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CROSS-TALK BETWEEN AUTOPHAGY AND APOPTOSIS AFTER RESVERATROL TREATMENT IN COLORECTAL CANCER

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INTRODUCTION

TYPES OF CELL DEATH

The maintenance of cellular homeostasis is fundamental for tissue integrity in multicellular organisms.

Balance between cell division and cell death is of almost importance for the development and maintenance of multicellular organism. Disorders of either process have pathologic consequences and can lead to disturb embryogenesis, neurodegenerative diseases or the development of cancer. Therefore, the equilibrium between life and death of cells is tightly controlled and faulty elements can effective be eliminated by a process called “programmed cell death” (PCD).

Three great categories of dynamic cellular activities that lead to cell death have been described in these years by researches: necrosis, apoptosis and autophagy.

This cell death classification is based on distinct biochemical and morphological characteristics present in the dying cell.

Two of these processes, apoptosis and autophagy, have been considered to be “programmed”, which refers to their strict genetic control (Broker et al., 2005; Danial and Korsmeyer, 2004).

Programmed cell death results in the disintegration of cellular components and their engulfment by surrounding cells.

Necrosis is generally considered a passive respons to massive cellular insult.

	APOPTOSIS	AUTOPHAGY	NECROSIS
CELL MEMBRANE	Blebbing, membrane integrity maintained	Blebbing	Loss of membrane integrity
NUCLEUS	Chromatin condensation, DNA laddering, nuclear fragmentation	Partial chromatin condensation, no DNA	Random DNA degradation
CYTOPLASM	Condensed membrane-bound cellular fragments, deploy-merization of cytoskeleton	Increased number of autophagic vesicles, degradation of Golgi, polyribosomes and ER	Swellind of cellular organelles

Fig.1: Scheme of cell death types

Apoptosis

Apoptosis, or programmed cell death type I, is a cell deletion process that physiologically accompany the development and health of multicellular organisms. Cells die in response to a variety of stimuli and during apoptosis they do so in a controlled, regulated fashion. This makes apoptosis distinct from another form of cell death called necrosis in which uncontrolled cell death leads to lysis of cells, inflammatory responses and, potentially, to serious health problems. Apoptosis, by contrast, is a process in which cells play an active role in their own death (which justifies why it is often referred to as “cell suicide”).

The intracellular machinery responsible for apoptosis seems to be similar in all animal cells. This machinery depends on a family of proteases called caspases that have a cysteine at their active site and cleave their target proteins at specific aspartic acids residues.

These proteases breakdown or cleave key cellular components that are required for normal cellular functioning including structural proteins in the cytoskeleton and nuclear proteins such as DNA repair enzymes. The

caspases can also activate other degradative enzymes such as DNases, which begin to cleave the DNA in the nucleus.

Necrosis

While apoptosis is increasingly well defined at the molecular level, necrosis seems to lack a molecular signature and therefore it has been referred to as a form of uncontrolled and pathological cell death. Recent studies suggest that necrosis is a regulated event that may be involved in multiple developmental, physiological and pathological scenarios (Majno and Joris, 1995). The principal features of necrosis include cellular energy depletion, damage to membrane lipids and loss of function of homeostatic ion pumps/channels. Unlike apoptosis, in which the Bcl-2 family of proteins and caspases play a key role, necrosis is induced by inhibition of cellular energy production, imbalance intracellular calcium flux, generation of ROS and activation of non apoptotic proteases. These events often potentiate each other and synergize to cause necrosis. It is a more chaotic way of dying, which results from circumstances outside the cell and is characterized by cytoplasmic and organelles with swelling and disruption of endosomal-lysosomal membranes and plasma membrane, leading to hydrolysis and release of cellular components. This release, in turn, results in massive inflammatory responses in the proximity of the dying cell (Scaffidi et al., 2002).

Moreover, it has been reported that necrotic cell death increases the probability of proto-oncogenic mutation. Necrotic cell death has been implicated in various pathological conditions, such as stroke, ischemia and several neurodegenerative diseases (Jiang et al., 2007; Stefani and Dobson, 2003).

Furthemore, it is often considered to be a causative factor of tumor promotion because excessive and prolonged inflammatory responses

create an environment rich of cytokines and hidrolytic enzymes that favour tumor growth, angiogenesis and invasion (Allavena et al., 2008) (Jiang et al., 2007).

As described above, one of the critical features of necrosis is the early rupture of the plasma membrane, which arises from ATP depletion, since reduction in the function of the ATP-dependent ion pumps on the plasma membrana due to energy depletion may disturb the intracellular homeostasis. Several membrane receptors have been implicated in triggering both necrosis and apoptosis. For example, TRAIL induces necrotic-like cell death at a lower extracellular pH in human HT29 colon carcinoma and HepG2 hepatocarcinoma cell lines (Meurette et al., 2005). In addition, FADD deficiency sensitizes Jurkat T cells to TNFα-dependent necrosis during activation-induced cell death (Bertazza and Mocellin, 2008) and ligation of DR4 and DR5 causes necrosis (Bodmer et al., 2000).

Moreover, among the secondary messengers, it has been reported that Ca^{2+} participates in receptor-mediated necrosis. Increased Ca^{2+} influx induces necrosis through MEC-4(d) channel-activated Ca^{2+} release from the endoplasmatic reticulum (ER) in neuron cells (Bianchi et al., 2004).

