancer chemotherapy, nowadays, is indeed the finding of a carrier system that would allow the delivery and site-specific uptake of the cytotoxic cargo in tumour tissue. Neuroblastoma is a particularly aggressive tumour very frequent in childhood. It has been proposed that neuronal cells are 'intrinsically' programmed to die and that they can be rescued from

such destiny only if they continuously receive survival signals from other cells. In this context, the endocytic system provides a unique pathway for neurons to internalize and metabolize extracellular nutrients and trophic factors. The essential role of such functions in the physiology of the nervous system is well illustrated by several neurodegenerative disorders (e.g., Alzheimer's disease, prion diseases, ataxia-teleangectasia) associated with abnormalities of the endosomal-lysosomal apparatus.

In the present work we analyzed the role of endosomal-lysosomal proteolysis in cell survival in two neuroblastoma cell lines (GI-LI-N and LAN-5) that differ in their susceptibility to apoptotic treatments by cytokines or antiblastic drugs and in their regulation of Cathepsins B and D expression.

Inhibition of lysosomal function by vacuolar alkalinizators such as ammonium chloride or chloroquine revealed to be cytotoxic for both neuroblastoma cell lines. This cell death showed morphological and biochemical features typical of apoptosis, including appearance of the hypodiploid peak (as assessed by cytofluorometric analysis) and participation of caspases.

We further investigated the consequences of lysosomal proteases inhibition on neuroblastoma cell survival. The treatment with E-64 or CA074-Me (two specific inhibitors of Cathepsin B) or with Pepstatin A (a specific inhibitor of Cathepsin D) revealed cytotoxicity for two neuroblastoma cell lines. Cell death was associated with condensation and fragmentation of chromatin, a hallmark of apoptosis. Concomitant inhibition of the caspase cascade protected neuroblastoma cells from cathepsin inhibitors-induced cytotoxicity.

These data indicate that prolonged inhibition of the lysosomal proteolytic pathway is incompatible with cell survival and that the cathepsin-mediated and caspase-mediated proteolytic systems are connected and cooperate in the regulation of neuronal cell death and survival. Of potential clinical relevance is that it appears possible to improve the cure of neuroblastoma by including cathepsin inhibitors in the chemotherapeutic regimen.

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LYSOSOMAL PROTEASES AS POTENTIAL TARGETS FOR THE INDUCTION OF APOPTOTIC CELL DEATH IN HUMAN NEUROBLASTOMAS

Roberta Castino, Deborah Pace, Marina Démoz, Marco Gargiulo, Chiara Ariatta, Elisabetta Raiteri and Ciro Isidoro*

Dipartimento di Scienze Mediche, Laboratorio di Patologia Molecolare, Università "A. Avogadro," Novara, Italy

Neuroblastoma is the most common type of cancer in infants. In children this tumor is particularly aggressive; despite various new therapeutic approaches, it is associated with poor prognosis. Given the importance of endosomal-lysosomal proteolysis in cellular metabolism, we hypothesized that inhibition of lysosomal protease would impact negatively on neuroblastoma cell survival. Treatment with E-64 or CA074Me (2 specific inhibitors of cathepsin B) or with pepstatin A (a specific inhibitor of cathepsin D) was cytotoxic for 2 neuroblastoma cell lines having different degrees of malignancy. Cell death was associated with condensation and fragmentation of chromatin and externalization of plasma membrane phosphatidylserine, 2 hallmarks of apoptosis. Concomitant inhibition of the caspase cascade protected neuroblastoma cells from cathepsin inhibitor-induced cytotoxicity. These data indicate that prolonged inhibition of the lysosomal proteolytic pathway is incompatible with cell survival, leading to apoptosis of neuroblastoma cells, and that the cathepsin-mediated and caspase-mediated proteolytic systems are connected and cooperate in the regulation of such an event. Since modern antitumor chemotherapy is aimed at restoring the normal rate of apoptosis in neoplastic tissues, the demonstration that endosomal-lysosomal cathepsins are involved in this process may constitute a basis for novel strategies that include cathepsin inhibitors in the therapeutic regimen.

Key words: *apoptosis; neuroblastoma; caspases; cathepsins; protease inhibitors*

Altered regulation of cell survival and death, including apoptosis, is considered an important factor in tumor development and progression, as well as in the response to antineoplastic therapy.[1,2] The molecular pathways controlling apoptosis include a complex network of intracellular proteases that act in an orderly sequence on cellular substrates and lead to characteristic modifications of cell morphology with eventual DNA cleavage and apoptotic body formation. Several proteolytic systems have been shown to participate in the apoptotic process, depending on the cell type and stimuli adopted. With few exceptions, the caspase system seems to be the most universally involved one.[3,4] Recently, proteases resident within the endosomal-lysosomal compartment (cathepsins) have also been associated with apoptosis.[5–9] These studies demonstrated the need for a cathepsin-mediated proteolytic event in the apoptotic pathway triggered by cytokines or antiblastic drugs. In addition, the active participation of the autophagic proteolytic pathway, particularly of lysosomal cathepsins B and D (CB, CD), has been envisaged in rat pheochromocytoma PC12 cell death after nutrient and serum factor deprivation. In this model of caspase-dependent apoptosis, CD acted as a death factor, whereas CB acted as a pro-survival factor.[10]

We followed an opposite approach, assuming that the endosomal-lysosomal proteolytic pathway serves crucial functions for cell viability. Indeed, experiments using cathepsin gene knockout and cathepsin inhibitors demonstrated the redundancy of proteolytic enzymes in the lysosomal apparatus so that overall protein turnover is not affected by single-cathepsin deficiency.[11,12] In neuronal cells, besides a role in intracellular protein turnover, the endosomal system provides a unique pathway to metabolize internalized extracellular nutrients and trophic factors that are essential for their survival.[13] We therefore tested the hypothesis that neither cathepsin is dispensable in nervous system-derived tumors. As model systems we employed 2 human neuroblastoma (NB) cell lines, namely, GILIN and LAN-5, that have previously been shown to differ in their malignant properties such as proliferation rate, degree of neuronal-like maturation and response to *in vitro* apoptogenic treatments with chemotherapeutic drugs and cytokines.[14–16] In this respect, LAN-5 cells appear more prone to undergo neuronal-like differentiation, whereas GILIN cells appear more susceptible to drug-induced apoptosis. We found that sustained inhibition of CB- or CD-mediated proteolysis is detrimental *per se* for NB cell survival. We also show that blocking the caspase cascade can largely prevent apoptosis by CD or CB inhibitors.

This observation suggests that the cathepsin- and caspase-mediated proteolytic systems are connected and cooperate in the regulation of neuronal cell death and survival. Current antitumor strategies are designed to induce tumor cell death via apoptosis, rather than necrosis, in order to avoid inflammatory side effects. In this respect, the present data are of potential clinical relevance, since it appears possible to improve the cure rate of neuroblastoma by including cathepsin inhibitors in the chemotherapeutic regimen.

MATERIAL AND METHODS

Cell cultures and treatments

NB cell lines, GILIN and LAN-5,[11] were grown in a 5% CO_2 atmosphere in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 4 mM glutamine and penicillin/streptomycin mix (1%). Culture reagents were purchased from Sigma (St. Louis, MO). For experiments, cells were seeded (5×10^4 cells/cm^2 in 25 cm^2 flasks) and allowed to adhere for 48 hr prior to any treatment. Protease inhibitors were added daily in fresh medium. Pepstatin A (PST) and E-64 (N-[L-3-trans-carboxyoxirane-2-carbonyl]-L-leucyl-3-methylbutylamide) were from Sigma. CA074Me (N-[L-trans-propylcarbamoyloxirane-2-carbonyl]-L-isoleucyl-L-proline) was from Bachem (Bubendorf, Switzerland). zVADfmk (benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone) and DEVDcho (benzyloxycarbonyl-Asp-Glu-Val-Asp aldheyde) were from Biomol (Plymouth Meeting, PA).

Abbreviations: CA074Me, N- (L-trans-propylcarbamoyloxirane-2-carbonyl)-L-isoleucyl-L-proline; CB, cathepsin B; CD, cathepsin D; DAPI, 4-6-diamidino 2-phenylindol-dihydrochloride; DEVDcho, benzyloxycarbonyl-Asp-Glu-Val-Asp aldehyde; E-64, N- (L-3-trans-carboxyoxirane-2-carbonyl)-L-leucyl-3-methylbutylamide; FITC, fluorescein isothiocyanate; NB, neuroblastoma; PST, pepstatin A; TB, trypan blue; zVADfmk, benzyloxycarbonyl-Val-Ala-Asp fluoromethylketone.

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*Correspondence to: Dipartimento di Scienze Mediche, "A. Avogadro" University at Novara, Via Solaroli 17, 28100 Novara, Italy.
Fax: +39-0321-620-421. E-mail: isidoro@med. unipmn.it

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Biochemical and morphologic evaluation of apoptosis

Cultures were observed at 12 hr intervals under a phase-contrast light microscope (Zeiss, Axiovert 25) and photographed (Contax 167MT). This allowed an objective evaluation of morphology and cell density, and the detection of apoptotic or necrotic cells. At the times indicated, adherent cells from control and treated NB cultures were harvested by trypsinization and counted. Occurrence of necrosis in detached cells was demonstrated by the trypan blue staining test or by cytofluorometric analysis after staining with propidium iodide. Occurrence of apoptosis was ascertained morphologically and cytofluorometrically. Chromatin alterations were demonstrated by staining the cells with the DNA-labeling fluorescent dye 4-6-diamidino 2-phenylindol-dihydrochloride (DAPI), at 1 μg/ml methanol). Moreover, the appearance of a subdiploid cell population, corresponding to apoptotic cells, was monitored by flow cytometry on whole cell populations (*i.e.*, monolayer plus cells recovered from the medium) fixed (30 min with 70% ethanol) and stained with propidium iodide (0.2 mg/ml) in the presence of DNAse-free RNAse.

As an independent method to assess apoptosis, the presence of cells expressing annexin V binding sites at the surface was evaluated by flow cytometry. Annexin V binds specifically to phosphatidylserine, which is known to be externalized on the plasma membrane at an early stage of apoptosis.[17] This method also allows an objective quantitation of apoptosis.[17] Staining of cells (which were not fixed) with annexin V by fluorescein isothiocyanate (FITC) and propidium iodide was performed using the Early Apoptosis detection kit (Bender MedSystems, Vienna, Austria) following the manufacturer's instructions. Under this condition propidium iodide enters only necrotic cells, either primary (annexin V-negative) or secondary to apoptosis (annexin V-positive). Fluorescently labeled cells were detected with a FacScan flow cytometer (Becton Dickinson, Mountain View, CA). At least 10,000 events were analyzed for each sample. In some experiments cytotoxicity was evaluated by colorimetric estimation of adherent cells stained with crystal violet. Briefly, cells were left to adhere in flat-bottomed 96-well plates after being exposed to protease inhibitors, washed twice with PBS and incubated for 10 min at 37°C with 50 μl of staining solution (crystal violet 0.5%, formaldehyde 4%, ethanol 30%, NaCl 0.17%). The staining solution was removed, and the cells were washed twice and left to dry for 1 hr at 50°C. Adherent stained cells were resuspended in 33% acetic acid, and the intensity of the stain was determined at 570 nm in a microplate reader (model 450, Bio-Rad, Hercules, CA) equipped with the software microplate manager/PC. If not otherwise specified, reagents were purchased from Sigma.

Statistical analysis

Statistical significance of differences between groups of data was determined by using the paired *t*-test (Instat-3-statistical software, Graphpad S, San Diego, CA).

RESULTS

Inhibition of either lysosomal cathepsin B or D is incompatible with neuroblastoma cell survival

Given the essential role of lysosomal proteolysis in the metabolism of extracellular nutrients and trophic factors internalized in the endocytic system by neuronal cells, we evaluated the consequences of CB or CD inhibition on NB cell viability. GILIN and LAN-5 cells were treated for up to 72 hr with 100 μM E-64 or 10 μM CA074Me (2 inhibitors of CB[18,19]) or 100 μM pepstatin A (an inhibitor of CD[20]). These concentrations of cathepsin inhibitors were chosen based on previous work from this and other laboratories demonstrating the effective inhibition of lysosomal protein degradation.[12,18] The results of this experiment are summarized in Table I. CD inhibition revealed cytotoxicity for NB cells, causing by 72 hr about 70 and 90% cell loss in GILIN and LAN-5 cells, respectively. The long incubation time required for pepstatin A to exert its cytotoxic effect is in agreement with the low rate of intracellular accumulation of this inhibitor in cultured cells.[21] CA074Me, which is highly membrane-permeable and highly specific for CB,[19] readily exerted its effects, causing about 40% cell loss within 24 hr and almost complete death of the cultures by 48 hr of treatment. In this experimental condition CA074Me caused cell death primarily by necrosis, as assessed by flow cytometry (not shown). In this respect, LAN-5 cells were more susceptible (see below). E-64, which is a broad-spectrum inhibitor of cysteine proteases, of which CB is one, was much less cytotoxic than CA074Me, causing about 40% cell loss by 72 hr of treatment. The discrepancy between the cytotoxic effects of CA074Me and E-64 can be partly explained by differences in their permeability and accumulation within acidic compartments.

Cathepsin inhibitors induce cell death with morphologic features typical of apoptosis

Apoptotic cell death is characterized by cell shrinkage, cleavage of nuclear DNA, condensation of chromatin and fragmentation of nucleus and cytoplasm with consequent formation of apoptotic bodies. To ascertain the occurrence of such features, we examined pepstatin A- and E-64-treated cells under a fluorescent microscope after chromatin staining with DAPI. As shown in Figure 1, treatment with either lysosomal protease inhibitor was associated with extensive chromatin alterations. These were clearly detectable in detached cells recovered from the medium, whereas they were not (or only slightly) apparent in treated cells still adherent to the plastic (not shown), suggesting that nuclear alterations were a late event of this apoptotic process.

Apoptosis by cathepsin inhibitors is caspase-dependent

In several models the apoptotic process was shown to depend on the sequential activation of cytosolic proteases belonging to the caspase family.[4] Whether the caspase cascade and the lysosomal proteolytic pathway intersect during onset of apoptosis is not known. We therefore looked for the involvement of caspases in the apoptotic pathway initiated by CB and CD inhibitors. To inhibit the activation of the caspase cascade, we used zVADfmk, which exhibits a broad-spectrum inhibitory potential (including initiator caspases), and DEVDcho, which inhibits caspase 3. Both these inhibitors are cell-permeable and accumulate in the cytosol within few hours.[22] In a first set of experiments, GILIN cells were treated for 24 or 48 hr with a cathepsin inhibitor, and a caspase inhibitor was added at the same time. In 48 hr treated cultures, the medium was renewed after the first 24 hr, and substances were re-added in order to avoid side effects from nutrient consumption and possible

TABLE I – CYTOTOXIC EFFECTS OF CD AND CB INHIBITORS[1]

Time (hr)	PST		E64		CA074Me	
	GILIN	LAN-5	GILIN	LAN-5	GILIN	LAN-5
24	92 ± 8	90 ± 9	100 ± 8	99 ± 9	59 ± 5	59 ± 4
48	37 ± 3	37 ± 4	90 ± 9	93 ± 6	5 ± 2	5 ± 3
72	30 ± 4	10 ± 1	60 ± 3	54 ± 6	0	0

[1]NB cells were incubated for up to 72 hr in the absence or presence of a CD inhibitor (100 μM pepstatin A [PST]) or a CB inhibitor (100 μM E-64 or 10 μM CA074Me) as indicated. Medium was changed daily and substances re-added. At 24 hr intervals, adherent viable cells were counted. Data are expressed as percentage of cells in treated cultures with respect to controls and represent the mean ± SD of 4 separate experiments.

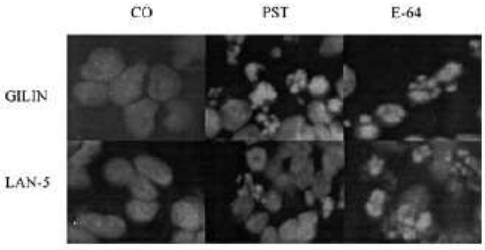

FIGURE 1 – Chromatin alterations induced by lysosomal protease inhibitors in NB cells. GILIN and LAN-5 cells were incubated for up to 72 hr in daily renewed medium with 100 μM pepstatin A (PST) or 100 μM E-64. Detached cells were recovered from medium of treated cultures and left to adhere on a glass coverslip. Parallel untreated cultures (Co) were set on sterile glass coverslips. Cells were then stained with DAPI to show DNA and observed and photographed under a fluorescent microscope. Nuclei of control cells were of regular size and shape and were evenly stained with the fluorescent dye. By contrast, nuclei of treated cells were smaller and clearly exhibited chromatin condensation and fragmentation. Original magnification ×1,000.

accumulation of secreted toxic factors. CA074Me and pepstatin A were used to specifically inhibit CB and CD, respectively. zVADfmk at 2 μM concentration prevented apoptosis induced by pepstatin A, but not by CA074Me, whereas at 10 μM concentration it protected NB cells from both pepstatin A- and CA074Me-induced apoptosis (Fig. 2*a*). At 5 μM, DEVDcho, a rather specific inhibitor of caspase 3, also protected NB cells from cytotoxicity induced by lysosomal inhibitors (not shown). We performed a flow cytometric analysis of annexin V^{FITC}-stained cells to monitor the occurrence of apoptosis.

As shown in Figure 2*b*, a large proportion of GILIN cells treated with a cathepsin inhibitor exhibited positivity for annexin V, which was abolished when the incubations were performed in the presence of the pan-caspase inhibitor zVADfmk. These data indicate that the inhibition of a lysosomal protease generates an apoptotic signal that alters the plasma membrane symmetry in a caspase-dependent mode. To quantitate the protective effect of zVADfmk in GILIN cells exposed to a cathepsin inhibitor, we performed a colorimetric test that indirectly measures the cells attached to the plastic. The data shown in Figure 3 confirm that cell loss induced by CA074Me or pepstatin A can be largely prevented provided that a caspase inhibitor is present during the incubation.

Finally, we extended the above observation to LAN-5 cells. In these experiments, cell death was evaluated by counting the trypan blue-positive cells (representing necrotic cells) and by flow cytometric analysis of annexin V^{FITC}-stained cells (Fig. 4). LAN-5 cells were highly susceptible to the cytotoxic action of cathepsin inhibitors. In this respect, CA074Me was the most effective, causing more than 50% cell death (largely by primary necrosis), when used at 10 μM concentration for 24 hr (not shown). For this reason, in LAN-5 cultures this drug was used at 5 μM final concentration for a period of 12 hr. Again, inhibition of a lysosomal cathepsin induced apoptotic cell death, as assessed by the outer appearance of annexin V binding sites, which could largely be blocked by cotreatment with a pan-caspase inhibitor.

DISCUSSION

Neuroblastoma is an extracranial tumor of the nervous system that is frequent in childhood. Due to the onset of metastatic dissemination and resistance to chemotherapy, most children with neuroblastoma have a low overall probability of survival.[23,24] In the present work we asked whether inhibition of selected lysosomal proteases would affect cell survival and trigger apoptotic death in human neuroblastoma cells. GILIN and LAN-5 cells, 2 NB cell lines that differ in their *in vitro* response to chemotherapeutic drugs and cytokines,[14–16] were cultivated in the presence of cathepsin inhibitors under conditions (concentration and time of incubation) that effectively impair the proteolytic activity of CB and CD.[18,20]

Two main conclusions can be drawn from our study: (*i*) the proteolytic function of endosomal-lysosomal organelles is essen-

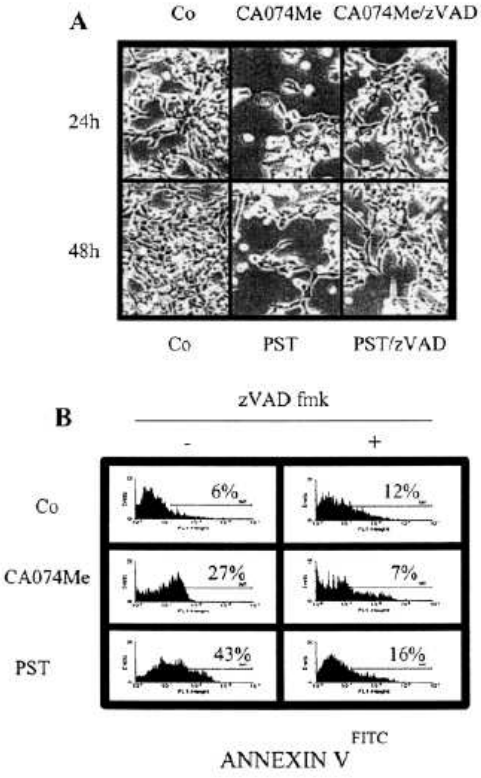

FIGURE 2 – The caspase inhibitor zVADfmk prevents apoptosis by pepstatin A or CA074Me in GILIN cultures. GILIN cells were incubated with CA074Me (10 μM for 24 hr) or pepstatin A (PST, 100 μM for 48 hr) in the absence or presence of the caspase inhibitor zVADfmk (10 μM) as indicated. Medium was changed and substances readded in fresh medium after the first 24 hr. Control cultures (Co) were incubated in a similar manner but without addition of proteases inhibitors. (*a*) Cultures were photographed under contrast phase microscopy. Cell loss is apparent in cultures treated with CB or CD inhibitors. The picture also shows that in the presence of zVADfmk, the monolayers of GILIN treated with CA074Me or PST were well preserved and resembled those of controls. The monolayer of cultures treated with zVADfmk alone resembled that of untreated controls (not shown). (*b*) Induction of annexin V positivity by cathepsin inhibitors is caspase-dependent. GILIN cells were incubated as described above. At the end, cells were labeled with annexin V by FITC and analyzed by flow cytometry. Increased FITC staining is evident in CA074Me- and PST-treated cells. This shift in FITC fluorescence is not observed in cells incubated with both the cathepsin inhibitor and zVAD. One of 3 separate experiments with similar results is shown.

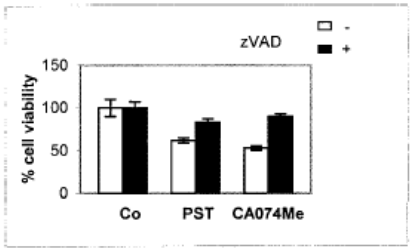

FIGURE 3 – Evaluation of the protective effect of zVADfmk in GILIN cells treated with cathepsin inhibitors. GILIN cells were essentially cultivated and treated as for the experiment reported in Figure 2, except that cells were plated in multiwell 96 microplates. Cellular viability was evaluated by the crystal violet staining test, as described in Material and Methods. Absorbance was determined in at least 10 multiwell per condition. Data represent the mean ± SD of 3 independent experiments and are expressed as percentage assuming the controls as 100%. Differences between paired groups (± zVAD) were extremely significant ($p < 0.0002$ for PST; $p < 0.0001$ for CA074Me). At the concentration used (10 μM), zVADfmk exhibited no cytotoxicity.

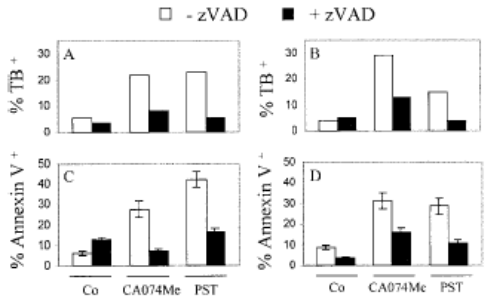

FIGURE 4 – The caspase inhibitor zVADfmk prevents NB cell death induced by the cathepsin inhibitors GILIN and LAN-5. Cells were cultivated in multiwell 6 plates and incubated with CA074Me (GILIN, 10 μM for 24 hr; LAN-5, 5 μM for 12 hr) or pepstatin A (PST, 100 μM for 48 hr) in the absence or presence of the caspase inhibitor zVADfmk (10 μM) as indicated. Cell death was calculated as percentage of trypan blue-positive cells in cultures (*a*, LAN-5; *b*, GILIN). Values represent the mean of 2 parallel determinations. Similar results were obtained in 4 other independent experiments. As a measure of early onset of apoptosis, positivity for annexin V binding sites was determined by flow cytometry. (*c*) (LAN-5) and (*d*) (GILIN) report the percentage of annexin V^{FITC}-positive cells. Data represent the mean ± SD of 3 parallel determinations. One of 4 independent experiments with similar results is shown. Differences between paired groups (± zVAD) were highly significant ($p < 0.002$ for GILIN treated with PST and $p < 0.006$ for others).

tial for NB cell survival; and (*ii*) inhibition of CB or CD triggers the caspase-dependent apoptosis of NB cells. Strikingly, LAN-5 cells, compared with GILIN cells, are less sensitive to cytokine- and retinoid-induced apoptosis[15,16] were highly susceptible to lysosomal cathepsin inhibitors. It should be noted that in these cells CA074Me could induce cell death by necrosis or by apoptosis, depending on concentration used and time of incubation. This is indeed not surprising, since it is well known that the type and extent of cell damage vary with the intensity of the toxic treatment. Accordingly, many other cytotoxic agents including cytokines and antiblastic drugs have been shown to trigger either necrosis or apoptosis depending on the experimental conditions.

The stereotypic morphologic and biochemical changes that are the hallmarks of apoptosis have been described in a wide variety of experimental models, thus suggesting the existence of a common execution pathway. The group of cytosolic proteases termed caspases is believed to serve this function.[22] Depending on the apoptotic phase in which they act, members of the caspase family are classified as *initiators* (the prototypes being caspases 8 and 9) or *effectors* (the prototype being caspase 3). Based on the protective effect of zVADfmk, it appears that lysosomal protease inhibitors trigger the activation of initiator caspases. To our knowledge, ours is the first report to document such an effect of lysosomal protease inhibitors.

The present data are in contrast with the recent reports attributing to lysosomal cathepsins an active role in the induction of apoptosis triggered by cytokines or antiblastic drugs.[5,8,9] Why should inhibition of just one cathepsin be detrimental for NB cell survival? It has been previously shown that deficiency of CD or inhibition of either CD or CB does not compromise overall turnover of long-lived proteins.[11,12] Therefore, it appears unlikely that in our model apoptosis resulted from a complete block of bulk protein degradation. It should be mentioned, however, that cathepsins resident within endosomes also accomplish a limited, maturation-like proteolysis that leads to the activation of the precursor form of biologically active substances.[25] In this regard, experiments with gene knockout have shown that CD is a key enzyme for the development of vital tissues and organs, suggesting a role for this protease in the generation of specific growth factors or the inactivation of growth inhibitors that control cell turnover.[11] In fact, CD can affect either the activation of hormones and growth factor precursors by limited proteolysis within endosomes or the inactivation of mature growth factors by endocytosis and subsequent extensive degradation within lysosomes.[25] It is conceivable that proteolysis within the endosomal-lysosomal system also plays such a vital role in neuronal cells.[13]

The fact that inhibition of just 1 of the 2 cathepsins is cytotoxic to NB cells indicates that CB and CD affect distinct proteolytic events that are essential for cell viability. We hypothesize that in cultured NB cells CB and CD are sequentially involved in the the degradation of proapoptotic factors and that the inhibition of either enzyme leads to accumulation of substrates that eventually impact on the caspase cascade. The primary mechanism by which most novel antitumor chemotherapeutic agents induce cell death is induction of apoptosis.[2] The present observations imply that specific therapies designed to enhance drug-induced apoptosis that include cathepsin inhibitors could form the basis for treatment of neuroblastomas.

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REFERENCES

1. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science 1995; 267:1456–62.
2. Fisher DE Apoptosis in cancer therapy: crossing the threshold. Cell 1994;78:539–42.
3. Salvesen GS, Dixit VM. Caspases: intracellular signaling by proteolysis. Cell 1997;91:443–6.
4. Sukharev SA, Pleshakova OV, Sadovnikov VB. Role of proteases in activation of apoptosis. Cell Death Diff. 1997;4:457–62.
5. Deiss LP, Hamutal G, Berissi H, et al. Cathepsin D protease mediates programmed cell death induced by interferon-γ, Fas/APO-1 and TNF-α. EMBO J 1996;15:3861–70.
6. Wu GS, Saftig P, Peters CD, et al. Potential role for cathepsin D in

p53-dependent tumor suppression and chemosensitivity. Oncogene 1998;16:2177–83.
7. Shibata M, Kanamori S, Isahara K, et al. Participation of cathepsin B and D in apoptosis of PC12 cells following serum deprivation. Biochem Biophys Res Commun 1998;251:199–203.
8. Guicciardi ME, Deussing J, Miyoshi H, et al. Cathepsin B contributes to TNF-alpha-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome c. J Clin Invest 2000;106:1127–37.
9. Zang Y, Beard RL, Chandraratna RA, et al. Evidence of a lysosomal pathway for apoptosis induced by the synthetic retinoid CD437 in human leukemia HL-60 cells. Cell Death Differ 2001;8:477–85.
10. Isahara K, Ohsawa Y, Kanamori S, et al. Regulation of a novel pathway for cell death by lysosomal aspartic and cysteine proteinases. Neuroscience 1999;91:233–49.
11. Saftig P, Hetman M, Schmahl W, et al. Mice deficient for lysosomal proteinase cathepsin D exhibit progressive atrophy of the intestinal mucosa and profound destruction of lymphoid cells. EMBO J 1995; 14:3599–608.
12. Kominami E, Ueno T, Muno D, et al. The selective role of cathepsins B and D in the lysosomal degradation of endogenous and exogenous proteins. FEBS Lett 1991;287:189–92.
13. Nixon RA, Cataldo AM. The endosomal-lysosomal system of neurons: new roles. Trends Neurosci 1995;18:489–96.
14. Ponzoni M, Casalaro A, Panciotti M, et al. The combination of γ-interferon and tumor necrosis factor causes a rapid and extensive differentiation of human neuroblastoma cells. Cancer Res 1992;52: 931–9.
15. Ponzoni M, Bocca P, Chiesa V, et al. Differential effect of N-(4-hydroxyphenyl)retinammide and retinoic acid on neuroblastoma cells: apoptosis versus differentiation. Cancer Res 1995;55:853–61.
16. Montaldo PG, Chiesa V, Bado M, et al. Induction of differentiation and apoptosis by interferon γ in human neuroblastoma cells in vitro as a dual and alternative early biological response. Cell Death Differ 1997;4:150–8.
17. Martin SJ, Reutelingsperger CP, McGahon AJ, et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. J Exp Med 1995;182:1545–56.
18. Oshita T, Nikawa, T, Towatari, T, et al. Effects of selective inhibition of cathepsin B and general inhibition of cysteine proteinases on lysosomal proteolysis in rat liver in vivo and in vitro. Eur J Biochem 1992;209:223–31.
19. Buttle DJ, Murata M, Knight CG, et al. CA074 methyl ester: a proinhibitor for intracellular cathepsin B. Arch Biochem Biophys 1992;299:377–80.
20. Knight CG, Barrett AJ. Interaction of human cathepsin D with the inhibitor pepstatin. Biochem J 1976;155:117–25.
21. Shields PP, Gonzales TA, Charles D, et al. Accumulation of pepstatin in cultured endothelial cells and its effect on endothelin processing. Biochem Biophys Res Commun 1991;177:1006–12.
22. Cohen GM. Caspases: the executioners of apoptosis. Biochem J 1997;326:1–16.
23. Berthold F, Brandeis WE, Lampert F. Neuroblastoma: diagnosis advances and therapeutic results in 370 patients. Monogr Paediatr 1985; 18:210–5.
24. Philip T, Ladenstein R, Zucher JM, et al. Double megatherapy and autologous bone marrow transplantation for advanced neuroblastoma: the LMCE2 study. Br J Cancer 1993;67:119–23.
25. Berg T, Gjöen T, Bakke O. Physiological functions of endosomal proteolysis. Biochem J 1995;307:313–26.

2. BASI MOLECOLARI DELLA REGOLAZIONE RECIPROCA TRA AUTOFAGIA E MORTE CELLULARE NELLE MALATTIE NEURODEGENERATIVE: UNO STUDIO SPERIMENTALE SUL DIABETE INSIPIDO NEUROIPOFISARIO (FNDI)

Il sistema ipotalamo-neuroipofisario (HNS) è quello centrale per il mantenimento del bilancio idrico nei mammiferi, principalmente attraverso la produzione dell'ormone vasopressina (VP). La VP è sintetizzata come proormone nei neuroni magnocellulari dell'HNS. Da qui, viene trasportata lungo gli assoni, e durante questo trasporto viene processata a peptide biologicamente attivo, successivamente viene immagazzinata nei terminali nervosi dell'ipofisi posteriore prima di essere rilasciata nel circolo sanguigno quando è fisiologicamente richiesta.

La vasopressina è ampiamente conosciuta come l'ormone antidiuretico, la VP magnocellulare è coinvolta nel mantenimento dell'omeostasi idrica e salina promuovendo il riassorbimento di acqua nei reni. La vasopressina agisce sui recettori V2 (Lolait *et al*, 1992) che sono presenti nella lamina basale delle cellule dei tubuli prossimali e dei dotti dei reni (Chase *et al*, 1968). I recettori V2 sono accoppiati alle subunità α della proteina G stimolatoria, Gs (Orloff and Handker 1967). La stimolazione del recettore V2 determina l'attivazione dell'adenilato ciclasi e la produzione dell'AMP ciclico. L'aumento intracellulare di cAMP attiva la protein chinasi A, che, a sua volta determina il trasporto dei canali acquosi dell'acquaporina 2 dalle vescicole intracellulari sul lato apicale della plasmamembrana (Deen et al, 1994; Harris et al, 1991). I canali acquosi di acquaporina 2 aumentano enormemente la permeabilità osmotica della membrana permettendo il riassorbimento dell'acqua.

Il gene della vasopressina contiene tre esoni (Schmale *et al*, 1983; Ivell and Richter, 1984; Ruppert *et al*, 1984; Sausville *et al*, 1985; Hara *et al*, 1990). L'esone I codifica un peptide segnale, l'ormone, un sito di taglio di tre aminoacidi basici (-Gly-Lys-Arg-) e e primi nove aminoacidi dell'N-terminale delle neurofisina (NPI in OT, NPII in VP). L'esone II codifica l'altamente conservata porzione centrale della NP (aminoacidi 10-76). L'esone III del gene della VP codifica i restanti aminoacidi del C-terminale, un sito di taglio basico (-Arg-) e il glicopeptide al C-terminale (GP). GP contiene la sequenza consenso (Asn-X-Ser/Thr) richiesta per la glicosilazione.

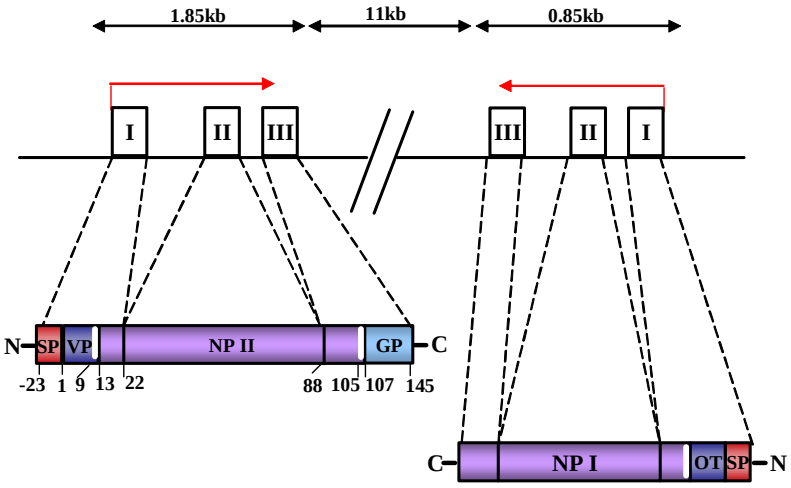

Figura 12.

Un aumento nella osmolarità nel plasma determina il rilascio di VP dall'ipofisi posteriore, e ciò porta ad una aumentata sintesi (Brownstein *et al*, 1980). E' ben noto che stimoli osmotici quali la deidratazione e l'apporto di sale aumentano la trascrizione del gene della VP (Murphy and Carter, 1990). Ciò causa un aumento nei livelli dell'mRNA della VP (Burbach et al, 1984; Herman et al, 1991; Lightman and Young, 1987; Murphy et al, 1989; Sherman et al, 1986; Zing et al, 1986). Lo stimolo osmotico acuto causato da una iniezione intraperitoneale di una soluzione salina ipertonica provoca un aumento della trascrizione del gene della VP di circa 6 volte (Murphy and Carter, 1990). Al contrario, uno stimolo iposmotico può inibire la secrezione e la biosintesi di VP (Robinson et al, 1990).

Il diabete insipido neuroipofisario (FNDI) è una malattia autosomica dominante che si presenta nei bambini con eccessiva sete e diuresi come conseguenza di una progressive perdita di secrezione dell'ormone vasopressina dai terminali nervosi post pituitari (Baylis and Robertson, 1981; Robertson, 1995). Studi hanno dimostrato che mutazioni nel gene della Vp possono essere la causa dell'FNDI. Nel laboratorio di David Murphy, a Bristol, è stato prodotto un modello animale di ratti transgenici che presentano una forma mutante di VP corrispondente all'FNDI (Nagasaki et al., 1995) e che codifica una proteina troncata (Cys67stop). Inoltre, nel corso del mio soggiorno di studio presso il laboratorio del Prof. David Murphy, è stato messo a punto un modello "*in vitro*" di FNDI mediante l'utilizzo di vettori ricombinanti adenovirali in cellule di

neuroblastoma murino (N2A). Queste cellule riproducono dal punto di vista fenotipico le alterazioni a livello cellulare che si osservano nei neuroni dei cervelli transgenici FNDI.

Negli articoli allegati sono descritti gli studi condotti sui modelli “in vivo” e in “in vitro” volti a chiarire la basi molecolari della regolazione reciproca tra autofagia e morte cellulare nelle malattie neurodegenerative.

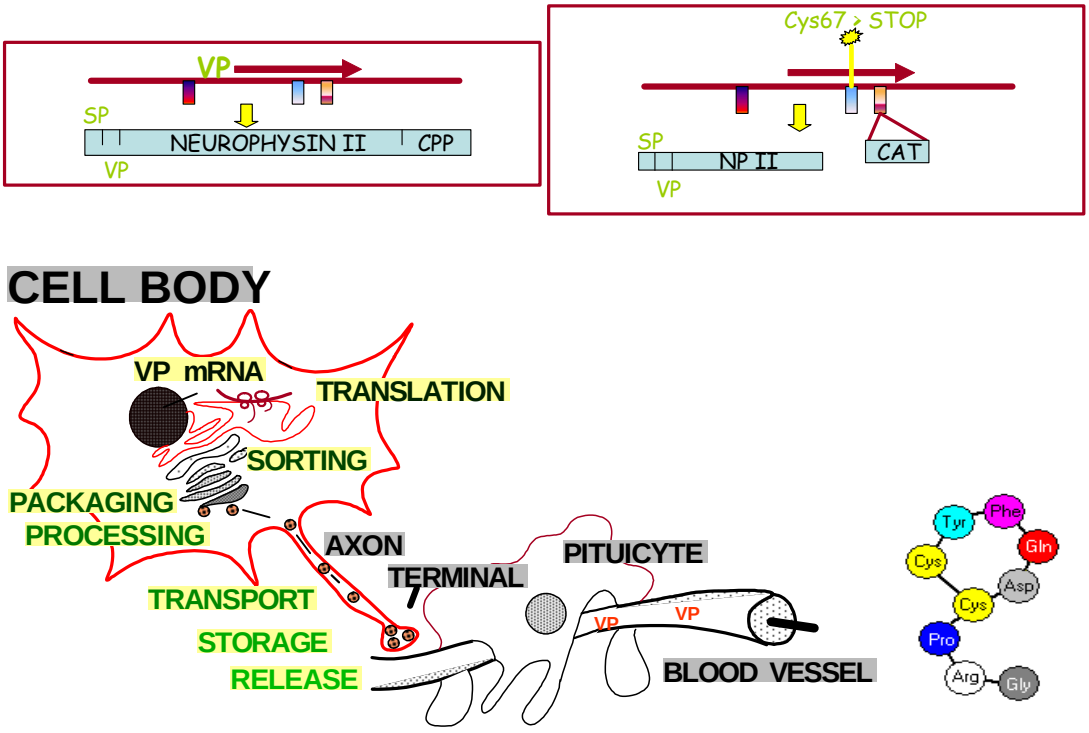

Fig. 13

ENGLISH VERSION

REGULATION BETWEEN AUTOPHAGY AND NEURONAL CELL DEATH IN NEURODEGENERATIVE DISEASE: A SPERIMENTAL APPROACH IN NEUROHYPOPHYSEAL DIABETES INSIPIDUS

Familial neurohypophyseal diabetes insipidus (FNDI) is an autosomal dominant, progressive, inherited neurodegenerative disorder, caused by mutations in the gene encoding the antidiuretic hormone vasopressin (VP), that presents as polydipsia and polyuria as a consequence of a loss of secretion of the antidiuretic hormone vasopressin (VP) from posterior pituitary nerve terminals. FNDI, like other protein aggregation neurodegenerative diseases, such as Alzheimer's, Parkinson's and Huntington's, is associated with autophagy - rats expressing an FNDI VP transgene (Cys67stop) show a neuronal pathology characterised by autophagic-like structures in the cell body - but the role of autophagy in these diseases is unclear. We have studied the relationships between mutant protein accumulation, autophagy, cell survival, and cell death using a novel and tractable *in vitro* system. We have constructed adenoviral vectors (Ads) that express structural genes encoding either the Cys67stop mutant protein or an epitope-tagged wild-type VP precursor. Following infection of neuroblastoma cells, wild-type material enters neurite processes and accumulates in terminals, whilst the Cys67stop protein is confined to enlarged vesicles in the cell body. As in Cys67stop rats, these structures are of ER origin, and co-localise with markers of autophagy. Neither wild-type VP nor Cys67stop expression affected cell viability. However, inhibition of autophagy or lysosomal protein degradation significantly increased apoptotic cell death following Cys67stop expression. The activation of autophagy by an FNDI mutant protein is thus a pro-survival mechanism.

In the following papers we report our studies in a model of Familial Neurohypophyseal Diabetes Insipidus (FNDI). To address the relationships between mutant protein accumulation, autophagy, cell survival, and cell death, we have developed a novel and tractable *in vitro* model of adFNDI by infecting neuroblastoma Neuro2A cells with recombinant adenoviral vectors (Ads) expressing the truncated Cys67stop mutant form of VP (Ad-VCAT-Cys67stop) or an epitope-tagged wild-type VP precursor (Ad-VCAT).