Another factor that triggers necrosis is the excessive production of reactive oxygen species (ROS).

In some cases, a specific type of ROS appears to be relevant for determination of the mode of cell death. For example, superoxide induces apoptosis, whereas hydrogen peroxide induces non-apoptotic cell death and JNK-1mediated PARP-1 activation (Zhang et al., 2007).

Currently, though it is not clear why ROS generation leads to apoptosis in some cells and necrosis in other ones, the amount or the type of ROS are believed to be critical factors involved in determining the mode of cell death; yet other regulatory factors can not be excluded.

Autophagy

Unlike apoptosis and necrosis, autophagy is not synonymous with cell death. Autophagy (from the Greek, “auto” oneself, “phagy” to eat) is evolutionarily conserved and occurs in all eukaryotic cells, from yeast to mammals (Levine and Klionsky, 2004).

Autphagy is activated in response to nutrient starvation, differentiation and developmental triggers. It is an adaptive process responding to metabolic stresses that results in degradation of intracellular proteins and organelles (Lum et al., 2005; Shintani and Klionsky, 2004).

During autophagy, portions of the cytoplasm are encapsulated in a double-membrane structure referred to as an autophagosome. Autophagosomes then fuse with lysosomes to form an autophagolysosomes where in the content is degraded by lysosomal hydrolases. Under physiological conditions, autophagy occurs at basal levels in practically all tissues, contributing to the routine turnover of cytoplasmic components. It can promote cell adaptation and survival during stresses such as starvation; still, under sustained stressful conditions the cells may die because of excessive autophagy.

In yeast, a cassette of autophagy-related genes have been identified that regulate autophagy induction, autophagosome formation and expansion, fusion with lysosomes and the recycling of autophagosome contents (Levine and Klionsky, 2004).

Cross-Talk between Apoptosis and Autophagy

Several lines of evidence indicate that a cross-talk exists between autophagic and apoptotic pathways. In general terms, it appears that similar stimuli can induce either apoptosis and autophagy. Whereas a mixed phenotype of autophagy and apoptosis can sometime be detected in response to these common stimuli, in many other instances, autophagy

and apoptosis develop in a mutually exclusive manner, perhaps as a result of variable triggers threshold for both processes, or as a result of a cellular “decision” between the two responses that may be linked to a mutual inhibition of the two phenomena.

Autophagy and apoptosis share many common inducers. The type of the initiating stimulus might determine which process will dominate. For example, during nutrient deprivation the default pathway would be autophagy, which creates a metabolic state with high (or increased) ATP that is anti-apoptotic (Katayama et al., 2007).

Successful removal of the damaged organelles followed by repair and adaption would allow for survival, while failure to restore homeostasis would result in deleyed apoptosis. By contrast, the default pathway that is triggered by other signals such as DNA damage or death-receptor activation would be immediate apoptosis, which occurs in a rapid, self-amplifying process, precluding simultaneous autophagic responses.

Other examples of cross-talk between apoptosis and autophagy are knockout or knockdown of ATG5, that abolishes autophagy and reduces the incidence of apoptotic events in human cancer cells treated with staurosporin (Yousefi et al., 2006).

BAX and BAK are required for mitochondrial outer permeabilization. It has been shown that mouse embryonic fibroblasts (MEFs) from double-knockout $Bax^{-/-}$ $Bak^{-/-}$ mice are resistant to a range of apoptosis inducers. When treated with DNA-damaging agents such as etoposide, $Bax^{-/-}$ $Bak^{-/-}$ MEFs failed to undergo apoptosis and instead manifested massive autophagy followed by deleyed cell death .

Beclin 1, the mammalian ortholog of yeast Atg6, was originally identified through its interaction with Bcl-2 (Shimizu et al., 2004).

Recent findings have shown that Bcl-2 and Bcl-xL expression can sensitize cells to autophagic death induced by etoposide (Shimizu et al.,

2004), and that Bcl-2 inhibits Beclin 1-mediated autophagy in response to starvation (Pattingre et al., 2005). These contradictory findings suggest that the outcome of the autophagic response may vary depending on the type of insult or cellular stress.

When HeLa or HCT116 cancer cells are cultured in the absence of nutrients, they rapidly induce autophagy in order to recycle essential metabolites, such as lipids and aminoacids, for fuelling the bioenergetic machinery. In these circumstances, the inhibition of autophagy results in an accelerated cell death that manifests the hallmarks of apoptosis, including chromatin condensation, major outer membrane protein (MOMP) and activation of caspases (Boya et al., 2005). When autophagy is blocked at an early stage by depletion of Beclin-1, ATG5, ATG10, ATG12 or Vps34, no autophagic vacuoles were found in the cells, which underwent a typical type I cell death. By contrast, when the fusion of autophagosomes and lysosomes was blocked by the addition of lysosomal inhibitors (chloroquine or the vacuolar ATPase inhibitor bafilomycin A) or by depletion of the lysosomal protein LAMP2, autophagic vacuoles accumulated and the cells manifested a mixed type I-type II morphology before the cells succumbed to death (Boya et al., 2005; Gonzalez-Polo et al., 2005).