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Autophagy is a prosurvival mechanism in cells expressing an autosomal dominant familial neurohypophyseal diabetes insipidus mutant vasopressin transgene

Roberta Castino,[*,†] Janet Davies,[*,‡] Stephanie Beaucourt,[*] Ciro Isidoro,[†] and David Murphy[*]

[*]Molecular Neuroendocrinology Research Group, Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology, University of Bristol, Dorothy Hodgkin Building, Whitson Street, Bristol, England; and [†]"Amedeo Avogadro" University, Laboratory of Molecular Pathology, Department of Medical Sciences, Via Solaroli 17, 28100 Novara, Italy

[‡]Present address: Cambridge Institute for Medical Research (CIMR), Wellcome Trust/MRC Building, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2XY, England

Corresponding author: David Murphy, Molecular Neuroendocrinology Research Group, Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology, University of Bristol, Dorothy Hodgkin Building, Whitson Street, Bristol BS1 3NY, England. E-mail: d.murphy@bristol.ac.uk

ABSTRACT

Autosomal dominant familial neurohypophyseal diabetes insipidus (adFNDI) is a progressive, inherited neurodegenerative disorder that presents as polydipsia and polyuria as a consequence of a loss of secretion of the antidiuretic hormone vasopressin (VP) from posterior pituitary nerve terminals. VP gene mutations cause adFNDI. Rats expressing an adFNDI VP transgene (Cys67stop) show a neuronal pathology characterized by autophagic structures in the cell body. adFNDI has thus been added to the list of protein aggregation diseases, along with Alzheimer's, Parkinson's and Huntington's, which are associated with autophagy, a bulk process that delivers regions of cytosol to lysosomes for degradation. However, the role of autophagy in these diseases is unclear. To address the relationships between mutant protein accumulation, autophagy, cell survival, and cell death, we have developed a novel and tractable in vitro system. We have constructed adenoviral vectors (Ads) that express structural genes encoding either the Cys67stop mutant protein (Ad-VCAT-Cys67stop) or an epitope-tagged wild-type VP precursor (Ad-VCAT). After infection of mouse neuroblastoma Neuro2a cells, Ad-VCAT encoded material enters neurite processes and accumulates in terminals, while the Cys67stop protein is confined to enlarged vesicles in the cell body. Similar to the intracellular derangements seen in the Cys67stop rats, these structures are of ER origin, and colocalize with markers of autophagy. Neither Ad-VCAT-Cys67stop nor Ad-VCAT expression affected cell viability. However, inhibition of autophagy or lysosomal protein degradation, while having no effect on Ad-VCAT-expressing cells, significantly increased apoptotic cell death following Ad-VCAT-Cys67stop expression. These data suggest that activation of autophagy by the stress of the expression of an adFNDI mutant protein is a prosurvival mechanism.

Key words: apoptosis • transgenic • adFNDI • antidiuretic hormone • VP

A crucial mediator of osmotic stability in mammalian organisms is the antidiuretic hormone vasopressin (VP) (1). VP is synthesized as a prepropeptide in the cell bodies of hypothalamic neurons (2). After signal peptide removal and disulphide bond formation in the endoplasmic reticulum (ER), the propeptide is sorted (3) into the regulated secretory pathway in the trans-Golgi network (TGN) (3, 4). Dense core granules are transported to storage in nerve terminals in the posterior pituitary (4) from where peptide is mobilized for secretion into the circulation by electrical activities evoked by osmotic cues. At the level of the kidney, VP increases the permeability of the collecting ducts, reducing water excretion.

Autosomal dominant familial neurohypophyseal diabetes insipidus (adFNDI) is caused by mutations in the VP gene and presents as excessive drinking and urination as a consequence of a progressive loss of secretion of VP from posterior pituitary nerve terminals (5). We have recently shown that expression of an adFNDI mutant transgene encoding a truncated precursor (Cys67stop) in rat VP neurons induces autophagy (6, 7), a bulk process, found in all eukaryotes from yeast to humans, that delivers regions of cytosol to lysosomes for degradation (8, 9). Thus, adFNDI, like other neurodegenerative diseases, such as Parkinson's (10), Alzheimer's (11) and Huntington's (12), is associated with autophagy, although its role in these disorders is unclear.

To study the role of autophagy in protein aggregation disease, we have now used adenoviral vectors (Ads) to deliver the Cys67stop mutant protein or an epitope-tagged wild-type VP precursor into neuroblastoma cells. While wild-type material entered neurite processes and accumulated in terminals, the Cys67stop protein was confined to autophagic vesicles of ER origin in the cell body. Neither Cys67stop nor wild-type VP expression affected cell viability. However, inhibition of any crucial step of autophagy induced apoptotic cell death in Cys67stop-expressing cells. We conclude that the activation of autophagy by the cellular stress of the expression of an adFNDI mutant protein gene is thus a prosurvival mechanism.

MATERIALS AND METHODS

Transgenic rats

Animals were cared for in accord with U.K. law. 3-VCAT-3 and 3-VCAT-3-Cys67stop transgenic rats have been described (6). Rats (n=4) were killed between 12 and 18 months of age. Animals were perfused transcardially with 60 ml of PBS followed by 60 ml of 4% (wt/vol) paraformaldehyde. Dissected brains were left overnight in 4% (wt/vol) paraformaldehyde then cryopreserved in 30% (wt/vol) sucrose and frozen prior to sectioning.

Adenoviral vectors

Ads were made using the Cre-*lox* recombination method (13). Fragments encompassing the rat VP structural gene were excised from the 3-VCAT-3 and 3-VCAT-3-Cys67stop constructs (6) by complete digestion with HindIII (3′ end) and partial digestion with Pst1 (5′ end). Insertion into HindIII and PstI digested pSP72 generated pSP72-VCAT and pSP72-VCAT-Cys67stop. Complete digestion of these constructs with XhoI and partial digestion with Pst1 excised the transgene fragments, which were recloned into the Pst1 and Sal1 sites of the recombination

vector pAdloxTRE to generate pAdloxTRE-VCAT and pAdloxTRE-VCAT-Cys67stop. pAdloxTRE is a derivative of pAdlox containing a tetracycline response element (TRE) upstream of the minimal CMV promoter. pAdloxTRE-VCAT and pAdloxTRE-VCAT-Cys67stop were then cotransfected with Ψ5 DNA into Cre8 helper cells to generate recombinant virus. Ad-CMV-TetOff encodes the TetOff transactivator under the constitutive control of the CMV promoter-enhancer (14). TetOff interacts with the TRE to drive the expression of transgenes in the absence of tetracycline. Neuro2a cells were infected with Ad-CMV-TetOff and either Ad-VCAT or Ad-VCAT-Cys67stop (ratio of 1:1) at a multiplicity of infection of 50.

Cell culture and treatments

Mouse neuroblastoma Neuro 2a cells were grown in a humidified 5% (vol/vol) CO_2 atmosphere in DMEM medium supplemented with 10% (vol/vol) FCS and penicillin/streptomycin (1% wt/vol). Cells were seeded at 5×10^4 cells/cm^2 in six-well multiplates and allowed to adhere for 24 h before any treatment. For morphological studies, cells were seeded at a density of 15,000 cells/cm^2 on noncoated sterile cover slips. To dissect the autophagic pathway, we employed drugs to interfere with the three fundamental steps—autophagosome formation, fusion of the autophagic vacuoles with lysosomes, and intra-autophagolysosomal proteolysis. Inhibitors (Sigma-Aldrich, Gillingham, Dorset, UK) were added at the same time as viral infection and were used at the lowest, most effective nontoxic concentrations. 3-methyladenine (3MA; 100 μM in DMSO) inhibits the formation of autophagososmes (15). Asparagine (Asn; 20 mM in DMEM) prevents the delivery of autophagocytosed material to lysosomes (16). Pepstatin A (Pst; 100 μM in DMSO) inhibits the lysosomal protease cathepsin D (17). The final concentration of DMSO in the medium (<0.1% vol/vol) is nontoxic.

Immunofluorescence

Cells grown on cover slips and brain sections were fixed with paraformaldehyde (4% wt/vol), blocked with 1% (wt/vol) BSA, permeabilized with 0.5% (vol/vol) Triton X-100 for 20 min, then incubated with primary antibody at an appropriate dilution. After washing, samples were incubated with FITC- or TRITC-conjugated secondary antibodies at room temperature in the dark. FITC- and TRITC-labeled cells were viewed using standard fluorescein and rhodamine filters (excitation 488 nm and 568, respectively) under an upright confocal microscope (TCS-NT, Leica Microsystems, Milton Keynes, UK). Primary rabbit polyclonal antisera recognizing the Cys67stop mutant protein (αCX67; 6, 7) and the DR-12-EK C-terminal epitope (αDR-12-EK; 18) have been described. The antiserum recognizing Beclin 1 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). BD Biosciences (Oxford, UK) supplied antisera recognizing calnexin, TGN38, cathepsin D, Rab24 and cytochrome c. Antisera directed against protein disulphide isomerase (PDI) were the kind gift of Dr. S. Fuller (European Molecular Biology Laboratory, Heidelberg, Germany), while anti-Vps34 was generously donated by Dr. H.W. Davidson, CIMR, University of Cambridge, UK).

Western blot analysis

Cells were lysed in water containing 1% (wt/vol) Na deoxycholate. 30 μg of cell homogenate was fractionated by in a 12.5% (wt/vol) polyacrylamide gel, and then electroblotted onto PDVF membrane (Millipore Corporation, Bedford, MA, USA). Beclin 1 was detected by

chemiluminescence reaction using a specific polyclonal antiserum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) followed by horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories Ltd., Hemel Hemstead, UK). The membrane was then stripped for 30 min at 55°C in 100 mM β-mercaptoethanol, 2% (wt/vol) SDS, 62.5 mM Tris-HCl pH 6.8, then reincubated with a specific anti-β-tubulin monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) to control for sample, integrity, loading, and transfer. Image analysis and signal quantification was performed using NIH Image J (RSB, NIMH, Bethesda, MD, USA).

Labeling of low-pH organelles with acridine orange

The lysosomal compartment of living cells was identified with acridine orange (AO), a fluorochrome that accumulates in acid organelles and, once protonated, emits red light under UV excitation. Adherent cells on cover slips were incubated for 5 min with AO (1 mg/ml), washed 3 times with PBS, and then immediately examined by fluorescence microscopy.

Labeling of autophagic vacuoles with monodansylcadaverine

Monodansylcadaverine (MDC) is a spontaneously fluorescent dye that is incorporated selectively into autophagosomes and autolysosomes (19). Cells were incubated with 0.05 mM MDC in PBS at 37°C for 1 h. After incubation, cells were washed twice with PBS and immediately analyzed by fluorescence microscopy (excitation: 380-420, barrier filter 450 nm).

Cell viability and death

Dead cells were identified using the trypan blue staining test. Adherent cells were detached, resuspended in DMEM, and then mixed with an equal volume of 0.04% (wt/vol) trypan blue. The percentage of cells stained was determined by haemocytometer counting. Apoptosis was ascertained morphologically and cytofluorometrically. Nuclear condensation and fragmentation were detected using 4,6-diamidino 2-phenylindole (DAPI; Sigma-Aldrich, Gillingham, Dorset, UK). Cells adherent on glass cover slips were washed with PBS, fixed with paraformaldehyde (4% wt/vol), and then stained with DAPI (10 ng/ml) in PBS for 30 min at 37°C, before visualization using fluorescence microscopy with UV excitation (350 nm; 20). The appearance of a hypo-diploid cell population (subG1) was monitored by flow cytometry on the whole cell population (i.e., monolayer plus cells recovered from the medium), fixed for 30 min with 70% (vol/vol) ethanol, and stained with propidium iodide (PI; 0.2 mg/ml) in the presence of DNase-free RNase (to avoid RNA labeling). Apoptosis was further objectively assessed using a method based on the expression of cell surface Annexin V binding sites (20, 21). Annexin V binds specifically to phosphatidylserine, which is externalized on the plasma membrane during the early stages of apoptosis. The whole cell population (i.e., adherent and detached cells) were labeled with FITC-conjugated Annexin V without prior fixation (22). Fluorescent cells were detected with a FacsScalibur fluorescence-activated cell-sorter (BD Biosciences, Oxford, UK). At least 10,000 events were analyzed for each sample. Cytochrome c release (23) was examined by immunofluoresence (see above).

Caspase activity was measured using the Caspase Detection Kit (Merck Biosciences Ltd, Nottingham, UK) using FITC-ZVAD-FMK as a substrate. Stained cells were analyzed by flow cytometry using FL1 channel and by fluorescence microscopy.

Statistical Analysis

All experiments were independently replicated at least 3 times. Data are presented as means ± SD. Statistical significance of differences between groups of data was determined by using the two-way ANOVA (ANOVA using SPSS software). A *P* value less 0.05 was taken as denoting significance (represented by an asterisk in the figures).

RESULTS

Cys67stop mutant protein accumulates in cathepsin-D positive compartments

Transgene VCAT encodes the wild-type VP precursor c-terminally tagged with a unique hexadecapeptide (DR-12-EK) (18). In the VCAT-Cys67stop transgene, a C to A transition in Exon II replaces a Cys (TGC) at position 67 of the neurophysin portion of the VP precursor with a translation termination codon (TGA) (6). These transgenes were incorporated into replication-deficient adenoviral vectors (Fig. 1*A*) that mediate a high rate of infection (>90%) of target mouse neuroblastoma Neuro2a cells with consequent robust transgene expression. We first used phase contrast microscopy to examine the morphology of Ad-VCAT- and Ad-VCAT-Cys67stop-infected Neuro2a cells (Fig. 1*B*). Cells infected with Ad-VCAT were indistinguishable from noninfected cells, both showing characteristic neuritic processes (Fig. 1*B*). In contrast, a large number of Ad-VCAT-Cys67stop infected cells had a "rounded" appearance, characterized by neurite withdrawal, along with pronounced cellular inclusions (Fig. 1*B*). Similar pronounced vesicles were revealed in Ad-VCAT-Cys67stop infected cells stained with Acridine Orange (AO), a dye that identifies acid vacuolar compartments (Fig. 1*C*). In noninfected and Ad-VCAT-infected cells, the dye reveals a diffuse punctate staining throughout the cytoplasm, as expected for lysosmes. In contrast, Ad-VCAT-Cys67stop infection caused the accumulation and redistribution, in the perinuclear region, of enlarged acidic compartments. We then used specific antisera that recognize the Cys67stop mutant protein (6, 7) and the VCAT DR-12-EK C-terminal epitope (18) to ask about the fate of transgene proteins in Ad-infected Neuro2a cells, and this was compared with rats expressing the same transgenes in VP-expressing MCNs. In 3-VCAT-3 rats (18), transgene proteins follow a parallel path with the endogenous wild-type VP through the regulated secretory pathway. Similarly, in Neuro2a cells, Ad-VCAT expression elicits DR-12-EK staining in the cell body, with material entering neurite processes and accumulating in terminals (Fig. 1*D*). In contrast, and similar to 3-VCAT-3-Cys67stop transgenic rats (12, 13), the truncated protein encoded by Ad-VCAT-Cys67stop is confined to the cell body, often within enlarged vacuolar compartments (Fig. 1*D*).

We then assessed the subcellular localization of the Ad-VCAT- and Ad-VCAT-Cys67stop-encoded proteins using dual-labeling immunocytochemistry (Fig. 2). Wild-type VP precursor is targeted to the regulatory secretory pathway, and although it passes through the ER and TGN, there is little accumulation therein. Similar, DR-12-EK immunoreactivity is not found in quantity in the ER nor the trans-Golgi network (TGN, Fig. 2). In contrast, and as in the 3-VCAT-3-Cys67stop transgenic rats (6, 7), Cys67stop protein appears to accumulate in ER-derived elements, as revealed by substantial colocalization with calnexin (Fig. 2) and protein disulphide isomerase (PDI; Fig. 2). Some Cys67stop protein also appears to be contained in the TGN (Fig. 2). Importantly, there is marked colocalization of the Cys67stop protein with the lysosomal marker cathepsin D (Fig. 2). These data suggest that, in Neuro2a cells, as in the MCNs of

transgenic rats, the mutant Cys67stop protein accumulates in the ER, and in acidic degradative compartments, perhaps associated or derived from the TGN.

Cys67stop expression triggers autophagy

Monodansylcadaverine (MDC), a spontaneously fluorescent dye, selectively labels autophagosomes and autolysosomes, and is therefore considered a valuable tool to morphologically monitor the autophagic process (19). In noninfected and Ad-VCAT-infected Neuro2a cells, MDC staining revealed a diffuse punctate pattern (Fig. 2), which probably is indicative of basal, constitutive autophagy. In contrast, Ad-VCAT-Cys67stop infection results in the accumulation of large MDC-stained vesicles (Fig. 3*A*), suggesting up-regulation of autophagy. This was supported by the examination of expression of Beclin-1, the mammalian analog of yeast autophagy gene Atg6, a regulator of the autophagic pathway that associates with apoptosis inhibitor bcl-2 (24, 25). Western blot analysis revealed significant up-regulation of Beclin 1 in Ad-VCAT-Cys67stop-infected cells (Fig. 3*B* and 3*C*), consistent with an up-regulation of autophagic processes.

We then characterized the intracellular distribution of key regulators of autophagy (26), comparing 3-VCAT-3 and 3-VCAT-3-Cys67stop transgenic rats with Neuro2a cells infected with Ad-VCAT or Ad-VCAT-Cys67stop. We examined Beclin 1, the class III phosphatidylinositol 3-kinase (PI3K) Vps34, and Rab24. Beclin-1 interacts with Vps34, and, together, these molecules control autophagy as a complex at the TGN (27, 28). The GTPase Rab24 is thought to be involved in the regulation of vesicular transport associated with autophagy (29). In 3-VCAT-3 transgenic rats (Fig. 4*A*) and in Ad-VCAT-infected cells (Fig. 4*B*), Beclin 1, Vps34 and Rab24 show a typical perinuclear reticular localization. There is no colocalization with DR-12-EK-like immunoreactivity. In contrast, expression of the Cys67stop protein, either in 3-VCAT-3-Cys67stop transgenic rats (Fig. 4*A*) or following infection of Neuro2a cells with the Ad-VCAT-Cys67stop viral vector (Fig. 4*B*), dramatically alters the distribution of these markers, which now strongly localize with either the Cys67stop protein or the ER marker PDI in large vesicles.

Inhibition of the autophagic-lysosomal pathway in Cys67stop-expressing cells induces apoptosis

We then asked whether expression of the Cys67stop mutant protein and activation of autophagy in Neuro2a cells affected cell viability. On its own, Ad-VCAT-Cys67 had no effect on the viability of Neuro2a cells as assessed by visual microscopic examination (not shown), cell growth assessed by counting (Fig. 5*A*) or the incorporation of trypan blue (Fig. 5*B*) over a time course of 2 (Fig. 5) or 8 days (not shown). However, treatment of cells infected for 2 days with the autophagy inhibitors 3MA or Asn, or the cathepsin D inhibitor Pst, dramatically induced cell death in Ad-VCAT-Cys67stop-, but not Ad-VCAT-, infected cultures, as assessed visually (Fig. 6*A*), by cell counting (Fig. 6*B*) and by trypan blue staining (Fig. 6*C*), suggesting that the degradation of mutant protein in lysosomes following autophagic delivery is a prosurvival mechanism.

To ascertain whether the cell death induced by the inhibition of autophagic-lysosomal protein degradation in Ad-VCAT-Cys67stop infected Neuro2a cells was classically apoptotic in nature,

we used flow cytometry to quantify cell populations labeled with either FITC-annexin V, to identify early apoptotic cells (20, 21), or PI, to identify the subG1 hypodiploid population (20). Inhibition of the autophagic-lysosomal pathway with 3MA, Asn, or Pst had no effect on Annexin-V binding in Neuro2a cells infected with Ad-VCAT (Fig. 7A). However, blockade of the autophagy-lysosomal pathway in Cys67stop expressing cells doubled the association of FITC-Annexin V with the surface phosphatidylserine (Fig. 7*A*), indicative of early apoptosis. Similarly, treatment of Ad-VCAT-Cys67stop-infected Neuro2a cells with Asn or Pst significantly increased the subG1 population, while having no effect on Ad-VCAT-infected cells (Fig. 7*B*). The presence of characteristic chromatin alterations was also evidenced by DAPI staining (20), which revealed nuclear condensation and fragmentation in Asn- and Pst-treated Ad-VCAT-Cys67stop infected, but not Ad-VCAT-infected cells (Fig. 7*C*).

To demonstrate that the cell death prompted by the blockade of autophagy and lysosome degradative pathways is indeed classical apoptosis, we assessed cytochrome c release from mitochondria (Fig. 7*D*) and caspase activation (Fig. 7*E*). Asn and Pst treatment resulted in a reduction in mitochondrial integrity, as assessed by cytochrome c immunocytochemistry (23; Fig. 7*D*) in Ad-VCAT-Cys67stop-infected, but not Ad-VCAT-infected cells. Further, 3MA, Asn- and Pst-treatments all activated caspases (23) in Ad-VCAT-Cys67stop-infected Neuro2a cells, but not in Ad-VCAT-infected cells (Fig. 7*E*).

DISCUSSION

We have demonstrated that the expression of an adFNDI mutant VP transgene induces autophagy in transgenic rat neurons (6, 7). However, the role of autophagy remains unclear (30). While it is not difficult to imagine that autophagy, a process involving massive intracellular degradation, might promote neuronal death (31), this is contradicted by the similarly axiomatic concept that mammalian autophagy, as in yeast (32), is a prosurvival mechanism. To address the relationships between mutant protein accumulation, autophagy, cell survival, and cell death, we have efficiently delivered an adFNDI mutant protein (Cys67stop), or an epitope-tagged wild-type VP precursor, into mouse neuroblastoma Neuro2a cells using Ad vectors. While the wild-type material enters neurite processes and accumulates in terminals, the Cys67stop protein is confined to enlarged vesicles in the cell body, that appear to be of ER origin, and that are subsequently targeted for auophagic degradation. The partial colocalization of the Cys67stop protein with the TGN marker TGN38 (Fig. 2) is of interest given the role of this structure in the origin of autophagososmes. Indeed, it is known that autophagy can be triggered by a Beclin 1-phosphatidylinositol 3-kinase complex that functions at the trans-Golgi network (28)

Cys67stop expression had no effect on cell viability, but inhibition of autophagy or lysosomal protein degradation significantly increased apoptotic cell death. 3MA, Asn, and Pst, which affect the autophagic process at different steps (autophagosome formation, autolysosome formation and protein degradation, respectively), all exerted the same effect on the viability of Ad-VCAT-Cys67stop expressing cells. This indicates that once the mutant protein in the ER has triggered a signal to initiate autophagy, the process cannot be arrested. Our data with Pst also indicates that cathepsin D is a main protease involved in autophagic degradation of the mutant VP. Thus we suggest that the activation of autophagy by the cellular stress of the expression of an adFNDI mutant protein gene is a prosurvival mechanism. By analogy, we can hypothesize that the activation of autophagy seen in Parkinson's, Huntington's, and Alzheimer's neurons (10–12)

may also have a prosurvival role, at least in the early stages of these diseases (33). Indeed, recent evidence suggests that, as in our adFNDI model, the induction of autophagy in Huntington's disease decreases the accumulation (34) and toxicity (35) of polyglutamine aggregates.

We have previously speculated as to the role of autophagy in the etiology of adFNDI (6, 7). We have suggested that the accumulation of mutant Cys67stop protein in the ER causes insoluble aggregates to form. This results in the development of a pathology characterized by a grossly deranged ER that accumulates both mutant and trapped wild-type protein. Autophagy, acting as a cell survival mechanism, removes the deranged structures. Wild-type prohormone will be eliminated when the deranged organelle is destroyed, resulting in progressive VP deficiency. However, this hypothesis fails to explain the paucity of VP neurons seen in the hypothalami of some adFNDI patients (36–39). Similarly, a recent report (40) has described Cys67stop "knock-in" mice exhibiting a progressive loss of VP-expression, although no direct evidence of neuronal death was presented. We suggest that neuronal atrophy, although not necessarily the primary cause of adFNDI, might be a long-term consequence. The progressive reduction of VP secretion caused by autophagy would lead to a chronic increase in plasma osmolality. Consequent chronic over-stimulation of VP neurons might trigger a pathologic sequence of neurotoxic events that ultimately lead to apoptosis, as has been observed in a rat diabetes mellitus model (41). Whether autophagy has a role in long-term death, as has been hypothesized (33), remains to be determined. In this context, we note that the expression in transgenic mice of exon I of the human Huntington's disease gene carrying a CAG repeat expansion had no effect on the death of cultured striatal neurons (42). However, transgenic neurons exposed to neurotoxic concentrations of dopamine exhibited elevated cell death compared with wild-type neurons. The mutant neurons exposed to dopamine also exhibited lysosome-associated responses, including induction of autophagic vacuoles. We thus hypothesize that frail neurons, already undergoing autophagy in order to clear mutant proteins, might be more prone to the toxic effects of a second stress, and we are presently using our Ad-mediated gene transfer system to investigate the possibility that autophagy plays such a role in long-term neurodegeneration.

An interesting and thus far unexplored avenue of research will relate to the cell "rounding" and neurite withdrawal phenomenon that we have observed following Cys67stop expression in vitro. It is interesting to note that a common feature of human FNDI is the disappearance of the characteristic posterior pituitary magnetic resonance imaging bright spot (e.g., 43). Although this has often been taken to indicate cell death (43), it has actually been shown to relate to a lack of accumulated VP gene products in axon terminals (44). Could it be that this is a consequence of VP neuron axon withdrawal or degeneration in the absence of cell body death (see, 45)? The implications of this possibility from the point of view of the etiology of FNDI, and perhaps other neurodegenerative diseases, are indeed profound.

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REFERENCES

1. Reeves, W. B., and Andreoli, T. E. (1992) The posterior pituitary and water metabolism. In *Williams Textbook of Endocrinology*, Wilson, J. D., and Foster, D. W., eds, Philadelphia: W. B. Saunders, pp. 311-356,

2. Burbach, J. P. H., Luckman, S. M., Murphy, D., and Gainer, H. (2001) Gene regulation in the magnocellular hypothalamo-neurohypophyseal system. *Phys. Rev.* **81,** 1197–1267

3. de Bree, F. M., Knight, D., and Murphy, D. (2000) Sorting of the vasopressin prohormone into the regulated secretory pathway. *FEBS Lett.* **475,** 175–180

4. Brownstein, M. J., Russell, J. T., and Gainer, H. (1980) Synthesis, transport, and release of posterior pituitary hormones. *Science* **207,** 373–378

5. Christensen, J.H., Siggaard, C., and Rittig, S. (2003) Autosomal dominant familial neurohypophyseal diabetes insipidus. *APMIS Suppl 109* **111,** 92-95

6. Si-Hoe, S.-L., de Bree, F. M., Nijenhuis, M., Davies, J. E., Howell, L. M. C., Tinley, H., Waller, S. J., Zeng, Q., Zalm, R., Sonnemans, M., et al. (2000) Endoplasmic reticulum derangement in hypothalamic neurons of rats expressing a familial neurohypophyseal diabetes insipidus mutant vasopressin transgene. *FASEB J. Express* 10.1096/fj.99-0892fje (http://www.fasebj.org/cgi/reprint/99-0892fjev1). *FASEB J.* **14,** 1680–1684

7. Davies, J., and Murphy, D. (2002) Autophagy in hypothalamic neurons of rats expressing a familial neurohypophysial diabetes insipidus transgene. *J. Neuroendocrinol.* **14,** 629–637

8. Yoshimori, T. (2003) Autophagy: a regulated bulk degradation process inside cells. *Biochem. Biophys. Res. Commun.* **313,** 453–458

9. Mizushima, N., Ohsumi, Y., and Yoshimori, T. (2002) Autophagosome formation in mammalian cells. *Cell Struct. Funct.* **27,** 421–429

10. Anglade, P., Vyas, S., Javoy-Agid, F., Herrero, M. T., Michel, P. P., Marquez, J., Mouatt-Prigent, A., Ruberg, M., Hirsch, E. C., and Agid, Y. (1997) Apoptosis and autophagy in nigral neurons of patients with Parkinson's disease. *Histol. Histopathol.* **12,** 25–31

11. Nixon, R. A., Cataldo, A. M., and Mathews, P. M. (2000) The endosomal-lysosomal system of neurons in Alzheimer's disease pathogenesis: a review. *Neurochem. Res.* **25,** 1161–1172

12. Kegel, K. B., Kim, M., Sapp, E., McIntyre, C., Castano, J. G., Aronin, N., and DiFiglia, M. (2000) Huntingtin expression stimulates endosomal-lysosomal activity, endosome tubulation, and autophagy. *J. Neurosci.* **20,** 7268–7278

13. Hardy, S., Kitamura, M., Harris-Stansil, T., Dai, Y., and Phipps, M. L. (1997) Construction of adenovirus vectors through Cre-lox recombination. *J. Virol.* **71,** 1842–1849

14. Harding, T. C., Geddes, B. J., Noel, J. D., Murphy, D., and Uney, J. (1997) Tetracycline-regulated transgene expression in hippocampal neurons following transfection with adenoviral vectors. *J. Neurochem.* **69,** 2620–2623

15. Seglen, P. O. and Gordon, PB. (1982) 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. *Proc. Natl. Acad. Sci. USA* **79.** 1889-1892

16. Hoyvik, H., Gordon, P. B., Berg, T. O., Stromhaug, P. E., and Seglen, P. O. (1991) Inhibition of autophagic-lysosomal delivery and autophagic lactolysis by asparagine. *J. Cell Biol.* **113,** 1305–1312

17. Knight, C. G., and Barrett, A. J. (1975) Interaction of human cathepsin D with the inhibitor pepstatin. *Biochem. J.* **155,** 117–125

18. Waller, S., Fairhall, K. M., Xu, J., Robinson, I. C. A. F., and Murphy, D. (1996) Neurohypophyseal and fluid homeostasis in transgenic rats expressing a tagged rat vasopressin prepropeptide in vasopressinergic magnocellular neurons. *Endocrinology* **137,** 5068–5077

19. Biederbick, A., Kern, H. F., and Elsasser, H. P. (1995) Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. *Eur. J. Cell Biol.* **66,** 3–14

20. Castino, R., Pace, D., Demoz, M., Gargiulo, M., Ariatta, C., Raiteri, E., and Isidoro, C. (2002) Lysosomal proteases as potential targets for the induction of apoptotic cell death in human neuroblastomas. *Int. J. Cancer* **97,** 775–779

21. Martin, S. J., Reutelingsperger, C. P., McGahon, A. J., Rader, J. A., van Schie, R. C., LaFace, D. M., and Green, D. R. (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis, regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J. Exp. Med.* **182,** 1545–1556

22. Demoz, M., Castino, R., Cesaro, P., Baccino, F. M., Bonelli, G., and Isidoro, C. (2002) Endosomal-lysosomal proteolysis mediates death signalling by TNFalpha, not by etoposide, in L929 fibrosarcoma cells: evidence for an active role of cathepsin D. *Biol. Chem.* **383,** 1237–1248

23. Chen, Q., Chai, Y., Mazuder, S., Drazba, J., Chisolm, G., and Almasan, A. (2001) The late increase of free radicals during genotoxic-stress induced apoptosis is associated with cytochrome c release from mitochondria induced by caspase-mediated feedback loop amplification. *Scientific World Journal* **1,** 142–153

24. Liang, X. H., Kleeman, L. K., Jiang, H. H., Gordon, G., Goldman, J. E., Berry, G., Herman, B., and Levine, B. (1998) Protection against fatal Sindbis virus encephalitis by beclin, a novel Bcl-2-interacting protein. *J. Virol.* **72,** 8586–8596

25. Liang, X. H., Jackson, S., Seaman, M., Brown, K., Kempkes, B., Hibshoosh, H., and Levine, B. (1999) Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* **402,** 672–676

26. Petiot, A., Pattingre, S., Arico, S., Meley, D., and Codogno, P. (2002) Diversity of signalling controls of macroautophagy in mammalian cells. *Cell Struct. Funct.* **27,** 431–441

27. Petiot, A., Ogier-Denis, E., Blommaart, E. F., Meijer, A. J., and Codogno, P. (2000) Distinct classes of phosphatidylinositol 3′-kinases are involved in signaling pathways that control macroautophagy in HT-29 cells. *J. Biol. Chem.* **275,** 992–998

28. Kihara, A., Kabeya, Y., Ohsumi, Y., and Yoshimori, T. (2001) Beclin- phosphatidylinositol 3-kinase complex functions at the trans-Golgi network. *EMBO Rep.* **2,** 330–335

29. Munafo, D. B., and Colombo, M. I. (2002) Induction of autophagy causes dramatic changes in the subcellular distribution of GFP-Rab24. *Traffic* **3,** 472–482

30. Yuan, J., Lipinski, M., and Degterev, A. (2003) Diversity in the mechanisms of neuronal cell death. *Neuron* **40,** 401–413

31. Lockshin, R. A., and Zaken, Z. (2002) Caspase-independent cell deaths. *Curr. Opin. Cell Biol.* **14,** 727–733

32. Ohsumi, Y. (2001) Molecular dissection of autophagy: two ubiquitin-like systems. *Nat. Rev. Mol. Cell Biol.* **2,** 211–216

33. Maria Cuervo, A. (2004) Autophagy: in sickness and in health. *Trends Cell Biol.* **14,** 70–77

34. Ravikumar, B., Duden, R., and Rubinsztein, D. C. (2002) Aggregate prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. *Hum. Mol. Genet.* **11,** 1107–1117

35. Ravikumar, B., Vacher, C., Berger, Z., Davies, J. E., Luo, S., Oroz, L. G., Scaravilli, F., Easton, D. F., Duden, R., O'Kane, C. J., et al. (2004) Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nat. Genet.* **36,** 585–595

36. Braverman, L. E., Mancini, J. P., and McGoldrick, D. M. (1965) Hereditary idiopathic diabetes insipidus: A case report with autopsy findings. *Ann. Intern. Med.* **63,** 503–508

37. Green, J. R., Buchan, G. C., Alvord, E. C. J., and Swanson, A. G. (1967) Hereditary and idiopathic types of diabetes insipidus. *Brain* **90,** 707–714

38. Nagai, I., Li, C. H., Hsieh, S. M., Kizaki, T., and Urano, Y. (1984) Two cases of hereditary diabetes insipidus, with an autopsy finding in one. *Acta Endocrinol.* **105,** 318–323

39. Bergeron, C., Kovacs, K., Ezrin, C., and Mizzen, C. (1991) Hereditary diabetes insipidus: an immunohistochemical study of the hypothalamus and pituitary gland. *Acta Neuropathol* **81,** 345–348

40. Russell, T. A., Ito, M., Ito, M., Yu, R. N., Martinson, F. A., Weiss, J., and Jameson, L. J. (2003) A murine model of autosomal dominant neurohypophyseal diabetes insipidus reveals progressive loss of vasopressin-producing neurons. *J. Clin. Invest.* **112,** 1697–1706

41. Klein, J. P., Hains, B. C., Craner, M. J., Black, J. A., and Waxman, S. G. (2004) Apoptosis of vasopressinergic hypothalamic neurons in chronic diabetes mellitus. *Neurobiol. Dis.* **15,** 221–228

42. Petersen, A., Larsen, K. E., Behr, G. G., Romero, N., Przedborski, S., Brundin, P., and Sulzer, D. (2001) Expanded CAG repeats in exon 1 of the Huntington's disease gene stimulate dopamine-mediated striatal neuron autophagy and degeneration. *Hum. Mol. Genet.* **10,** 1243–1254

43. Mahoney, C. P., Weinberger, E., Bryant, C., Ito, M., Jameson, L. J., and Ito, M. (2002) Effect of aging on vasopressin production in a kindred with autosomal dominant neurohypophyseal diabetes insipidus due to the DeltaE47 mutation. *J. Clin. Endocrinol. Metab.* **87,** 870–876

44. Kurokawa, H., Fujisawa, I., Nakano, Y., Kimura, H., Akagi, K., Ikeda, K., Uokawa, K., and Tanaka, Y. (1998) Posterior lobe of the pituitary gland: correlation between signal intensity on T1-weighted MR images and vasopressin concentration. *Radiology* **207,** 79–83

45. Raff, M. C., Whitmore, A. V., and Finn, J. T. (2002) Axonal self-destruction and neurodegeneration. *Science* **296,** 868–871

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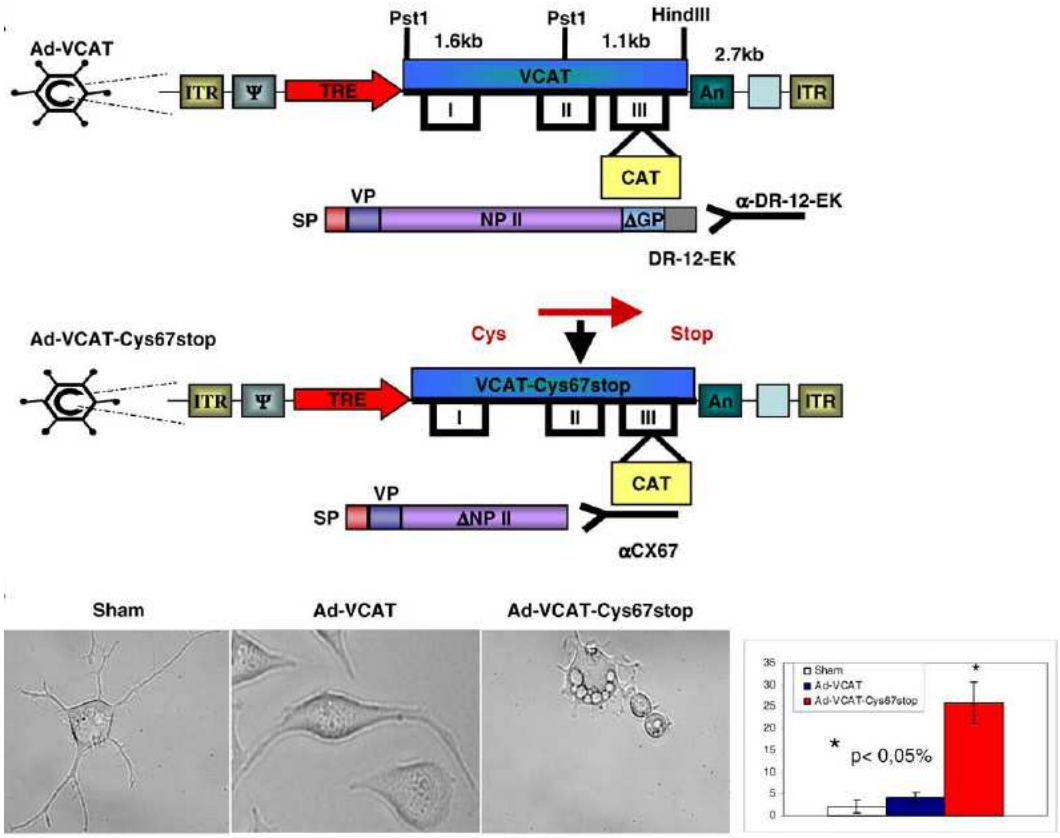
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NP II
ΔGP
α-DR-12-EK
DR-12-EK
Ad-VCAT-Cys67stop
Cys
Stop
VCAT-Cys67stop
ΔNP II
αCX67
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Ad-VCAT
Ad-VCAT-Cys67stop
p< 0,05%

Fig. 1 (cont)

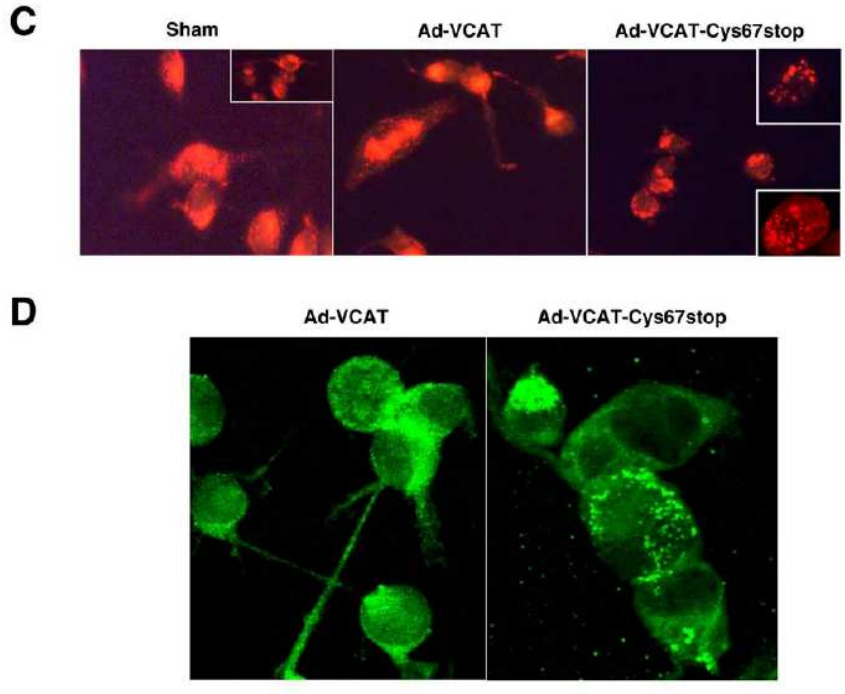
C
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Figure 1. An in vitro model of FNDI. ***A***) Schematic diagram of the Ad-VCAT and Ad-VCAT-Cys67stop viral vectors described in this study and the proteins that they encode. VCAT encompasses the three exons (I, II, and III) of the rat VP structural gene containing a chloramphenicol acetyl transferase (CAT) reporter in exon III. VCAT encodes a modified VP precursor consisting of the signal peptide (SP) from the N terminus; the VP hormone, neurophysin (NP II), a truncated glycoprotein (ΔGP); and a unique hexadecapeptide (DRSAGYYGLFKDRKEK, abbreviated to DR-12-EK) at the C terminus (18). DR-12-EK is recognized by specific antisera (αDR-12-EK; 18). In the VCAT-Cys67stop transgene, a C to A transition in Exon II replaces a Cys (TGC) at position 67 of NP-II with a translation termination codon (TGA). The transgene encodes a mutant truncated prepropeptide lacking the C terminus of the neurophysin II (ΔNP II), and all of the C-terminal glycopeptide. This is specifically recognized by antisera αCX67 (6, 7). ITR, inverted terminal repeats; TRE, tetracycline responsive element; A_n, transcription termination and polyadenylation signal. ***B***) While infection of Neuro2a cells with Ad-VCAT has no effect on morphology, Ad-VCAT-Cys67stop causes cell rounding and neurite withdrawal associated with pronounced intracellular inclusions, as viewed under phase contrast microscopy. The graph to the right shows the quantification of the cell-rounding effect. Cells were counted under phase contrast microscopy, and the percentage of cells with a "round" phenotype is reported as a percentage of the total. ***C***) Cells plated on cover slips were incubated with AO, a fluorochrome that accumulates within acid organelles and that, once protonated, emits red light under UV excitation. In noninfected and Ad-VCAT-infected cells, the dye reveals a diffuse punctate stain throughout the cytoplasm. In contrast, Ad-VCAT-Cys67stop infection caused the accumulation and the redistribution in the perinuclear region of the acid compartments, which appear enlarged. ***D***) Cells grown on cover slips were incubated with primary antibody at the appropriate dilution and then with a specific FITC- and TRITC-labeled secondary antibody. Ad-VCAT expression elicits DR-12-EK staining in the cell body, with material entering neurite processes and accumulating in terminals. In contrast the truncated protein encoded by Ad-VCAT-Cys67stop is confined to the cell body, often within enlarged vacuolar compartments.

Fig. 3

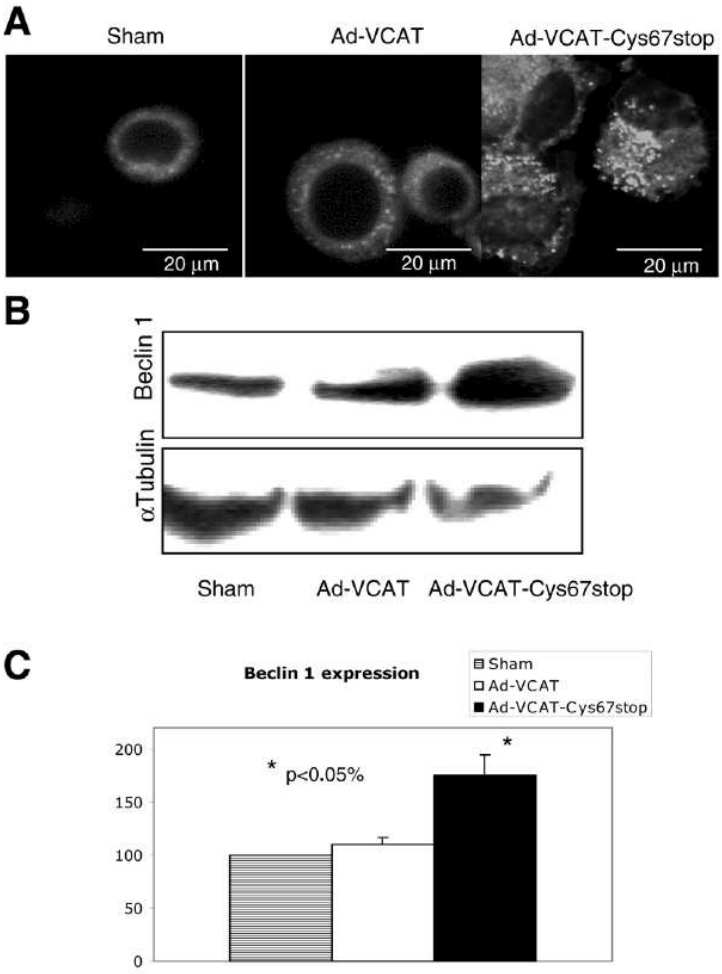

Figure 3. Expression of mutant Cys67stop VP protein triggers autophagy. ***A***) Neuro2a cells infected with Ads for 24 h were incubated with MDC then analyzed by fluorescence microscopy. In noninfected and Ad-VCAT-infected Neuro2a cells, MDC reveals diffuse punctate pattern. In contrast, Ad-VCAT-Cys67stop infection results in the accumulation of large MDC-stained autophagosomes. Infection for 6 or 48 h revealed the same pattern (not shown). ***B***) Extracts of uninfected (sham) Neuro2a cells, or Neuro2a cells infected with either Ad-VCAT or Ad-VCAT-Cys67stop for 24 h, were fractionated by PAGE, then electroblotted onto PDVF membrane. Beclin 1 was detected by chemiluminescence reaction using a specific polyclonal antiserum followed by horseradish peroxidase-conjugated secondary antibody. The membrane was then stripped and reincubated with a specific monoclonal antibody against β-tubulin as a normalization control. ***C***) Quantification of 3 separate experiments revealed significant up-regulation of Beclin 1 expression following Ad-VCAT-Cys67stop infection.

Fig. 4

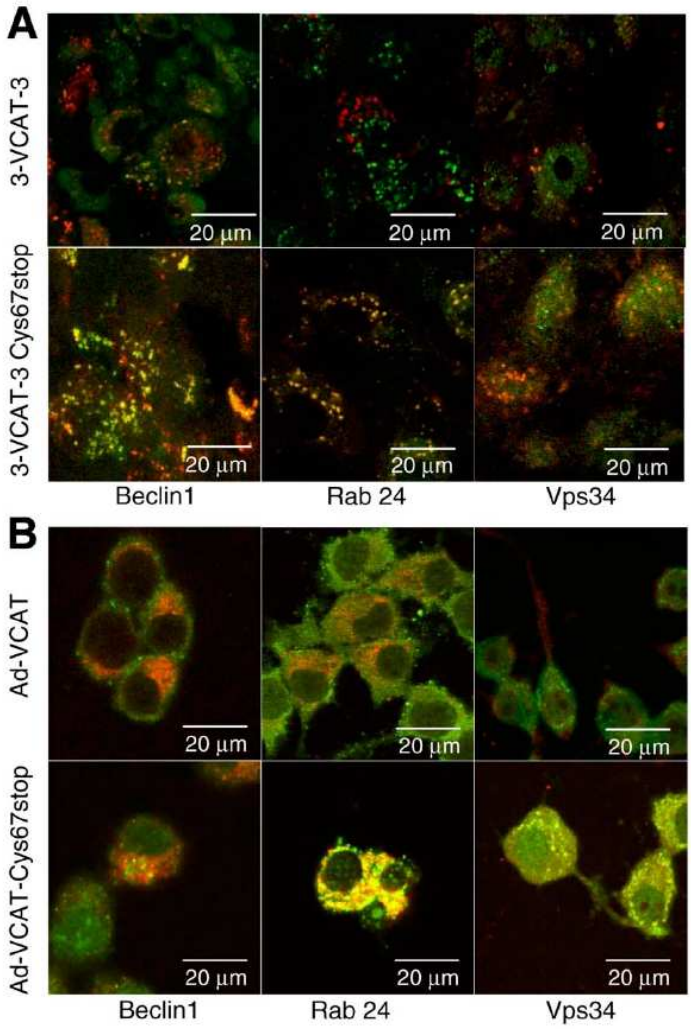

Figure 4. Colocalization of transgene proteins with autophagy markers in (*A*) hypothalamic (supraoptic nucleus) neurons of 3-VCAT-3 and 3-VCAT-3-Cys67stop transgenic rats or (*B*) Neuro2a cells infected with Ad-VCAT or Ad-VCAT-Cys67stop for 48 h. In 3-VCAT-3 transgenic rats or Ad-VCAT-infected Neuro2a cells, there is little colocalization of Beclin-1 (red) with PDI (green), or of Rab24 or Vps34 (red) with DR-12-EK-like immunoreactivity (green). In contrast, in 3-VCAT-3-Cys67stop transgenic rats, or following infection of Neuro2a cells with Ad-VCAT-Cys67stop, the distribution of these markers becomes vesicular, with marked colocalization with either Cys67stop or PDI.

Fig. 5

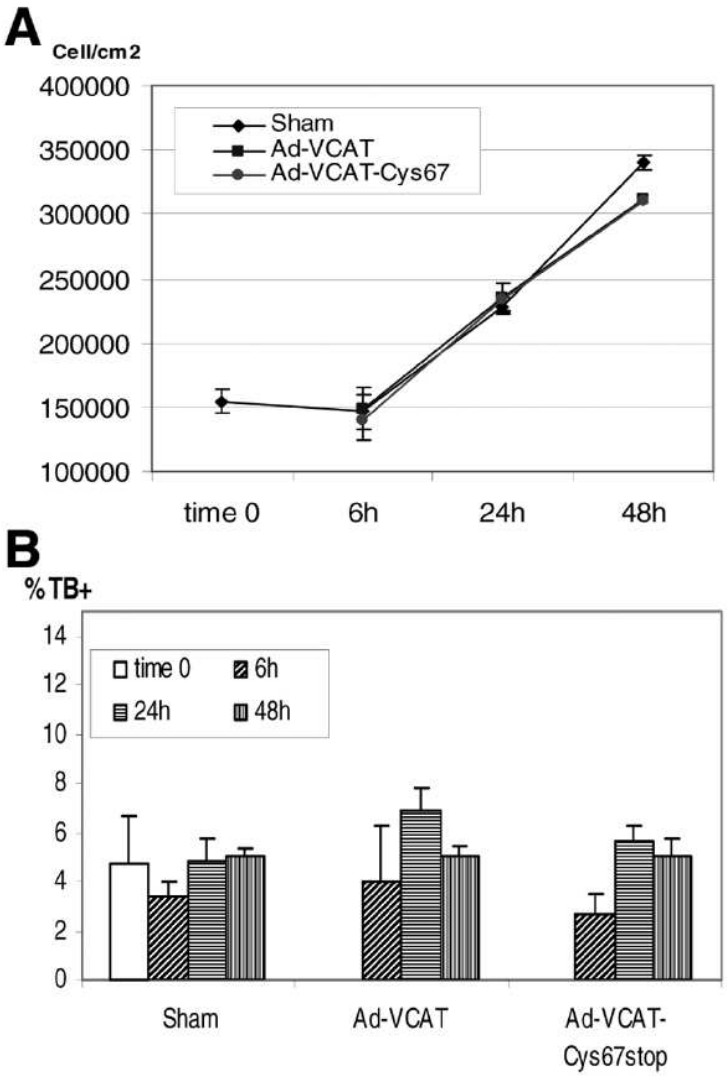

Figure 5. Neither Ad-VCAT nor Ad-VCAT-Cys67 infection affect the viability of Neuro2a cells. Neuro2a cells were infected with either Ad-VCAT or Ad-VCAT-Cys67stop, or were sham-infected. At the times indicated, cell growth was assessed by counting using a haemocytometer (*A*) or the incorporation of trypan blue (*B*) to quantify dead cells.

Fig. 6

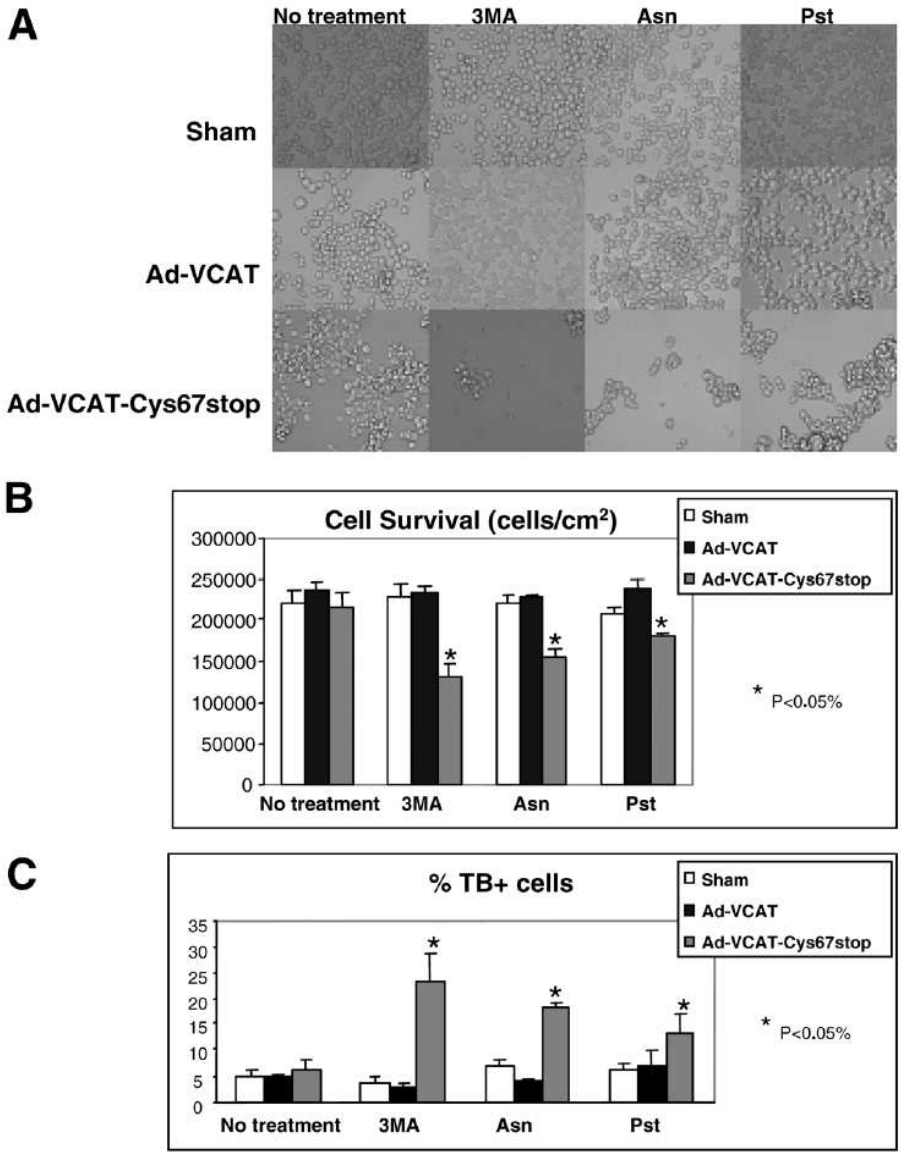

Figure 6. Inhibition of the autophagic-lysosomal pathway in Ad-VCAT-Cys67stop-infected cells induces cell death. Simultaneous treatment of cells infected with Ads for 36 h with the autophagy inhibitors Asn and 3MA or with the inhibitor of lysosomal cathepsin D, Pst, dramatically induced cell death in Ad-VCAT-Cys67stop, but not Ad-VCAT, infected cultures, as assessed by phase contrast microscopy (***A***) cell counting (***B***) and trypan blue staining (***C***).

Fig. 7

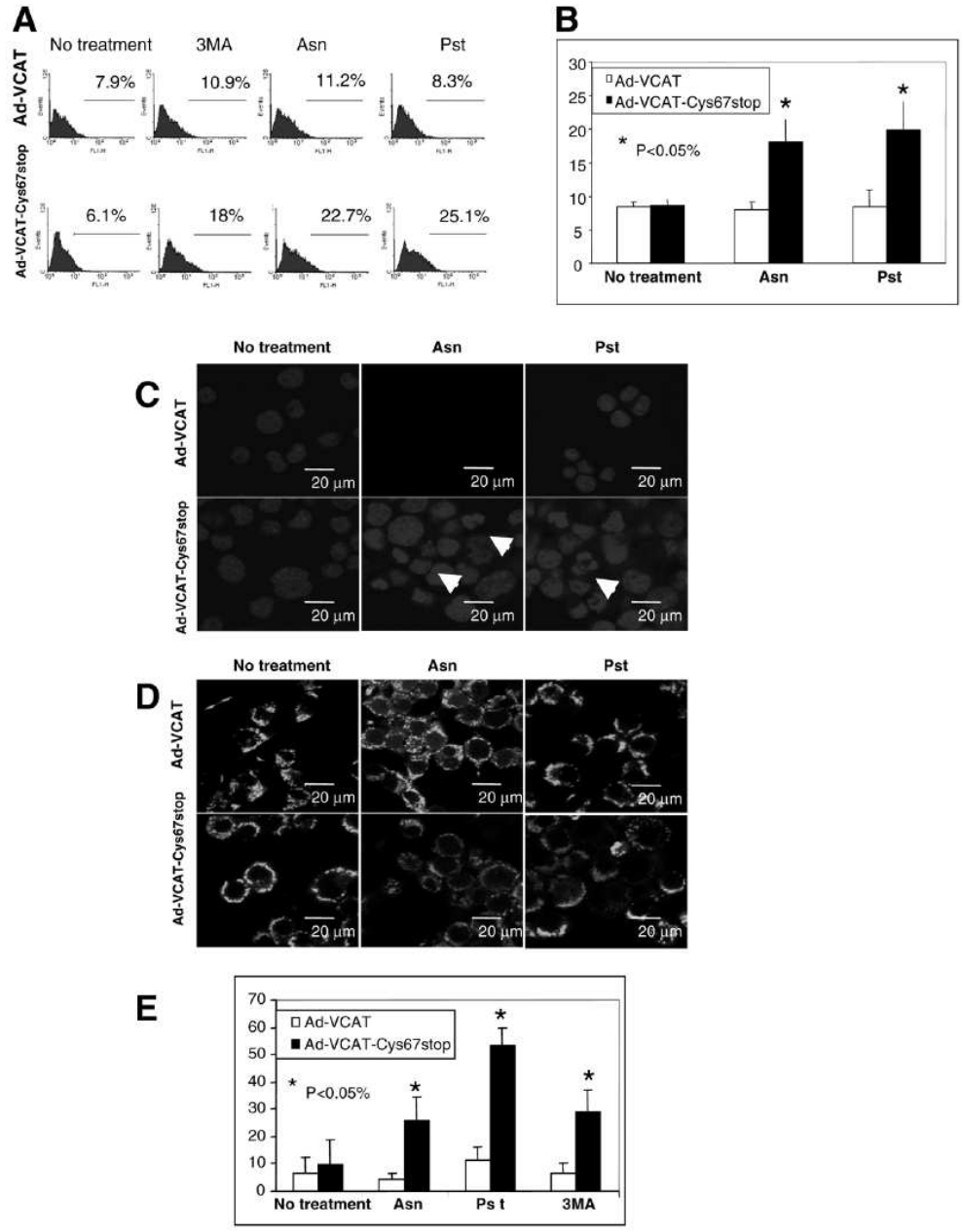
A
No treatment
3MA
Asn
Pst
Ad-VCAT
7.9%
10.9%
11.2%
8.3%
Ad-VCAT-Cys67stop
6.1%
18%
22.7%
25.1%
B
Ad-VCAT
Ad-VCAT-Cys67stop
* P<0.05%
No treatment
Asn
Pst
C
No treatment
Asn
Pst
Ad-VCAT
Ad-VCAT-Cys67stop
20 μm
D
No treatment
Asn
Pst
Ad-VCAT
Ad-VCAT-Cys67stop
20 μm
E
Ad-VCAT
Ad-VCAT-Cys67stop
* P<0.05%
No treatment
Asn
Ps t
3MA

Fig. 7 (cont)

Figure 7. Inhibition of the autophagic-lysosomal pathway in cells expressing the Cys67stop protein leads to apoptosis. Cells were infected with Ads and treated with inhibitors for 36 h. ***A*)** Cytofluorometric detection of cells expressing cell-surface Annexin V binding sites. Treatment of Ad-VCAT-Cys67stop-infected Neuro2a cells, but not Ad-VCAT-infected cells, with inhibitors of the autophagic-lysosomal pathway increases the percentage of Annexin V-positive cells. ***B*)** The appearance of a hypodiploid (subG1) cell population was monitored by flow cytometry on the whole cell population (i.e., monolayer plus cells recovered from the medium). Treatment with inhibitors of the autophagic-lysosomal pathway significantly increased the subG1 population following infection of Neuro2a cells with Ad-VCAT-Cys67stop, but not with Ad-VCAT. ***C*)** Chromatin alterations were demonstrated by the appearance of condensed and fragmented nuclei as revealed by DAPI staining. Arrows show fragmented cells with condensed DNA. ***D*)** Release of cytochrome *c* by permeabilized mitochondria revealed by immunofluorescence using a specific monoclonal antibody. ***E*)** Activation of caspases was demonstrated by fluorescent staining of adherent cells with FITC-VAD-FMK. Labeled cells were observed and counted under a fluorescence microscope and further analyzed by flow cytometry. The graph shows that, in the presence of 3MA, Asn, or Pst, there is a significant increase in the percentage of activated caspase-positive cells following Ad-VCAT-Cys67stop, but not Ad-VCAT infection.

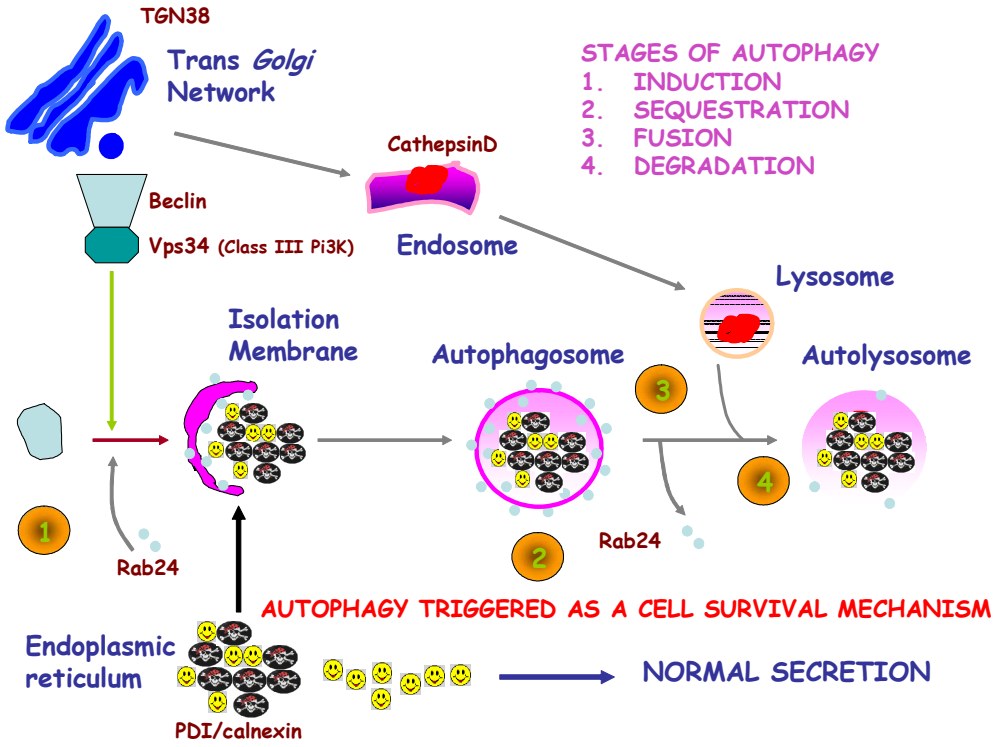

Fig. 14. Schema interpretativo

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Autophagy-dependent cell survival and cell death in an autosomal dominant familial neurohypophyseal diabetes insipidus in vitro model

Roberta Castino,*,† Ciro Isidoro,† and David Murphy*

*Molecular Neuroendrocrinology Research Group, Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology, Bristol, England; and †Amedeo Avogadro University, Laboratory of Molecular Pathology, Department of Medical Sciences, Novara, Italy

Corresponding author: David Murphy, Henry Wellcome Laboratories for Integrative Neuroscience and Endocrinology, Dorothy Hodgkin Building, Whitson Street, Bristol BS1 3NY, England. E-mail: d.murphy@bristol.ac.uk

ABSTRACT

Mutations in the human gene encoding the antidiuretic hormone vasopressin (VP) cause autosomal dominant familial neurohypophyseal diabetes insipidus (adFNDI), a rare inherited disorder that presents as polydipsia and polyuria as a consequence of a loss of secretion of VP from posterior pituitary nerve terminals. Work from our laboratories has shown that adFNDI, like other neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's, is associated with autophagy. We have recently shown that the activation of autophagy in mouse neuroblastoma Neuro2a cells after adenoviral vector-mediated delivery of an adFNDI mutant VP transgene (Cys67stop) is a cell survival mechanism; its inhibition induces apoptosis. We now show that expression of Cys67stop sensitizes Neuro2a cells to the lethal effects of dopamine. This mode of cell death exhibits features typically associated with classical apoptosis. Yet inhibition of autophagy reversed these effects and rescued cell viability. We propose that autophagy-mediated cell death is a "two-hit" process: Following the cellular stress of the accumulation of a misfolded mutant protein, autophagy is prosurvival. However, a second insult triggers an autophagy-dependent apoptosis.

Key words: vasopressin • apoptosis • dopamine • adFNDI

Osmotic homeostasis is aggressively defended in mammalian organisms. A crucial mediator of osmotic stability is the neuropeptide antidiuretic hormone vasopressin (VP) (1). VP is synthesized as part of a prepropeptide precursor in the cell bodies of hypothalamic neurons involved in osmoregulation (2). Following signal peptide removal and disulphide bond formation in the endoplasmic reticulum (ER), the propeptide is sorted (3) and packaged into dense core granules of the regulated secretory pathway in the trans-Golgi network (TGN) (3, 4). Further processing and maturation events occur as the granules are transported from the cell body to storage in nerve terminals in the posterior pituitary (4). Here, the mature peptide is stored until mobilized for secretion into the circulation by electrical activities evoked

by hyperosmolality. VP travels through the bloodstream to specific receptor targets located in the kidney, where it increases the permeability of the collecting ducts to water, reducing the renal excretion of water, and promoting water conservation.

VP-mediated osmotic homeostasis breaks down in familial neurohypophyseal diabetes insipidus (adFNDI) (5), an autosomal dominant disorder caused by mutations in the vasopressin (VP) gene (6, 7). adFNDI presents as excessive drinking and urination as a consequence of a progressive loss of secretion of VP from posterior pituitary nerve terminals (5). Carriers are born normal, and the disease develops during childhood, despite the presence of a normal allele. A limited number of autopsy examinations have described a paucity of VP neurons in the hypothalami of adFNDI patients (8–11). Similarly, a recent report has shown that "knock-in" mice expressing an adFNDI mutant VP gene exhibit a progressive loss of VP-expression (12). These data suggest that progressive degeneration might be involved in the pathogenesis of adFNDI.

Neurodegeneration and neuronal cell death underlie the symptoms of many neurological diseases (13). The best-understood mechanism of neuronal cell death, classically defined as "apoptosis," is mediated via caspase 9 activation and a mitochondrial route, or directly via caspase 8 to the primary effector, caspase 3 (14, 15). However, it is now recognized that other cell death pathways exist, including autophagy (15), a bulk process that delivers regions of cytoplasm, or whole organelles, to lysosomes for degradation and recycling (16). These pathways are not mutually exclusive. For example, it has been demonstrated that the same apoptotic signals that cause mitochondrial dysfunction, cytochrome *c* release and caspase activation also activate autophagy (17).

We have shown that rat VP hypothalamic neurons (18–20), and mouse neuroblastoma Neuro2a cells (20), exhibit autophagy following the expression of an adFNDI transgene (Cys67stop) corresponding to a mutant VP allele originally identified in a Japanese family (21). Mutant adFNDI protein accumulates in the ER, which is then targeted for autophagic degradation (20). Thus adFNDI, like a number of other clinically important protein aggregation neurodegenerative diseases (22, 23), such as Parkinson's (24, 25), Alzheimer's (26–28) and Huntington's (29), is associated with the accumulation of misfolded protein aggregates within cellular compartments that are subsequently targeted for degradation by autophagy. However, the role of autophagy in the etiology of these disorders is unclear. While it is not difficult to imagine that autophagy, a process involving massive intracellular degradation, might promote cell death (30, 31), this is contradicted by the similarly axiomatic concept that autophagy (30, 31) is a prosurvival mechanism under conditions of cellular stress. Indeed, we have shown that inhibition of autophagy in Neuro2a cells expressing a virally delivered adFNDI mutant transgene (Cys67stop) induces apoptosis, suggesting that autophagy plays a role in the prevention of cell death following the stress of mutant protein accumulation (20).

In the present report, we have further explored the relationship between mutant protein accumulation, autophagy, cell survival, and cell death. These studies were based on the premise that neuronal cells in vivo are continuously exposed to environmental and metabolic insults, such as neurotransmitter stimulation or oxidative stress. We hypothesized that frail neurons, already undergoing autophagy in order to clear mutant proteins, might be more prone to cell suicide as a consequence of exposure to such stressors. Neuro2a cells expressing the Cys67stop mutant protein were subjected to oxidative stress using dopamine (DA). We found that this additional

insult did indeed lead to increased cell death, manifested by the typical morphological and biochemical features of classical apoptosis. Surprisingly, inhibition of autophagy blocked cytochrome *c* release from mitochondria and caspase activation, and, importantly, rescued cell viability. These data suggest that autophagy has a bifunctional role in cell survival and death following mutant protein accumulation. Initially, autophagy has a prosurvival role, presumably by clearing potentially toxic protein aggregates. However, following a second insult, autophagy switches to a death mode that shares biochemical and morphological features with classical apoptosis.

MATERIALS AND METHODS

Adenoviral vectors

The Ad-VCAT and Ad-VCAT-Cys67stop recombinant adenoviral vectors have previously been described (20). Ad-VCAT-Cys67stop encodes the Cys67stop truncated VP precursor and is implicated as the cause of adFNDI in a Japanese family (21), under the control of the tetracycline-responsive element (TRE) (32). Ad-VCAT encodes a wild-type VP precursor C-terminally tagged with a 16 amino acid DR-12-EK epitope (33), also under TRE control. Ad-CMV-TetOff encodes the TetOff transactivator under the constitutive control of the CMV promoter-enhancer (34). TetOff interacts with the TRE to drive the expression of transgenes in the absence of tetracycline (32, 34). Neuro2a cells were infected with Ad-CMV-TetOff and either Ad-VCAT or Ad-VCAT-Cys67stop (ratio of 1:1) at a multiplicity of infection (MOI) of 50. Both Ad vectors mediate a high rate of infection (>90%) of target mouse neuroblastoma Neuro2a cells, irrespective of treatment.

Cell culture and treatments

Mouse neuroblastoma Neuro2a cells were grown in humidified 5% (vol/vol) CO_2 atmosphere in DMEM medium supplemented with 10% (vol/vol) FCS and penicillin/streptomycin mix (1% wt/vol). Cells were seeded at 5×10^4 cells/cm^2 in six-well multiplates and allowed to adhere for 24 h before any treatment. For morphological studies, cells were seeded at a density of 15,000 cells/cm^2 on noncoated sterile cover slips. Autophagy inhibitors were added at the same time as viral infection and were used at the lowest, most effective nontoxic concentrations. 3-methyladenine (3MA; Sigma-Aldrich, Gillingham, Dorset, UK; 100 μM in DMSO) inhibits the formation of autophagosomes (35, 36). Asparagine (Asn; Sigma-Aldrich; 20 mM in DMEM) prevents the delivery of autophagocytosed material to lysosomes (37). The final concentration of DMSO in the medium (<0.1% vol/vol) is not toxic to Neuro2a cells. Ten hours after infection, some cultures were treated with DA (1 mM; Sigma-Aldrich). The pan-caspase inhibitor ZVAD-fmk (Alexis Corporation, Lausen, Switzerland; 20 μM) was added 3 h before DA treatment to ensure cell permeation.

Labeling of autophagic vacuoles with monodansylcadaverine

Monodansylcadaverine (MDC) is a spontaneously fluorescent dye that is incorporated selectively into autophagosomes and autolysosomes (38, 39). Cells were incubated with 0.05 mM MDC in PBS at 37°C for 1 h. After incubation, cells were washed two times with PBS and immediately analyzed by fluorescence microscopy (excitation: 380-420, barrier filter 450 nm).

Cell viability and cell death

At the end of the incubation period, cultures were observed under a phase-contrast light microscope (Leica DM IRB, Leica Microsystems, Milton Keynes, UK) and photographed using a Leica DC 300F camera (Leica Microsystems, Milton Keynes, UK). At the times indicated, control and treated cells were harvested and counted. Dead cells were identified using the trypan blue staining test. Adherent cells were detached and resuspended in DMEM. Subsequently, they were mixed with 0.04% (wt/vol) trypan blue in the ratio 1:1. The percentage of cells stained was determined by hemocytometer counting. Apoptosis was ascertained morphologically and cytofluorometrically. The appearance of a hypodiploid cell population (subG1) was monitored by flow cytometry on the whole cell population (i.e., monolayer plus cells recovered from the medium) fixed for 30 min with 70% (vol/vol) ethanol and stained with propidium iodide (PI; 0.2 mg/ml) in the presence of DNase-free RNase (to avoid RNA labeling). Data obtained represent the hypodiploid content as a proportion of the total labeled DNA. The settings of the instrument (forward and sidescatter parameters) should exclude mechanically damaged cells. Apoptosis was further objectively assessed using a method based on the expression of cell surface Annexin V binding sites (40, 41). Annexin V binds specifically to phosphatidylserine, which is externalized on the intact plasma membrane during the early stages of apoptosis. The whole cell population (i.e., adherent and detached cells) were labeled with fluorescein isothiocyanate (FITC)-conjugated Annexin V without prior fixation (42) to detect phosphatidylserine exposed on the outer leaflet of the plasma membrane. Fluorescent cells were detected with a FacsScalibur fluorescence-activated cell-sorter (BD Biosciences, Oxford, UK). At least 10,000 events were analyzed for each sample.

Mitochondrial membrane integrity and caspase activity

Cytochrome *c* release from mitochondria (43, 44) was examined using a mouse monoclonal antibody (BD Biosciences, Oxford, UK). Cells were seeded on cover slips, fixed with 4% (wt/vol) paraformaldehyde then permeabilized with 0.5% (vol/vol) Triton X-10 for 20 min. Incubation with the anticytochrome *c* monoclonal antibody for 3 h at room temperature was followed by incubation with FITC-conjugated anti-mouse secondary antibody. Stained cells were observed using a confocal microscope (LeicaDMIRE 2. Leica Microsystems, Milton Keynes, UK) with an excitation wavelength of 488 nm. Caspase activity was measured with the Caspase Detection Kit (Merck Biosciences Ltd., Nottingham, UK) using FITC-VAD-FMK as a substrate. Stained cells were analyzed by flow cytometry (FacsScalibur fluorescence-activated cell-sorter, BD Biosciences, Oxford, UK) using FL1 channel and by fluorescence microscopy, using a FITC filter (44).

Statistical analysis

All experiments were independently replicated at least 3 times. Data are presented ± SD. Statistical significance of differences between groups of data was determined by using the two-way ANOVA (Anova using SPSS software). A *P* value less than 0.05 was considered significant (denoted by an asterisk in the figures).

RESULTS

The expression of Cys67Stop sensitizes Neuro2a cells to a second insult

We have tested the hypothesis that Cys67stop expression sensitizes the cell to the effects of a second insult. Mouse neuroblastoma Neuro2a cells were infected for 10 h with Ad-VCAT-Cys67stop, which encodes the Cys67stop truncated VP precursor, or the control virus Ad-VCAT, encoding a C-terminally tagged wild-type VP precursor. Cells were incubated with DA for a further 8, 14, and 40 h, and cell viability was assessed visually (not shown), by cell counting (not shown) and by trypan blue staining to assess cell death (Fig. 1). We have previously shown (20) that the expression of Cys67stop on its own has no effect on cell viability, a finding confirmed by this time course experiment. However, simultaneous treatment with DA sensitized the Ad-VCAT-Cys67stop-infected cells to the toxic effects of DA (Fig. 1); cell death was faster and more pronounced at all time points compared with cells infected with the Ad-VCAT control virus (Fig. 1). Sham (uninfected) cells behaved as Ad-VCAT-infected cells (not shown).

Cell death induced by DA in Cys67stop expressing cells is autophagy-dependent

We then asked whether the cell death induced by DA in Neuro2a cells expressing the Cys67stop protein is related to autophagy. As previously demonstrated (20), blockade of autophagy using either Asn, which prevents fusion of autophagosomes with lysosomes (37), or 3MA, which blocks autophagosome formation (35, 36), has no effect on the viability of Ad-VCAT-infected cells at any time point (Fig. 1). However, both 3MA and Asn induce cell death in cells infected with Ad-VCAT-Cys67stop-for 50 h (i.e., 10 h plus 40 h) (Fig. 1), suggesting that autophagy is a prosurvival mechanism in cells accumulating a mutant protein (20). DA toxicity in Ad-VCAT-infected cells becomes apparent only after 40 h of treatment, but simultaneous treatment with 3MA or Asn has no effect on viability, suggesting that DA-induced death in these cells is autophagy-independent. In contrast, blockade of autophagy dramatically reduced cell death in DA-treated cultures infected with the Ad-VCAT-Cys67stop virus, as demonstrated by a significant increase in cell number (not shown) and by a significant decrease in trypan blue staining (Fig. 1). Although evident at all time points, the prevention of DA-induced cell death by the blockade of autophagy in cells expressing the Cys67stop mutant protein is most apparent 24 h (i.e., 10 h plus 14 h) after vector infection (Fig. 1). Subsequent experiments to characterize the mechanisms of cell death in DA-treated cultures infected with the Ad-VCAT-Cys67stop virus focused on this time point.

Cell death induced by DA in Cys67stop expressing cells has apoptotic characteristics

We asked whether the cell death induced by DA in Cys67stop-expressing cells was related to classic apoptosis. Flow cytometry was used to quantify cell populations labeled with FITC-Annexin V that identify early apoptotic cells. Treatment of Cys67stop-expressing cells with DA quadrupled the association of FITC-Annexin V with the surface phosphatidylserine, indicative of early apoptosis (Fig. 2*A*). Flow cytometry of PI-labeled cells was used to quantify the subG1 hypodiploid population (Fig. 2*B*). Again, an increase in the subG1 populations in Ad-VCAT-Cys67stop-infected Neuro2a suggested the involvement of an apoptotic pathway. Apoptosis is associated with activation of caspases by a cytosolic multiprotein complex formed upon

cytochrome *c* release from permeabilized mitochondria (45). Immunocytochemistry revealed massive cytochrome *c* release from mitochondria in DA-treated Ad-VCAT-Cys67stop-infected cells (Fig. 2*C* and 2*D*). Interestingly, DA significantly increased Annexin V binding to Ad-VCAT-infected cells (Fig. 2*A*) and cytochrome *c* release (Fig. 2*C* and 2*D*), but DNA fragmentation (the SubG1 population) was not induced (Fig. 2*B*).

Apoptosis induced by DA in Cys67stop expressing cells is autophagy dependent

Autophagy inhibitors 3MA and Asn both blocked the classically apoptotic cell death characteristics seen in DA-treated Neuro2a cells expressing Cys67stop. The autophagy inhibitors significantly reduced the Annexin V FITC-positive population (Fig. 2*A*), decreased the subG1 population (Fig. 2*B*), and inhibited the release of cytochrome *c* (Fig. 2*C* and 2*D*). By contrast, 3MA and Asn reduced Annexin V binding, in DA-treated control Ad-VCAT-infected cells (Fig. 2*A*), but had no effect on cytochrome *c* release (Fig. 2*C* and 2*D*).

Cell death induced by DA in Cys67stop expressing cells is caspase dependent

Next, we checked whether the caspase cascade was involved in DA-triggered cell death. Flow cytometry revealed a strong activation of caspases in DA-treated cells expressing Cys67stop, which was completely abolished by concomitant treatment with 3MA or Asn (Fig. 3*A*). By contrast, in control Ad-VCAT-infected cells, DA treatment was associated with only a slight increase of caspase activity, which was both 3MA- and Asn-insensitive (Fig. 3*A*). We then used the specific inhibitor ZVAD-fmk to show that the death of DA-treated Ad-VCAT-Cys67stop-infected cells was indeed caspase-dependent. As expected, ZVAD-fmk blocks caspase activity in DA-treated cells expressing Cys67stop (Fig. 3*B*), and this results in the rescue of cell viability, as assessed by the trypan-blue exclusion test (Fig. 3*C*). Apparently, the small increase in trypan blue-positive cells in DA-treated VCAT-expresing cultures is not abolished by caspase inhibition.

Autophagosome formation and apoptosis

We further investigated the functional link between autophagy and caspase-dependent cell death induced by DA. MDC is a spontaneously fluorescent dye that is thought to be incorporated selectively into autophagosomes and autolysosomes (38, 39). We used MDC staining to reveal the presence of autophagolysosmes in cells infected with either the control Ad-VCAT virus or the Ad-VCAT-Cys67stop vector, with or without subsequent DA treatment (Fig. 4). As previously demonstrated (20), pronounced autophagolysosomes were not seen in Ad-VCAT-infected cells (Fig. 4*A*), nor in uninfected cells (not shown); the weak MDC staining in Ad-VCAT-infected cells probably reflects the basal activity of the autophagy-lysosomal system, which apparently is not inhibited by the dose of 3MA used in the present study (see also Fig. 4*B*). In contrast, Cys67stop expression induced the appearance of large, dense punctate vesicles (Fig. 4*B*). In our previous work we have shown that these large vacuoles correspond to autophagolysosomes on the basis of their acid pH and the presence of autophagy markers such as Vps34, rab24, Beclin-1 and cathepsin D (20). DA-treatment also induced vesicle formation in Ad-VCAT-infected cells, and further increased the degree of autophagolysosome formation in Cys67stop-expressing cells. A similar picture was obtained by staining the cells with acridine orange, a fluorochrome that labels acid vacuolar compartments (not shown). It should be pointed

out, however, that immunofluorescent co-localization studies revealed that only the truncated mutant transgenic VP was derouted to the autophagic pathway (20). As expected, blockade of autophagy initiation with 3MA reduced the level of autophagolysosomes in Cys67stop-expressing cells, with or without DA (Fig. 4). However, ZVAD-fmk treatment increased autophagosome formation in both Ad-VCAT- or Ad-VCAT-Cys67stop-infected cells, with or without DA (Fig. 4), suggesting that caspases may inhibit the initiation of autophagy.