APOPTOSIS

Alhough the phenomenon was known for almost a century, in 1972, Kerr, Wyllie and Currie first coined the term “apoptosis” in order to differentiate naturally occurring cell death from necrotic cell death that results from acute tissue injury (Kerr et al., 1972).

They also noted that apoptosis was responsible for maintaining the equilibrium between cell proliferation and cell death. Morphologic characteristics of apoptosis include cell membrane blebbing, cell shrinkage, chromatin condensation and nucleosomal fragmentation. Under normal conditions, cells undergoing apoptosis are reconized by macrophages or neighboring cells that consume the cells’ fractionated carcasses. Apoptosis has been considered a major mechanism of chemotherapy-induced cell death and pathways regulating apoptosis are the focus of many preclinical drug discovery investigation.

The critical signature that define the apoptotic cell death is the activation of caspases. These are synthesized in the cell as inactive precursors, or pro-caspases, which are usually activated by cleavage at aspartic acids by other caspases. Once activated, caspases cleave, and thereby activate the pro-caspases, thus amplifying the proteolytic cascade (Earnshaw, 1999).

In mammals, a wide array of external signals may triggers one or more of the three major apoptotic cell death:

-the *intrinsic* or mitochondria-mediated pathway

-the *extrinsic* or extracellular activated pathway (death receptor pathway)

-the *endosomal-lysosomal pathway*

The *intrinsic* pathway is usually activated in response to intracellular stress signal, which include DNA damage and high levels of reactive oxygen species (ROS), as well as viral infection and activation of

oncogenes. The *extrinsic* pathway is triggered by the binding of an extracellular ligand to a receptor on the plasma membrana. The endosomic pathway that involes the lysosomal cathepsins. A third pathway leads to the *transient permeabilization of endosomal-lysosomal organelles* with consequent release of cathepsins that may in turn trigger the permeabilization of mitochondria. This pathway can be troggered directly by lysosomotropic agents or may follow the extrinsic pathway. These pathways end up with the activation of proteolytic enzymes called caspase, that mediate the rapid dismantling of cellular organelles and architecture.

The intrinsic pathway

The most important turning point in the course of the intrinsic apoptotic process occurs in the mitochondria. A pivotal event in the mitochondrial pathway is MOMP, that is mainly mediated and controlled by Bcl-2 family members.

Bcl-2 family members act by regulating the efflux of apoptogenic proteins from mitochondria. They have four BH domain. The number and the combination of the BH domains dictate if the proteins are pro- or anti-apoptotic.

Antiapoptotic Bcl-2 members contain all four BH domain and include Bcl-2, Bcl-xL, Mcl-1, Bcl-w and Bf1-1/A1.

Instead proapoptotic members lack the BH4 domain and are divided into two groups, the BH3-only members and the multidomain BH1-3 proapoptotic members Bax and Bak. In mouse cells, deletion of these two protein is sufficient to prevent MOMP induced by upstream apoptotic events (Lindsten et al., 2000; Wei et al., 2001).

Bax and Bak normally exist as inactive monomers. Bax resides in the cytosol or loosely attached to intracellular membranes (Suzuki et al.,

2000), and Bak is found by Mcl-1, Bcl-xL or voltage-dependent anion channel protein 2 (VDAC-2) in the mitochondrial anion membrane (Scorrano and Korsmeyer, 2003; Scorrano et al., 2003; Willis et al., 2005).

The generalized scheme of intrinsic pathway activation is the oligomerization of Bax and Bak in the mitochondrial outer membrane cousing its permeabilization and the release of apoptogenic factors such as cytochrome c, second mitochondria-derived activator of caspases/direct IAP-binding (Smac-DIABLO) and Omi stress-regulated endoprotease/high temperature requirement protein A2 (Omi/HtrA2).

Once released, cytochrome c binds apoptotic protease-activating factor 1 (Apaf-1), which recruits pro-caspase 9, promoting its self-activation. Activated caspase-9 then cleaves the downstream effectors caspase-3 and caspase-7, which rapidly cleave intracellular substrates. Protein of the inhibitor of apoptosis proteins (IAP family), including X-linked IAP (XIAP), c-IAP1 and c-IAP2 can bind and inhibit the active sites of caspase-3 and caspase-7 and caspase-9. When released from mitochondria, Smac/DIABLO and Omi/HtrA2 can bind these IAPs and prevent their inhibition of the activated caspases (Du et al., 2000) (Suzuki et al., 2001) (Verhagen et al., 2000).

Antiapoptotic Bcl-2 proteins block oligomerization of bax and bak , thuspreventing the mitochondrial membrane permeabilization. However, it has been shown that Bax oligomerization on mitochondrial and lysosomal membranes is cathepsin D-dependent (Bidere et al. 2005, Castino et al., 2008).

Members of the BH3-only family include Bid, Bad, Bim, Puma, Noxa, Bmf and several others. Their activity appears to be kept in check by either transcriptional events. For example, cellular stresses, such as ionizing radiation (IR) or chemotherapy activate a DNA damage response

that stabilizes the p53 tumor suppressor protein. p53 acts to either arrest the cell division cycle by transcriptionally activating the cyclin-dependent kinase inhibitor p21, giving the cell time to repair the demage or else it helps to mediate apoptotic cell death. p53 also activates proapoptotic genes, including those encoding Bax and the BH3-only protein Puma, Noxa and Bid (El-Deiry, 2003).