DISCUSSION

We have explored the relationships between mutant protein accumulation, activation of autophagy, cell survival, and cell death in the transgenic rat (18, 19) and cell culture (20) models of adFNDI. We have previously shown that the specific expression of an adFNDI mutant transgene (Cys67stop) in rat VP hypothalamic neurons activates autophagy, a process that is mimicked following the viral-delivery of Cys67stop to Neuro2a cells (20). In the latter model, inhibition of autophagy triggers apoptosis, suggesting a role for this bulk degradation process in cell survival following the accumulation of a toxic mutant protein. Although autophagy is triggered as early as 6 h after infection with Ad-VCAT-Cys67stop (unpublished), we show here that an overt autophagy-dependent effect on cell protection does not become apparent until after 24 h of infection in the presence of DA (Fig. 1). In the absence of DA, this effect is strongly delayed to 50 h. However, as early as 18 h (10 h plus 8 h) after infection (Fig. 1), autophagy, induced as a consequence of mutant Cys67stop protein expression (20) (Fig. 4), sensitizes the cell to the lethal effects of DA. No longer is autophagy protective; rather, a "two-hit" autophagy-dependent cell death is observed in Cys67stop-expressing Neuro2a cells exposed to a second insult, in this case DA. Death has features akin to classically defined apoptosis, such as the release of cytochrome *c* into the cytosol, caspase activation, the appearance of a hypodiploid subG1 population and an increase in Annexin V binding sites (Fig. 2). All of these features are dependent upon the process of autophagy, as shown by their reduction or elimination in the presence of autophagy inhibitors. The precise mechanism whereby autophagy triggers or mediates cell death remains to be determined. However, it is interesting to note that cell death depends upon caspase activation (Fig. 3). Similarly, it has recently been shown that caspases function in autophagic programmed cell death in steroid-treated *Drosophila* salivary gland cells (46). However, in contrast to a recent report demonstrating caspase-8 induction of autophagic cell death in L929 fibroblastic cells (47), it would appear that caspases act downstream of autophagy in DA-treated Cys67stop-expressing cells; inhibition of autophagy blocks caspase activation (Fig. 3*A*). Interestingly, blockade of caspase activity seems to promote autophagosome formation (Fig. 4), suggesting that caspases might inhibit autophagy in this system.

Treatment of Neuro2a cells infected with the control virus Ad-VCAT induces cell death after 40 h of DA treatment (Fig. 1). Indeed, signatures of cell death—the elaboration of cell surface Annexin V binding sites and cytochrome *c* release, but not DNA fragmentation (Fig. 2)—are evident earlier. DA treatment also induces the appearance of structures in the cell that stain with MDC, possibly autophagosomes or autolysosomes (38, 39), but DA-mediated death is not significantly sensitive to inhibitors of autophagy (Fig. 1), and although cytochrome *c* release is independent of autophagy, the appearance of Annexin V binding sites is blocked by autophagy inhibitors (Fig. 2). Further, cell death induced by DA is insensitive to blockade of caspases (Fig. 3). In contrast, treatment of Neuro2a cells expressing Cys67stop induces an autophagy- and caspase-dependent apoptosis characterized by cell surface binding sites for Annexin V, DNA

fragmentation, and cytochrome *c* release as early as 8 h after DA addition (Fig. 1). Thus, on the basis of the biochemical and morphological features, DA activated two different death pathways in Ad-VCAT- and in Ad-VCAT-Cys67stop-infected cells: autophagy (MDC staining) and Annexin V-positivity are observed in both cases, whereas the subG1 population is seen only in the latter case. It should be pointed out that Annexin V-positivity is a feature shared by both types of programmed cell death, that is, apoptosis and autophagic cell death, while the subG1 peak reflects DNA fragmentation, which is a downstream (or late) event of the caspase cascade activation (48). Our data can be interpreted as follows (see Fig. 5): *1)* In VCAT-Cys67stop expressing cells, DA induces a cell death with all of the characteristics of apoptosis (caspase-dependence, Annexin V-positivity and subG1-positivity) that is, however, autophagy-dependent (cytochrome *c* release, caspase activation, and downstream events are prevented by autophagy inhibitors 3MA or Asn); *2)* In control Ad-VCAT-infected cells, DA induces an autophagic cell death, which is caspase-independent and, in fact, it is not inhibited by ZVAD and does not show the appearance of the subG1 peak. Nonetheless, DA induces an autophagy-independent release of cytchrome *c* and a slight activation of caspases which is, however, not instrumental in the progress to cell death.

We have previously speculated as to the role of autophagy in the etiology of adFNDI (18–20). We suggested that the accumulation of mutant Cys67stop protein in the ER causes insoluble aggregates to form. This results in the development of a pathology characterized by a grossly deranged ER that accumulates both mutant and trapped wild-type protein. Under these circumstances, autophagy, acting as a cell survival mechanism, removes the deranged structures. Wild-type prohormone will be eliminated when the deranged organelle is destroyed, resulting in progressive VP-deficiency. However, this hypothesis failed to directly explain the paucity of VP neurons seen in the hypothalami of some adFNDI patients (8–11). We had suggested that neuronal atrophy, although not necessarily the primary cause of the disease, might be a long-term consequence of adFNDI, yet we were, until now, unable to bolster this hypothesis with a proposed mechanism. We noted that neuronal cells in vivo are continuously exposed to environmental and metabolic insults, such as neurotransmitter stimulation, oxidative stress, prosurvival factor depletion, or nutrient starvation. Here, we tested the hypothesis that frail neurons, already undergoing autophagy in order to clear mutant proteins, might be more prone to cell suicide as a consequence of exposure to such stressors. Indeed, we have now shown that Neuro2a cells expressing the Cys67stop mutant protein are less viable following DA exposure. We thus suggest that, in adFNDI, a progressive reduction of VP secretion, caused by autophagy, would lead to a chronic increase in plasma osmolality. Overstimulation of VP neurons by endogenous, cell-autonomous mechanisms and by excitatory afferents (49) might then trigger a pathologic sequence of neurotoxic events that ultimately leads to autophagy-dependent cell death.

Although adFNDI is a rare disease, we suggest that our studies on this intriguing model have broader implications for our understanding of more common, and more devastating, neurodegenerative disorders. We have shown that autophagy has a bimodal role in the etiology and pathogenesis of protein aggregation disease. Initially, autophagy is triggered to destroy potentially toxic aggregates. For example, as in our adFNDI model, the induction of autophagy by polyglutamine aggregates in Huntington's disease models decreases their accumulation (50) and toxicity (51). However, this ongoing autophagic activity sensitizes the neuron to the toxic

effects of a second insult. Under these circumstances, autophagy switches from a prosurvival to a prodeath mode. The signaling pathways that flick this switch are currently under investigation.

ACKNOWLEDGMENTS

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REFERENCES

1. Reeves, W. B., and Andreoli, T. E. (1992) The posterior pituitary and water metabolism. In *Williams Textbook of Endocrinology* (Wilson, J.D. and Foster, D.W., eds) Philadelphia: W. B. Saunders, pp 311–356

2. Burbach, J. P. H., Luckman, S. M., Murphy, D., and Gainer, H. (2001) Gene regulation in the magnocellular hypothalamo-neurohypophysial system. *Phys. Rev.* **81,** 1197–1267

3. de Bree, F. M., Knight, D., and Murphy, D. (2000) Sorting of the vasopressin prohormone into the regulated secretory pathway. *FEBS Lett.* **475,** 175–180

4. Brownstein, M. J., Russell, J. T., and Gainer, H. (1980) Synthesis, transport, and release of posterior pituitary hormones. *Science* **207,** 373–378

5. Christensen, J.H., Siggaard, C., and Rittig, S. (2003) Autosomal dominant familial neurohypophyseal diabetes insipidus. *APMIS Suppl 109* **111,** 92–95

6. Christensen, J. H., Siggaard, C., Corydon, T. J., Robertson, G. L., Gregersen, N., Bolund, L., and Rittig, S. (2004) Impaired trafficking of mutated AVP prohormone in cells expressing rare disease genes causing autosomal dominant familial neurohypophyseal diabetes insipidus. *Clin. Endocrinol. (Oxf.)* **60,** 125–136

7. Christensen, J. H., Siggaard, C., Corydon, T. J., deSanctis, L., Kovacs, L., Robertson, G. L., Gregersen, N., and Rittig, S. (2004) Six novel mutations in the arginine vasopressin gene in 15 kindreds with autosomal dominant familial neurohypophyseal diabetes insipidus give further insight into the pathogenesis. *Eur. J. Hum. Genet.* **12,** 44–51

8. Braverman, L. E., Mancini, J. P., and McGoldrick, D. M. (1965) Hereditary idiopathic diabetes insipidus: A case report with autopsy findings. *Ann. Intern. Med.* **63,** 503–508

9. Green, J. R., Buchan, G. C., Alvord, E. C. J., and Swanson, A. G. (1967) Hereditary and idiopathic types of diabetes insipidus. *Brain* **90,** 707–714

10. Nagai, I., Li, C. H., Hsieh, S. M., Kizaki, T., and Urano, Y. (1984) Two cases of hereditary diabetes insipidus, with an autopsy finding in one. *Acta Endocrinol. (Copenh.)* **105,** 318–323

11. Bergeron, C., Kovacs, K., Ezrin, C., and Mizzen, C. (1991) Hereditary diabetes insipidus: an immunohistochemical study of the hypothalamus and pituitary gland. *Acta Neuropathol. (Berl.)* **81,** 345–348

12. Russell, T. A., Ito, M., Ito, M., Yu, R. N., Martinson, F. A., Weiss, J., and Jameson, L. J. (2003) A murine model of autosomal dominant neurohypophyseal diabetes insipidus reveals progressive loss of vasopressin-producing neurons. *J. Clin. Invest.* **112,** 1697–1706

13. Mattson, M. P. (2000) Apoptosis in neurodegenerative disorders. *Nat. Rev. Mol. Cell Biol.* **1,** 120–129

14. Yuan, J., Lipinski, M., and Degterev, A. (2003) Diversity in the mechanisms of neuronal cell death. *Neuron* **40,** 401–413

15. Lockshin, R. A., and Zakeri, Z. (2002) Caspase-independent cell deaths. *Curr. Opin. Cell Biol.* **14,** 727–733

16. Yoshimori, T. (2003) Autophagy: a regulated bulk degradation process inside cells. *Biochem. Biophys. Res. Commun.* **313,** 453–458

17. Xue, L., Fletcher, G. C., and Tolkovsky, A. M. (1999) Autophagy is activated by apoptotic signalling in sympathetic neurons: an alternative mechanism of death execution. *Mol. Cell. Neurosci.* **14,** 180–198

18. Si-Hoe, S.-L., de Bree, F. M., Nijenhuis, M., Davies, J. E., Howell, L. M. C., Tinley, H., Waller, S. J., Zeng, Q., Zalm, R., Sonnemans, M., et al. (2000) Endoplasmic reticulum derangement in hypothalamic neurons of rats expressing a familial neurohypophyseal diabetes insipidus mutant vasopressin transgene. *FASEB J. Express* 10.1096/fj.99-0892fje (http://www.fasebj.org/cgi/reprint/99-0892fjev1)/ *FASEB J.* **14,** 1680–1684

19. Davies, J., and Murphy, D. (2002) Autophagy in hypothalamic neurons of rats expressing a familial neurohypophysial diabetes insipidus transgene. *J. Neuroendocrinol.* **14,** 629–637

20. Castino, R., Davies, J., Beaucourt, S., Isidoro, C., and Murphy, D. (2005) Autophagy is a prosurvival mechanism in mouse neuroblastoma cells expressing an autosomal dominant familial neurohypophyseal diabetes insipidus mutant vasopressin transgene. *FASEB J. Express* doi:10.1096/fj.04-3162fje

21. Nagasaki, H., Ito, M., Yuasa, H., Saito, H., Fukase, M., Hamada, K., Ishikawa, E., Katakami, H., and Oiso, Y. (1995) Two novel mutations in the coding region for neurophysin-II associated with familial central diabetes insipidus. *J. Clin. Endocrinol. Metab.* **80,** 1352–1356

22. Jellinger, K. A., and Stadelmann, C. H. (2000) The enigma of cell death in neurodegenerative disorders. *J. Neural Transm. Suppl.* **60,** 21–36

23. Jellinger, K. A., and Stadelmann, C. (2000) Mechanisms of cell death in neurodegenerative disorders. *J. Neural Transm. Suppl.* **59,** 95–114

24. Anglade, P., Vyas, S., Javoy-Agid, F., Herrero, M. T., Michel, P. P., Marquez, J., Mouatt-Prigent, A., Ruberg, M., Hirsch, E. C., and Agid, Y. (1997) Apoptosis and autophagy in nigral neurons of patients with Parkinson's disease. *Histol. Histopathol.* **12,** 25–31

25. Jellinger, K. A. (2000) Cell death mechanisms in Parkinson's disease. *J. Neural Transm.* **107,** 1–29

26. Cataldo, A. M., Hamilton, D., Barnett, J. L., Paskevich, P. A., and Nixon, R. A. (1996) Properties of the endosomal-lysosomal system in the human central nervous system: disturbances mark most neurons in populations at risk to degenerate in Alzheimer's disease. *J. Neurosci.* **16,** 186–199

27. Adamec, E., Mohan, P. S., Cataldo, A. M., Vonsattel, J. P., and Nixon, R. A. (2000) Up-regulation of the lysosomal system in experimental models of neuronal injury: implications for Alzheimer's disease. *Neuroscience* **100,** 663–675

28. Nixon, R. A., Cataldo, A. M., and Mathews, P. M. (2000) The endosomal-lysosomal system of neurons in Alzheimer's disease pathogenesis: a review. *Neurochem. Res.* **25,** 1161–1172

29. Kegel, K. B., Kim, M., Sapp, E., McIntyre, C., Castano, J. G., Aronin, N., and DiFiglia, M. (2000) Huntingtin expression stimulates endosomal-lysosomal activity, endosome tubulation, and autophagy. *J. Neurosci.* **20,** 7268–7278

30. Bursch, W., Ellinger, A., Gerner, C., Frohwein, U., and Schulte-Hermann, R. (2000) Programmed cell death (PCD). Apoptosis, autophagic PCD, or others? *Ann. N. Y. Acad. Sci.* **926,** 1–12

31. Cuevo, A. M. (2004) Autophagy: in sickness and in health. *Trends Cell Biol.* **14,** 70–77

32. Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995) Transcriptional activation by tetracyclines in mammalian cells. *Science* **268,** 1766–1769

33. Waller, S., Fairhall, K. M., Xu, J., Robinson, I. C. A. F., and Murphy, D. (1996) Neurohypophyseal and fluid homeostasis in transgenic rats expressing a tagged rat vasopressin prepropeptide in vasopressinergic magnocellular neurons. *Endocrinology* **137,** 5068–5077

34. Harding, T. C., Geddes, B. J., Noel, J. D., Murphy, D., and Uney, J. (1997) Tetracycline-regulated transgene expression in hippocampal neurons following transfection with adenoviral vectors. *J. Neurochem.* **69,** 2620–2623

35. Seglen, P. O., and Gordon, P. B. (1982) 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. *Proc. Natl. Acad. Sci. USA* **79,** 1889–1892

36. Blommaart, E. F., Krause, U., Schellen, J. P., Vreeling-Sindelarova, H., and Meijer, A. J. (1997) The phosphatidyl 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. *Eur. J. Biochem.* **243,** 240–246

37. Hoyvik, H., Gordon, P. B., Berg, T. O., Stromhaug, P. E., and Seglen, P. O. (1991) Inhibition of autophagic-lysosomal delivery and autophagic lactolysis by asparagine. *J. Cell Biol.* **113,** 1305–1312

38. Biederbick, A., Kern, H. F., and Elsasser, H. P. (1995) Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. *Eur. J. Cell Biol.* **66,** 3–14

39. Munafo, D. B., and Colombo, M. I. (2001) A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation. *J. Cell Sci.* **114,** 3619–3629

40. Martin, S. J., Reutelingsperger, C. P., McGahon, A. J., Rader, J. A., van Schie, R. C., LaFace, D. M., and Green, D. R. (1995) Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J. Exp. Med.* **182,** 1545–1556

41. Castino, R., Pace, D., Demoz, M., Gargiulo, M., Ariatta, C., Raiteri, E., and Isidoro, C. (2002) Lysosomal proteases as potential targets for the induction of apoptotic cell death in human neuroblastomas. *Int. J. Cancer* **97,** 775–779

42. Demoz, M., Castino, R., Cesaro, P., Baccino, F. M., Bonelli, G., and Isidoro, C. (2002) Endosomal-lysosomal proteolysis mediates death signalling by TNFalpha, not by etoposide, in L929 fibrosarcoma cells: evidence for an active role of cathepsin D. *J. Biol. Chem.* **383,** 1237–1248

43. Chen, Q., Chai, Y., Mazuder, S., Drazba, J., Chisolm, G., and Almasan, A. (2001) The late increase of free radicals during genotoxic stress-induced apoptosis is associated with cytochrome *c* release from mitochondria induced by caspase-mediated feedback loop amplification. *Sci. World J.* **1,** 142–153

44. Chen, C., Lin, H., and Chen, B. D. (2000) Bcl-2 antibodies induce hemoglobin release by red blood cells loaded with in vitro translated Bcl-2 and its cleaved fragment. *Biochem. Biophys. Res. Commun.* **270,** 816–820

45. Lim, M. L., Lum, M. G., Hansen, T. M., Roucou, X., and Nagley, P. (2002) On the release of cytochrome *c* from mitochondria during cell death signaling. *J. Biomed. Sci.* **9,** 488–506

46. Martin, D. N., and Baehrecke, E. H. (2004) Caspases function in autophagic programmed cell death in *Drosophila*. *Development* **131,** 275–284

47. Yu, L., Alva, A., Su, H., Dutt, P., Freundt, E., Welsh, S., Baehrecke, E. H., and Lenardo, M. J. (2004) Regulation of an ATG7-beclin 1 program of autophagic cell death by caspase-8. *Science* **304,** 1500–1502

48. Lockshin, R. A., and Zakeri, Z. (2004) Apoptosis, autophagy, and more. *Int J Biochem Cell Biol.* **36,** 2405-2419

49. Sharman, S., Ghorbel, M., Leroux, M., Beaucourt, S., Wong, L-F., and Murphy, D. (2004) Deciphering the mechanisms of homeostatic plasticity in the hypothalamo-

neurohypophyseal system–genomic and gene transfer strategies. *Progr. Biophys. Molec. Biol.* **84,** 151–182

50 Ravikumar, B., Duden, R., and Rubinsztein, D.C. (2002) Aggregate prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. *Hum. Mol. Genet.* **11,** 1107–1117

51 Ravikumar, B., Vacher, C., Berger, Z., Davies, J.E., Luo, S., Oroz, L.G., Scaravilli, F., Easton, D.F., Duden, R., O'Kane, C.J. and Rubinsztein, D.C. (2004) Inhibition of mTOR induces autophagy and reduces the toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. *Nat. Genet.* **36,** 585–595

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Fig. 1

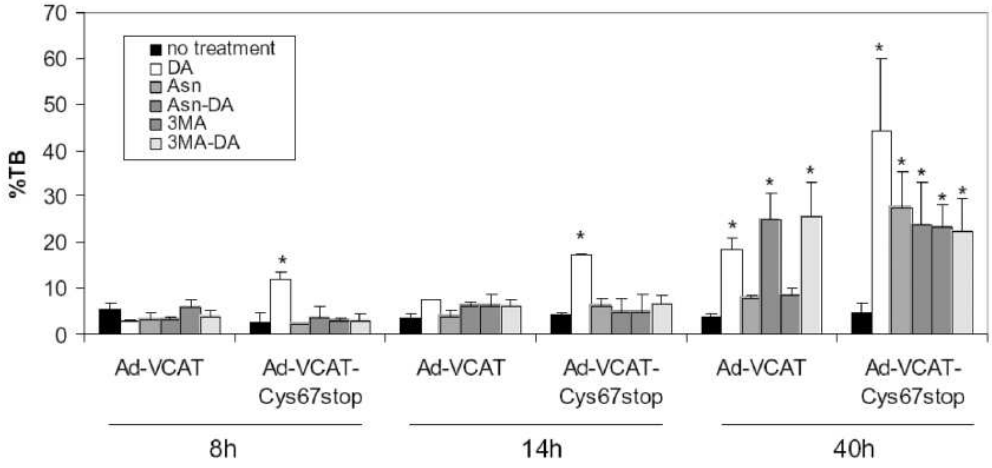

Figure 1. Cys67stop expression sensitizes Neuro2a cells to the toxic effects of DA. Neuro2a cells were infected with either the Ad-VCAT or Ad-VCAT-Cys67stop vectors, bearing wild-type or truncated VP transgenes, respectively. Ten hours later, cells were further incubated with or without DA (1 mM) for 8, 14, and 40 h. Some cultures were treated with the autophagy inhibitors Asn or 3MA. Cell viability was assessed by trypan blue (TB) staining, to quantify dead cells. Cys67stop expression has no effect on cell viability, but inhibition of the autophagy-lysosomal pathway in cells expressing the Cys67stop protein induces cell death at 50 h postinfection. Treatment of Cys67stop-expressing cells with DA increases death, which is prevented by treatment with autophagy inhibitors. In control Ad-VCAT-infected cells an autophagy-independent DA toxicity became apparent only at a late time point. Sham (uninfected) cells behaved as Ad-VCAT-infected cells (not shown).

Fig. 2

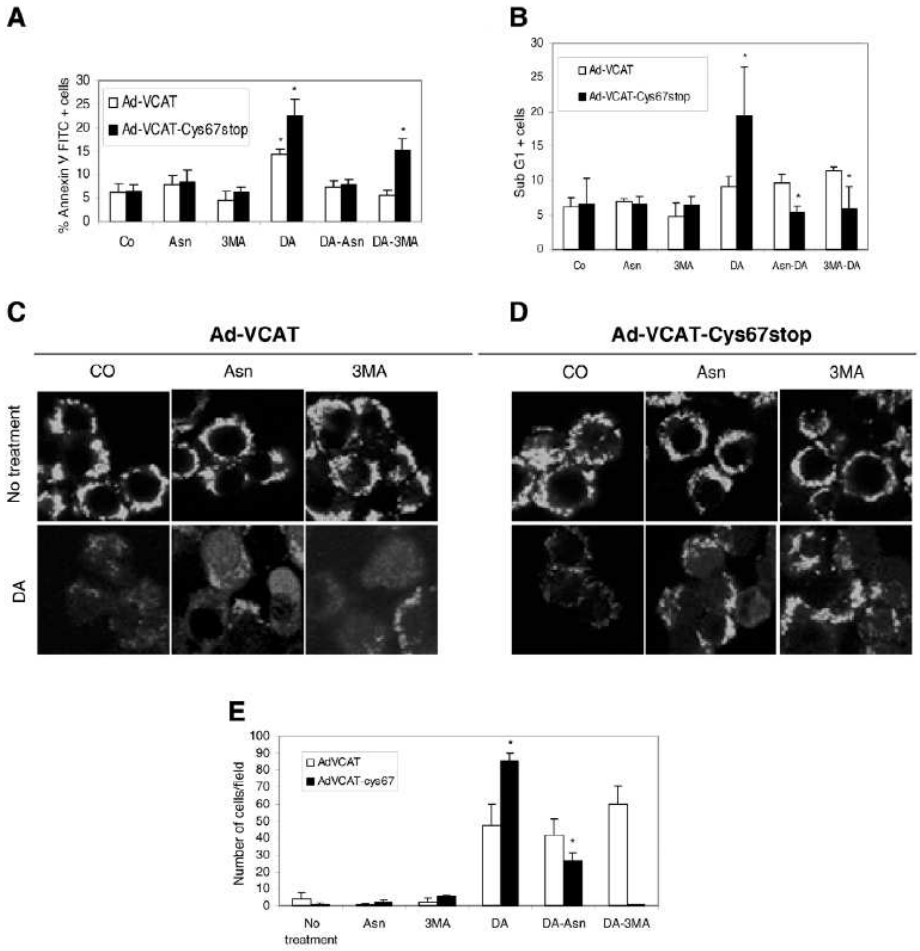

Figure 2. DA activates an autophagy-dependent apoptotic pathway in cells expressing the Cys67stop protein. 10 h after viral infection, cells were treated with DA for a further 14 h. Inhibitors were added at the same time as viral infection. ***A***) Apoptosis was ascertained cytofluorometrically by detection of cells expressing Annexin V binding sites on the surface. Treatment of Ad-VCAT-Cys67stop- and Ad-VCAT-infected Neuro2a cells with DA increases the percentage of Annexin V-positive cells, and this is reversed by inhibition of autophagy with 3MA or Asn. ***B***) The subG1 hypodiploid population was quantified by flow cytometry of PI-labeled cells. Treatment of Ad-VCAT-Cys67stop-infected Neuro2a cells, but not Ad-VCAT infected cells, with DA increases the subG1 population, and this is reversed by inhibition of autophagy with 3MA or Asn. ***C***) DA-induced cell death is associated with release of cytochrome *c* from mitochondria. Ad-VCAT or Ad-VCAT-Cys67stop-infected N2A cells were treated with DA for 14 h. The immunofluorescence shows the release of cytochrome *c* by permeabilized mitochondria. In Ad-VCAT-Cys67stop-infected cells, but not in Ad-VCAT-infected cells, the release of cytochrome *c* is prevented by 3MA and Asn. ***D***) This was quantified by counting cytoplasm-stained cells in at least four fields under the fluorescent microscope. Data are given as mean ± SD of the percentage of stained cell per field.

Fig. 3

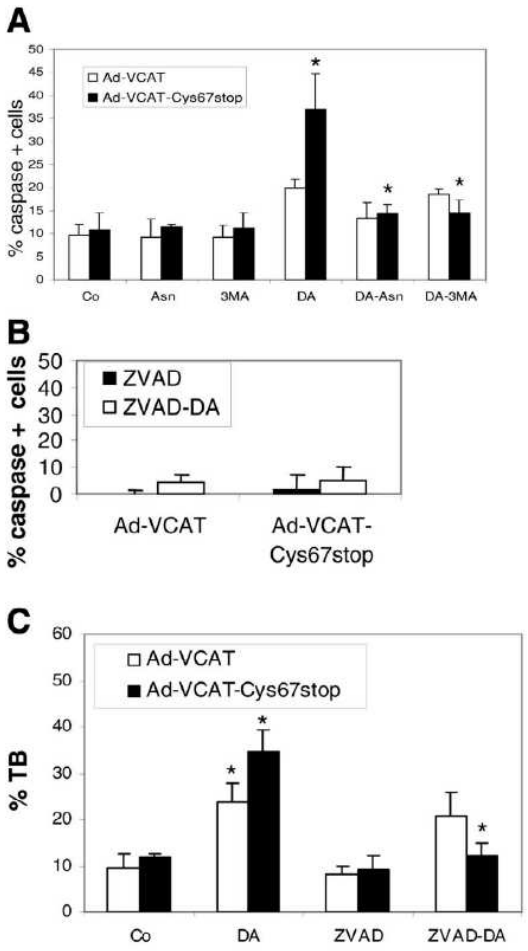

Figure 3. Cell death in DA-treated Neuro2a cell expressing Cys67stop is caspase-dependent. Neuro2a cells were infected with either the Ad-VCAT or Ad-VCAT-Cys67stop vectors. Ten hours later, cells were further incubated with or without DA for a further 14 h. Some cultures were treated with the pan-caspase inhibitor ZVAD-fmk. ***A***) Activation of caspases was demonstrated by fluorescent staining of adherent cells with FITC-ZVAD-fmk. Labeled cells were observed and counted under a fluorescence microscope and further analyzed by flow cytometry. In the presence of DA, there is a significant increase in the percentage of activated caspase-positive cells following Ad-VCAT-Cys67stop expression, and the effect is reversed by 3MA or Asn. ***B***) Caspase activity in Ad-VCAT- and Ad-VCAT-Cys67stop-infected cells in the absence and presence of DA is abolished by treatment with ZVAD-fmk. ***C***) Cell viability was assessed by trypan blue staining to quantify dead cells. Cell death mediated by DA is caspase-dependent in Cys67stop-expressing cells, whereas in control Ad-VCAT-expressing cells DA toxicity is not significantly prevented by caspase inhibition.

Fig. 4

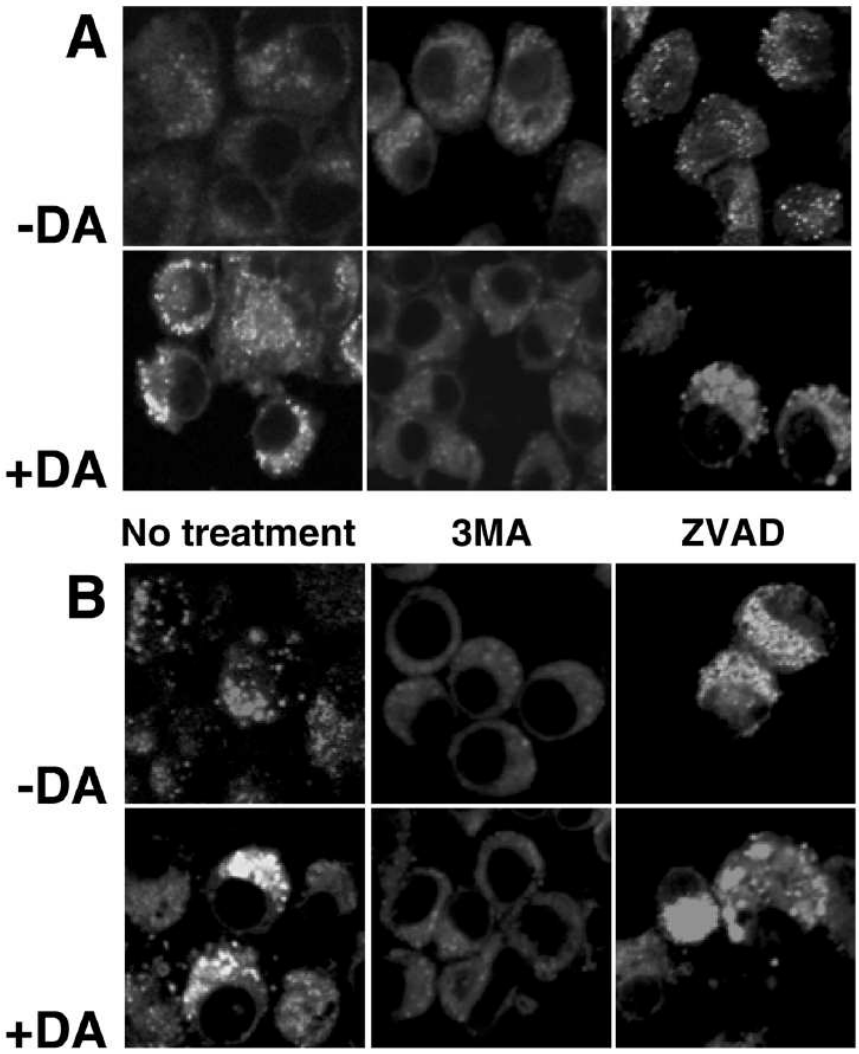

Figure 4. Autophagosome formation in Neuro2a cells infected for 10 h with either the control Ad-VCAT virus (A) or the Ad-VCAT-Cys67stop vector (B), with further incubation for 14 h in the presence (+) or absence (–) of DA. Some cultures were treated with the autophagy inhibitor 3MA or the pan-caspase inhibitor ZVAD-fmk. Autophagosomal vesicles were identified using MDC. A basal level of autophagy is seen in Ad-VCAT-infected cells expressing the wild-type VP, whereas Cys67stop expression induces the appearance of large, dense punctuate vesicles. DA-treatment increased vesicle formation in both Ad-VCAT- and Ad-VCAT-Cys67stop-infected cells. 3MA reduces the degree of autophagolysosome formation, whereas ZVAD-fmk treatment increases it.

Fig. 5

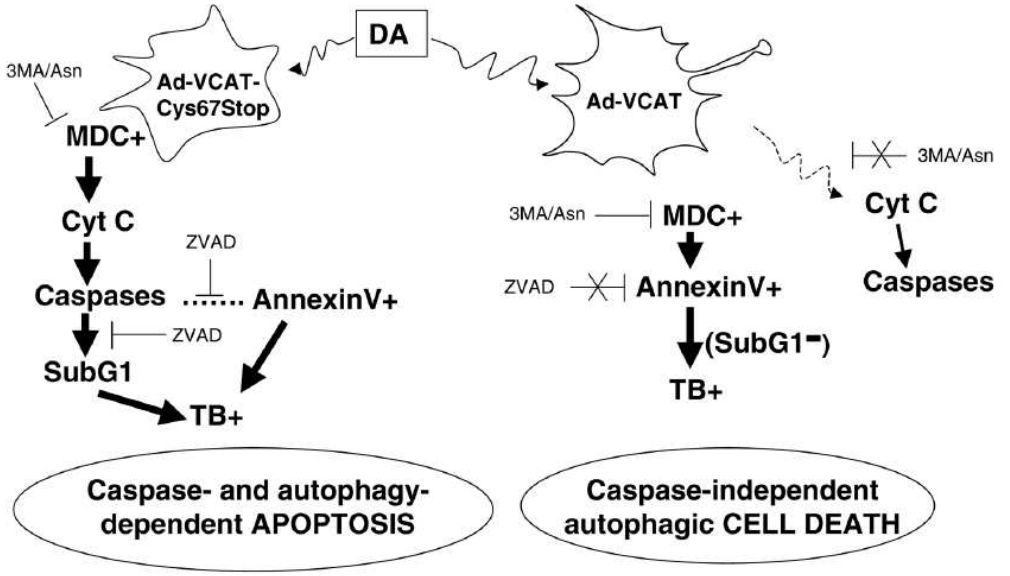

Figure 5. DA activates two different death pathways in Ad-VCAT and in Ad-VCAT-Cys67stop-infected cells. In VCAT-Cys67stop-expressing cells, DA induces a cell death with all of the characteristics of apoptosis (caspase-dependence, Annexin V-positivity and subG1-positivity) that is, however, autophagy-dependent. Cytochrome *c* release, caspase activation, and downstream events, including cell death as measured by TB incorporation, are prevented by autophagy inhibitors 3MA or Asn. In control VCAT-expressing cells, DA induces an autophagic cell death, which is caspase-independent and is not inhibited by ZVAD and does not show the appearance of the subG1 peak. Nonetheless, DA induces an autophagy-independent release of cytchrome *c* and a slight activation of caspases that is not involved in cell death.

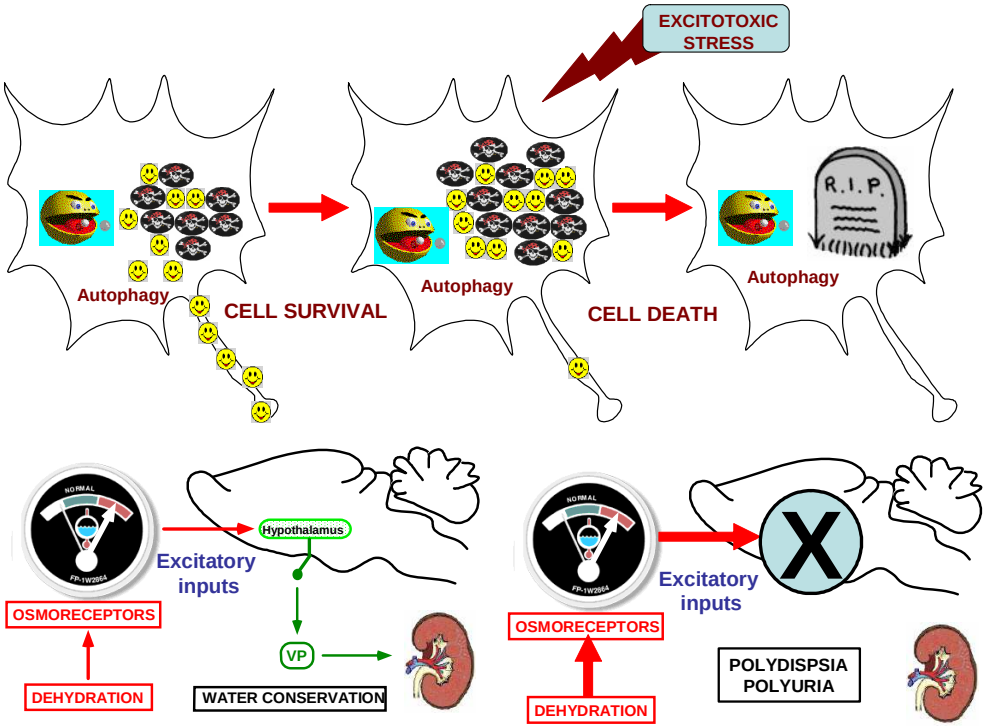

Fig. 15 Schema interpretativo

3. REGOLAZIONE DELLA PROTEOLISI LISOSOMICA E MORTE NEURONALE DA STRESS OSSIDATIVO

L'incidenza delle malattie neurodegenerative (sindrome di Alzheimer, morbo di Parkinson, corea di Hungtinton, sclerosi laterale amiotrofica) è aumentata notevolmente negli ultimi anni. Le cause e i meccanismi che sono alla base della patogenesi di queste malattie neurodegenerative cominciano ad essere delineati, grazie soprattutto alla disponibilità di strumenti e metodi biotecnologici sempre più sofisticati e potenti e ai modelli in "vitro" di malattie di più facile manipolazione. Tra i molti fattori che causano danno neuronale possiamo certamente includere lo stress ossidativo. L'elevato utilizzo di ossigeno e l'elevata attività mitocondriale dei neuroni rende, infatti, le cellule neuronali particolarmente sensibili allo stress ossidativo mediato dai radicali liberi dell'ossigeno (ROS). Lo stress ossidativo sembra indurre la morte delle cellule neuronali sia direttamente agendo su più bersagli (membrana plasmatica, DNA, RNA) e su alcune vie di trasduzione dei segnali di morte sia indirettamente favorendo un accumulo intracellulare di materiale lipo-proteico non degradabile di frequente riscontro nelle malattie neurodegenerative).

Nell'articolo allegato (sottoposto per la pubblicazione a Journal of Neurochemistry) sono descritti i risultati di esperimenti svolti a chiarire il ruolo delle catepsine lisosomiche nella morte cellulare indotta da perossido di idrogeno in cellule di neuroblastoma umano.

ENGLISH VERSION

REGULATION OF LYSOSOMAL PROTEOLYSIS AND PEROXIDE-INDUCED CELL DEATH

Neuronal cells are highly susceptible to reactive oxigen species (ROS)-induced damage. Low levels of ROS are produced in all organs during normal physiological conditions, yet endogenous antioxidant systems protect from any lethal consequence. Oxidative stress in the brain occurs when generation of ROS overrides the ability of antioxidant systems to remove excess of ROS. Oxidative stress manifests as increased levels of oxidized lipids, proteins and nucleic acids. In particular, protein oxidation of enzymes would negatively reflect on multiple cellular functions including protein synthesis, signal transduction, production of energy, ion homeostasis and intracellular trafficking. Moreover, oxidized proteins tend to form aggregates, as a consequence of their increased hydrofobicity and misfolding (Squier, 2001).

We have studied the complex interplay between these two death pathways in human neuroblastoma cells subjected to various oxidoradical stresses. In this study we have investigated the involvement of the lysosomal pathway in the cytotoxic mechanism of hydrogen peroxide in human neuroblastoma cells SH-SY5Y, a widely used model cell system for studying neuronal cell death. Cell death by hydrogen peroxide was preceded by alteration of lysosomal and mitochondrial membrane integrity. Leakage from lysosomes, permeabilization of mitochondria and cell death in hydrogen peroxide-treated neuroblastoma cells were prevented by desferrioxamine, an iron chelator that abolishes the formation of reactive oxigen species within lysosomes. Inhibition of cathepsin D, not of cathepsin B, as well as small-interference RNA-mediated silencing of cathepsin D gene protected from hydrogen peroxide-induced injury of lysosomes and mitochondria and prevented caspase activation and TUNEL-positive cell death.

ESSENTIAL ROLE OF CATHEPSIN D IN OXIDORADICAL-INDUCED NEURONAL CELL DEATH

Roberta Castino, Natascia Bellio, Giuseppina Nicotra, Carlo Follo, Nicol F. Trincheri and Ciro Isidoro[1]

Laboratorio di Patologia Molecolare, Università del Piemonte Orientale "A. Avogadro", Dipartimento di Scienze Mediche, Novara (Italy)

Abbreviations: CA074Me, L-trans-Epoxysuccinyl-Ile-Pro-OMe; CB, cathepsin B; CD, cathepsin D; DAPI, 4-6-diamidino 2-phenylindol-dihydrochloride; DFO, desferrioxamine; FITC, fluorescein isothiocyanate; FITC-VAD-fmk, FITC-Val-Ala-Asp-fluoromethylketone; Pst, Pepstatin A; PI, propidium iodide; ROS, reactive oxygen species; siRNA, small interference RNA; TUNEL, terminal deoxinucleotidyl transferase-mediated dUTP-biotin nick end labeling.

Corresponding author:

Università del Piemonte Orientale "A. Avogadro", Dipartimento di Scienze Mediche, Via Solaroli 17, 28100 Novara; tel + 39 (0)321 660607; fax +39 (0)321620421

e-mail: Isidoro@med.unipmn.it

ABSTRACT

Hydrogen peroxide, the major oxidoradical species in the central nervous system, has been implicated in neuronal cell death and associated neurodegenerative diseases. In this study we have investigated the involvement of the lysosomal pathway in the cytotoxic mechanism of hydrogen peroxide in human neuroblastoma cells SH-SY5Y, a widely used model cell system for studying neuronal cell death. Cell death by hydrogen peroxide was preceded by alteration of lysosomal and mitochondrial membrane integrity. Leakage from lysosomes, permeabilization of mitochondria and cell death in hydrogen peroxide-treated neuroblastoma cells were prevented by desferrioxamine, an iron chelator that abolishes the formation of reactive oxigen species within lysosomes. Inhibition of cathepsin D, not of cathepsin B, as well as small-interference RNA-mediated silencing of cathepsin D gene protected from hydrogen peroxide-induced injury of lysosomes and mitochondria and prevented caspase activation and TUNEL-positive cell death. The present study identifies the lysosome as the primary target and cathepsin D as the principal mediator of hydrogen peroxide lethal activity in neuronal cells. Our data indicate that lysosome-targeted antioxidant drugs or cathepsin D inhibitors could have great therapeutic potential to avoid neuronal cell death by oxidoradical injury.

Running title: Cathepsin D and neuronal cell death

Key words: oxidative stress, lysosomes, mitochondria, caspases, apoptosis, neurodegeneration.

INTRODUCTION

Neuronal cells are highly susceptible to reactive oxigen species (ROS)-induced damage. Low levels of ROS are produced in all organs during normal physiological conditions, yet endogenous antioxidant systems protect from any lethal consequence. Oxidative stress in the brain occurs when generation of ROS overrides the ability of antioxidant systems to remove excess of ROS. Several factors contribute to elevated ROS generation in the nervous tissue: first, the brain has the highest metabolic consumption of oxygen in the body (Malese, 2002); second, it contains high levels of iron, a reactive metal that promotes the formation of ROS (Koeppen, 1995; Brun and Brunk, 1970; Herbert et al., 1994; Smith et al., 2002); third, it is targeted by ROS-inducing neurotransmitters (Thakar and Hassan, 1988; Langeveld et al., 1995) and, forth, it is enriched in polyunsatured fatty acids that can propagate cell injury by oxygen free radicals (Keller and Mattson, 1998). Oxidative stress manifests as increased levels of oxidized lipids, proteins and nucleic acids. In particular, protein oxidation of enzymes would negatively reflect on multiple cellular functions including protein synthesis, signal transduction, production of energy, ion homeostasis and intracellular trafficking. Moreover, oxidized proteins tend to form aggregates, as a consequence of their increased hydrofobicity and misfolding (Squier, 2001). These protein aggregates are potentially toxic for neurons because of their adverse effects on the cytoskeleton dynamic and the vesicular traffic along the axon and the dendrites (Sohal, 2002; Lee et al., 2003). Two principal proteolytic systems are in charge to remove oxidized proteins and protein aggregates: the proteasome and the lysosome (Goldberg, 2003; Keller et al., 2004). It has been reported that highly oxidized proteins can inhibit proteolysis, thus promoting the progressive deposition of toxic undegradable proteinaceous aggregates. This explains the deleterious effects of oxidative stress in the pathogenesis and progression of neurodegenerative disorders such as Alzheimer's, Parkinson's, Huntington's and prion diseases (Maiese and Chong, 2004; Barnham et al., 2004; Brown, 2005; Goswami et al., 2006). Exposure to ROS can precipitate apoptosis in both neuronal cells and astrocytes (Coyle and Puttfarcken, 1993; Robb and Connor, 1998). The signaling pathways

involved in oxidative stress-induced neuronal apoptotic cell death are complex and not fully understood (Chandra et al., 2000; Sastry et al., 2000). The aim of the present study was to dissect the death pathway activated by hydrogen peroxide, the most represented ROS in the CNS. As a neuronal cell model, we chose the human neuroblastoma cell line SH-SY5Y, a model cell system widely employed in studies on neuronal cell death by oxidative stress (Zhang et al., 1997; Misonou et al., 2000). We focused, in particular, on the lysosome-mitochondrion axis of death signaling. In this report we show that hydrogen peroxide leads to lysosome permeabilization via iron-catalyzed formation of ROS. Moreover, we demonstrate that cathepsin D (CD), not cathepsin B (CB), triggers the mitochondrial caspase-dependent intrinsic pathway of cell death in hydrogen peroxide injured neuronal cells. A novel and interesting finding of the present study is that CD, not CB, is indispensable to induce the permeabilization of lysosome membrane, indicating that beside lipid peroxidation a proteolytic event is needed for the formation of micropores. This is the first demonstration of the direct involvement of CD in this process. Altogether, the present data lend support to the view that lysosome-targeted antioxidant drugs as well as genetic or pharmacological manipulation of CD activity could have great therapeutic potential to avoid neuronal cell death induced by oxidative stress.

MATERIALS AND METHODS

Unless otherwise specified all chemicals and antibodies were from Sigma-Aldrich Corp., St. Luis, MO, USA.

Cell culturing, treatments and evaluation of cytotoxicity

Human neuroblastoma SH-SY5Y cell line was obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultivated under standard culture conditions (37°C; 95% air:5% CO_2) in 50% Minimal Essential medium and 50% F12 Nutrient Medium supplemented with 10% heat- inactivated fetal bovine serum (Invitrogen Corp., Carlsbad, CA, USA), 2mM L-

glutamine and 1% of a penicillin-streptomycin solution. Cells were seeded and let adhere on sterile plastic dishes for 24 h prior to start any treatment. Treatments included 200 μM hydrogen peroxide, 100 μM Pepstatin A (Pst), 10 μM CA074Me (Bachem AG, Bubendorf, Switzerland), and 1 mM desferrioxamine (DFO). In some cases, before starting the incubation with hydrogen peroxide the culture medium was supplemented with DFO (3 h in advance), Pst (12 h in advance) or CA074Me (1 h in advance). Pst was diluted from a stock solution in dimethylsulfoxide so that final concentration of the solvent in the incubation medium did not exceed 0.1 % (v/v), a concentration that was not toxic to cells. At the end of incubation, adherent and suspended cells were collected, diluted in a solution containing trypan blue and counted. Alternatively, cells were collected, washed in PBS and incubated for 15 min at room temperature with 2 μl annexin V-FITC (Alexis Laboratories, S. Diego, USA), 5 μl propidium iodide (PI) and 98 μl of buffer (10mM Hepes/NaOH pH 7.4, 140mM NaCl, 2.5mM $CaCl_2$). Cells (at least 10,000 per sample) were analyzed in a FacScan flow cytometer (Becton Dickinson, Mountain View, Ca, USA) equipped with the winMDI software.

SubG1 analysis

At the end of incubation, the cells were pooled, collected by centrifugation (1000 g for 15 min), washed twice with cold PBS and fixed in ice-cold 70% ethanol for 1h at 4°C. Cells were then washed twice with PBS and incubated with RNase A (0.4mg/ml) for 30 min at 37°C and with PI (0.18 mg/ml) for 15 min in the dark, at room temperature. In this case only necrotic cells could be labeled. DNA analysis was performed with a FacScan flow cytometer (Becton Dickinson, Mountain View, Ca, USA) equipped with a 488 nm argon laser. Cells with hypodiploid content of DNA (subG1 peak) were assumed as apoptotic.

siRNA transfection

Post-translational silencing of CD expression was achieved by the small interference RNA (siRNA) technology. Duplexes of 21-nucleotide siRNA including two 3'-overhanging TT were synthesized by MWG Biotech AG (Washington, DC). The sense strand of siRNA was

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GAACAUCUUCUCCUUCUAC, corresponding to the positions 724-742 relative to the start codon of the CD mRNA (Faust et al., 1985). An inefficient CD9 oligonucleotide corresponding to the AGGUAGUGUAAUCGCCUUG sequence was used as a negative control of transfection (referred to as control-duplex). Transfection was performed with Lipofectamine 2000 (Invitrogen Corp.) following the manufacturer's protocol. After a first round of transfection, cells were incubated for 48 h in fresh medium, then subjected to a second round of transfection and incubated for further 48 h in fresh medium prior to any treatment.

Cathepsin D activity

CD activity was assayed at pH 3.65 using an aminomethylcoumarine-conjugated hemoglobin as substrate (Démoz et al., 2006). An aliquot (40 µl) of cell homogenate was incubated for 12 h at 37°C in a formate-acetate buffer (12.5 mM, pH 3.65), 1 µg aminomethylcoumarine-hemoglobin in 50 µl final volume. The assay was run on a multiwell plate and fluorescence was read in a spectrafluorometer at 365 and 460 nm excitation and emission wavelengths, respectively. To prove that substrate was hydrolyzed by CD, the specific inhibitor Pst was added to parallel samples.

Protein expression analysis

CD protein levels were evaluated by standard immunoblotting procedure (Démoz et al., 2006). Cells were homogenized in buffer containing detergents and protease inhibitors. 30 µg of cell proteins were denatured with Laemmli sample buffer, separated by electrophoresis on a 12.5% polyacrylamide gel and then electroblotted onto nitrocellulose membrane (Biorad, Hercules, CA, USA). CD was detected by a rabbit polyclonal antiserum anti-CD (Démoz et al., 2006) and Tubulin by a mouse monoclonal antibody followed by peroxidase-conjugated appropriate secondary antibody and subsequent peroxidase-induced chemiluminescence reaction (Biorad). Intensity of the bands was estimated by densitometric analysis (Quantity one software).

Assessment of caspases activity

Caspases activity was measured with the Caspases Detection kit (Merck Biosciences Ltd, Nottingham, UK) using FITC-VAD-fmk as a substrate following the manufacturer's protocol.

Stained cells (at least 10,000 per sample) were analyzed by flow cytometry. Data were interpreted using the winMDI software.

Fluorescence microscope imaging

Coverslips with adherent cells stained as detailed below were mounted in mowiol (1% in PBS). Images were captured with a Zeiss fluorescence microscope equipped with a digital camera or with Leica DMIRE2 confocal fluorescence microscope (Leica Microsystems AG, Wetzlad, Germany) equipped with Leica Confocal Software v. 2.61. For each experimental condition three coverslips were prepared. At least four fields in each coverslip were examined by two independent investigators. Representative images of selected fields are shown. Data were reproduced in at least three independent experiments.

Cathepsin D and Tubulin immunostaining

Immunofluorescence staining was performed as previously reported (Démoz et al., 2006). CD was detected by a rabbit polyclonal antiserum anti-CD (Démoz et al., 2006) followed by a Tretamethyl-rodhamine-Isothiocyanate-conjugated goat-anti-rabbit IgG. Tubulin was detected using a mouse monoclonal antibody followed by a FITC-conjugated goat-anti-mouse IgG.

DAPI and TUNEL staining

Apoptosis-associated chromatin alterations were detected by staining the cells with the DNA-labeling fluorescent dye 4-6-diamidino 2-phenylindol-dihydrochloride (DAPI, 1:500 in PBS/0.1% Triton X-100, 4% fetal bovine serum). Alternatively, apoptotic cells were revealed by *in situ* Terminal deoxinucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay performed with the "In situ Cell Death Detection" fluorescent Kit (Roche Diagnostics Corporation Indianapolis, IN, USA) following manufacturer's instructions.

Lysosomes and mitochondria permeabilization studies

For studies on lysosomal membrane integrity, at the end of treatments cells on coverslips were incubated with the lysosomotropic fluorochrome Acridine Orange (AO, 1:200 from 0.5 mg/ml in distilled water) for 10 min at 37°C. AO is a metachromatic fluorophore that emits a yellow-green or

a red fluorescence depending on its un-charged native or its proton-charged form, respectively. Living cells were immediately observed and photographed under the confocal fluorescence microscope. Mitochondrial membrane integrity was tested by using the fluorescent dye Rhodamine 123 (50 nM in culture medium; 10 min) or with Mitotracker Red (Invitrogen Corp.). For Mitotracker, cells on coverslips were fixed in 3.7% paraformaldehyde for 1h, permeabilized with 0.2% Triton X-100 for 20 min and incubated with 0.2 µl/ml of mitotracker solution for 15 min at 37°C.

Statistical analysis

The Instat-3 Statistical software (Graphpad Software Inc, San Diego, CA, USA) was used. Data are given as mean ± S.D.

RESULTS

Cell death by hydrogen peroxide is preceded by lysosomes and mitochondria permeabilization

The incubation of human neuroblastoma SH-SY5Y culture with 200 µM hydrogen peroxide for 2 h resulted in a dramatic cell loss in the monolayer amounting to approximately 50 % of adherent cells (Figure 1A). Cytofluorometric analysis of cells double-labeled with Annexin V-FITC, which binds to phosphatidylserine, and with propidium iodide, which labels DNA in necrotic cells, indicated that approximately 50 % of treated cells died by apoptotic-like cell death and that half of this population underwent secondary necrosis (Figure 1B). To assess the involvement of the apoptosis intrinsic pathway in hydrogen peroxide-induced cell death we examined the integrity of mitochondria as tested by retention of the fluorescent dye rhodamine. The images in Figure 1C clearly indicated loss of mitochondrial membrane integrity in cells exposed to hydrogen peroxide. Mithocondrial dysfunction, as tested by rhodamine staining, started to become apparent after 1 h of exposure to hydrogen peroxide. We further investigated whether hydrogen peroxide also affected the integrity of lysosomes, organelles that have been shown to play a role in apoptotic-like cell death pathways (Castino et al., 2003; Jaattela et al., 2004). Cells were treated or not with hydrogen peroxide for 0 to

2 h and at designated time were pre-loaded with the fluorescent dye AO and immediately observed under the microscope. AO is a lysosomotropic and metachromatic fluorophore that emits red fluorescence once it is protonated in acidic compartments. In control cells, AO fluorescence appeared as definite and intense red spots, indicative of acid organelles staining, whereas in cells exposed to hydrogen peroxide AO staining appeared weak, diffuse and yellow/green-like colored indicating cytosolic localization (Figure 1D). This staining feature was evident already after 10 min of incubation with hydrogen peroxide and the number of cells showing it increased with time of incubation. The present observations suggest that hydrogen peroxide cytotoxicity in neuronal cells first involves the lysosomes, followed by damage of mitochondria and eventually leads to cell death. The following experiments are aimed to assess and prove this sequence and to define the molecular actors linking this cascade of events.

Chelation of lysosomal free iron prevents lysosome and mitochondria permeabilization and protects from hydrogen peroxide-induced cell death

It has been suggested that transient pores in the lysosomal membrane arise from hydroxyl radicals-induced lipid peroxidation. These oxidoradicals are generated within lysosomes in the presence of hydrogen peroxide by iron-catalyzed Fenton reaction (Graf et al., 1984; Brunk et al., 1995). Desferrioxamine (DFO), a Fe^{2+}-chelator that accumulates within the lysosomal compartment by endocytosis, has been shown to protect lysosomes from oxidant-induced damage (Laub et al., 1985; Ollinger and Brunk, 1995; Cable and Lloyde, 1999; Persson et al., 2003). We assayed the ability of DFO to prevent lysosome destabilization and occurrence of apoptosis associated with hydrogen peroxide treatment in SH-SY5Y cells. Cells were exposed to hydrogen peroxide for 2 h in the absence or the presence of DFO, then loaded with AO and observed under the fluorescence microscope. Images in Figure 2A show that DFO effectively protected lysosomes from hydrogen peroxide damage, as demonstrated by the fact that AO fluorescence while weak and diffuse in hydrogen peroxide-treated cells, appears red-intense and punctuate, like in controls, in cells that had

been treated in the presence of DFO. We then checked whether such protection extended to mitochondria. A parallel set of cells, treated as above, were fluorescently stained for nuclei with DAPI and for mitochondria with mitotracker. DAPI staining gives information about the state of chromatin, whether condensed and fragmented (as occurs in apopotic cells) or not. Mitotracker staining gives information about the integrity of the mitochondrial membranes. Double-staining allowed to assess the morphological state of mitochondria in parallel with the healthy state of cells. In hydrogen peroxide-treated cultures, cells in which chromatin appeared condensed and fragmented showed a diffuse and weak staining with mitotracker, indicating that in apoptotic cells the integrity of mitochondria was disrupted (Figure 2B). These staining features were not apparent in treated cultures in which DFO was present. In this case, in fact, chromatin and mitochondria appeared intact as also observed in control cultures. We finally checked whether such protection on organelles integrity by DFO reflected on preservation of cell viability. For this purpose, cell monolayers were treated with hydrogen peroxide in the absence or the presence of DFO and at the end of incubation (2 h) adherent vital cells were counted. As shown in Figure 2C, DFO afforded complete protection from hydrogen peroxide-induced cell loss. To further substantiate the capability of DFO to prevent neuronal cell death by hydrogen peroxide, we quantified by cytofluorometry the cell population with a hypodiploid content of DNA (so-called 'subG1 peak') corresponding to apoptotic cells. Cytofluorograms in Figure 2D confirm that DFO effectively protects from hydrogen peroxide-induced apoptosis in neuronal cells.

Hydrogen peroxide triggers the intrinsic pathway of caspase activation: inhibition by Pepstatin A, not by CA074Me

We searched for a functional link between lysosome leakage, mitochondrial dysfunction and cell death by hydrogen peroxide. The intrinsic pathway of apoptosis is initiated by alteration of outer mitochondrial membrane with release of pro-apoptotic factors that promote the activation of pro-caspase 9 and, eventually, lead to the activation of pro-caspase 3 (Danial and Korsemeyer, 2004).

Lysosomal cathepsins B and D (CB, CD) have been shown able to initiate the intrinsic pathway by promoting the insertion of proteins of the Bcl2 superfamily into the outer mitochondrial membrane resulting in the formation of transient pores (Stoka et al., 2001; Bidere et al., 2003; Heinrich et al., 2004). We reasoned that CB and CD could have leaked out from permeabilized lysosomes and thus triggered the mitochondrial events of the intrinsic apoptotic pathway. To assess the possible involvement of CB and/or CD in hydrogen peroxide-induced mitochondrial permeabilization we employed two specific inhibitors of these proteases, namely CA074Me for CB and Pepstatin A (Pst) for CD. SH-SY5Y cells were treated with hydrogen peroxide in the absence or the presence of either inhibitor and then double-labeled with DAPI and mitotracker as above. As previously seen (Figures 1C and 2B), hydrogen peroxide-induced apoptosis (as shown by chromatin alterations) was associated with mitochondrial permeabilization (Figure 3A). However, the staining features of both chromatin and mitochondria were similar to controls in cultures treated with hydrogen peroxide in the presence of Pst; CA074Me, by contrary, did not prevent the chromatin alterations nor the mitochondrial membrane destabilization induced by hydrogen peroxide (Figure 3A). We then investigated whether the protective effects of Pst were epiphenomenal or causally linked to neuronal cell fate. Caspase 3-mediated hydrolysis of poly(ADP-ribose) polymerase, an enzyme involved in DNA repair, results in the accumulation of nicked DNA in nuclei of apoptotic cells that can be evidenced with the TUNEL technique. We fluorescently stained the nuclei of SH-SY5Y cells by TUNEL in control and treated cultures. In parallel cultures we also added Pst or CA074Me to assess the direct involvement of CD or CB, respectively, in caspase 3-dependent cell death. As expected in case of true apoptosis, neuronal cells exposed to hydrogen peroxide showed TUNEL positive (Figure 3B). CA074Me did not avoid caspase 3-dependent accumulation of DNA damage induced by hydrogen peroxide, as demonstrated by positive TUNEL staining (Figure 3B). However, when hydrogen peroxide treatment was performed in the presence of Pst the cell monolayer appeared well preserved and no nucleus stained for TUNEL (Figure 3B), indicating that in these cells caspase 3 was not operative. To have an objective estimation of the inhibitory effect of Pst on

the caspase-dependent pathway of cell death, we measured the potential to cleave a fluorogenic peptide substrate of different caspases by cell homogenates of cultures treated or not with hydrogen peroxide in the absence or the presence of this inhibitor. Data shown in Figure 3C indicate that activation of caspases occurs in cells treated with hydrogen peroxide, yet this activation does not occur if Pst is present. Thus, pharmacological inhibition of CD, not of CB, prevented mitochondrial membrane permeabilization, caspase activation and TUNEL-positive cell death. The present data further confirm that in hydrogen peroxide-treated neuronal cells activation of the CD–mediated proteolytic pathway precedes that of the caspase cascade.

Neuronal cell death by hydrogen peroxide is strictly CD-dependent

Next, we verified that CD activity was indeed indispensable for hydrogen peroxide-induced cell death in SH-SY5Y neuronal cells. As shown in Figure 4A, in cultures exposed to hydrogen peroxide along with Pst cell loss from the monolayer was largely suppressed. Pst also prevented the externalization of phospatidylserine on plasma-membrane of neuronal cells exposed to hydrogen peroxide, as tested by labeling with Annexin-V-FITC (Figure 4B). Pst is known to inhibit not only CD but also cathepsin E, another aspartic protease found in endosomes and lysosomes of neuronal cells (Nakanishi et al., 1997). To definitely prove the active role of CD in the death pathway activated by hydrogen peroxide we specifically down-regulated the expression of this protease by transient transfection with an siRNA 21-mer duplex. Negative controls consisted of cells transfected with an inefficient siRNA not targeting CD mRNA (control duplex). The extent of CD down-regulation was monitored by assaying the proteolytic activity at acid pH on fluorogenic substrate and by immunoblotting determination of CD protein level (Figure 4C). At the time of treatment in siRNA-transfected cells CD activity was almost completely absent and CD protein level, normalized against tubulin protein level, was down-regulated by >85% (densitometry average of three independent experiments). A parallel set of cultures was used to estimate cell vitality. Transfection in itself was not harmful, as cell viability in control duplex-transfected cultures was

not dissimilar from that reported in non-transfected cultures (not shown). In siRNA-transfected cultures toxicity by hydrogen peroxide was negligible, the number of adherent viable cells being almost similar to that of control cultures (not shown). Cytofluorometric analysis of annexin V-labeled cells indicated that protection by CD-siRNA against hydrogen peroxide cytotoxicity was practically complete and effective in the early phase of apoptosis (Figure 4D). The fact that Pst and siRNA elicited the same protective effect rules out the involvement of any aspartic protease other than CD in hydrogen peroxide cytotoxicity.

CD is needed for lysosomal permeabilization by hydrogen peroxide

CD is normally confined within endosomal-lysosomal organelles, yet it has been shown to relocate into the cytosol under cytotoxic circumstances associated with lysosome leakage (e.g., Roberg et al., 1999; Bidere et al., 2003), which can explain its ability to activate the intrinsic apoptosis pathway. We verified by immunofluorescence that cytosolic relocation of CD also occurred in dying neuronal cells exposed to hydrogen peroxide. The images in Figure 5A show in control cells a definite and punctuate pattern of CD staining as expected for a protein confined within the lysosomal organelles, whereas in suffering cells exposed to hydrogen peroxide CD staining appears diffuse throughout the cytoplasm, indicating cytosolic spread-out of the protein. We finally focused on the possible involvement of cathepsin-mediated proteolysis in hydrogen peroxide-induced damage of lysosomal membrane. To this end, intralysosomal cathepsins were inhibited by either CA074Me or Pst in cells exposed to hydrogen peroxide and lysosome integrity was monitored by AO retention. As shown in Figure 5B, Pst, not CA074Me, prevented leakage of AO from lysosomes in hydrogen peroxide-treated cells, indicating the possible participation of CD in the formation of micropores in the lysosomal membrane. As this was somehow unexpected, we used a genetic and more specific approach to ascertain the active involvement of CD in hydrogen peroxide-induced lysosome permeabilization. For this purpose, we repeated the observation in cells in which the CD gene had been post-translationally silenced by specific siRNA transfection. Again, it was found that

AO fluorescence, which appeared as intense red spots in control cells, was instead of yellow-green color and diffused in the cytoplasm in hydrogen peroxide-treated cells (Figure 5C). However, in cells that had been transfected with a specific CD siRNA the organule-like pattern of AO fluorescence resembled that in control cells, whether or not treated with hydrogen peroxide. This data strongly supports the hypothesis that lysosome membrane permeabilization requires both a lipid peroxidation and a CD-mediated proteolysis events.

DISCUSSION

Hydrogen peroxide is the most abundant and highly diffusible oxidant specie generated within the CNS. Hydrogen peroxide is a physiological product of the metabolism in neurons (Smith et al., 2002) and its production is increased upon stimulation by neurotransmitters and also under ischemic conditions (Thakar and Hassan, 1988; Langeveld et al., 1995; Hyslop et al., 1995). Hydrogen peroxide has a relative short half-life, being almost completely metabolized within 30 min (Hampton and Orrenius, 1997; Persson et al., 2003). Hydrogen peroxide freely crosses membranes and accumulates within organelles such as mitochondria and lysosomes. The latter are of particular interest for the catabolism of hydrogen peroxide. In these organelles, in fact, free Fe^{2+} rapidly catalyzes the conversion of hydrogen peroxide molecules into free hydroxyl radicals as dictated by the Fenton chemistry (Graf et al., 1984). Lysosomes degrade many iron-containing macromolecules (mitochondrial cytochromes, ferritin, etc) and therefore is probably the biggest reservoir of chelatable Fe^{2+}, also because of its acid and cysteine-rich reducing environment (Brun and Brunk, 1970; Zdolsek et al., 1993). As a consequence, lysosomes are highly vulnerable to oxidative stress and associated lipid peroxidation of the membrane (Ollinger and Brunk, 1995; Brunk et al., 1995; 2001). In this study we examined the involvement of a lysosome signaling pathway in the cytotoxic mechanism of hydrogen peroxide in SH-SY5Y neuronal-like cells. Oxidants induce cell death by both apoptosis and necrosis, depending on the concentration of free oxidoradicals (Dypbukt et al., 1994). We have assessed time- and concentration-conditions of hydrogen peroxide treatment that

lead SH-SY5Y cells to non-necrotic cell death. SH-SY5Y cultures exposed to 200 μM hydrogen peroxide showed about 50 % cell loss after 2 h of incubation. We found that free-radical-induced lysosome membrane destabilization is an early and indispensable event in the lethal cascade activated by hydrogen peroxide in neuronal cells. Lysosome permeabilization and cell death by hydrogen peroxide were in fact completely prevented by DFO, an iron chelator that localizes almost exclusively within the lysosomal compartment (Ollinger and Brunk, 1995; Laub et al., 1985; Cable and Lloyde, 1999). Free radicals-associated injury provoked leakage of CD from lysosomes. Likely, other lysosomal hydrolases, including cathepsins B and L, leaked out from destabilized lysosomes in hydrogen peroxide-treated cells. Both CD and CB have been shown able to mediate programmed cell death by a variety of death stimuli (Deiss et al., 1996; Guicciardi et al., 2000; Démoz et al., 2002; Boya et al., 2003; Bidere et al., 2003). However, studies with cathepsin specific inhibitors and siRNA demonstrated that CD, not CB, was responsible for the activation of the intrinsic pathway of caspase activation in neuronal cells exposed to hydrogen peroxide. These data are in agreement with other reports showing the cytosolic relocation of CD and its direct involvement in mitochondrial permeabilization in fibroblasts, cardiomyocytes and macrophages subjected to oxidative stress (Roberg et al., 1999; Roberg and Ollinger, 1998; Persson et al., 2003). Apoptosis intrinsic pathway proceeds via permeabilization of the outer mitochondrial membrane and release of pro-apoptotic molecules that activate the caspase cascade and ends up with characteristic alterations of the chromatin that manifest as condensation and fragmentations (evidenced by DAPI staining), nicked DNA (evidenced by TUNEL staining) and hypodiploid content of DNA (evidenced as subG1 peak). An early morphological hallmark of apoptosis is the externalization of phospatidylserine on plasma-membrane that can be evidenced by annexin-V labeling. We found that either Pst and CD-siRNA not only preserved the monolayer from hydrogen peroxide cytotoxicity, but also prevented all the above typical features of apoptosis (Figure 6), indicating that CD proteolysis is an up-stream effector of the hydrogen peroxide cytotoxic mechanism in neuronal cells. How CD can affect mitochondrial integrity and start the apoptosis pathway off is now

becoming clear. CD has been shown able to interact with both Bax and Bid, promoting their post-translational modification and subsequent insertion on the outer membrane of mitochondria (Bidere et al., 2003; Heinrich et al., 2004). What remains elusive is how the lysosomal enzyme crosses the lysosomal membrane and localizes in the cytosol in apoptotic cells. Several mechanisms have been proposed to explain the transient formation of micropores in the membrane of lysosomes under cytotoxic stress. Iron-catalyzed ROS generation within the lysosome is surely an important factor contributing to lipid peroxidation and lysosome membrane damage (Brunk et al., 1995; Antunes et al., 2001; Persson et al., 2003). In addition, intralysosome accumulation of sphingosine can determine membrane permeabilization via a detergent mechanism (Kakedal et al., 2001). More recently, it has been shown that overexpression of Bcl2, which is known to opposite the release of cytochrome c from mitochondria, also prevents lysosomal membrane permeabilization (Zhao et al., 2000). On the other hand, truncated Bid was found essential to allow cytosolic relocation of CB from lysosomes (Werneburg et al., 2004). Similarly, Bax has been suggested as a crucial mediator of cytosolic relocation of CD from lysosomes in fibroblasts treated with staurosporine (Kakedal et al., 2005). Thus, it appears that lysosomes and mitochondria share the same mechanisms that regulate the permeabilization of their membrane . Here we show that pharmacological inhibition of CD or post-translational down-regulation of its expression largely prevented occurrence of lysosome leakage in cells exposed to hydrogen peroxide, a protection shown also by DFO. The fact that Pst and siRNA elicited the same protective effect rules out the involvement of any aspartic protease other than CD in hydrogen peroxide cytotoxicity and permits to exclude that protection by Pst could be attributed to the hydroxyl-scavenger properties of dimethylsulfoxide (Rao et al., 1988), in which Pst was dissolved. Thus, our data suggest that formation of transient micropores in lysosomal membrane requires two actions: a ROS-induced peroxidation of membrane lipids and a CD-mediated proteolytic event. As yet, the substrate of this proteolysis is not known though Bid or Bax, which have been found associated with the membrane of acid organelles (Heinrich et al., 2004; Kakedal et al., 2005) are obvious potential candidates. On the whole, the present data support the

need for developing new lysosome-targeted antioxidant drugs and for designing genetic or pharmacological strategies aimed to abrogate CD activity as additional therapeutical tools for the treatment of neurodegenerative diseases associated with ROS-induced lysosome rupture.

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REFERENCES

Antunes F., Cadenas E., Brunk U.T. (2001) Apoptosis induced by exposure to a low steady-state concentration of H2O2 is a consequence of lysosomal rupture. Biochem. J. 365:549-555.

Barnham K. J., Masters C. L., Bush A. I. (2004) Neurodegenerative diseases and oxidative stress. Nat Rev Drug Discov. 3(3):205-14.

Bidere N., Lorenzo H.K., Carmona S., Laforge M., Harper F., Dumont C., Senik A. (2003) Cathepsin D triggers Bax Activation, Resulting in Selective Apoptosis-inducing Factor (AIF) Relocation in T Lymphocytes Entering the Early Commitment Phase to Apoptosis. J. Biol Chem 278:31401-31411.

Boya P, Andreau K, Poncet D et al. Lysosomal membrane permeabilization induces cell death in a mitochondrion-dependent fashion. J Exp Med 2003;197:1323-34.

Brown P. (2005) Pathogenesis and transfusion risk of transmissible spongiform encephalopathies. Dev Biol ;120:27-33.

Brun A. and Brunk U.T. (1970) Histochemical indications for lysosomal localization of heavy metals in normal rat brain and liver. J. Histochem. Cytochem. 18(11): 820-7.

Brunk U.T., Neuzil J., Eaton J.W. Lysosomal involvement in apoptosis (2001) Redox Report 6(2):91-97.

Brunk U.T., Zhang H., Roberg K., Ollinger K. (1995) Lethal hydrogen peroxide toxicity involves lysosomal membranes iron-catalyzed reactions with membrane damage. Redox Report. 1:267-277.

Cable H.,Lioyd J.B. (1999) Cellular uptake and release of two contrasting iron chelators. J Pharm Pharmacol. 51(2):131-4.

Castino R., Demòze M. and Isidoro C.(2003) Destination 'lysosome': a target organelle for tumour cell killing? J. Mol. Recognit. 16(5):337-48.

Chandra J., Samali A., Orrenius S. (2000) Triggering and modulation of apoptosis by oxidative stress. Free Radic Biol Med.29(3-4):323-33.

Coyle J.T., Puttfarcken P. (1993) Oxidative stress, glutamate, and neurodegenerative disorders. Science. 262(5134):689-95.

Danial N.N., Korsmeyer S.J. (2004) Cell death: critical control points. Cell. 23;116(2):205-19.

Deiss LP, Galinka H, Berissi H, Cohen O, Kimchi A. (1996) Cathepsin D protease mediates programmed cell death induced by interferon-gamma, Fas/APO-1 and TNF-alpha. EMBO J 15:3861-70.

Demòz M, Castino R, Cesaro P, Baccino FM, Bonelli G, Isidoro C. (2002) Endosomal-lysosomal proteolysis mediates death signalling by TNFalpha, not by etoposide, in L929 fibrosarcoma cells: evidence for an active role of cathepsin D. Biol Chem 383:1237-48.

Demòz M., Castino R., Follo C., Hasilik A., Sloane B.F., Isidoro C. (2006) High yield synthesis and characterization of phosphorylated recombinant human procathepsin D expressed in mammalian cells. Protein Expr Purif. 45(1):157-67.

Dypbukt J. M., Ankarcrona M., Burkitt M., Sjoholm A., Strom K., Orrenius S., Nicotera P. (1994)Different prooxidant levels stimulate growth, trigger apoptosis, or produce necrosis of insulin-secreting RINm5F cells. The role of intracellular polyamines. J Biol Chem. 1994 269(48):30553-60.

Goldsberg A.L. (2003) Protein degradation and protection against misfolded or damaged proteins. Nature 426:895-899.

Goswami A., Dikshit P., Mishra A., Mulherkar S., Nukina N., Jana N.R. (2006) Oxidative stress promotes mutant huntingtin aggregation and mutant huntingtin-dependent cell death by mimicking proteasomal malfunction. Biochem Biophys Res Commun 342(1):184-90.

Graf E, Mahoney JR, Bryant RG, Eaton JW. (1984). Iron-catalyzed hydroxyl radical formation. Stringent requirement for free iron coordination site.J Biol Chem. 259(6):3620-4.

Guicciardi ME, Deussing J, Miyoshi J et al. Cathepsin B contributes to TNF-alpha-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome c. (2000) J Clin Invest 106:1127-37.

Hampton M.B., Orrenius S. (1997) Dual regulation of caspase activity by hydrogen peroxide: implications for apoptosis. FEBS Lett. 414(3):552-6.

Heinrich M., Neumeyer J., Jakob M., Hallas C., Tchikov V., Winoto-Morbach S., Wickel M., Schneider-Brachert W., Trauzold A., Hethke A., Schutze S. (2004) Cathepsin D links TNF-induced acid sphingomyelinase to Bid-mediated caspase-9 and -3 activation. Cell Death Differ. 11(5):550-63.

Herbert V., Shaw S., Jayatilleke E., Stopler-Kasdan T. (1994) Most free-radical injury is iron-related: it is promoted by iron, hemin, holoferritin and vitamin C, and inhibited by desferoxamine and apoferitin. Stem Cells 12: 289-303.

Hyslop P.A., Hinshaw D.B., Scraufstatter I.U., Cochrane C.G., Kunz S., Vosbeck K. (1995) Hydrogen peroxide as a potent bacteriostatic antibiotic: implications for host defense. Free Radic Biol Med. 19(1):31-7.

Jaattela M., Cande C., Kroemer G. (2004) Lysosomes and mitochondria in the commitment to apoptosis: a potential role for cathepsin D and AIF.Cell Death Differ.11(2):135-6.
Kakedal K., Johansson U.,and Ollinger K. (2001) The lysosomal protease cathepsin D mediates apoptosis induced by oxidative stress. FASEB J. 15: 1592-1594.

Kakedal K., Johansson A.C., Johansson U., Heimlich G., Roberg K., Wang N.S., Jurgensmeier J.M and Ollinger K. (2005) Lysosomal membrane permeabilization during apoptosis-involvement of Bax? Int. J. Exp. Pathol. 86 (5): 309-313.

Keller J.N., Mattson M.P. (1998) Roles of lipid peroxidation in modulation of cellular signalling pathways, cell dysfunction, and death in the nervous system. Rev Neurosci. 9:105-16.

Keller J.N., Dimayuga E., Chen Q., Thorpe J., Gee J., Ding Q. (2004) Autophagy, proteosomes, lipofuscin ad oxidative stress in the aging brain. Int J Biochem Cell Biol 36:2376-91.

Koeppen AH. (1995) The history of iron in the brain. J Neurol Sci. 134:1-9.

Laub R.,Schneider Y.J., Octave J.N., Trouet A.,Crichton R.R. (1985) Cellular pharmacology of deferrioxamine B and derivatives in cultured rat hepatocytes in relation to iron mobilization. Biochem Pharmacol. 34(8):1175-83.

Langeveld C.H., Schepens E., Stoof J.C., Bast A., Drukarch B. (1995) Differential sensitivity to hydrogen peroxide of dopaminergic and noradrenergic neurotransmission in rat brain slices. Free Radic Biol Med. 9(2):209-17.

Lee C.S., Han E.S., Lee W.B. (2003) Antioxidant effect of phenelzine on MPP+-induced cell viability loss in differentiated PC12 cells. Neurochem Res. 28(12):1833-41.

Maiese K., Chong Z.Z. (2004) Insights into oxidative stress and potential novel therapeutic targets for Alzheimer disease. Restor Neurol Neurosci 22:87-104.

Misonou H., Morishima-Kawashima M., Ihara Y. (2000) Oxidative stress induces intracellular accumulation of amyloid beta-protein (Abeta) in human neuroblastoma cells. Biochemistry. 39(23):6951-9.

Malese D.R. (2002) Healty people 2010—leading health indicators for women. Womens Health Issues 12, 155-64.

Nakanishi H., Amano T., Sastradipura D.F., Yoshinime Y., Tsuksba T., Tanabe K., Hirotsu I., Ohono T., Yamamoto K. (1997) Incresead expression of cathepsins E and D in neurons of the aged rat brain and their colocalization with lipofuscin and carboxy-terminal fragments of Alzheimer amyloid precursor protein. J. Neurochem. 68: 739-49.

Ollinger K.,Brunk U.T. (1995) Cellular injury induced by oxidative stress is mediated through lysosomal damage. Free Radic Biol Med. 19(5):565-74

Rao P.S., Luber J.M., Milinowicz J., Lalezari P. and Mueller H.S. (1988) Specificity of oxygen radical scavenger efficiency using luminol enhanced chemiluminescence.

Persson H.L., Yu Z., Tirosh O., Eaton J.M., Brunk U.T. (2003) Prevention of oxidant-induced cell death by lysosomotropic iron chelators. Free Radic. Biol. Med. 34.1295-1305

Robb S. J, Connor J. R. (1998) An in vitro model for analysis of oxidative death in primary mouse astrocytes.Brain Res. 30;788(1-2):125-32.

Roberg K., and Ollinger K. (1998). Oxidative stress causes relocation of the lysosomal enzyme cathepsin D with ensuing apoptosis in neonatal rat cardiomiocytes. Am. J. Pathol. 152:1151-56.

Roberg K., Johansson U., Ollinger K. (1999). Lysosomal release of cathepsin D precedes relocation of cytocrome c and loss of mitochondrial transmembrane potential during apoptosis by oxdative stress. Free Radic. Biol. Med. 27:1228-1237.

Sastry P.S., Rao K.S. (2000) Apoptosis and the nervous system. J Neurochem. 74:1-20.

Smith M.A., Nunomura A., Zhu X., Takeda A., Perry G. (2002) Metabolic, metallic, and mitotic sources of oxidatives stress in Alzheimer disease. Antioxid Redox Signal 2,413-20.

Sohal R.S., Mockett R.J., Orr W.C. (2002) Mechanisms of aging: appraisal of the oxidative stress hypothesis. Free Radic Biol Med 33, 575-86.

Squier T.C. (2001) Oxidative stress and protein aggregation during biological aging. Exp Gerontol 36, 1539-50.

Stoka V., Tutk B., Schendel S.L. Kim T.H., Cirman T., Snipas S.J., Ellerby L.M., Bredesen D., Freeze H., Abrahamson M., Bromme D., Krajewski S., Reed J.C., Yin X.M. Turk V.,Salvesen G.S. (2001) Lysosomal protease pathways to apoptosis. Cleavage of bid, not pro-caspases, is the most likely route.J Biol Chem. 2;276(5):3149-57. Epub 2000 Nov 9.

Thakar JH, Hassan MN. (1988) Effects of 6-hydroxydopamine on oxidative phosphorylation of mitochondria from rat striatum, cortex, and liver. Can J Physiol Pharmacol. 1988 Apr;66(4):376-9.

Werneburg N., Guicciardi M.E., Yin X.M., Gores G.J. (2004) TNF-alpha-mediated lysosomal permeabilization is FAN and caspase 8/Bid dependent. Am. J. Physiol. Gastrointest. Liver Physiol. 287: 436-443.

Zhang L., Zhao B., Yew D.T., Kusiak J.W., Roth G.S. (1997) Processing of Alzheimer's amyloid precursor protein during H2O2-induced apoptosis in human neuronal cells. Biochem. Biophys. Res. Commun. 235:845-8.

Zdolsek J.M., Roberg K., Brunk U.T. (1993) Visualization of iron in cultured macrophages: a cytochemical light and electron microscopic study using autometallography. Free Radic Biol Med. 15(1):1-11.

Zhao M., Eaton J.W., Brunk U.T. (2000) Protection against oxidant-mediated lysosomal rupture: a new anti-apoptotic activity of bcl-2? FEBS Lett. 485:104-108.

FIGURE LEGENDS

Figure 1. **Peroxide-induced cell death is preceded by lysosomal leakage. (A)** The cells were incubated for 2 h with 200 μM of hydrogen peroxide (perox). At the end of treatment living cells were counted. Data represent the mean ± SD of three independent experiments in triplicate. **(B)** AnnexinV-FITC and PI staining in cells treated or not with hydrogen peroxide for 2 h. The percentage of positivity for annexin V-FITC and PI is indicated. Data are representative of three independent experiments. (**C**) Cells were stained with the mitochondria-affine rhodamine fluorochrome. Diffuse red fluorescence in cells exposed for 2 h to hydrogen peroxide is indicative of mitochondria permeabilization. **(D)** Cells incubated with hydrogen peroxide for 30 min and then stained with the lysosomotropic AO fluorochrome. Intense red fluorescent spots are indicative of intralysosomal retention of AO. After hydrogen peroxide incubation cells show a yellow-green like fluorescence indicative of cytosolic diffusion of the fluorochrome. Images in (C) and (D) are representative of four independent experiments.

Figure 2. **The iron chelator desferrioxamine prevents hydrogen peroxide cytotoxicity.** Cells incubated with hydrogen peroxide (perox, 2 h) in the absence or the presence of desferrioxamine (DFO, 1 mM; added 3 h prior to the treatment). **(A)** Cells stained with the lysosomotropic AO fluorochrome. DFO prevents hydrogen peroxide-induced lysosome leakage. Images are representative of four independent experiments. **(B)** Cells double-stained for chromatin with DAPI (blue fluorescence) and for mitochondria with mitotracker (red fluorescence). Weak and diffuse mitotracker staining is visible in hydrogen peroxide-treated cells with picnotic nuclei and condensed and fragmented chromatin. This staining pattern is not observed in cells treated in the presence of DFO. Images in (A) and (B) are representative of four separate experiments. (**C**) Cells were plated on dishes and treated as above. At the end of treatments adherent living (trypan blue-excluding) cells were counted. Data represent the mean ± SD of three independent experiments in triplicate. **(D)** Cells treated as in (C) were collected and stained with PI for cytofluorometric analysis of the

hypodiploid (SubG1) population. Representative cytofluorograms (out of three independent experiments) are shown (the percentage of SubG1 positive cells is indicated).