The extrinsic pathway

This pathway activated by apoptotic stimuli comprising extrinsic signals such as the binding of death inducing ligands to cell surface receptors.

It is activated by members of two protein families, the tumor necrosis factor (TNF) and the receptors for these ligands (TNFR) are involved in the activation of this pathway (Locksley et al., 2001).

Most TNF family members bind receptors that activate signals involved in proinflammatory responses and do not signal cell death. The TNF ligands that can induce apoptosis are TNF-α, FasL (also known as CD95L) and TNF receptor apoptosis-inducing ligand (TRAIL, also known as Apo2L) (LeBlanc and Ashkenazi, 2003; Peter and Krammer, 2003).

After extraxellular ligand binding, the cytoplasmic end of the TNFR recruits initiating caspases. TRAIL binding to its death-inducing receptors acts in a manner similar to FasL, while TNF-mediated signalling is more complex. The ligand-bound Fas or TRAIL death receptors (DR4 and DR5) recruit the adapter protein Fas-associating death domain-containing protein (FADD) (Chinnaiyan et al., 1995).

Bound FADD recruits initiator caspase-8 and caspase-10, and this assembly of protein (receptor, FADD, and caspases) is termed the death-inducing signalling complex (DISC) (Kischkel et al., 1995).

Recruitment of caspase-8/10 to the DISC leads to their autoproteolytic cleavage (Boatright and Salvesen, 2003; Donepudi et al., 2003).

Caspase-8/10 activity can be blocked by a protein with which they share high homology, FLIP (FADD-like interleukin-1β -converting enzyme inhibitory protein). FLIP can oligomerize with caspase-8/10 but lacks critical residues in its caspase domain, including the catalytic cysteine, so acting as a dominant-negative inhibitor.

The BH3-only protein Bid connects the extrinsic pathway to mitochondria. Infact, Bid is cleaved by caspase-8, resulting in its myristyolization of a newly-exposed glycine residue to form tBid. tBid is then targeted to mitochondrial membranes where it promotes Bax and Bak oligomerization (Li H et al. 1998, Luo X et al. 1998).

The extrinsic pathway is involved in maintaining immune homeostasis and preventing the emergence of lymphomas or the development of autoimmunity (Bidere N et al. 2006). Cell derived from the hematopoietic progenitor ($CD8^+$ T cells, natural killer cells and dendritic cells) have the capacity to mediate cell death through the extrinsic pathway. For example, antigen stimulation of T cells causes the induction of FasL, TNF and TRAIL (Brunner T et al. 1995, Zheng L et al. 1995, Janssen EM et al. 2005).

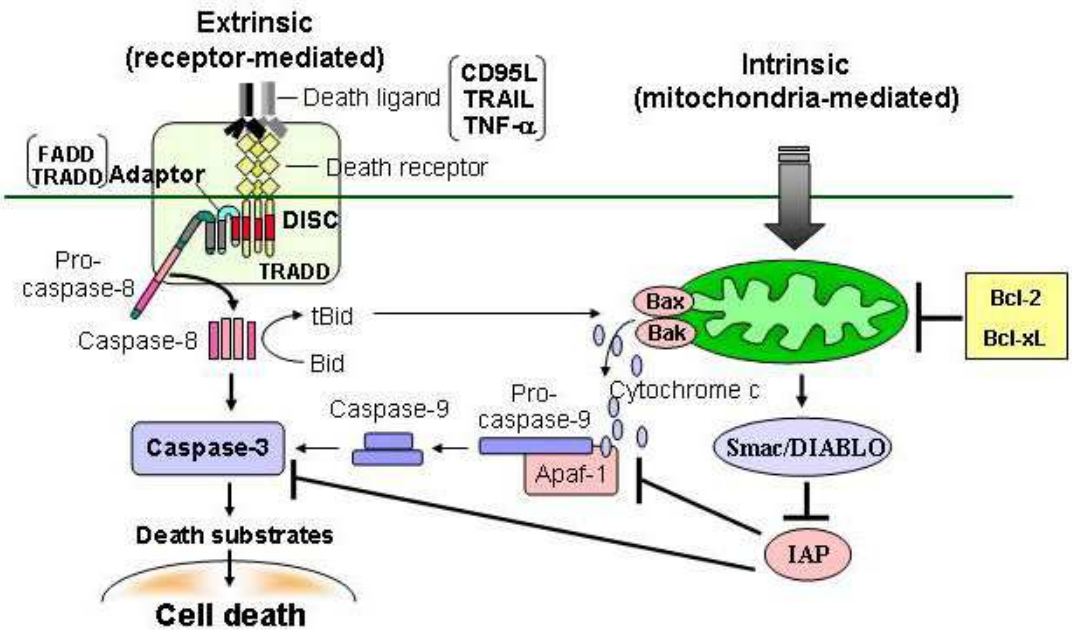


Fig 2: Scheme for the extrinsic and intrinsic pathways of apoptosis

The endosomal-lysosomal pathway

Although caspases are known to play a central role in apoptotic cell death, an emerging body of evidence has also implicated non-caspase proteases, including cathepsin proteases. The cathepsin proteases reside primarily in endosomes and lysosomes (Kirshke H et al. 1987, Chapman HA et al. 1997). Cathepsins can be divided into three categories based on their catalytic amino acid:

the aspartate proteases (cathepsins D and E)

the cysteine proteases (cathepsins B, C, F, H, K, L, O, S, T,V, W, and X),

the serine proteases (cathepsins A and G)

It has long been thought that the primary function of cathepsins is the terminal degradation of proteins in the lysosomal compartment.

Recent studies have determined that cathepsins B and D relocalize to the cytoplasm in response to certain apoptotic stimuli, and participate in the

execution of apoptosis (Guicciardi ME et al. 2004, Johnson DE 2000, Demoz et al. 2002).

Early studies showed that inhibition of cathepsin B with CA-074-Me abrogated bile salt-induced apoptosis in hepatocytes (Roberts LR et al. 1997). Subsequently, cathepsin B was shown to be released into the cytosol following treatment of WEHI-S fibrosarcoma cells with tumor necrosis factor-a (TNFa) (Foghsgaard L et al. 2001) or treatment of non–small cell lung cancer cells with microtubule stabilizing agents (Broker LE et al. 2004). Inhibition of cathepsin B with pharmacologic inhibitors or antisense directed against cathepsin B mRNA-attenuated microtubule stabilizing agent- and TNFa-mediated apoptosis.

Moreover, Guicciardi et al. have shown that hepatocytes from cathepsin B–deficient mice are markedly resistant to TNFa.

Cathepsin D is the major intracellular aspartate protease and is expressed in all human tissues. The cathepsin D enzyme is synthesized as a 52 kDa precursor, which undergoes processing to active single chain (48 kDa) and double chain (31 and 14 kDa subunits) forms (Hasilik and Neufeld, 1980; Faust PL et al. 1985).

Mice that are deficient in cathepsin D die on postnatal day 26 due to atrophy of the intestinal mucosa and consequent anorexia (Saftig P et al. 1995). Cathepsin D has been shown to be relocalized to the cytoplasm following treatment of cells with hydrogen peroxide (Yin L et al. 2005, Castino R et al. 2007), oxidized low-density lipoprotein (Yuan XM et al. 2000) and the protein kinase C inhibitor staurosporine (Johansson AC et al. 2003; Bidere et al, 2005) and here it can induce the alteration of lysosomal and mitochondrial membrane integrity.

Reports demonstrating participation of cathepsin D in apoptosis execution have relied heavily on the pharmacologic inhibitor pepstatin A.

Unfortunately, pepstatin is not entirely specific, and is also known to inhibit cathepsin E, pepsin, and renin.

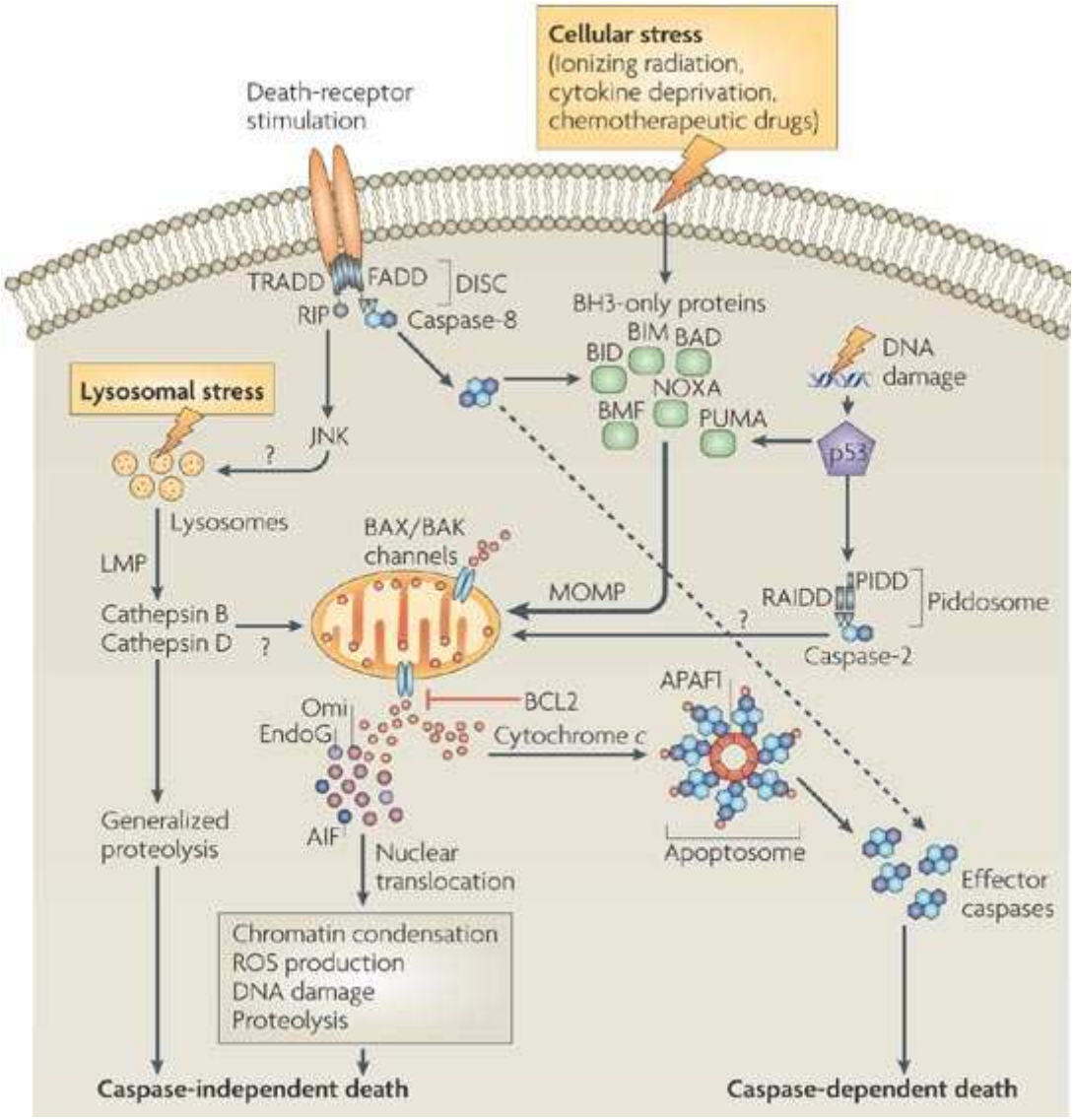
Moreover it has been shown that there is an involvement of Cathepsin D in the TNF-α –apoptosis induction (Demoz M et al. 2002).