Figure 3. **Pharmacological inhibition of CD, not of CB, prevents hydrogen peroxide-induced activation of intrinsic caspase-dependent apoptosis. (A)** Cells on coverslips were exposed or not to hydrogen peroxide (perox, 2 h) in the absence or the presence of the CB inhibitor CA074Me or the CD inhibitor (Pst). At the end of incubation, cells were double-stained for chromatin with DAPI (blu fluorescence) and for mitochondria with mitotracker (red fluorescence). Protection from mitochondria permeabilization (as tested by mitotracker retention) by hydrogen peroxide is observed only when Pst is present. **(B)** Cells treated as in (A) and TUNEL-stained to evidence the occurrence of caspase 3-mediate apoptosis. Pst, not CA074Me, prevented TUNEL-positive staining induced by hydrogen peroxide treatment. (**C**) Total caspase activity was measured in cell homogenates by employing a fluorogenic substrate. A 2.8-fold increase of caspase activity was reported in cells treated with hydrogen peroxide (perox) for 2 h. This increase was largely suppressed in cells treated with hydrogen peroxide in the presence of Pst. Data are the means of two separate experiments in double.

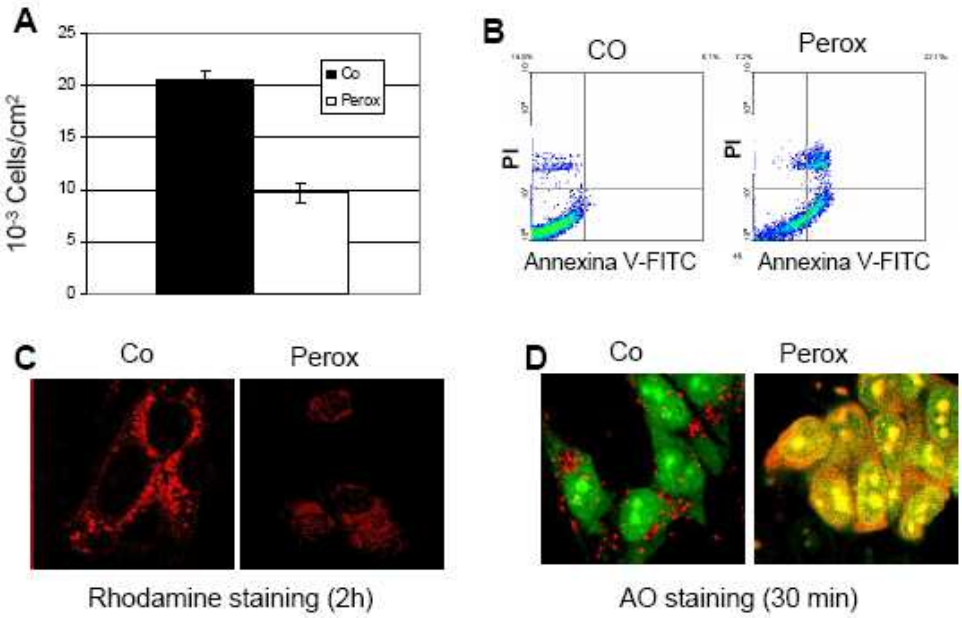
Figure 4. **Hydrogen peroxide cytotoxicity requires CD activity. (A)** Adherent SH-SY5Y cultures were exposed or not to 200 μM hydrogen peroxide for 2 h. In parallel cultures Pst was added 12 h before and was present throughout the incubation with hydrogen peroxide. At the end of treatments living trypan blue-excluding cells were counted. Data represent the mean ± SD of three independent experiments in triplicate. **(B)** Externalization of phosphatidylserine, an early apoptotic event, was assayed by AnnexinV-FITC staining of cells treated as in (A) and analyzed by cytofluorometry. The percentage of positivity is indicated. **(C)** Post-translational silencing of CD expression by siRNA as tested by CD activity assay and immunoblotting of CD mature peptide. Cultures were un-transfected (Control, Co) or transfected in two rounds with either a control duplex or a specific CD siRNA and CD expression evaluated at 48 h (after the first round of transfection) and at 96 h (i.e., 48 h after the second round of transfection). Complete down-regulation of CD activity was achieved

at 96 h. The expression level of the double-chain mature CD resident in lysosomes (only the large chain of 34 kDa is visible on gel) was assessed by immunoblotting. The filter was stripped and re-probed with an anti-tubulin antibody to reveal the protein sample loading. **(D)** Adherent cultures transfected as in (C) were exposed or not to hydrogen peroxide and apoptosis evaluated by cytofluorometry in cells stained with annexin V-FITC. Data demonstrate that abolishing CD expression renders the cells resistant to hydrogen peroxide toxicity. The cytofluorograms shown in panels B and D are representative of three independent experiments.

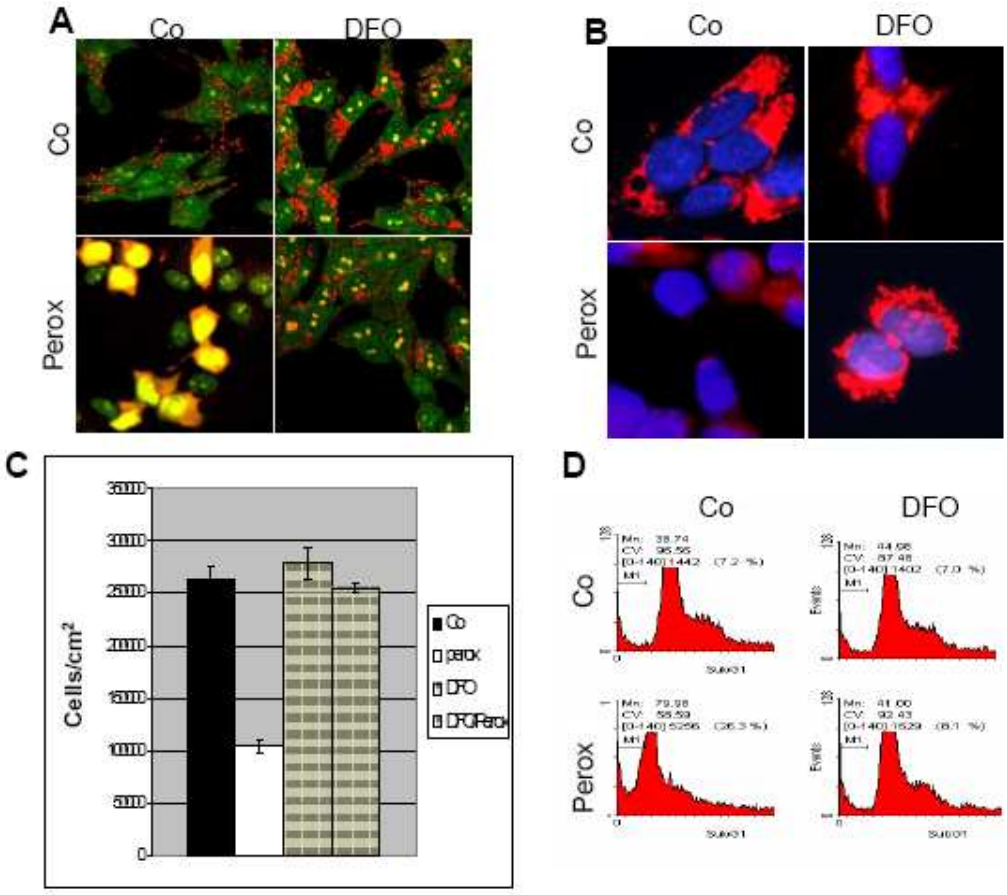
Figure 5. **Lysosome permeabilization by hydrogen peroxide is CD-dependent. (A)** Immunofluorescence staining of CD (red) and cytoskeletal tubulin (green) in control and hydrogen peroxide-treated cells grown on coverslips. Differently from controls, treated cells show a diffuse cytosolic staining of CD. **(B)** Cells adherent on coverslips were exposed or not to hydrogen peroxide under conditions in which either CB or CD had been inhibited with CA074Me or Pst, respectively. Cells were then labeled with AO and immediately observed under the fluorescence microscope. Intense red fluorescent spots are indicative of intralysosomal retention of AO. Leakage of AO from lysosomes (as indicated by weak and cytoplasm diffuse yellow-green fluorescence) occurs in hydrogen peroxide-treated cells. Pst, not CA074Me, prevents this effect. **(C)** Cells transfected as described in the legend to Figure 4 were exposed to hydrogen peroxide and lysosome integrity was assessed by testing AO retention. The images demonstrate that siRNA-mediate down-regulation of CD expression protects the lysosomes from hydrogen peroxide-induced damage. Images are representative of four independent experiments

Figure 6. **Interpretative scheme of the results.** Hydrogen peroxide leads to intralysosomal generation of hydroxyl radicals (via Fenton reaction) that cause lipid peroxidation of lysosome membrane and consequent lysosome leakage. DFO, an intralysosomal Fe^{2+}-chelator, avoids generation of ROS and lysosome leakage. Cytosolic relocation of CD triggers the intrinsic apoptosis pathway characterized by the following steps: permeabilization of mitochondria, activation of caspases and appearance of annexin V-positive and TUNEL-positive cell death.

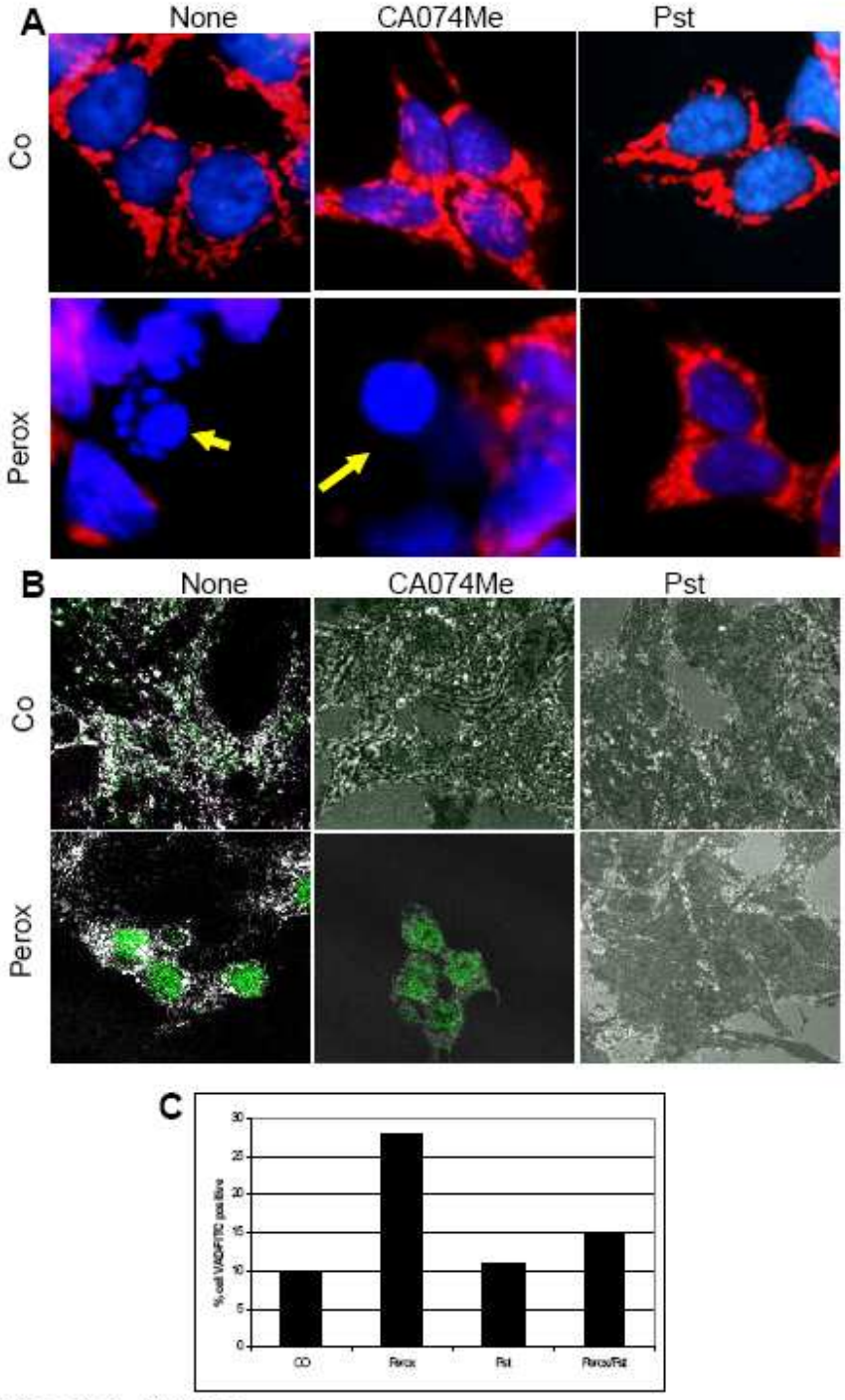
Inhibition or down-regulation of CD blocks this pathway at crucial steps: it suppresses the formation of transient micropores in lysosomal membrane and the permeabilization of mitochondria.



Castino et al., figure 1



Castino et al., figure 2



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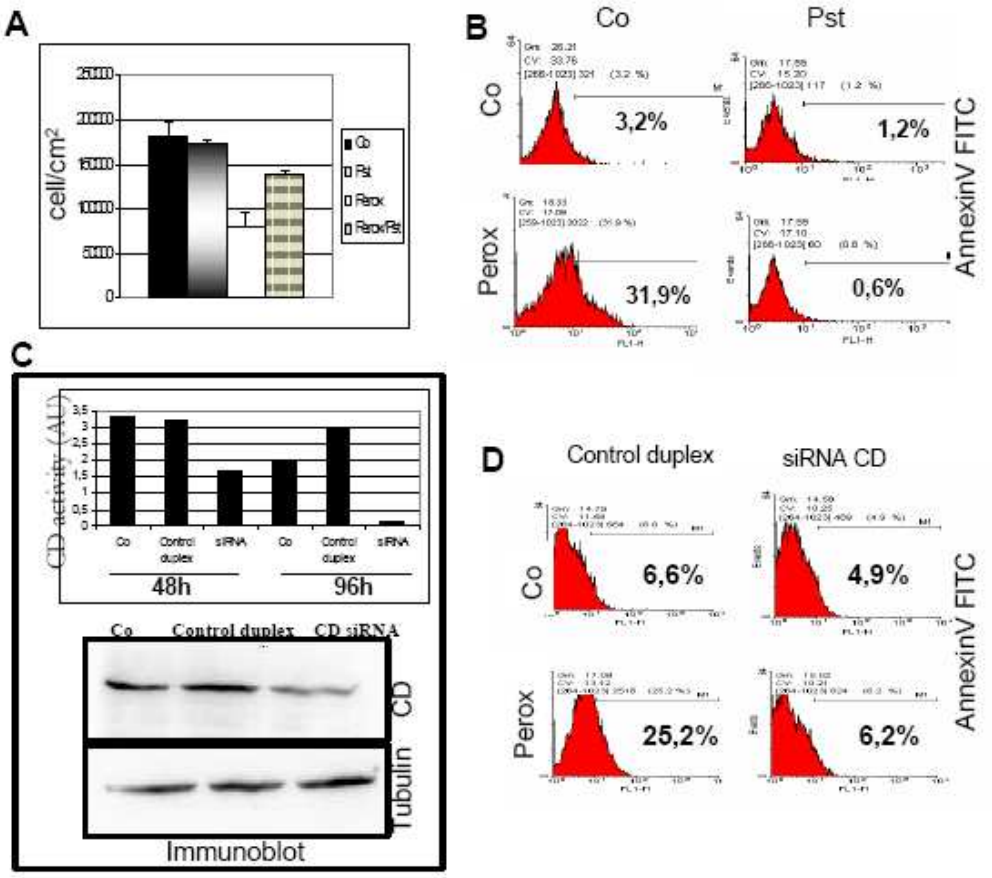

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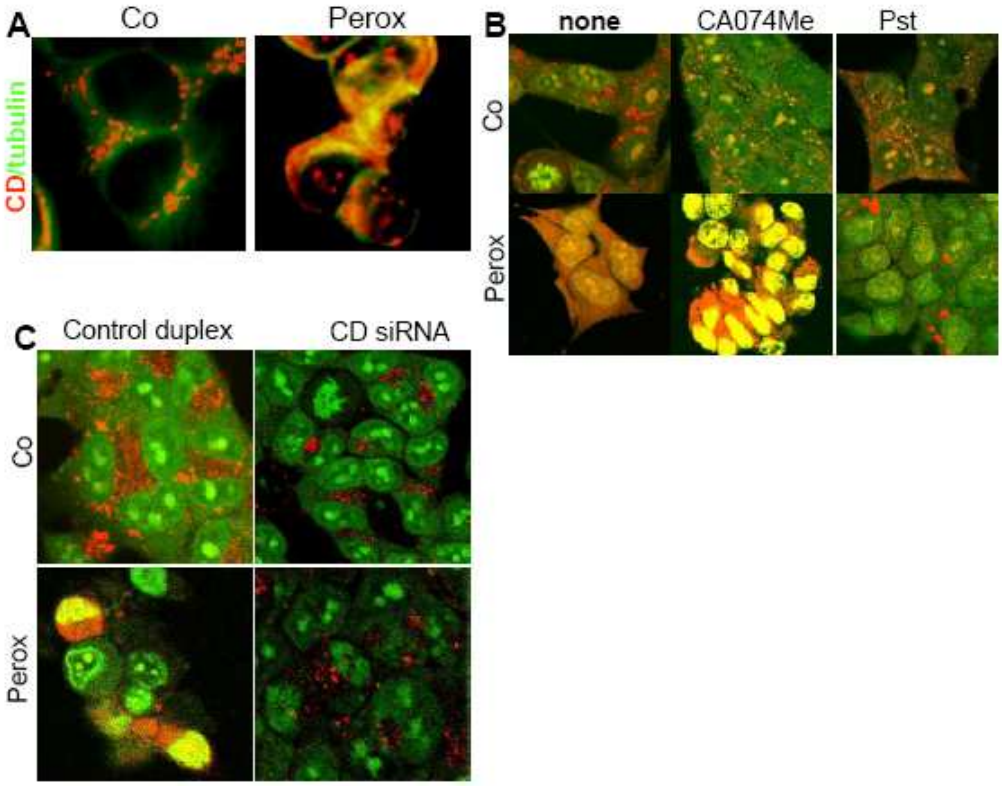
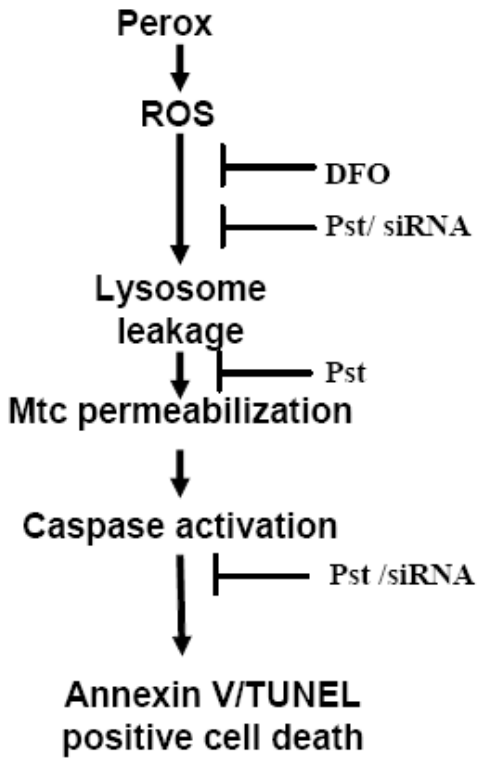


Castino et al., figure 5



Castino et al., figure 6

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BIBLIOGRAFIA

1. Abbott L.F., Nelson S.B. (2000) Synaptic plasticity: taming the beast. Nat. Neurosci. 3:1178-1183.

2. Agarwal S., Sohal R. S. (1994). Aging and proteolysis of oxidized proteins. Archives of Biochemistry Biophysics 309:24-28.

3. Aita V.M., Liang X.H., Murty V.V., Pincus D.L., Yu W., Cayanis E., Kalachikov S., Gilliam T.C., Levine B. (1999). Cloning and genomic organization of beclin 1, a candidate tumor suppressor gene on chromosome 17q21. Genomics 59:59-65.

4. Alakurtti K., Weber E., Rinne R., Theil G., de Haan G.J., Lindhout D., Salmikangas P., Saukko P., Lahtinen U., Lehesjoki A.E. (2005). Loss of lysosomal association of cystatin B proteins representing progressive myoclonus epilepsy, EPM1, mutations. Eur. J. Hum. Genet. 13(2):208-15.

5. Amenta JS, Sargus MJ, Baccino FM. (1978). Inhibition of basal protein degradation in rat embryo fibroblasts by cycloheximide: correlation with activities of lysosomal proteases. J. Cell Physiol. 97(3 Pt 1):267-83.

6. Amenta J.S., Baccino F.M (1989). Proteolysis and cell death. Revis Biol Cellular 21:401-422.

7. Anglade P., Vyas S., Javoy F. et al. (1997). Apoptosis and autophagy innigral neurons of patients with Parkinson's disease. Histol. Histophatol. 12:25-31.

8. Arbatova J., D'Amato E., Vaarmann A., Zharkovsky A., Reeben M. (2005). Reduced serotonin and 3-hydroxyanthranilic acid levels in serum of cystatin B-deficient mice, a model system for progressive myoclonus epilepsy. Epilepsia. 46 Suppl 5:49-51.

9. Ardley H.C., Hung C.C., Robinson P.A. (2005). The aggravating role of the ubiquitin-proteasome system in neurodegeneration. FEBS Lett. 31:579(3):571-6.

10. Arees E.A., Astrom K.E. (1977). Cell death in the optic tectum of the developing rat. Anat. Embryol. 9:151(1):29-34.

11. Arico S., Petiot A., Bauvy C. et al. (2001). The tumor suppressor PTEN positively regulates macroautophagy by inhibiting the phosphatidylinositol 3-kinase/protein kinase B pathway. J. Biol. Chem. 38:35243-35246.

12. Artal-Sanz M, Tavernarakis N. (2005). Proteolytic mechanisms in necrotic cell death and neurodegeneration. FEBS Lett. 13: 579(15):3287-96.

13. Baccino F.M., Tessitore L., Bonelli G. (1984). Control of degradation and growth phase in normal and neoplastic cells. Toxicol. Pathol. 12:281-287.

14. Bahr B. A., Bendiske J. (2002). The neurophathogeni contributions of lysosomal dysfuction. Journal of Neurochemistry 83:481-489.
15. Banda N.K., Bernier J., Kurahara D.K. et al. (1992). Crosslinking CD4 by human immunodeficienty virus gp 120 primers T cells for activation-induced apoptosis. J. Exp Med 176:109-1106.
16. Barone P., Bravi D., Bermejo-Pareja F. et al. (1999). Pergolide monotherapy in the tratment of esrly PD: a randomized, controlled study. Neurology 53:573-579.
17. Bauvy C., Gane P., Arico S., Cotogno P., Ogier-Denis E. (2001). Autophagy delays sulfide-induced apoptosis in the human intestinl colon cancer cell line HT-29. Exp. Cell Res. 268:139-149.
18. Barbacid M. (1995). Structural and functional properties of the TRK family of neurotrophin receptors.Ann. N Y Acad. Sci. 7:766:442-58.
19. Beaulaton J., Lockshin R. A. (1977). Ultrastructural study of the normal degeneration of the intersegmental musles of Anthereae polyphemus and Manduca sexta with particular reference of cellular autophagy. J. Morphol. 154:39-57.
20. Barde Y.A., Edgar D., Thoenen H. (1982). Purification of a new neurotrophic factor from mammalian brain. EMBO J. 1(5):549-53.
21. Baylis P.H., Robertson G.L. (1981). Vasopressin function in familial cranial diabetes insipidus. Postgrad. Med. J. 57(663):36-40.
22. Beaulaton J, Lockshin RA. (1982). The relation of programmed cell death to development and reproduction: comparative studies and an attempt at classification. Int Rev. Cytol. 79:215-35.
23. Beck T., Hall M. N. (1999). The TOR signalling pathway controls nuclear loclization of nutrient- regulated transcription factors. Nature 402: 689-692.
24. Behrens T.W., Muller D.L. (1996). Bcl-X and the regulationof survival in the immune system. Immunol. Res 16:149-160.
25. Bednarsky E., Lynch G. (1996). Cytosolic proteolysis of tau by cathepsin D in hippocampus following suppression of cathepsin B and L. Journal of Neurochemistry 67:1846-1855.
26. Berg T., Gjoen T., Bakke O. (1995). Physiological functions of endosomal proteolysis. Biochem. J. 307 (Pt 2):313-26.
27. Bertini F., Bari D.R. (1970). The effect of drugs on the rate of proteolysis in vitro within secondary liver lysosomes. J Cell Physiol. 1970 Oct;76(2):225-9.

28. Blommaart E.F., Luiken J.J. et al. (1995). Phosphorilation of ribosomal protein S6 is inhibitory for autophagy in isolated rat hepatocytes. J. Biol. Chem. 270 :2320-2326.
29. Blommaart EF, Krause U, Schellens JP, Vreeling-Sindelarova H, Meijer AJ. (1997). The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. Eur. J. Biochem. 243(1-2):240-6.
30. Boise L.H., Gonzales-Garcia M., Postema C.E.et al (1993) Bcl-x, a bcl-2-related gene that functions as a dominant regulator of apoptotic cell death. Cell. 74:597-608.
31. Bohley P., Seglen P.O. (1992). Proteases and proteolysis in the lysosome. Experientia 48:151-157.
32. Borner C., Monney L. (1999). Apoptosis without caspases: an inefficient molecular guillotine? Cell Death Differ. 6(6):497-507.
33. Bouillet P., Strasser A. (2002). BH3-only proteins - evolutionarily conserved proapoptotic Bcl-2 family members essential for initiating programmed cell death. J. Cell Sci. 115(8):1567-74.
34. Borgonovo B., Cocucci E., Racchetti G., Podini P., Bachi A., Meldolesi J. (2002). Regulated exocytosis: a novel, widely expressed system. Nat. Cell. Biol. 4(12):955-62.
35. Bossi G., Griffiths G.M. (1999). Degranulation plays an essential part in regulating cell surface expression of Fas ligand in T cells and natural killer cells. Nat. Med. 5(1):90-6.
36. Bradley M.O. (1977). Regulation of protein degradation in normal and transformed human cells. J. Biol. Chem. 252:53105315.
37. Brown E. J. et al (1995). Control of p70 s6 kinase by kinase activity of FRAP in vivo. Nature 377 : 441-446.
38. Brown G.C., Bal-Price A. (2003) Inflammatory neurodegeneration mediated by nitric oxide, glutamate and mitochondria. Mol. Neurobiol 27:325-355.
39. Browne S.E., Ferrante R.J., Beal M.F. (1999). Oxidative stress in Huntington's disease.
Brain Pathol. 9(1):147-63.
40. Brownstein M.J., Russell J.T., Gainer H. (1980). Synthesis, transport, and release of posterior pituitary hormones. Science. 207(4429):373-8.
41. Brunet A., Bonni A., Zigmond M.J., Lin M.Z., Juo P., Hu L.S., Anderson M.J., Arden K.C., Blenis J., Greenberg M.E., 1999. Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. Cell 96: 857–868.

42. Brunk U.T., Svensson (1999).Oxidative stress, growth factors starvation Redox. Rep.4:3-11.

43. Brunk U. T., Terman A. (2002). Lipofuscin: mechanism of age-related accumulation and influnce on cell function. Free Radical Biology & Medicine 33:611-619.

44. Brawn K., Fridovich I (1981). DNA strand scission by enzimically generated oxigen radicals. Arch Biochem Biophys 206:414-419.

45. Beckam K. B., Ames B.N. (1998). The free radical theory of aging matures. Physiological Reviews 78:547-581.

46. Bunker T. (1982). Preparing a leaflet for patient education. Br. Med. J. 5: 284(6330):1710.

47. Burbach J.P., De Hoop M.J., Schmale H., Richter D., De Kloet E.R., Ten Haaf J.A., De Wied D. (1984). Differential responses to osmotic stress of vasopressin-neurophysin mRNA in hypothalamic nuclei. Neuroendocrinology. 39(6):582-4.

48. Burger A.M., Jenkins T.C., Double J.A., Bibby M.C. (1999). Cellular uptake, cytotoxicity and DNA-binding studies of the novel imidazoacridinone antineoplastic agent C1311. Br. J. Cancer. 81:367-375.

49. Bursch W., Hochegger K., Torok L., Marian B., Ellinger A. and Hermann R.S. (2000) Autophagic and apoptotic types of programmed cell death exhibit different fates of cytoskeletal filaments. J. Cell Sci. 113, 1189–1198.

50. Bursch W. (2001). The autophagosomal-lysosomal compartment in programmed cell death. Cell Death Differ. 8:569-581.

51. Bursch W., Ellinger A., Kienzl H., Torok L., Pandey S., Sikorska M., Walker R., Hermann R.S. (1996). Active cell death induced by the anti-estrogens tamoxifen and ICI 164 384 in human mammary carcinoma cells (MCF-7) in culture: the role of autophagy. Carcinogenesis. 17:1595-607.

52. Burnside B. (1975). The form and arrangement of microtubules: an historical, primarily morphological, review. Ann. N Y Acad. Sci. 30:253:14-26.

53. Butterfield D. A., Kanski, J. (2001).Brain protein oxidation in age-related neurodegenerative disorders that are associated with aggregated proteins. Mechnical Ageing and development 122:945-962.

54. Camp L.A., Verkruyse L.A., Afendis S.J., Slaughter C.A., Hofmann S.L. (1994). Molecular cloning and expression of palmitoyl-protein thioesterase. J. Biol. Chem. 16;269(37):23212-9.

55. Cardone M.H., Smith B.L., Song W., Mochly-Rosen D., Mostov K.E. (1994). Phorbol myristate acetate-mediated stimulation of transcytosis and apical recycling in MDCK cells. J. Cell. Biol. 124(5):717-27.
56. Castino R., Pace D., Demoz M., Gargiulo M., Ariatta C., Raiteri E., Isidoro C. (2002). Lysosomal proteases as potential targets for the induction of apoptotic cell death in human neuroblastomas. Int. J. Cancer. 97:775-779.
57. Castino R., Demoz M., Isidoro C. (2003) Destination “Lysosome”: a target organelle for humour cell killing? J. Mol. Recognit 16:337-348.
58. Cataldo A.M., Barnett J.L., Mann D.M., Nixon R.A. (1996). Colocalization of lysosomal hydrolase and beta-amyloid in diffuse plaques of the cerebellum and striatum in Alzheimer's disease and Down's syndrome. J. Neuropathol. Exp. Neurol. 55(6):704-15.
59. Caughey B., Raymond G.J., Ernst D., Race R.E. (1991). N-terminal truncation of the scrapie-associated form of PrP by lysosomal protease(s): implications regarding the site of conversion of PrP to the protease-resistant state. J. Virol. 65(12):6597-603.
60. Cecconi F., Alvarez-Bolado G., Meyer B.I., Roth K.A., Gruss P. (1998). Apaf1 (CED-4 homolog) regulates programmed cell death in mammalian development. Cell. 18:94(6):727-37.
61. Charriaut-Marlangue C., Dessi F., Ben-Ari Y. (1996). Use of two-dimensional gel electrophoresis to characterize protein synthesis during neuronal death in cerebellar culture. Electrophoresis. 17(11):1781-6.
62. Chase L.R., Aurbach G.D. (1968). Renal adenyl cyclase: anatomically separate sites for parathyroid hormone and vasopressin. Science. 159(814):545-7.
63. Chesebro B., Trifilo M., Race R., Meade-White K., Teng C., LaCasse R., Raymond L., Favara C., Baron G., Priola S., Caughey B., Masliah E., Oldstone M. (2005). Anchorless prion protein results in infectious amyloid disease without clinical scrapie. Science. 308(5727):1435-9.
64. Brown N.A., Seabrook G.R. (1995). Phosphorylation- and voltage-dependent inhibition of neuronal calcium currents by activation of human D2(short) dopamine receptors. Br. J. Pharmacol. 115(3):459-66.
65. Chiarpotto E., Domenicotti C., Paola D., Vitali A., Nitti M., Pronzato M.A., Biasi F., Cottalasso D., Marinari U.M., Dragonetti A., Cesaro P., Isidoro C., Poli G. (1999). Regulation of rat hepatocyte protein kinase C beta isoenzymes by the lipid peroxidation

product 4-hydroxy-2,3-nonenal: A signaling pathway to modulate vesicular transport of glycoproteins. Hepatology. 29(5):1565-72.

66. Chinnaiyan A.M., O'Rourke K., Tewari M. and Dixit V.M. (1995). FADD, a novel death domain-containing protein, interacts with the death domain of Fas and initiates apoptosis. Cell 81: 505–512.

67. Chinnaiyan A.M., Chaudhary D., O'Rourke K., Koonin E.V., Dixit V.M. (1997). Role of CED-4 in the activation of CED-3. Nature. 388(6644):728-9.

68. Cho S., Dawson P.E., Dawson G. (2000). Antisense palmitoyl protein thioesterase 1 (PPT1) treatment inhibits PPT1 activity and increases cell death in LA-N-5 neuroblastoma cells. J. Neurosci. Res. 15;62(2):234-40.

69. Chou M.M., Blenis J. (1995). The 70 kDa S6 kinase: regulation of a kinase with multiple roles in mitogenic signalling. Curr. Opin. Cell. Biol. 7:806-814.

70. Chung J., Kuo C.J., Crabtree G.R., Blenis J. (1992). Rapamycin-FKBP specifically blocks growth-dependent activation of and signaling by the 70 kd S6 protein kinases. Cell. 69:1227-1236.

71. Clarke PG. (1982). The genuineness of isthmo-optic neuronal death in chick embryos. Anat. Embryol. (Berl). 165(3):389-404.

72. Clarke PG. (1985). Neuronal death during development in the isthmo-optic nucleus of the chick: sustaining role of afferents from the tectum. J. Comp. Neurol. 234(3):365-79.

73. Clarke PG, Clarke S. (1996). Nineteenth century research on naturally occurring cell death and related phenomena. Anat. Embryol. (Berl). 193(2):81-99.

74. Cuervo A.M. (2005). Autophagy: many paths to the same end. Mol. Cell. Biochem. 263(1-2):55-72.

75. Chinnaiyan A.N., O'Rourke K., Tewari M., Dixit V.M. (1995). FADD, a novel death domain-containing protein, interacts with death domain of Fas and initiates apoptosis. Cell 81:505-512.

76. Codogno P., Ogier-Denis E., Houri J.J. (1997). Signal transduction pathways in macroautophagy. Cell Signal. 9(2):125-30.

77. Cohen J.J., Duke R.C. (1992). Apoptosis and programmed cell death in immunity. Annu. Rev. Immunol. 10:267-293.

78. Cohen G.M., Park J.C., Grasso J.S. (1990). Comparison of demyelination and neural degeneration in spiral and Scarpa's ganglia of C57BL/6 mice. J. Electron. Microsc. Tech. 15(2):165-72.

79. Cole K.K., Perez-Polo J.R. (2002). Poly (ADP-ribose) polymerase inhibition prevents both apoptotic-like delayed neuronal death and necrosis after H2O2 injuri. J. Neurochem. 82 :19-29.

80. Collins S., Boyd A., Fletcher A., Gonzales M.F., McLean C.A., Masters C.L. Recent advances in the pre-mortem diagnosis of Creutzfeldt-Jakob disease. J. Clin. Neurosci. 7(3):195-202.

81. Comans P.E., McLennan I.S., Mark R.F. (1987). Mammalian motoneuron cell death: development of the lateral motor column of a wallaby (Macropus eugenii). J. Comp. Neurol. 22:260(4):627-34.

82. Cory S., Adams J.M. (2002). The Bcl2 family: regulators of the cellular life-or-death switch. Nat. Rev. Cancer. 2(9):647-56.

83. Cover C., Mansouri A., Knight T.R., Bajt M.L. et al (2005) Peroxynitrite-induced mitochondrial and endonuclease-mediated nuclear DNA damage in acetaminophen hepatotoxicity. J. Pharmacol. Exp. Ther. 315(2):879-87.

84. Cryns V.L., Byun Y., Rana A., Mellor H., Lustig K.D., Ghanem L., Parker P.J., Kirschner M.W., Yuan J. (1997). Specific proteolysis of the kinase protein kinase C-related kinase 2 by caspase-3 during apoptosis. Identification by a novel, small pool expression cloning strategy. J. Biol. Chem. 21:272(47):29449-53.

85. Cuervo A.M., Dice J.F. (1996). A receptor for the selective uptake and degradation of proteins by lysosomes. Science. 26;273(5274):501-3.

86. Cuervo A.M., Dice J.F. (1996). How do intracellular proteolytic systems change with age? Front Biosci. 1:3:d25-43.

87. Cuervo A.M., Dice J.F. (1998). Lysosomes, a meeting point of proteins, chaperones, and proteases. J. Mol. Med. 76(1):6-12.

88. Cuervo A.M., Dice J.F. (2000). Age-related decline in chaperone-mediated autophagy. J Biol Chem. 275(40):31505-13.

89. Cuervo A. M., Dice J. F. (2000). When lysosome get old. Experimental Gerontology 35:119-131.

90. Cunningham T.J. (1982). Naturally occurring neuron death and its regulation by developing neural pathways. Int. Rev. Cytol. 74:163-86.

91. Datta K., Biswal S.S., Kehrer J.P. (1999). The 5-lipoxygenase-activating protein (FLAP) inhibitor, MK886, induces apoptosis independently of FLAP. Biochem J. 340 :371-5.

92. Davies K. J.(1987). Protein damage and degradation by oxygen radicals. The Journal of Biological Chemistry 262:9895-9901.

93. Dawson G., Glaser P.T. (1988). Abnormal cathepsin B activity in Batten disease. Am. J. Med. Genet. Suppl. 1988;5:209-20.

94. Dawson V.L., Dawson T.M. (1998) Nitric oxide in neurodegeneration. Prog Brain Res 118:215-229.

95. Dechant G., Barde Y.A. (2002). The neurotrophin receptor p75(NTR): novel functions and implications for diseases of the nervous system. Nat. Neurosci. 5(11):1131-6.

96. de Duve C. (1983). Lysosomes revisited. Eur J Biochem. 137(3):391-7.

97. Deckwerth T.L., Elliott J.L., Knudson C.M., Johnson E.M. Jr, Snider W.D., Korsmeyer S.J. (1996). BAX is required for neuronal death after trophic factor deprivation and during development. Neuron. 17(3):401-11.

98. Deen P.M., Verdijk M.A., Knoers N.V., Wieringa B., Monnens L.A., van Os C.H., van Oost B.A. (1994). Requirement of human renal water channel aquaporin-2 for vasopressin-dependent concentration of urine. Science. 264(5155):92-5.

99. Degenhardt K, Chen G, Lindsten T, White E. (2002). BAX and BAK mediate p53-independent suppression of tumorigenesis. Cancer Cell. 2(3):193-203.

100. Deiss LP, Galinka H, Berissi H, Cohen O and Kimchi A (1996) Cathepsin D protease mediates programmed cell death induced by interferon-g, Fas/APO-1 and TNF-a. EMBO J. 15: 3861–3870.

101. Demoz M., Castino R., Cesaro P., et al. (2002). Endosomal-lysosomal proteolisysis mediates death signalling by TNF-alpha, not by etoposide, in L929 fibrosarcoma cells: evidence for active role of cathepsin D. Biol Chem 383:1237-1248.

102. Dennis P.B., Fumagalli S., Thomas G. (1999). Target of rapamycin (TOR): Balancing the opposing forces of protein synthesis and degradation. Curr. Opn. Genet. Dev. 9:49-54.

103. Deshmukh M., Johnson E.M. Jr. (1998). Evidence of a novel event during neuronal death: development of competence-to-die in response to cytoplasmic cytochrome c. Neuron. 21(4):695-705.

104. De Stefanis D., Démoz M., Dragonetti A., Houri J.J., Codogno O.D., Baccino F.M., Isidoro C. (1997). Differentiation-induced changes in the content, secretion, and subcellular distribution of lysosomal cathepsins in the human colon cancer HT-29 cell line. Cell Tissue Res. 289:109-117.

105. Di Cristofano A., Pandolfi P.P. (2000). The multiple roles of PTEN in tumor suppression. Cell. 18:100(4):387-90.

106. Ding Q., Dimayuga E., Martin S., Bruce-Keller A.J., Nukala V., Cuervo A.M., Keller J.N. (2003). Characterization of chronic low-level proteasome inhibition on neural homeostasis. J Neurochem. 86(2):489-97.

107. Dragonetti A., Baldassarre M., Castino R., Demoz M., Luini A., Buccione R., Isidoro C. (2000). The lysosomal protease cathepsin D is efficiently sorted to and secreted from regulated secretory compartments in the rat basophilic/mast cell line RBL. J. Cell. Sci. 113 (Pt 18):3289-98.

108. Dreher B., Potts R.A., Bennett M.R. (1983). Evidence that the early postnatal reduction in the number of rat retinal ganglion cells is due to a wave of ganglion cell death. Neurosci Lett. 29:36(3):255-60.

109. Dubois-Dauphin M., Frankowski H., Tsujimoto Y., Huarte J., Martinou J.C. (1994). Neonatal motoneurons overexpressing the bcl-2 protooncogene in transgenic mice are protected from axotomy-induced cell death. Proc. Natl. Acad. Sci. U S A. 91(8):3309-13.

110. Dunlop R. A., Rodgers K. J., Dean R. T. (2002). Recent development in tha intracellular degradation of oxidized proteins. Free Radical Biology & Medicine 33:894-906.

111. Dunn WA. Jr (1990). Studies on the mechanism of autophagy: formation of the autophagic vacuole. J. Cell. Biol. 110:1923-1933.

112. Earnshaw W.C., Martins L.M., Kaufmann S.H. (1999). Mammalian caspase: structure, activation, substrates, and functions during apoptosis. Annu Rev Biochem 68:383-424.

113. Ege T., Reisbig R.R., Rogne S. (1984). Enhancement of DNA-mediated gene transfer by inhibitors of autophagic-lysosomal function. Exp. Cell Res. 155(1):9-16.

114. Estus S., Zaks W.J., Freeman R.S., Gruda M., Bravo R., Johnson E.M. Jr. (1994). Altered gene expression in neurons during programmed cell death: identification of c-jun as necessary for neuronal apoptosis. J. Cell Biol. 127(6):1717-27.

115. Ezaki J., Wolfe L.S., Ishidoh K., Muno D., Ueno T., Kominami E. (1996). Lysosomal proteinosis based on decreased degradation of a specific protein, mitochondrial ATP synthase subunit C: Batten disease. Adv. Exp. Med. Biol. 389:121-8.