However, antisense-mediated inhibition of cathepsin D has served to verify a role for this enzyme in IFN-g- and Fas-mediated apoptosis (Deiss LP et al. 1996). Furthermore, Bidere et al. have shown that down-regulation of cathepsin D expression using small interfering RNA (siRNA) inhibits early apoptotic events associated with staurosporine-induced apoptosis in

human T lymphocytes.

Although previous reports have indicated the importance of cathepsin D during apoptosis caused by the agents and stimuli described above, less is known about the involvement of this enzyme in chemotherapy-induced apoptosis.

Intriguing findings were provided by Wu and collaborators , who showed that fibroblasts derived from cathepsin D knockout mice are more resistant to Adriamycin and etoposide (VP- 16). Very recently it has been show na model in which on treatment with a cytotoxic drug the activation of a cathepsin D-bax loop leads to the generalized permeabilization of lysosomes and eventually of mitochondria, thus reaching the point-of-non-return, and culminates with the activation of the caspase cascade (Castino et al. 2008).



Endosomal-lysosomal pathway

Fig 3: The endosomal-lysosomal pathway scheme

AUTOPHAGIC CELL DEATH

The Autophagy pathway

This form of cell death follows the hyperactivation of the autophagy degradative process. Autophagy is generally defined as a lysosome-dependent mechanism of intracellular degradation that is used for the turnover of aged cellular components.

Three pathways of lysosome-mediated degradation have been identified:

-chaperone-mediated autophagy

-microautophagy

-macroautophagy

These pathways differ with respect to their physiological functions and the mode of cargo delivery to the lysosome.

The chaperone-mediated autophagy (CMA) consists in selective delivering of soluble cytosolic proteins that contain a chaperone-mediated autophagy targeting motif for incorporation into lysosomes (the amino acid sequence KFERQ) (Kaushik and Cuervo, 2006). In this case a chaperone-cochaperone complex (hsc70) transports the specific substrate to the lysosomal membrane for translocation into the lysosome following binding to lysosomal-associated membrane protein type 2A (Lamp2A).

The microautophagy, whereby cytoplasmic constituents are engulfed by invagination of the lysosomal membrane and subsequently degraded (Klionsky, 2007). The function of this process in higher eukaryotes is not known, whereas microautophagy-like processes in fungi are involved in selective organelle degradation.

In the case of macroautophagy the cargoes are sequestered within a unique double-membrane cytosolic vesicle named autophagosome. Sequestration can be either non-specific, involving the engulfment of bulk cytoplasm, or selective, targeting specific cargoes such as organelles

or invasive microbes. The engulfment of peroxisomes and mitochondria by autophagosomes for transport to lysosomal degradation is referred to as pexophagy and mitophagy, respectively.

The molecular machinary of macroautophagy

We will focus our attention on macroautophagy (herein referred to as autophagy), the major regulated catabolic mechanism that eukaryotic cells use to degrade long-lived proteins and organelles.

This form of autophagy involves the delivery of cytoplasmic cargo sequestered inside double-membrane vesicles to the lysosome.

Autophagy begins with a *signal* (or stimuli), which can originate from an extracellular stress, for example a shortage of essential nutrients, growth factors, ATP or oxygen, or an intracellular stress, such as an unfolded protein response (UPR) in the endoplasmic reticulum (ER), or an increase in unfolded cytoplasmic proteins. The signal to maintain basal autophagy might be a quality control sensor monitoring protein and organelle function. How these diverse signals are generated, integrated and propagated to downstream targets, including the Atg (Autophagy-related) proteins is not completely known, although some key regulators have been identified, including TOR (target of rapamycin), a negative regulator of autophagy. Downstream of mammalian TOR (mTOR) are Ulk (Unc51-like kinase) 1 and 2, which are the orthologues of yeast Atg1. Ulk1/2 are serine/threonine kinases required for autophagy that propagate the initiating signal via phosphorylation of sofar unknown substrates, and possibly non-catalytic effectors (Chan et al. 2009).