116. Ezaki J., Tanida I., Kanehagi N., Kominami E. (1999). A lysosomal proteinase, the late infantile neuronal ceroid lipofuscinosis gene (CLN2) product, is essential for degradation of a hydrophobic protein, the subunit c of ATP synthase. J. Neurochem. 72(6):2573-82.

117. Farlie P.G., Dringen R., Rees S.M., Kannourakis G., Bernard O. (1995). bcl-2 transgene expression can protect neurons against developmental and induced cell death. Proc. Natl. Acad Sci U S A. 9:92(10):4397-401.

118. Feng L., Xia Y., Seiffert D., Wilson C.B. (1995). Oxidative stress-inducible protein tyrosine phosphatase in glomerulonephritis. Kidney Int. 48(6):1920-8.

119. Ferrante R.J., Andreassen O.A., Jenkins B.G., et al. (2000). Neuroprotective effects of creatine in a transgenic mouse model of Huntington's disease. J. Neurosci. 20:4389-97.

120. Ferrer I., Tunon T., Soriano E., del Rio A., Iraizoz I., Fonseca M., Guionnet N. Calbindin immunoreactivity in normal human temporal neocortex. Brain Res. 14:572(1-2):33-41.

121. Ferri K.F, Kroemer G. (2001). Mitochondria--the suicide organelles. Bioessays. 23(2):111-5.

122. Finlay B.L., Berg A.T., Sengelaub D.R. (1982). Cell death in the mammalian visual system during normal development: II. Superior colliculus. J Comp Neurol. 1:204(4):318-24.

123. Finlay B.L., Pallas S.L. (1989). Control of cell number in the developing mammalian visual system. Prog. Neurobiol. 32(3):207-34.

124. Foghsgaard L, Wissing D, Mauch D, Lademann U, Bastholm L, Boes M, Elling F, Leist M and Ja¨a¨ ttela¨ M (2001) Cathepsin B acts as a dominant execution protease in tumor cell apoptosis induced by tumor necrosis factor. J. Cell Biol. 153: 999–1009.

125. Fossgreen A., Bruckner B., Czech C., Masters C.L., Beyreuther K., Paro R. (1998). Transgenic Drosophila expressing human amyloid precursor protein show gamma-secretase activity and a blistered-wing phenotype. Proc. Natl. Acad. Sci. U S A. 10:95(23):13703-8.

126. Frade J.M., Rodriguez-Tebar A., Barde Y.A. (1996). Induction of cell death by endogenous nerve growth factor through its p75 receptor. Nature. 12:383(6596):166-8.

127. Fruman D.A., Meyers R.E., Cantley L.C. (1998). Phosphoinositide kinases. Annu Rev Biochem. 67:481-507.

128. Garcia R., Aguiar J., Alberti E., de la Cuetara K., Pavon N. (2004). Bone marrow stromal cells produce nerve growth factor and glial cell line-derived neurotrophic factors. Biochem. Biophys. Res. Commun. 9:316(3):753-4.

129. Giasson B. I., Duda J. E., Murray I. V., Chen Q., Souza J. M., Hurtig H. I. et al. (2000). Oxidative damage linked to neurodeneration by selective alpha-synuclein nitration in synucleinophathy lesions. Science 290:985-989.

130. Glucksmann A. (1965). Cell death in normal development. Arch Biol (Liege). 76(2):419-37.

131. Goate A., Chartier-Harlin M.C., Mullan M., Brown J., Crawford F., Fidani L., Giuffra L., Haynes A., Irving N., James L., et al. (1991). Segregation of a missense mutation in the amyloid precursor protein gene with familial Alzheimer's disease. Nature. 21:349(6311):704-6.

132. Goebel H.H. (1995). The neuronal ceroid-lipofuscinoses. J. Child. Neurol. 10(6):424-37.

133. Gomez-Santos C., Ferrer I., Santidrian A. F., Barrachina M., Gil J., Ambrosio S. (2003). Dopamina induces autophagic cell death and alpha-synuclein increase in human neuroblastma SH-SY5Y cells. Journal of Neuroscience Research 73:341-350.

134. Gonzalez-Garcia M., Garcia I., Ding L., O'Shea S., Boise L.H., Thompson C.B., Nunez G. (1995). Bcl-x is expressed in embryonic and postnatal neural tissues and functions to prevent neuronal cell death. Proc. Natl. Acad. Sci. U S A. 9:92(10):4304-8.

135. Greenbaum L.M., Esumi H., Sato S. (1979). Further studies of the effect of pepstatin on ascites accumulation in tumor-bearing mice. Cancer Lett. 7:91-6.

136. Grinde B., Seglen P.O. (1981). Effects of amino acid analogues on protein degradation in isolated rat hepatocytes. Biochim. Biophys. Acta. 5:676:43-50.

137. Grune T., Shringarpure R., Sitte N., Davies K. J. (2004). Age-related changes in protein oxidation in protein oxidation and proteolysis in mammalian cells. Journal of Gerontology 56:B459-B467.

138. Gruss-Fischer T., Fabian I (2002). Protection by ascorbic acid from denaturation and release of cytochrome C, alteration of mitochondrial membrane potential and ativation of multiple caspases induced by H2O2 in human leukemia cells. Biochem. Pharmacol. 63:1325-1335.

139. Gu F., Gruenberg J. (1999). Biogenesis of transport intermediates in the endocytic pathway. FEBS Lett. 452(1-2):61-6.

140. Guicciardi M.E., Deussing J., Miyoshi H., Bronk S.F., Svingen P.A., Peters C., Kaufmann S.H., Gores G.J. (2000). Cathepsin B contributes to TNF-alpha-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome c. J. Clin. Invest. 106(9):1127-37.

141. Hay J.C., Scheller R.H. (1997). SNAREs and NSF in targeted membrane fusion. Curr. Opin. Cell. Biol. 9(4):505-12.

142. Ham J., Babij C., Whitfield J., Pfarr C.M., Lallemand D., Yaniv M., Rubin L.L. (1995). A c-Jun dominant negative mutant protects sympathetic neurons against programmed cell death. Neuron. 14(5):927-39.

143. Hara Y., Battey J., Gainer H. (1990). Structure of mouse vasopressin and oxytocin genes. Brain Res. Mol. 8(4):319-24.

144. Harris A.J, McCaig C.D. (1984). Motoneuron death and motor unit size during embryonic development of the rat. J. Neurosci. 4(1):13-24.

145. Harris C.A., Johnson E.M. Jr. (2001). BH3-only Bcl-2 family members are coordinately regulated by the JNK pathway and require Bax to induce apoptosis in neurons. J. Biol. Chem. 12:276(41):37754-60.

146. Harris H.W. Jr, Strange K., Zeidel M.L. (1991). Current understanding of the cellular biology and molecular structure of the antidiuretic hormone-stimulated water transport pathway. J. Clin. Invest. 88(1):1-8.

147. Heinrich M., Neumeyer J., Jakob M., Hallas C., Tchikov V., Winoto-Morbach S., Wickel M., Schneider-Brachert W., Trauzold A., Hethke A., Schutze S. (2004). Cathepsin D links TNF-induced acid sphingomyelinase to Bid-mediated caspase-9 and -3 activation. Cell Death Diff. 11(5):550-63.

148. Heyes F.A., Smith E.I., Pizzo P.A., Poplack D.G., Lippincot J.P. (1989). Philadelphia 623-634.

149. Hellsten E., Vesa J., Olkkonen V.M., Jalanko A., Peltonen L. (1996). Human palmitoyl protein thioesterase: evidence for lysosomal targeting of the enzyme and disturbed cellular routing in infantile neuronal ceroid lipofuscinosis. EMBO J. 1;15(19):5240-5.

150. Heppner G.H. (1984). Tumor heterogeneity. Cancer Res. 44:2259-2265.

151. Herman J.P., Schafer M.K., Watson S.J., Sherman T.G. (1991). In situ hybridization analysis of arginine vasopressin gene transcription using intron-specific probes. Mol. Endocrinol. 5(10):1447-56.

152. Holen I., Gordon P. B., Seglen P.O. (1993). Inhibition of hepatocytic autophagy by okadaic acid and other protein phosphatase inhibitors. Eur. J. Biochem. 215:113–122.

153. Holen I., Gordon P.B., Seglen P.O. (1992). Protein kinase-dependent effects of okadaic acid on hepatocytic autophagy and cytoskeletal integrity. Biochem. J. 284:633-636.

154. Holen I., Gordon P.B., Stromhaug P.E., Seglen P.O. (1996). Role of cAMP in the regulation of hepatocytic autophagy. Eur. J. Biochem. 15:236(1):163-70.

155. Hopgood M.F., Clark M.G., Ballard F.J. (1980). Protein degradation in hepatocyte monolayers. Effects of glucagon, adenosine 3':5'-cyclic monophosphate and insulin. Biochem. J. 15:186(1):71-9.

156. Hopfner M., Maaser K., Theiss A., Lenz M., Sutter A.P., Kashtan H., Von Lampe B., Riecken E.O., Zeitz M., Scherubl H. (2003). Hypericin activated by an incoherent light source has photodynamic effects on esophageal cancer cells. Int. J. Colorectal. Dis. 18:239-47.

157. Houseweart M.K., Vilaythong A., Yin X.M., Turk B., Noebels J.L., Myers R.M. (2003). Apoptosis caused by cathepsins does not require Bid signaling in an in vivo model of progressive myoclonus epilepsy (EPM1). Cell Death Differ. 10(12):1329-35.

158. Hsu S.Y., Lin P., Hsueh A.J. (1998). BOD (Bcl-2-related ovarian death gene) is an ovarian BH3 domain-containing proapoptotic Bcl-2 protein capable of dimerization with diverse antiapoptotic Bcl-2 members. Mol. Endocrinol. 12(9):1432-40.

159. Hurle J.M. (1988). Cell death in developing systems. Methods. Achiev. Exp. Pathol. 13:55-86.

160. Isahara K, Ohsawa Y, Kanamori S, Shibata M, Waguri S, Sato N, Gotow T, Watanabe T, Momoi T, Urase K, Kominami E and Uchiyama Y (1999). Regulation of a novel pathway for cell death by lysosomal aspartic and cysteine proteinases. Neuroscience 91: 233–249.

161. Ishihara I., .Minami Y., Nishizaki T., Matsuoka T., Yamamura H. (2000). Activation of calpain precedes morphological alterations during hydrogen peroxide-induced apoptosis in neuronally differentiated mouse embryonal carcinoma p19 cell line. Neurosi Lett.279:97-100.

162. Ishisaka R., Utsumi T., Yabuki M., Kanno T., Furuno T., Inoue M. and Utsumi K. (1998) Activation of caspase-3-like protease by digitonin-treated lysosomes. FEBS Lett. 435: 233–236.

163. Ishisaka R., Utsumi T., Kanno T., Arita K., Katunuma N., Akiyama J. and Utsumi K. (1999) Participation of a cathepsin-L-type protease in the activation of caspase-3. Cell Struct. Funct. 24: 465–470.

164. Isidoro C., Démoz M., De Stefanis D., Baccino F.M., Monelli G. (1995a). Synthesis, maturation and extracellular release of procathepsin D as influenced by cell proliferation or transformation. Int. J. Cancer 63:866-871.

165. Isidoro C., de Stefanis D., Démoz M., Ogier-Denis E., Codogno P., Baccino F.M. (1997a). Expression and Posttranslational fate of cathepsin D in HT-29 tumor cells depend on their enterocytic differentiation state. Cell. Growth and Diff. 8:1029-1037.

166. Ito T, Deng X, Carr BK, May WS. Bcl-2 phosphorylation required for anti-apoptosis function. J Biol Chem 1997;272:11671–3.

167. Inoki K., Ouyang H., Li Y., Guan K.L. (2005). Signaling by target of rapamycin proteins in cell growth control. Microbiol. Mol. Biol. Rev. 69(1):79-100.

168. Ivell R., Richter D. (1984). Structure and comparison of the oxytocin and vasopressin genes from rat. Proc. Natl. Acad. Sci. U S A. 81(7):2006-10.

169. Jackson G.S., Collinge J. (2001). The molecular pathology of CJD: old and new variants. Mol. Pathol. 54(6):393-9.

170. Jaiswal J.K., Andrews N.W., Simon S.M. (2002). Membrane proximal lysosomes are the major vesicles responsible for calcium-dependent exocytosis in nonsecretory cells. J. Cell. Biol. 159(4):625-35.

171. Janowsky J.S., Finlay B.L. (1983). Cell degeneration in early development of the forebrain and cerebellum. Anat. Embryol. (Berl). 167(3):439-47.

172. Jelinek R., Friebova Z. (1966). Influence of mitotic activity on neurulation movements. Nature. 19:209(25):822-3.

173. Jellinger K.A. e Stadelmann C. (2000). Mechanisms of cell death in neurodegenerative disorders. J. Neural. Transm. Suppl. 59:95-114.

174. Jeyakumar M., Dwek R.A, Butters T.D., Platt F.M. (2005). Storage solutions: treating lysosomal disorders of the brain. Nat. Rev. Neurosci. 6(9):713-25.

175. Jacobson M.D., Weil M., Raff M.C. (1997). Programmed cell death in animal development. Cell 88:347-354.

176. Jia L., Dourmashkin R.R., Gray A.B., Newland A.C., Kesley S.M. (1997). Inhibition of autophagy abrogates tumot necrosis factor alpha induced apoptosis in human T-lymphoblastic Leukaemic cells. Br.J.Haematol. 98:673-685,1997.

177. Jia L., Dourmashkin R.R., Allen P.D., Gray A.B., Newland A.C., Kelsey S.M. (1997). Inhibition of autophagy abrogates tumour necrosis factor alpha induced apoptosis in human T-lymphoblastic leukaemic cells. Br. J. Haematol. 98:673-685.

178. Jiang D., Boonplueang R., Andersen K. (2001). Caspase 3 inhibition attnuates hydrogen peroxide-induced DNA fragmentation but not cell death in neuronal PC12 cells. J. Neurochem. 76:1745-1755.

179. Johnson G.V., Simonato M., Jope R.S. (1988). Dose- and time-dependent hippocampal cholinergic lesions induced by ethylcholine mustard aziridinium ion: effects of nerve growth factor, GM1 ganglioside, and vitamin E. Neurochem. Res. 13(8):685-92.

180. Kabeya Y., Mizushima N., Ueno T., Yamamoto A., Kirisako T., Noda T., Kominami E., Ohsumi Y., Yashimoro T.et (2000). LC3, a mammalian homilogue of yeast Apg8p, is localized in autophagosome membranes after processing. EMBO J. 19:5720-5728.

181. Kabeya Y., Mizushima N., Yamamoto A., Oshitani-Okamoto S., Ohsumi Y., Yoshimori T. Kabeya et al., (2004) LC3, GABARAP and GATE16 localize to autophagosomal membrane depending on form-II formation. J. Cell Sci. 1:117:2805-12.

182. Ka¨gedal K, Johansson U and O¨ llinger K (2001) The lysosomal protease cathepsin D mediates apoptosis induced by oxidative stress. FASEB J. 15:
1592–1594.

183. Kang S.J., Wang S., Hara H., Peterson E.P., Namura S., Amin-Hanjani S., Huang Z., Srinivasan A., Tomaselli K.J., Thornberry N.A., Moskowitz M.A., Yuan J. (2000). Dual role of caspase-11 in mediating activation of caspase-1 and caspase-3 under pathological conditions. J. Cell Biol. 1:149(3):613-22.

184. Kang P.M., Haunstetter A., Aoki H., Usheva A., Izumo S. (2000). Morphological and molecular characterization of adult cardiomyocyte apoptosis during hypoxia and reoxygenation. Circ Res. 21;87(2):118-25.

185. Kanzawa T., Kondo Y., Ito H., Kondo S., Germano I. (2003). Induction of autophagic cell death in malignant glioma cells by arsenic trioxide. Cancer Res. 632103-632108.

186. Karfunkel P. (1974). The mechanisms of neural tube formation. Int. Rev. Cytol. 38(0):245-71.

187. Karlsson K., Carlsson S.R. (1998). Sorting of lysosomal membrane glycoproteins lamp-1 and lamp-2 into vesicles distinct from mannose 6-phosphate

receptor/gamma-adaptin vesicles at the trans-Golgi network. J. Biol. Chem. 273(30):18966-73.

188. Katunuma N, Matsui A, Le QT, Utsumi K, Salvesen G and Ohashi A (2001) Novel procaspase-3 activating cascade mediated by lysoapoptases and its biological significances in apoptosis. Adv. Enzyme Regul. 41: 237–250.

189. Kaufmann SH and Gores GJ (2000) Cathepsin B contributes to TNF-amediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome c. J. Clin. Invest. 106: 1127–1137.

190. Kegel K.B., Kim M., Sapp E., McIntyre C., Castano J.G., Aronin N., DiFiglia M. (2000). Huntingtin expression stimulates endosomal-lysosomal activity, endosome tubulation, and autophagy. J. Neurosci. 1:20(19):7268-78.

191. Kelekar A. Thompson CB. Bcl-2-family proteins: the role of the BH3 domain in apoptosis. Trends Cell Biol 1998;8:324–30.

192. Kerr J.F, Harmon B., Searle J. (1974). An electron-microscope study of cell deletion in the anuran tadpole tail during spontaneous metamorphosis with special reference to apoptosis of striated muscle fibers. J. Cell Sci. 14(3):571-85.

193. Kessel D, luo Y., Mathieu P., Reiners jr J.J. (2000). Determinants of the apoptotic response to lysosomal photodamage. Photochem.Photobiol. 71:196-200.

194. Kheslelava N., Seeger R.C., Reynolds C.P. (1997). Drug resistance in human neuroblastoma cell lines correlates with clinical therapy. Eur. J. Cancer 33:2002-2006

195. Kiechle T, Dedeoglu A, Kubilus J, et al. (2002). Cytochrome c and caspase-9 expression in Huntington's disease. Neuromolecular Med. 1:183-95.

196. Kihara A., Noda T., Ishihara N., Oshumi Y. (2001). Two distinct Vps34 phopphatidylinosotol 3-kinase complexes function i autophagy and carboxypeptidase Y sortine i Saccharomyces cerevisiae. J. Cell. Biol. 152:519-530.

197. Kim J., Kliosky D.J. (2000). Autophagy, Cytoplasma-to-vacuole targeting pathway, and pexophagy in yeast and mammalian cells. Annu.Rev.Biochem. 69:303-342.

198. Kirkland R.A., Adibhatla R.M., Hatcher J.F., Franklin J.L. (2002). Loss of cardiolipin and mithocondria during aprogrammed neuronal death: evidence of a role for lipid proxidation and autophagy. Neuroscience 115:587-602.

199. Kischkel F.C., Lawrence D.A., Tinel A., LeBlanc H., Virmani A., Schow P., Gazdar A., Blenis J., Arnott D., Ashkenazi A., Kisckel et al., (2001). Death receptor

recruitment of endogenous caspase-10 and apoptosis initiation in the absence of caspase-8. J. Biol. Chem. 7;276(49):46639-46.

200. Kisen G.O., Tessitore L., Costelli P., Gordon P.B., Schwarze P.E., Baccino F.M., Seglen P.O. (1993). Reduced autophagic activity in primary rat hepatocellular carcinoma and ascites hepatoma cells. Carcinogenesis 14:2501-2505.

201. Kitanaka C., Kuchino Y. (1999). Caspase-indipendent programmed cell death with necrotic morphology. Cell. Death Differ 6: 508-515.

202. Klionsky D.J. et al. (2003). A unified nomenclature for yeast autophagy-related genes. Dev. Cell 5: 539–545.

203. Klionsky D.J., Emr S.D. (2000). Autophagy as a regulated pathway of cellular degradation. Science 290:1717-21.

204. Knecht E., Hernandez-Yago J., Grisolia S. (1984). Regulation of lysosomal autophagy in transformed and non-transformed mouse fibroblasts under several growth conditions. Exp Cell Res 154: 130-138.

205. Koike T., Martin D.P., Johnson E.M. Jr. (1989). Role of Ca2+ channels in the ability of membrane depolarization to prevent neuronal death induced by trophic-factor deprivation: evidence that levels of internal Ca2+ determine nerve growth factor dependence of sympathetic ganglion cells. Proc. Natl. Acad. Sci. U S A. 86(16):6421-5.

206. Kopitz J., Kisen G.O., Gordon P.B., Bohley P., Seglen P.O. (1990). Nonselective autophagy of cytosolic enzymes by isolated rat hepatocytes. J. Cell. Biol. 111(3):941-53.

207. Kornfeld S., Mellman I. (1989). The biogenesis of lysosomes. Annu. Rev. Cell. Biol. 5:483-525.

208. Koskiniemi M., Hyyppa M., Sainio K., Salmi T., Sarna S., Uotila L. (1980). Transient effect of L-tryptophan in progressive myoclonus epilepsy without Lafora bodies: clinical and electrophysiological study. Epilepsia. 21(4):351-7.

209. Kostic V., Jackson-Lewis V., de Bilbao F., Dubois-Dauphin M., Przedborski S. (1997). Bcl-2: prolonging life in a transgenic mouse model of familial amyotrophic lateral sclerosis. Science. 25:277(5325):559-62.

210. Kovacs A.L., Molnar K., Seglen P.O. (1981). Inhibition of autophagic sequestration and endogenous protein degradation in isolated rat hepatocytes by methylated adenosine derivatives. FEBS Lett. 16:134(2):194-6.

211. Kuida, K., Zheng, T.S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P. & Flavell, R.A. (1996) Decreased apoptosis in the brain and premature premature lethalityin CPP32-deficient mice. Nature 384:368–372.

212. Kuida K., Haydar T.F., Kuan C.Y., Gu Y., Taya C., Karasuyama H., Su M.S., Rakic P., Flavell RA. (1998). Reduced apoptosis and cytochrome c-mediated caspase activation in mice lacking caspase 9. Cell. 7:94(3):325-37.

213. Lance-Jones C. , (1982). Motoneuron cell death in the developing lumbar spinal cord of the mouse. Brain Res. 256(4):473-9.

214. Langman J., Guerrant R.L., Freeman B.G. (1966). Behavior of neuro-epithelial cells during closure of the neural tube. J. Comp. Neurol. 127(3):399-411.

215. Larsen K., Sulzer D. (2002). Autophagy in neurons: a review. Histology and Histophatology 17:987-908.

216. LeBlanc AC. (2005). The role of apoptotic pathways in Alzheimer's disease neurodegeneration and cell death. Curr. Alzheimer Res. 2(4):389-402.

217. Leino E., MacDonald E., Airaksinen M.M., Riekkinen P.J. (1980). Homovanillic acid and 5-hydroxyindoleacetic acid levels in cerebrospinal fluid of patients with progressive myoclonus epilepsy. Acta Neurol. Scand. 62(1):41-54.

218. Leino E., MacDonald E., Airaksinen M.M., Riekkinen P.J, Salo H. (1981). L-tryptophan-carbidopa trial in patients with long-standing progressive myoclonus epilepsy. Acta Neurol Scand. 1981 Aug;64(2):132-41.

219. Lemasters J.J., Nieminen A.L., Qian T., Trost L.C., Elmore S.P., Nishimura Y., Crowe R.A., Cascio W.E., Bradham C.A., Brenner D.A., Herman B. (1998). The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. Biochim. Biophys. Acta 1366:177-196.

220. Leto G., Pizzolanti G., Tumminello F.M., Gebbia N. (1994). Effects of E-64 (cysteine-proteinase inhibitor) and pepstatin (aspartyl-proteinase inhibitor) on metastasis formation in mice with mammary and ovarian tumors. In Vivo. 8:231-6.

221. Levy-Lahad E., Wasco W., Poorkaj P., Romano D.M., Oshima J., Pettingell W.H., Yu C.E., Jondro P.D., Schmidt S.D., Wang K., et al. (1995). Candidate gene for the chromosome 1 familial Alzheimer's disease locus. Science. 269(5226):973-7.

222. Lewis P.A., Properzi F., Prodromidou K., Clarke A.R., Collinge J., Jackson G.S. (2006). Removal of the glycosylphosphatidylinositol anchor from PrP(Sc) by cathepsin D does not reduce prion infectivity. Biochem J. 395(2):443-8.

223. Li W., Yuan X., Nordgren G., Dalen H., Dubowchik G.M., Firestone R.A., Brunk U.T. (2000). Induction of cell death by the lysosomotropic getergent MSDH. FEBS Lett. 470:35-39.

224. Li P., Nijhawan D., Budihardjo I., Srinivasula S.M., Ahmad M., Alnemri, E.S. Wang X. (1997) Cytochrome c and dATPdependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. Cell 91:479–489.

225. Liang X.H., Kleeman L.K., Jiang H.H. et al. (1998). Protection againts Fatal Sindbis Virus Encephalitis by Beclin, a novel Bcl-2-interacting Protein. Journal of virology 72:8586-8596.

226. Liang XH, Jackson S., Seaman M., Brown K., Kemkes B., Hibshoosh H., Levine B. (1999). Induction of autophagy anh inhibition of tumorigenesis by beclin 1. Nature 402:672-676.

227. Liberski P.P., Gajdusek D.C., Brown P. (2002). How do neurons degenerate in prion diseases or transmissible spongiform encephalopathies (TSEs): neuronal autophagy revisited. Acta Neurobiol. Exp. 62(3):141-7.

228. Liberski P.P., Sikorska B., Bratosiewicz-Wasik J., Gajdusek D.C., Brown P. (2004). Neuronal cell death in transmissible spongiform encephalopathies (prion diseases) revisited: from apoptosis to autophagy. Int. J. Biochem. Cell. Biol. 2004 36(12):2473-90.

229. Lightman S.L., Young W.S. (1987). Vasopressin, oxytocin, dynorphin, enkephalin and corticotrophin-releasing factor mRNA stimulation in the rat. J. Physiol. 394:23-39.

230. Lim C.S., Lee J.C., Kim S.D., Cheng D.J., Kaang B.K. (2002). Hydrogen peroxide-induced cell death in Aplasia sensory neurons. Brain Research 941:137-145.

231. Linseman D.A., Phelps R.A., Bouchard R.J., Le S.S., Laessig T.A., McClure M.L., Heidenreich K.A. (2002). Insulin-like growth factor-I blocks Bcl-2 interacting mediator of cell death (Bim) induction and intrinsic death signaling in cerebellar granule neurons. J Neurosci. 1:22(21):9287-97.

232. Linseman D.A., Butts B.D., Precht T.A., Phelps R.A., Le S.S., Laessig T.A., Bouchard R.J., Florez-McClure M.L., Heidenreich K.A. (2004). Glycogen synthase kinase-3beta phosphorylates Bax and promotes its mitochondrial localization during neuronal apoptosis. J. Neurosci. 3:24(44):9993-10002.

233. Liu B., Gao H.M., Wang J.Y., Jeohn G.H., Cooper C.L. Hong J.S. (2002) Role of nitric oxide in inflammation-mediated neurodegeneration. Ann n Y Acad Sci 962:318-331.

234. Lockshin R.A., Zakeri Z. (2002). Caspase-independent cell deaths. Curr. Opin. Cell. Biol. 14(6):727-33.

235. Lockwood T.D., Minassian I.A. (1982). Protein turnover and proliferation. Failure of SV-3T3 cells to increase lysosomal proteinases, increase protein degradation and cease net protein accumulation. Biochem. J. 206:251-258.

236. Lockwood T.D., Shier W.T. (1977). Regulation of acid proteases during growth, quiescence and starvation in normal and transformed cells. Nature 267:252-254.

237. Lockwood T.D., Minassian I.A., Roux L. (1982). Protein turnover and proliferation. Turnover kinetics associated with elevation of 3T3-cell acid-proteinase activity and cessation of net protein gain. Biochem J 206: 239-249.

238. Lolait S.J., O'Carroll A.M., McBride O.W., Konig M., Morel A., Brownstein M.J. (1992). Cloning and characterization of a vasopressin V2 receptor and possible link to nephrogenic diabetes insipidus. Nature. 357(6376):336-9.

239. Lynch G., Bi X. (2003). Lysosomes and brain aging in mammals. Neurochemical Research 28:1725-1734.

240. Lundvig D., Lindersson E., Jensen P.H. (2005) Pathogenic effects of alpha-synuclein aggregation. Brain Res Mol Brain Res 134:3-17.

241. Luzio J.P., Rous B.A., Bright N.A., Pryor P.R., Mullock B.M., Piper R.C. (2000). Lysosome-endosome fusion and lysosome biogenesis. J. Cell. Sci. 13 (Pt 9):1515-24.

242. Maehama T., Dixon J.E. (1998). The tumor suppressor, PTEN/MMAC1, dephosphorylates the lipid second messenger, phosphatidylinositol 3,4,5-trisphosphate. J .Biol. Chem. 29:273(22):13375-8.

243. Mahajan N.P., Linder K., Berry G., Gordon G.W., Heim R., Herman B. (1998). Bcl-2 and Bax interactions in mitochondria probed with green fluorescent protein and fluorescence resonance energy transfer. Nat. Biotechnol.16:547–52.

244. Mangiarini L., Sathasivam K., Seller M., et al. (1996). Exon 1 of the HD gene with an expanded CAG repeat is sufficient to cause a progressive neurological phenotype in transgenic mice. Cell.87:493-506.

245. Manev H., Cagnoli C.M., Atabay C., Kharlamov E. et al (1995). Neuronal apoptosis in an in vitro model of photochemically induced oxidative stress. Exp. Neurol. 133:19-206.

246. Mao Z., Bonni A., Xia F., Nadal-Vicens M., Greenberg M.E. (1999). Neuronal activity-dependent cell survival mediated by transcription factor MEF2. Science. 286(5440):785-90.

247. Martin-Morris L.E., White K. (1990). The Drosophila transcript encoded by the beta-amyloid protein precursor-like gene is restricted to the nervous system.

248. Development. 110(1):185-95.

249. Martinet W., Deroo T., Saelens X., Beirnaert E., Vanlandschoot P., Contreras R., Fiers W., Min Jou W. (1998). Evaluation of recombinant A/Victoria/3/75 (H3N2) influenza neuraminidase mutants as potential broad-spectrum subunit vaccines against influenza A. Arch. Virol.143(10):2011-9.

250. Martinou I, Fernandez PA, Missotten M, White E, Allet B, Sadoul R, Martinou JC. (1995). Viral proteins E1B19K and p35 protect sympathetic neurons from cell death induced by NGF deprivation. J. Cell. Biol. 128(1-2):201-8.

251. Mattson M. (1998). Modification of ion homeostasis by lipid peroxidation: roles in neuronal degeneration and adaptative plasticità. Trends Neurosi 21:53-57.

252. Matthews M.R., Raisman G. (1972). A light and electron microscopic study of the cellular response to axonal injury in the superior cervical ganglion of the rat. Proc. R. Soc. Lond. B. Biol. Sci. 181(62):43-79.

253. May W.S., Tyler P.G., Ito T., Armstrong D.K., Qatsha K.A., Davidson N.E. (1994). Interleukin-3 and bryostatin-1 mediate hyperphosphorylation of BCL2_ in association with suppression of apoptosis. J. Biol. Chem. 269:26865–70.

254. Mellman I. (1996). Membranes and sorting. Curr. Opin. Cell. Biol. 8(4):497-8. No abstract available.

255. Meresse S., Gorvel J.P., Chavrier P. (1995). The rab7 GTPase resides on a vesicular compartment connected to lysosomes. J. Cell. Sci. 108 (Pt 11):3349-58.

256. Merry D.E., Korsmeyer S.J. (1997). Bcl-2 gene family in the nervous system. Annu. Rev. Neurosci. 20:245-67.

257. Michalik A., Van Broeckhoven C. (2003). Pathogenesis of polyglutamine disorders: aggregation revisited. Hum. Mol. Genet. 12 Spec No 2:R173-86.

258. Michaelidis T.M., Sendtner M., Cooper J.D., Airaksinen M.S., Holtmann B., Meyer M., Thoenen H. (1996). Inactivation of bcl-2 results in progressive degeneration

of motoneurons, sympathetic and sensory neurons during early postnatal development. Neuron. 17(1):75-89.

259. Mitchener J.S., Shelburne J.D., Bradford W.D., Hawkins H.K. (1976). Cellular autophagocytosis induced by deprivation of serum and amino acids in HeLa cells. Am. J. Pathol. 83:485-498.

260. Mizushima N. et al. (1998). A protein conjugation system essential for autophagy. Nature 395:395–398 .

261. Mizushima N. et al (2001). Dissection of autophagosome formation using Apg-deficient mouse embryonic stem cells. J. Cell Biol. 152: 657–668.

262. Monney L., Olivier R., Otter I., Jansen B., Poirier G.G., Borner C. (1998). Role of an acidic compartment in tumor-necrosis-factor-alpha-induced production of ceramide, activation of caspase-3 and apoptosis. Eur. J. Biochem. 251:295-303.

263. Morata P., Vargas A.M., Sanchez-Medina F., Garcia M., Cardenete G., Zamora S. (1982). Evolution of gluconeogenic enzyme activities during starvation in liver and kidney of the rainbow trout (Salmo gairdneri). Comp. Biochem. Physiol. B. 71(1):65-70.

264. Mortimore G.E., Poso A.R. (1986). The lysosomal pathway of intracellular proteolysis in liver: regulation by amino acids. Adv. Enzyme Regul. 25:257-76.

265. Muriel M.P., Lambeng N., Darios F., Michel P.P., Hirsh E.C., Agid Y., Ruberg M. (2000). Mitochondrial free calcium levels (Rhod-2 fluorescence) and ultrastructural alterations in neuronally deifferentiated PC12 cells during ceramide-dependent cell death. J.Comp. Neurol. 426:297-315.

266. Myers M.P., Stolarov J.P., Eng C., Li J., Wang S.I., Wigler M.H., Parsons R., Tonks N.K. (1997). P-TEN, the tumor suppressor from human chromosome 10q23, is a dual-specificity phosphatase. Proc. Natl. Acad. Sci. U S A. 94(17):9052-7.

267. Munafo D.B, Colombo M.I. (2001). A novel assay to study autophagy: regulation of autophagosome vacuole size by amino acid deprivation. J. Cell. Sci. 114:3619-3629.

268. Murphy D., Levy A., Lightman S., Carter D. (1989). Vasopressin RNA in the neural lobe of the pituitary: dramatic accumulation in response to salt loading. Proc. Natl. Acad. Sci. U S A. 86(22):9002-5.

269. Murphy D., Carter D. (1990). Vasopressin gene expression in the rodent hypothalamus: transcriptional and posttranscriptional responses to physiological stimulation. Mol. Endocrinol. 4(7):1051-9.

270. Nagasaki H., Ito M., Yuasa H., Saito H., Fukase M., Hamada K., Ishikawa E., Katakami H., Oiso Y. (1995). Two novel mutations in the coding region for neurophysin-II associated with familial central diabetes insipidus. J. Clin. Endocrinol. Metab. 80(4):1352-6.

271. Nagata S., Golstein P. (1995). Fas death factor. Science 267:1449-1456.

272. Nakagawa T., Zhu H., Morishima N., Li E., Xu J., Yankner B.A., Yuan J. (2000). Caspase-12 mediates endoplasmic-reticulum-specific apoptosis and cytotoxicity by amyloid-beta. Nature. 6 :403(6765):98-103.

273. Naruse I., Keino H. (1995). Apoptosis in the developing CNS. Prog. Neurobiol. 47:135-155.

274. Neuzil J., Svensson I., Weber T., Weber C., Brunk U.T. (1999). Alpha-tocopheryl succinate-induced apoptosis in Jurkat T cells involves caspase-3 activation, and both lysosomal and mitochondrial destabilisation. FEBS Lett. 445:295-300.

275. Nilsson E., Yin D.(1997). Preparation of artificial ceroid/lipofuscn by oxidation of subcellular organelles. Mech Ageing Dev 99:61-78.

276. Nixon R.A., Cataldo A.M., Mathews P.M. (2000). The endosomal-lysosomal system of neurons in Alzheimer's disease pathogenesis: a review. Neurochem. Res. 25(9-10):1161-72.

277. Noda T., Oshumi Y. (1998). Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. J. Biol. Chem. 273:3963-3966.

278. Novick P., Zerial M.(1997). The diversity of Rab proteins in vesicle transport. Curr. Opin. Cell. Biol. 9(4):496-504.

279. Nuoffer C., Balch W.E. (1994). GTPases: multifunctional molecular switches regulating vesicular traffic. Annu. Rev. Biochem. 63:949-90.

280. Nurcombe V., NcGrath P.A., Bennett M.R. (1981). Postnatal death of motor neurons during the development of the brachial spinal cord of the rat. Neurosci. Lett. 23;27(3):249-54.

281. Ogier-Denis E., Couvineau A., Maoret J.J., Houri J.J., Bauvy C., De Stefanis D., Isidoro C., Laburthe M., Codogno P. (1995). A heterotrimeric Gi3-protein controls autophagic sequestration in the human colon cancer cell line HT-29. J. Biol. Chem. 6:13-16.

282. Ogier-Denis E., Houri J.J., Bauvy C., Codogno P. (1996). Guanine nucleotide exchange on heterotrimeric Gi3 protein controls autophagic sequestration in HT-29 cells. J. Biol. Chem. 27:28593-28600.

283. Olanow C.W., Tatton W.G. (1999). Etiology and pathogenesis of Parkinson's disease.
Annu Rev Neurosci. 22:123-44.
284. Olejnicka B.T., Dalen H., Brunk U.T. (1999). Minute oxidative stress is sufficient to induce apoptotic death of NIT-1 insulinoma cells. APMIS 107.747-761.
285. Ollinger K. (2000). Inhibition of cathepsin D prevents free-radical-induced apoptosis in rat cardiomyocites. Arch. Biochem.Biophys. 373:346-351.
286. Oltvai Z.N, Milliman C.L, Korsmeyer S.J. (1993). Bcl-2 heterodimerizes *in vivo* with a conserved homolog, Bax, that accelerates programmed cell death. Cell. 74:609–19.
287. Ona V.O., Li M., Vonsattel J.P., et al. (1999). Inhibition of caspase-1 slows disease progression in a mouse model of Huntington's disease. Nature 399:263-7.
288. Orloff J., Handler J. (1967). The role of adenosine 3',5'-phosphate in the action of antidiuretic hormone. Am. J. Med. 42(5):757-68.
289. Palmer D.N., Fearnley I.M., Medd S.M., Walker J.E., Martinus R.D., Bayliss S.L., Hall N.A., Lake B.D., Wolfe L.S., Jolly R.D. (1989). Lysosomal storage of the DCCD reactive proteolipid subunit of mitochondrial ATP synthase in human and ovine ceroid lipofuscinoses. Adv. Exp. Med. Biol. 266:211-22.
290. Pathak S.S., Blum J.S. (2000). Endocytic recycling is required for the presentation of an exogenous peptide via MHC class II molecules. Traffic. 1(7):561-9.
291. Pennacchio L.A., Lehesjoki A.E., Stone N.E., Willour V.L., Virtaneva K., Miao J., D'Amato E., Ramirez L., Faham M., Koskiniemi M., Warrington J.A., Norio R., de la Chapelle A., Cox D.R., Myers R.M. (1996). Mutations in the gene encoding cystatin B in progressive myoclonus epilepsy (EPM1). Science. 271(5256):1731-4.
292. Pennacchio L.A., Bouley D.M., Higgins K.M., Scott M.P., Noebels J.L., Myers R.M. (1998). Progressive ataxia, myoclonic epilepsy and cerebellar apoptosis in cystatin B-deficient mice. Nat. Genet. 20(3):251-8.
293. Pennypacker K.R., Kassed C.A., Eidizadeh S., O'Callaghan J.P. (2000) Brain injury: prolonged induction of transcription factors. Acta Neurobiol. Exp. 60:515-530.
294. Petersen A., Larsen K.E., Behr G.G., Romero N., Przedborski S., Brundin P., Sulzer D. (2001). Expanded CAG repeats in exon 1 of the Huntington's disease gene stimulate dopamine-mediated striatal neuron autophagy and degeneration. Hum. Mol. Genet. 10(12):1243-54.

295. Petiot A, Ogier-Denis E., Blommaart E.F., Meijer A.J., Codogno P. (2000).Distino classes of phonphatidylinositol 3'-kinase are involved in signalling pathway that control macdroautophagy in HT-29 cells. J. Biol.Chem. 275:992-998.

296. Plitz T., Pfeffer K. (2001). Intact lysosome transport and phagosome function despite kinectin deficiency. Mol. Cell. Biol. 21(17):6044-55.

297. Pfeifer U. (1977). Inhibition by insulin of the physiological autophagic breakdown of cell organelles. Acta Biol. Med. Ger. 36(11-12):1691-4.

298. Philpott K.L., McCarthy M.J., Klippel A., Rubin L.L. (1997). Activated phosphatidylinositol 3-kinase and Akt kinase promote survival of superior cervical neurons. J. Cell. Biol. 3:139(3):809-15.

299. Poern-Ares M. I., Ares M. P. S., Orrenius S. (1998) Calcium signalling and regulation of apoptosis. Toxicology in vitro 12:539-543.

300. Porter A.G., Ng P., Janicke R.U. (1997). Death substrates come alive. Bioessays 19:501-507.

301. Pranzatelli M.R., Huang Y., Tate E., Stanley M., Noetzel M.J., Gospe S.M. Jr., Banasiak K. (1995). Cerebrospinal fluid 5-hydroxyindoleacetic acid and homovanillic acid in the pediatric opsoclonus-myoclonus syndrome. Ann. Neurol. 37(2):189-97.

302. Provis J.M. (1986). Patterns of cell death in the ganglion cell layer of the human fetal retina. J. Comp. Neurol. 8;259(2):237-46.

303. Prusiner SB. (1998). Proc. Natl. Acad. Sci. U S A. 10;95(23):13363-83.

304. Pryor P.R., Liu S.C., Clark A.E., Yang J., Holman G.D., Tosh D. (2000). Chronic insulin effects on insulin signalling and GLUT4 endocytosis are reversed by metformin.Biochem J. 348 Pt 1:83-91.

305. Putcha G.V., Moulder K.L., Golden J.P., Bouillet P., Adams J.A., Strasser A., Johnson E.M. (2001). Induction of BIM, a proapoptotic BH3-only BCL-2 family member, is critical for neuronal apoptosis. Neuron. 29(3):615-28.

306. Putcha G.V., Harris C.A., Moulder K.L., Easton R.M., Thompson C.B., Johnson E.M. Jr. (2002). Intrinsic and extrinsic pathway signaling during neuronal apoptosis: lessons from the analysis of mutant mice. J. Cell. Biol. 157(3):441-53.