The cellular events that accomany the formation of the autophagosome follow distinct stages:

-vesicle nucleation (formation of the isolation membrane/phagophore)

could be considered as the defining event that creates an active phagophore (or isolation membrane), which precedes the foundation of the autophagosome. At the phagophore, Ulk1/2 function together with Atg9, a six-spanning membrane protein, and with the Class III phosphatidylinositol 3-kinase (PI3K) Vps34 complex, which comprises the Atg6 orthologue Beclin1 and its interactors, including Ambra1.

-vesicle elongation of an isolation membrane, which is called a phagophore. The edges of the phagophore then fuse (vesicle completion) to form autophagosome, a double-membraned vesicle that sequesters the cytoplasmic material and lysis of the autophagosome inner membrane and breakdown of its contents inside it.

-vesicle completion (growth and closure)

The expansion of the phagophore into an autophagosome requires two highly conserved ubiquitin (Ub)-like systems, which are essential for the posttranslational modification of Atg8. In mammalian cells, the most well studied homologue of Atg8 is MAP-LC3 (the microtubule associated protein light chain 3) or LC3. There are three forms of LC3, the newly synthesized full length protein, the Cterminally cleaved protein called LC3-I, and the PE-conjugated form of LC3-I, referred to as LC3-II which is generated by the ubiquitin-like system. The formation and recruitment of LC3-II during the expansion and sequestration step is dependent upon the Atg5-12-16 complex as well as the nucleation proteins. Sequestration of ubiquitinated cargo is facilitated by p62/SQSTM-1.

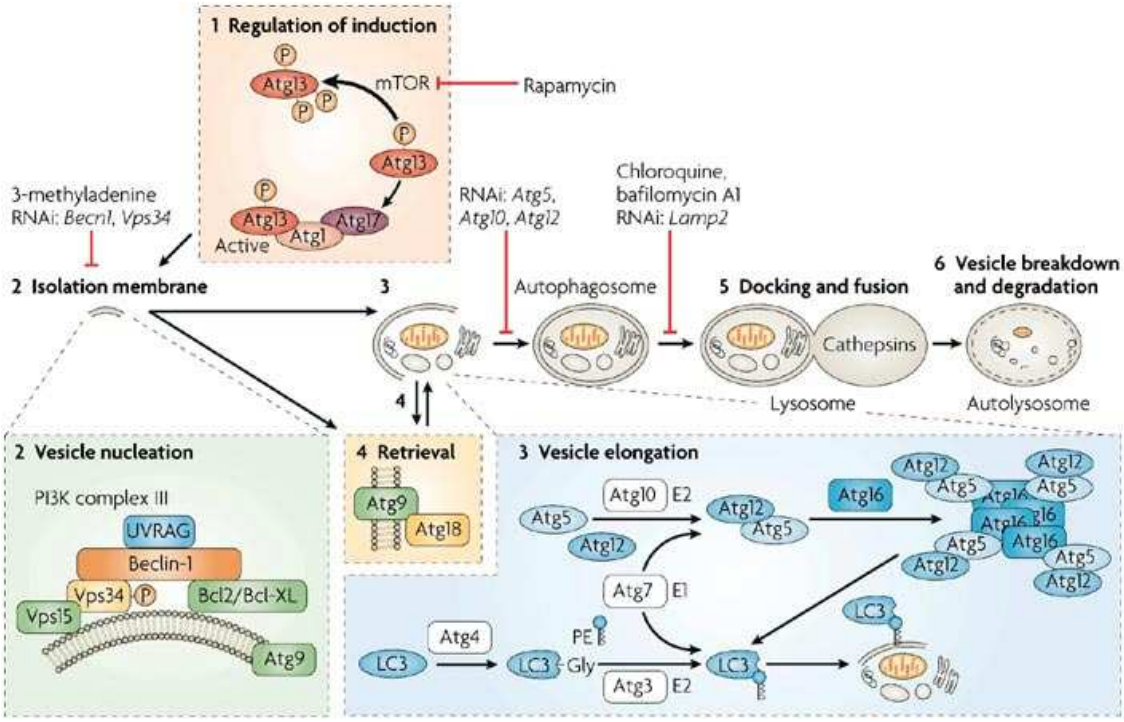


Fig 4: The three fases for the autophagy induction

Formation of the autophagosome followed by its fusion with a lysosome to form an autophagolysosome, where the captured material, together with the inner membrane, is degraded. The autophagy process occurs at low basal levels in virtually all cells to perform homeostatic functions such as protein and organelle turnover. It is rapidly upregulated when cells need to generate intracellular nutrients and energy, for example during starvation, growth factor withdrawal or high bioenergetic demands.

Autophagy is also upregulated when cells are preparing to undergo structural remodelling such as during developmental transitions or to rid themselves of damaging cytoplasmic components, for example during oxidative stress, infection or protein aggregate accumulation.

The identification of signals that regulate autophagy and genes that execute autophagy has facilitated detection and manipulation of the autophagy pathway.

Phosphatidylethanolamine (PE) conjugation of yeast Atg8 or mammalian LC3 during autophagy results in a non-soluble form of Atg8 (Atg8-PE) or LC3 (LC3-II) that stably associates with the autophagosomal membrane. Consequently, autophagy can be detected biochemically (by assessing the generation of Atg8-PE or LC3-II) or microscopically (by observing the localization pattern of fluorescently tagged Atg8 or LC3) (Mizushima and Klionsky, 2007).