307. Quignon F., De Bels F., Koken M., Feunteun J., Ameisen J.C., de Thè H. (1998). PML induces a novel caspase-independent death process. Nat. Gen. 20:259-265.

308. Qin Z.H., Wang Y., Kegel K.B., Kazantsev A., Apostol B.L, Thompson L.M., Yoder J., Aronin N., DiFiglia M. (2003). Autophagy regulates the processing of amino terminal huntingtin fragments. Hum. Mol. Genet. 12(24):3231-44.

309. Ravikumar B, Duden R, Rubinsztein DC. (2002). Aggregate-prone proteins with polyglutamine and polyalanine expansions are degraded by autophagy. Hum. Mol. Genet. 11(9):1107-17.

310. Ravikumar B., Vacher C., Berger Z., Davies J.E., Luo S., Oroz L.G., Scaravilli F., Easton D.F., Duden R., O'Kane C.J., Rubinsztein D.C. (2004). Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease. Nat. Genet. 36(6):585-95.

311. Ravikumar B., Rubinsztein D.C. (2004). Can autophagy protect against neurodegeneration caused by aggregate-prone proteins? Neuroreport. 15(16):2443-5.

312. Reddy P.H., Williams M., Charles V., et al. (1998). Behavioural abnormalities and selective neuronal loss in HD transgenic mice expressing mutated full-length HD cDNA. Nat. Genet. 20:198-202.

313. Reiners J.J. Jr, Caruso J.A., Mathieu P., Chelladurai B., Yin X.M., Kessel D. (2002). Release of cytochrome c and activation of pro-caspase-9 following lysosomal photodamage involves Bid cleavage. Cell. Death Differ. 9:934-44

314. Rez G., Palfia Z., Fellinger E. (1991). Occurence and inhibition by cycloheximide of apoptosis in vinblastine-treated murine pancreas. A role of autophagy? Acta Biol. Hung. 42:133-140.

315. Riederer M.A., Soldati T., Shapiro A.D., Lin J., Pfeffer S.R. (1994). Lysosome biogenesis requires Rab9 function and receptor recycling from endosomes to the trans-Golgi network. J. Cell. Biol. 125(3):573-82.

316. Riese R.J., Chapman H.A. (2000). Cathepsins and compartmentalization in antigen presentation. Curr. Opin. Immunol. 12(1):107-13.

317. Roberg K (2001) Relocalization of cathepsin D and cytochrome c early in apoptosis revealed by immunoelectron microscopy. Lab. Invest. 81: 149–158

318. Roberg K., Johansson U., Ollinger K. (1999). Lysosomal release of cathepsin D precedes relocation of cytocrome c and loss of mitochondrial transmembrane potential during apoptosis by oxdative stress. Free Radic. Biol. Med. 27:1228-1237.

319. Roberg K, Ka¨gedal K and Ollinger K (2002) Microinjection of cathepsin D induces caspase-dependent apoptosis in fibroblasts. Am. J. Pathol. 161:89–96.

320. Robertson G.L. (1995). Diabetes insipidus. Endocrinol. Metab. Clin. North Am. 24(3):549-72.

321. Robinson B.J., Stowell L.I., Johnson R.H., Palmer K.T. (1990). Is orthostatic hypotension in the elderly due to autonomic failure? Age Ageing. 19(5):288-96.

322. Rodemann H.P, Waxman L., Goldberg A.L. (1982). The stimulation of protein degradation in muscle by Ca2+ is mediated by prostaglandin E2 and does not require the calcium-activated protease. J. Biol. Chem. 10;257(15):8716-23.

323. Rogaev E.I., Sherrington R., Rogaeva E.A., Levesque G., Ikeda M., Liang Y., Chi H., Lin C., Holman K., Tsuda T., et al. (1995). Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene. Nature. 376(6543):775-8.

324. Rohn T.T., Hinds T.R., Vincenzi F.F. (1993) Ion transport ATPase as targets for free radical damage. Protection by an aminosteroid of the Ca2+ pump ATPase and Na+/K+ pump ATPase of human red blood cell membranes. Biochem. Pharmacol. 46:525-534.

325. Rothman J.E. (1994). Intracellular membrane fusion. Adv. Second Messenger Phosphoprotein Res. 29:81-96.

326. Rudd P.M., Wormald M.R., Wing D.R., Prusiner S.B., Dwek R.A. (2001). Prion glycoprotein: structure, dynamics, and roles for the sugars. Biochemistry. 2001 40(13):3759-66.

327. Ruppert S., Scherer G., Schutz G. (1984). Recent gene conversion involving bovine vasopressin and oxytocin precursor genes suggested by nucleotide sequence. Nature. 308(5959):554-7.

328. Saftig P., Hetman M., Schmahl W., Weber K., Heine L., Mossmann H., Koster A., Hess B., Evers M., von Figura K., et al. (1995). Mice deficient for the lysosomal proteinase cathepsin D exhibit progressive atrophy of the intestinal mucosa and profound destruction of lymphoid cells. EMBO J. 14:3599-608.

329. Sagot Y., Dubois-Dauphin M., Tan S.A., de Bilbao F., Aebischer P., Martinou J.C., Kato A.C. (1995). Bcl-2 overexpression prevents motoneuron cell body loss but not axonal degeneration in a mouse model of a neurodegenerative disease. J. Neurosci. 15(11):7727-33.

330. Sam Sik K., Ji Yeon L. (2004). Neuroprotective effects of flavones on hydrogen peroxide-induced apoptosis in SH-SY5Y neuroblastoma cells. Bioinorganic and Medical Chemistry Letters 14:2261-2264.

331. Sanchez I., Xu C.J., Juo P., Kakizaka A., Blenis J., Yuan J. (1999). Caspase-8 is required for cell death induced by expanded polyglutamine repeats. Neuron. 22:623-33.

332. Sang-Oh Y., Chang-Hyun Y., An-Sik Chung. (2002) Dose effect of oxidative stress on signal transduction in aging. Mechanisms of Ageing and development 123:1597-1604.

333. Saunders J.W. Jr. (1966). Death in embryonic systems. Science. 4:154(749):604-12.

334. Sausville E., Carney D., Battey J. (1985). The human vasopressin gene is linked to the oxytocin gene and is selectively expressed in a cultured lung cancer cell line. J. Biol. Chem. 260(18):10236-41.

335. Schmeizie T., Hall M.N. (2000). TOR, a central controller of cell growth. Cell. 103: 253-262.

336. Schendel SL, Montal M, Reed JC. Bcl-2 family proteins as ion-channels.
Cell Death Differ 1998;5:372–80.

337. Sherman T.G., McKelvy J.F., Watson S.J. (1986). Vasopressin mRNA regulation in individual hypothalamic nuclei: a northern and in situ hybridization analysis. J. Neurosci. 6(6):1685-94.

338. Schinder A.F., Poo M. (2000) The neurotrophin hypothesis for synaptic plasticity. Trends Neurosci. 23:639-645.

339. Schmale H., Heinsohn S., Richter D. (1983). Structural organization of the rat gene for the arginine vasopressin-neurophysin precursor. EMBO J. 2(5):763-7.

340. Schlesinger PH, Gross A, Yin XM, Yamamoto K, Saito M, Waksman G,
et al. Comparison of the ion channel characteristics of proapoptotic BAX
and antiapoptotic BCL-2. Proc Natl Acad Sci U S A 1997;94:11357–62.

341. Schweichel J.U., Marker H.J. (1973). The morphologyof various types of cell death in prenatal tissue. Teratology 7:253-266.

342. Seglen P.O., Gordon P.B. (1982) 3-Methyladenine: specific inhibitior of autophagi/lysosomal protein degradation in isolated rat hepatocytes. Proc. Nat. Acad. Sci USA 79:1889-1892.

343. Seglen P.O, Bohley P. (1992). Autophagy and other vacuolar protein degradation mechanisms. Experientia. 15;48(2):158-72.

344. Seglen P.O., Gordon P.B., Poli A. (1980). Amino acid inhibition of the autophagic/lysosomal pathway of protein degradation in isolated rat hepatocytes. Biochim. Biophys. Acta. 5:630:103-18.

345. Seglen P.O., Gordon P.B., Holen I. (1990). Non-selective autophagy. Semin. Cell. Biol.. 1(6):441-8.

346. Selbo P.K., Hogset A., Prasmickaite L., Berg K. (2002). Photochemical internalisation: a novel drug delivery system.Tumour Biol. 23:103-12.

347. Selbo P.K., Kaalhus O., Sivam G., Berg K. (2001). Aminolevulinic acid-based photochemical internalization of the immunotoxin MOC31-gelonin generates synergistic cytotoxic effects in vitro. Photochem. Photobiol. 74:303-10

348. Sherrington R., Rogaev E.I., Liang Y., Rogaeva E.A., Levesque G., Ikeda M., Chi H., Lin C., Li G., Holman K., et al. (1995). Cloning of a gene bearing missense mutations in early-onset familial Alzheimer's disease. Nature. 29;375(6534):754-60.

349. Shibata M., Kanamori S., Isahara K., Ohsawa Y., Konishi A., Kametaka S., Watanabe T., Ebisu S., Ishido K., Kominami E., Uchiyama Y. (1998). Partecipation of cathepsin B and D in apoptosis of PC12 cells following serum deprivation. Biochem. Biophys. Res. Commun. 251:199-203.

350. Shintani T., Klionsky D.J. (2004). Autophagy in health and disease: a double-edged sword. Science. 306(5698):990-5.

351. Simpson L., Parsons R. (2001). PTEN: life as a tumor suppressor. Exp. Cell. Res. 264:29-41.

352. Sleat D.E., Donnelly R.J., Lackland H., Liu C.G., Sohar I., Pullarkat R.K., Lobel P. (1999). Association of mutations in a lysosomal protein with classical late-infantile neuronal ceroid lipofuscinosis. Science. 19;277(5333):1802-5.

353. Sleat D.E., Gin R.M., Sohar I., Wisniewski K., Sklower-Brooks S., Pullarkat R.K., Palmer D.N., Lerner T.J., Boustany R.M., Uldall P., Siakotos A.N., Donnelly R.J., Lobel P. (1999). Mutational analysis of the defective protease in classic late-infantile neuronal ceroid lipofuscinosis, a neurodegenerative lysosomal storage disorder. Am. J. Hum. Genet. 64(6):1511-23.

354. Sohal R. S., Mockett R. J., Orr W.C. (2002). Mechanisms of aging: appraisal of the oxidative stress hypothesis. Free Radical Biology & Medicine 33:575-586.

355. Stadtman E. R., Berlett B. S. (1997). Reactive oxygen-mediated protein oxidation in aging and disease.Chemical Research and Toxicology 10:485-494.

356. Standtman, E. R., Levine, R. L.(2000). Protein oxidation. Annals of New York Academy of Sciences 899:191-208.

357. Squier T. C. (2001). Oxidative stress and protein aggregation during biological aging. Experimental Gerontology 36:1539-1550.

358. Stack J.H., De Wald D.B., Takegawa K., Emr S.D. (1995). Vescicle-mediated protein transport: regulatory interaction between the Vps15 protein kinase and the

Vps34 PtdIns 3-kinase essential for protein sorting to the vacuole in yeats. J. Cell. Biol. 129:321-334.

359. Stahl N., Borchelt D.R., Hsiao K., Prusiner S.B. (1987). Scrapie prion protein contains a phosphatidylinositol glycolipid. Cell. 51(2):229-40.

360. Stahl N., Prusiner S.B. (1991). Prions and prion proteins. FASEB J. 5(13):2799-807.

361. Stephanis L., Larsen K., Rideout H. J., Sulzer D., Greene L. A. (2001). Expression of A53T mutant but not wild-tipe alpha-synuclein in Pc12 cells induces alterations of the ubiquitin-dependent degradation system, loss of dopamine release, and autophagic cell death. Journal of Neuroscience 21:9549-9560.

362. Stevens C., Kennaway N.G., Fellman J.H. (1975). Ammonia intoxication: a hazard during rehabilitation of protein-deprived rats. J. Nutr. 105(11):1384-90.

363. Stinchcombe J.C., Griffiths G.M. (1999). Regulated secretion from hemopoietic cells. J. Cell. Biol. 147(1):1-6.

364. Stoka V., Turk B., Schendel SL., Kim TH., Cirman T., Snipas SL., Ellerby LM., Bredesen D., Freeze H., Abrahamso M., Bromme D., Krajewski S., Reed JC., Yin XM., Turk V., Salvesen GS. (2001). Lysosomal protease pathway to apoptosis: cleavage of Bid, not pro-caspases, is the most likely route. J. Biol. Chem. 276:3149-3157.

365. Strasser A., Puthalakath H., Bouillet P., Huang D.C., O'Connor L., O'Reilly L.A., Cullen L., Cory S., Adams J.M. (2000). The role of bim, a proapoptotic BH3-only member of the Bcl-2 family in cell-death control. Ann. N Y Acad. Sci. 917:541-8.

366. Stromhaug PE, Berg TO, Fengsrud M, Seglen PO. (1998). Purification and characterization of autophagosomes from rat hepatocytes. Biochem. J. 15;335-340.

367. Sundelin S. P., Nilsson S. E., Brunk U. T. (2001). Lipofuscin-formation in cultured retinal pigment epithelial cells is relaed to their melanin conten. Free Radical Biology & Medicine 30:74-81.

368. Szweda P.A., Camouse M., Lundberg K.C., Oberley T.D., Szweda L.I. (2003). Aging, lipofuscin formation, and free radical-mediated inhibition of cellular proteolytic systems. Ageing Res. Rev. 2(4):383-405.

369. Takayama S, Cazals-Hatem DL, Kitada S, Tanaka S, Miyashita T, Hovey LR 3d, et al. (1994). Evolutionary conservation of function among mammalian, avian, and viral homologs of the Bcl-2 oncoprotein. DNA Cell Biol 13:679–92.

370. Takeyama N., Miki S., Hirakawa A., Tanaka T. (2002). Role of mitochondrial permeability transition and cytochrome C release in hydrogen peroxide-induced apoptosis. Exp Cell. Res. 274:16-24.

371. Takeuchi Y., Kurohane K., Ichikawa K., Yonezawa S., Nango M., Oku N. (2003). Induction of intensive tumor suppression by antiangiogenic photodynamic therapy using polycation-modified liposomal photosensitizer. Cancer. 97:2027-34.

372. Tao K., Li J., Warner J., MacLeod K., Miller F.R., Sahagian G.G. (2001). Multiple lysosomal trafficking phenotypes in metastatic mouse mammary tumor cell lines. Int. J. Oncol. 19:1333-1339.

373. Tamura M., Gu J., Takino T., Yamada K.M. (1999). Tumor suppressor PTEN inhibition of cell invasion, migration, and growth: differential involvement of focal adhesion kinase and p130Cas. Cancer Res. 59:442-449.

374. Tapper H., Furuya W., Grinstein S. (2002). Localized exocytosis of primary (lysosomal) granules during phagocytosis: role of Ca2+-dependent tyrosine phosphorylation and microtubules. J. Immunol. 168(10):5287-96.

375. Terman A., Brunk U.T. (1998). Ceroid/lipofuscin formation in cultured human fibroblasts: the role of oxidative stress and lysosomal proteolysis. Mech. Ageing Dev. 104(3):277-91.

376. Tessitore L., Bonelli G., Cecchini G., Amenta L.S., Baccino F.M. (1987). Regulation of protein turnover versus growth state: ascites hepatoma as a model for studies both in the animal and in vitro. Arch Biochem Biophys 255:372-384

377. Tessitore L., Monelli G., Cecchini G., Augelli R., Amenta J.S., Baccino FM. (1988). Regulation of protein turnover versus growth state. Studies of the mechanism of initiation of acidic vacuolar proteolysis in cells of stationary ascites hepatoma. Biochem J 251: 483-490.

378. Thomas G., Hall M. N. (1997). TOR signalling and control of cell growth. Curr. Opin. Cell. Biol. 9:782-787.

379. Tikkanen R., Peltola M., Oinonen C., Rouvinen J., Peltonen L. (1997). Several cooperating binding sites mediate the interaction of a lysosomal enzyme with phosphotransferase. EMBO J. 16(22):6684-93.

380. Tyynela J., Suopanki J., Santavuori P., Baumann M., Haltia M. (1997). Variant late infantile neuronal ceroid-lipofuscinosis: pathology and biochemistry. J. Neuropathol. Exp. Neurol. 56(4):369-75.

381. Uberti D., Piccioni L. Colzi A. et al. (2002). Pergolide protects SH-SY5Y cells againts neurodegeneration induced by H2O2. European Journal of Pharmacology 434:17-20.

382. Ueno T., Kominami E. (1991). Mechanism and regulation of lysosomal sequestration and proteolysis. Biomed. Biochim. Acta. 50(4-6):365-71.

383. Vaillant A.R., Mazzoni I., Tudan C., Boudreau M., Kaplan D.R., Miller F.D. (1999). Depolarization and neurotrophins converge on the phosphatidylinositol 3-kinase-Akt pathway to synergistically regulate neuronal survival. J. Cell. Biol. 6:146(5):955-66.

384. Vaananen A., Buunk B.P., Kivimaki M., Pentti J., Vahtera J. (2005). When it is better to give than to receive: long-term health effects of perceived reciprocity in support exchange. J. Pers. Soc. Psychol. 89(2):176-93.

385. Van Dyke R.W., Steer C.J., Scharschmidt B.F. (1984). Clathrin-coated vesicles from rat liver: enzymatic profile and characterization of ATP-dependent proton transport. Proc. Natl. Acad. Sci. U S A. 81(10):3108-12.

386. Veis D.J., Sentman C.L., Bach E.A., Korsmeyer S.J. (1993). Expression of the Bcl-2 protein in murine and human thymocytes and in peripheral T lymphocytes. J. Immunol. 1:151(5):2546-54.

387. Venkatraman A., Shiva S., Davis A.J., Bailey S.M., Brookes P.S., Darley-Usmar V.M. (2003). Chronic alcohol consumption increases the sensitivity of rat liver mitochondrial respiration to inhibition by nitric oxide. Hepatology. 38(1):141-7.

388. Vesa J., Hellsten E., Verkruyse L.A., Camp L.A., Rapola J., Santavuori P., Hofmann S.L., Peltonen L. (1995). Mutations in the palmitoyl protein thioesterase gene causing infantile neuronal ceroid lipofuscinosis. Nature. 17;376(6541):584-7.

389. Vey M., Pilkuhn S., Wille H., Nixon R., DeArmond S.J., Smart E.J., Anderson R.G., Taraboulos A., Prusiner S.B. (1996). Subcellular colocalization of the cellular and scrapie prion proteins in caveolae-like membranous domains. Proc. Natl. Acad. Sci. USA. 93(25):14945-9.

390. Vickers J.C., Dickson T.C., Adlard P.A., Saunders H.L., King C.E., McCormack G. (2000). The cause of neuronal degeneration in Alzheimer's disease. Prog Neurobiol. 60(2):139-65.

391. Vines D.J, Warburton M.J. (1999). Classical late infantile neuronal ceroid lipofuscinosis fibroblasts are deficient in lysosomal tripeptidyl peptidase I. FEBS Lett. 443(2):131-5.

392. Vlahos C.J., Matter W.F., Hui K.Y., Brown R.F. (1994). A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J. Biol. Chem. 18:269(7):5241-8.

393. Xia Z., Dickens M., Raingeaud J., Davis R.J., Greenberg M.E. (1995). Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis. Science. 270(5240):1326-31.

394. Xue L., Fletcher G.C., Tolkovsky A.M. (1999). Autophagy is activated by apoptotic signalling in sympathetic neurons: an alternative mechanism of death execution. Mol. Cell. Neurosci. 14(3):180-98.

395. Waliany S., Das A.K., Gaben A., Wisniewski K.E., Hofmann S.L. (2000). Identification of three novel mutations of the palmitoyl-protein thioesterase-1 (PPT1) gene in children with neuronal ceroid-lipofuscinosis. Hum. Mutat. 15(2):206-7.

396. Watanable-Fukunaga R., Brannan C.I., et al. (1992). Lymphoproliferation disorder in mice explained by defect in Fas antigen that mediates apoptosis. Nature 356:314-317.

397. Webb J.L., Ravikumar B., Atkins J., Skepper J.N., Rubinsztein D.C. (2003). Alpha-Synuclein is degraded by both autophagy and the proteasome. J. Biol. Chem. 278(27):25009-13.

398. Wyllie A.H., Kerr J.F., Currie A.R. (1980). Cell death: the significance of apoptosis. Int. Rev. Cytol. 68:251-306.

399. Wei M.C., Zong W.X., Cheng E.H., Lindsten T., Panoutsakopoulou V., Ross A.J., Roth K.A., MacGregor G.R., Thompson C.B., Korsmeyer S.J. (2001). Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science. 292(5517):727-30.

400. Wei T., Ni Y., Hou J., Chen C., Zhao B., Xin W. (2000). Hydrogen peroxide-induced oxidative damage and apoptosis in cerebellar granule cell. Pharmacol. Res 41:427-433.

401. Wen S., Stolarov J., Myers M.P., Su J.D., Wigler M.H., Tonks N.K., Durden D.L. (2001). PTEN controls tumor-induced angiogenesis. Proc. Natl. Acad. Sci. USA 98:4622-4627.

402. Whitfield J., Neame S.J., Paquet L., Bernard O., Ham J. (2001). Dominant-negative c-Jun promotes neuronal survival by reducing BIM expression and inhibiting mitochondrial cytochrome c release. Neuron. 29(3):629-43.

403. Whittemore E. R., Loo D.T., Cotman C.W. (1994). Exposure to hydrogen peroxide induces cell death via apoptosis in cultural rat cortical neurons. Neuroreport 5:1485-1488.

404. Wiegmann K., Schutze S., Machleidt T., Witte D., Kronke M. (1994). Functional dichotomy of neutral and acidic sphingomyelinases in tumor necrosis factor signaling. Cell. 78:1005-1015.

405. Wilson-Annan J., O'Reilly L.A., Crawford S.A., Hausmann G., Beaumont J.G., Parma L.P., Chen L., Lackmann M., Lithgow T., Hinds M.G., Day C.L., Adams J.M., Huang D.C. (2003). Proapoptotic BH3-only proteins trigger membrane integration of prosurvival Bcl-w and neutralize its activity. J. Cell. Biol. 1:162(5):877-87.

406. Wright S.C., Zhong J., Larrick J.W. (1994). Inhibition of apoptosis as a mechanism of tumor promotion. FABES J. 8:654-660.

407. Wu D., Wallen H.D., Nuñez G. (1997). Interactions and regulation of subcellular localization of CED-4 and CED-9. Science 275:1126-1129.

408. Wu GS., Safting P., El-Deiry WS. (1998). Potential role for cathepsin D in p53-dependent tumor suppression and chemosensitivity. Oncogene 16: 2177-2183.

409. Wyllie (1980). Cell death: the significance of poptosis. Int Rev Cytol 68:251-306.

410. Zahrebelski G., Nieminem A.L., al-Ghoul K., Qian T.,Herman B. Lemaster J.J.(1995). Progression of subcellular chenges duringchemical hypoxia to cultured rat hepatocytes. A laser scannong confocal mocroscpic study. Hepatology 21:1361-1372.

411. Zang Y., Beard R.L., Chandraratna R.A., Kang J.X. (2001). Evidence of a lysosomal pathway for apoptosis induced by the synthetic retinoid CD437 in human leukemia HL-60 cells. Cell Death Differ. 8:477-85.

412. Zou H., Henzel W.J., Lutschg A., Wang X. (1997). Apaf-1, a human protein homolgous to C. Elegans CED-4, partecipates in cytochrome c-dependent avtivation on caspase-3. Cell 90:405-413.

413. Zhuang S., Demirs J.T., Kochevat I.E. (2000). p38 mitogen-activated protein kinase mediates bid cleavage, mitochondrial dysfuction, and caspase-3 activation during apoptosis induced by singlet oxygen but not by hydrogen peroxide. J. Biol. Chem 275:25939-25948.

414. Yamakawa, Ito Y., Naganawa T., Banno Y. et al. (2000). Activation of caspasi-9 and -3 during H2O2-induced apoptosis of PC12 cwlls independent of ceramide formation. Neurol.Res 22:556-564.

415. Yao R., Cooper G.M. (1995). Requirement for phosphatidylinositol-3 kinase in the prevention of apoptosis by nerve growth factor. Science. 31:267(5206):2003-6.

416. Yin D., Yuan X. (1995). Test-tube simulated lipofuscinogenesis.Effect ofoxidative stress on autophagy degradation. Mech Ageing Dev 81:37-50.

417. Yin D. (1996). Biochemical bass of lipofuscin ceroid, and age pigment-like fluorophores. Free Radic Biol Med 21:871-888.

418. Yu J., Zhang L. (2005) The transcriptional targets of p53 in apoptosis control. Biochem. Biophys. Res. Commun. 331:851-8.

419. Yuan J., Zhang J., Wong B.W., Si X., Wong J., Yang D., Luo H. (2005). Inhibition of glycogen synthase kinase 3beta suppresses coxsackievirus-induced cytopathic effect and apoptosis via stabilization of beta-catenin. Cell Death Differ. 12(8):1097-106.

ELENCO DELLE PUBLICAZIONI

1) Ulrike Bening, **Roberta Castino**, Norbert Harth, Ciro Isidoro, and Andrej Hasilik. Lisosomal Segregation of a mannose- rich glycoprotein imparted by the prosequence of myeloperoxidase. J. Cell. Biochem. 71(2): 158-68 (1998).

2) Marina Demoz, **Roberta Castino**, Antonella Dragonetti, Francesco M. Baccino e Ciro Isidoro. Transformation by oncogene Ras-p21 alters the processing and subcellular localization of the lysosomal protease cathepsin D. J. Cell. Biochem. 73: 370-378 (1999).

3) A. Dragonetti, M. Baldassarre, **R. Castino**, M. Demoz, A. Luini, R. Buccione, C. Isidoro. The lysosomal protease Cathepsin D is efficiently sorted to and secreted from regulated secretory compartments in the Rat Basophilic/Mast cell Line RBL.
J. Cell Science 2000, 113:3289-3298

4) P. Cesaro, E. Raiteri, M. Demoz, **R. Castino**, F.M. Baccino, G. Bonelli and C. Isidoro. Expression of Protein Kinase C α1 confers resistance to TNFα - and taxol-induced apoptosis in HT-29 coloncarcinoma cells.
Int. J. Cancer 2001, 93:179-184

5) R. Castino, D. Pace, M. Demoz, M. Gargiulo, C. Ariatta, E. Raiteri e C. Isidoro. Lysosomal proteases as potential targets for the induction of apoptotic cell death in human Neuroblastoma. Int. J. Cancer 2002, 97:775-779

6) M. Demoz, **R. Castino**, P. Cesaro, F.M. Baccino, G. Bonelli and C. Isidoro. Endosomal-lysosomal proteolysis mediates death signalling by TNF□, not by etoposide in L929 fibrosarcoma cells: evidence for an active role of Cathepsin D.
Biol. Chem 2002, 383: 1237-1248.

7) R. Castino, **M. Demoz and C. Isidoro.**
Destination "lysosome": a target organelle for tumour cell killing?
J. Mol. Recognition 2003, 16:1-13.

8) R. Carini, **R. Castino**, M. G. De Cesaris, R. Splendore, M. Demoz, E. Albano and C. Isidoro. Preconditioning-induced cytoprotection in hepatocytes requires Ca2+-dependent exocytosis of lysosomes. J. Cell Sci. 2003, 1065-1077.

9) M. Lkhider, **R. Castino**, C. Isidoro and M. Ollivier-Bousquet.
Cathepsin D secreted by lactating rat mammary epithelial cells processes Prolactin in physiological conditions. J. Cell Sci. 2004. 1;117:5155-64.

10) **R. Castino**, J. Davies, S.Beaucourt, C. Isidoro and D.Murphy
Autophagy is a pro-survival mechanism in mouse neuroblastoma cells expressing a familial neurohypophyseal diabetes insipidus mutant vasopressin transgene.
http://www.fasebj.org/cgi/reprint/04-3162fjev1?ijkey=K2Dc9Gi3nmRgI&keytype=ref&siteid=fasebj FASEB J. 2004.

11) **R. Castino**, C. Isidoro and D. Murphy. Autophagy –dependent cell survival and cell death in a Familial Neurohypophyseal Diabetes Insipidus *in vitro* modelhttp://www.fasebj.org/cgi/reprint/04-3163fjev1?ijkey=VCXLqVFV5bDSs&keytype=ref&siteid=fasebj FASEB J. 2004.

12) M. Démoz, **R. Castino**, C. Follo, A. Hasilik, B. F. Sloane, C. Isidoro
Purification and Structural and Functional Characterisation of Recombinant Human Procathepsin D expressed in Mammalian Cells.
Prot.Expr.Purif. 2005,45(1):157-67.

13) G. Valente, F. Manfroi, C. Peracchio, G. Nicotra, **R. Castino**, G. Nicosia, S. Kerim and C. Isidoro
CFLIP expression correlates with tumour progression and patient outcome in Non Hodgkin's Lymphomas of low-grade of malignancy.
Brit. J. Haem. 132 (5): 560-570

14) Carini R., Trincheri N.F., Alchera E., De Cesaris M.G., **Castino R.,** Splendore R.**,** Albano E., Isidoro C. PI£K-dependent lysosome exocytosis in nitric oxide-preconditioned hepatocytes. In Press Free Radical Biology and Medicine.

15) **Roberta Castino**, Natascia Bellio, Giuseppina Nicotra, Carlo Follo, Nicol F. Trincheri and Ciro Isidoro. Essential role of cathepsin d in oxidoradical-induced neuronal cell death. Submitted to Journal of Neurochemistry .

16) Nicol F. Trincheri, Giuseppina Nicotra, Carlo Follo, **Roberta Castino** and Ciro Isidoro Resveratrol induces cell death in colorectal cancer cells by a novel pathway involving lysosomal cathepsin D. Submitted to Molecular Cancer Therapy.

17) Follo C., Nicotra G., Trincheri F., Castino R., Isidoro C. Transport and processing of human cathepsin D mutated at the maturation proteolytic region. Submitted to Traffic

PARTECIPAZIONE A CONGRESSI DURANTE IL CORSO DI DOTTORATO

1. M. DEMOZ, P. CESARO, **R. CASTINO**, E. RAITERI, C. ARIATTA, M. GARGIULO, D. PACE, F. M. BACCINO, G. BONELLI, C. ISIDORO (2001)
Differential role of cathepsins B and D in TNF*alfa*- and etoposide-mediated signalling of apoptosis in l929 fibrosarcoma cells. VIIth International Symposium on "PROTEINASE INHIBITORS AND BIOLOGICAL CONTROL", Portroz (Brdo) by Ljubljana, Slovenia, June 16-20, 2001 (abstract book, p.60)

2. **R.CASTINO,** M. DEMOZ, E. RAITERI, D. PACE, M. GARGIULO, C. ARIATTA, M. PONZONI, C. ISIDORO (2001)
Inhbition of lysosomal function induces caspase-dependent cell death in human neuroblastoma cells. VIIth International Symposium on "PROTEINASE INHIBITORS AND BIOLOGICAL CONTROL", Portroz (Brdo) by Ljubljana, Slovenia, June 16-20, 2001 (abstract book, p. 61)

3. M. DEMOZ, M. GARGIULO, **R. CASTINO**, E. RAITERI, C. GIANINAZZI, C. ISIDORO (2001)
Generation of a recombinant vaccinia virus for the production of functional human proCathepsin D. 5° Congresso Nazionale Biotecnologie, L'Aquila, 13-15 Settembre 2001 (Atti, p62)

4. M. DEMOZ, P. CESARO, **R. CASTINO**, E. RAITERI, C. ARIATTA, M. GARGIULO, D. PACE, F. M. BACCINO, G. BONELLI, C. ISIDORO (2001)
Death signalling by TNFα, not by etoposido follows an endocytic pathway: evidence that the lysosomal protease Cathepsin D acts as a dominant executioner. 5° Congresso Nazionale Biotecnologie, L'Aquila, 13-15 Settembre 2001 (Atti, p73)

5. E. RAITERI, P. CESARO, M. DEMOZ, **R. CASTINO**, F. M. BACCINO, G. BONELLI, C. ISIDORO (2001)
Apoptosis by TNFα and taxol in HT-29 coloncarcinoma cells: hyperexpression of protein kinase C β1 confers resistance. 5° Congresso Nazionale Biotecnologie, L'Aquila, 13-15 Settembre 2001 (Atti, p84)

6. **R. CASTINO**, M. DEMOZ, E. RAITERI, D. PACE, M. GARGIULO, C. ARIATTA, M. PONZONI, C. ISIDORO (2001)
Lysosomes as potential targets for neuroblastoma chemotherapy. 5° Congresso Nazionale Biotecnologie, L'Aquila, 13-15 Settembre 2001 (Atti, p124)

7. C. ISIDORO, E. RAITERI, **R. CASTINO**, C. ARIATTA, M. DEMOZ (2001)
Stimulation of TNFR1 by human recombinant TNFα alters the lysosomal segregation of cathepsin D: role of second messengers and temporal-function relationship with apoptosis. Convegno CNR pf Biotecnologie, Genova 28-30 Ott 2001, abstract book pag. 299

8. E. RAITERI, D. DE STEFANIS , P. REFFO, **R. CASTINO**, F. BACCINO, G. BONELLI, C. ISIDORO (2001)
Regulation of cathepsin D targeting by ceramide and PKC: is there a functional relationship with TNFα-induced apoptosis ? 2nd General Meeting of International Proteolysis Society, Freising by Munich (D) Oct 31st-Nov 4th 2001, (lecture) abstract book L41

9. **R. CASTINO**, M. DEMOZ, E. RAITERI, D. PACE, M. GARGIULO, C. ARIATTA, C. ISIDORO (2001)

Inhibitors of lysosomal cathepsins as apoptotic inducers in human neuroblastomas. 2nd General Meeting of International Proteolysis Society, Freising by Munich (D) Oct 31st-Nov 4th 2001, abstract book (Poster) P122

10. M. DEMOZ, **R. CASTINO**, C. ISIDORO (2001)
Cathepsin D as a glycoprotein marker of vesicular traffic.European COST Action, joint meeting WG 1, 3, 5. Ayr (Scotland) 7-8 Dec 2001, (lecture) abstract book p. 4

11. C. ISIDORO, **R. CASTINO**, A. SALINI, M. DÉMOZ, C. GIANINAZZI, M. MIRABELLI, N. TRINCHERI, C. FOLLO (2002)
Prosurvival and proapoptotic signaling by lysosomal proteolysis in cancer cells: p53 as a determinant factor? 1st Cell Death and Differentiation Conference & 4th International SASS Foundation Conference "Apoptosis in Cancer and Infection" Capri (I) 6-9 October 2002, (lecture) Abstract book p. 26

12. **R. CASTINO**, M. DÉMOZ C. GIANINAZZI, M. MIRABELLI, N. TRINCHERI, C. FOLLO, C. ISIDORO (2002)
The endosomal pathway of TNFalfa apoptotic signaling. Joint Meeting of "The International Society for Interferon and Cytokine Research", Torino 6-10 October 2002, J. Interf & Cytok Res, vol 22, suppl 1 (2002) S155 (poster 12-1)

13. **ROBERTA CASTINO**, ALESSANDRIA SALINI, MARINA DÉMOZ, CAMILO GIANINAZZI, MARZIA MIRABELLI, NICOL TRINCHERI, CARLO FOLLO, GIANCARLO CASTELLANO, ANNA ASPESI AND CIRO ISIDORO (2003)
Lysosomal cathepsin D as potential targets for chemotherapy. International Workshop on "Cellular Transport Strategies for Targeting of Epitopes, Drugs and Reporter Molecules", Budapest 6-9 Marzo 2003 (Lecture), Abstract Book p.6-7

14. CAMILO GIANINAZZI, NICOL TRINCHERI, CARLO FOLLO, ANNA ASPESI, MARINA DÉMOZ, **ROBERTA CASTINO** AND CIRO ISIDORO (2003)
The impact of ceramide on lysosomal targeting: a study with wild-type and glycosylation knock-out mutated cathepsin D-green fluorescent protein chimeras. 2nd

Meeting SPHINGOLIPID CLUB, Sale Marasino (BS) 3-4 Giugno 2003, abstract book p. 6

15. ANNA ASPESI, **ROBERTA CASTINO**, CAMILO GIANINAZZI, MARZIA MIRABELLI, CARLO FOLLO, MARINA DÉMOZ, AND CIRO ISIDORO (2003) Modifying the cellular response to ceramide-mediated stress by gene manipulation of lysosomal cathepsin D expression. 2nd Meeting SPHINGOLIPID CLUB, Sale Marasino (BS) 3-4 Giugno 2003, abstract book p. 12

16. CARLO FOLLO, NICOL TRINCHERI, CAMILO GIANINAZZI, ANNA ASPESI, MARINA DÉMOZ, **ROBERTA CASTINO** AND CIRO ISIDORO (2003) Biogenesi degli enzimi lisosomici: uno studio con la proteina chimerica Catepsina D-Green Fluorescent Protein. 6° Congresso Nazionale CIB, Consorzio Interuniversitario Biotecnologie, Padova 4-6 Giugno 2003, abstract book p. 122

17. CAMILO GIANINAZZI, ANNA ASPESI, **ROBERTA CASTINO**, MARZIA MIRABELLI, CARLO FOLLO, MARINA DÉMOZ AND CIRO ISIDORO (2003) L'iper-espressione di catepsina d esalta la risposta cellulare allo stress mediato da ceramide. 6° Congresso Nazionale CIB, Consorzio Interuniversitario Biotecnologie, Padova 4-6 Giugno 2003, abstract book p. 123

18. **ROBERTA CASTINO**, MARZIA MIRABELLI, GIUSEPPINA NICOTRA, ANNA ASPESI, MARINA DÉMOZ AND CIRO ISIDORO (2003) Il ruolo dei lisosomi nella immuno- e chemio-resistenza dei tumori. 6° Congresso Nazionale CIB, Consorzio Interuniversitario Biotecnologie, Padova 4-6 Giugno 2003, abstract book p. 124

19. **R. CASTINO**, M. DEMOZ, M. LKHIDER, M. OLLIVIER-BOUSQUET, C. ISIDORO (2003) Characterisation and localisation of Cathepsin D in lactating rat mammary epithelial cell: role in prolactin processing under physiological conditions. European COST Action Meeting on "Risk factors of Breast Cancer", Montpellier (France) 4-5 July, 2003, (lecture), Abstract book p.

20. **ROBERTA CASTINO**, DAVID MURPHY AND CIRO ISIDORO (2004)
'In vitro' model of a neuroendocrine disorder by infection of neuroblastoma cells with modified adenoviral vectors: the role of Autophagy in the pathogenesis of FNDI.CIB Catania Abstract Congresso CNB7, Catania 8-10 Ottobre 2004, Abstract book p. 136

21. **ROBERTA CASTINO**, DAVID MURPHY AND CIRO ISIDORO (2004)
Over-expression of Cys67 Stop Vasopressin in neuroblastoma cells triggers autophagy: a case of Unfolded Protein Response leading to neuronal cell death. 4° Convegno Scientifico "Meccanismi Molecolari in Neuroscienze", Milano 17-18 Giugno 2004 (www.neuroscienze molecolari.it/abstracts)

22. MIRABELLI M., TRINCHERI N. F., NICOTRA G., MANFROI F., **CASTINO R**., VALENTE G. AND ISIDORO C. (2005)
Expression of lysosomal cathepsin D and of FLIP in human lymphomas: relationship with tumour progression. XLVI Congresso Nazionale della Società Italiana di Cancerologia, Pisa, 24-27 Ottobre 2004, Tumori, 4 (2), 2005, p.89

23. TRINCHERI N. F., NICOTRA G., MIRABELLI M., FOLLO C., **CASTINO R**., ISIDORO C. (2005)
Resveratrol Induces Growth Arrest And Cell Death In Human Colorectal Cancer Cells By Modulating Autophagocytosis. XLVI Congresso Nazionale della Società Italiana di Cancerologia, Pisa, 24-27 Ottobre 2004, Tumori, 4 (2), 2005, p.36

24. **CASTINO R**., FOLLO C., THEPPARIT C., BEAUCOURT S., BELLIO N., MURPHY D., AND ISIDORO C**.** (2005)
The complex regulation of autophagy and cell death in neurodegeneration: dissection of the signalling pathways by genetic manipulations. VIII Congresso Nazionale di Biotecnologie- Università degli Studi di Siena (sessione A) 8 Settembre, 2005.

25. ISIDORO C., **CASTINO R**., THEPPARIT C., BEAUCOURT S., BELLIO N., AND MURPHY D (2005)
The complex regulation of autophagy and apoptosis in neurodegeneration: a genetic approach to dissect the signalling pathways. 6th Meeting of the Slovenian Biochemical Society with International Participation. Lipica, September 21-25, 2005, p.70.

26. **CASTINO R.**, BELLIO N., FOLLO C., MURPHY D., AND ISIDORO C. (2005)
Involvement of Cathepsin D in oxidoradicals-induced caspase-dependent cell death in neuroblastoma cells. 4th General Meeting of the International Proteolysis Society. Quebec City, October 15-19, 2005

27. **CASTINO R**., THEPPARIT C., BEAUCOURT S., BELLIO N., MURPHY D., AND ISIDORO C. (2005)
Cross-talk between Cathepsin D and Caspase in the Unfolded Protein Response. 4th General Meeting of the International Proteolysis Society. Quebec City, October 15-19, 2005

28. TRINCHERI N.F., NICOTRA G., PERACCHIO C., FOLLO C., BELLIO N., **CASTINO R**., ISIDORO C. (2005)
Resveratrol kills colon cancer cells by activating both Caspase-dependent and Cathepsin-dependent death pathways. 4th General Meeting of the International Proteolysis Society. Quebec City, October 15-19, 2005

29. **CASTINO R**., BELLIO N., FOLLO C., MURPHY D., AND ISIDORO C. (2005)
Oxidative stress activates both autophagy- and caspase-dependent pathways of cell death. I Congress of Physiological Sciences of Serbia and Montenegro with International participation. November, 9-12, 2005.

30. **CASTINO R.,** THEPPARIT C., BEAUCOURT S., BELLIO N., MURPHY D., AND ISIDORO C. (2005)
Regulation and function of the autophagy-lysosomal degradative pathway in the pathogenesis of familial neurohypohyseal diabetes insipidus. I Congress of Physiological Sciences of Serbia and Montenegro with International participation. November, 9-12, 2005.

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