Autophagy can be pharmacologically induced by inhibiting negative regulators such as TOR with rapamycin (Rubinsztein et al., 2007); the antiapoptotic proteins Bcl-2 and Bcl-X_L that bind to the mammalian ortholog of yeast Atg6, Beclin1, with ABT-737 (Maiuri et al., 2007b) ; IP_3R with xestospongin B, an IP_3R antagonist; or lithium, a molecule that lowers IP_3R levels (Criollo et al., 2007).

Autophagy can be pharmacologically inhibited by targeting the class III PI3K involved in autophagosome formation with 3-methyladenine or by targeting the fusion of autophagosomes with lysosomes, using inhibitors of the lysosomal proton pump such as bafilomycin A1 or lysosomotropic alkalines such as chloroquine and 3-hydroxychloroquine (Rubinsztein et al., 2007). It should be noted that all of these pharmacological agents lack specificity for the autophagy pathway. Therefore, although some of these agents such as rapamycin, lithium and chloroquine are clinically available and may be helpful for treating diseases associated with autphagy deregulation, genetic approaches to inhibiting autophagy, for example knockout of ATG genes by homologous recombination or knockdown by small-interfering RNA have yelded more conclusive information about the biologic roles of autophagy in health and disease.

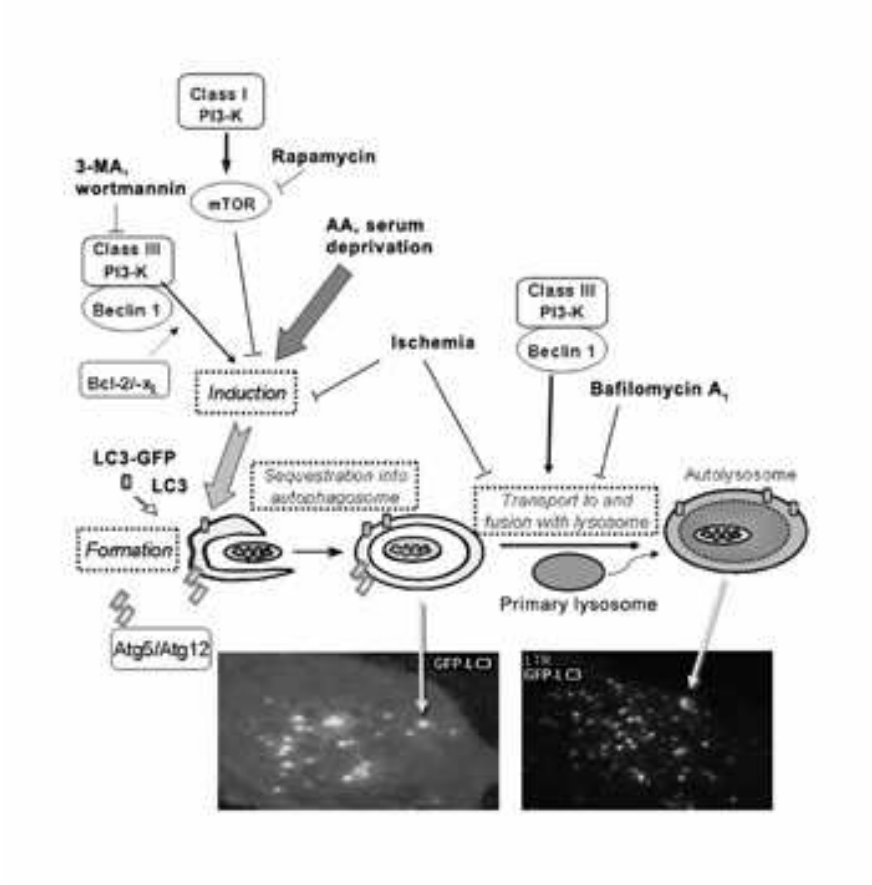


Fig 5: The LC3-lipidation

PHYSIOLOGICAL FUNCTION OF AUTOPHAGY

Autophagy defends against metabolic stress

Autophagy is activated as an adaptive catabolic process in response to different forms of metabolic stress, including nutrient deprivation, growth factor depletion and hypoxia. This form of bulk degradation generates free amino and fatty acids that can be recycled in a cell-autonomous fashion or delivered systematically to distant sites within the organism. Presumably, the amino acids generated are used for "*the novo*" synthesis of protein that are essential for stress adaption. The molecular basis for the recycling function of autophagy has only recently begun to be defined with the identification of yeast Atg22 as a vacuolar permease required for the efflux into the cytosol of amino acids resulting from autophagic degradation (Mizushima and Klionsky, 2007). The amino acids liberated from autophagic degradation can be further processed and together with the fatty acids, used by the tricarboxylic acid cycle (TCA) to maintain cellular ATP production. The importance of autophagy in fueling the TCA cycle is supported by studies showing that certain phenotypes of autophagy-deficient cells can be reversed by supplying them with a TCA substrate such as pyruvate (or its membrane-permeable derivative methylpyruvate).

This role of autophagy in maintaining macromolecular synthesis and ATP production is likely a critical mechanism underlying its evolutionarily conserved prosurvival function. Gene knock-out and knoc-down studies provide strong evidence that autophagy plays an essential function in organismal during nutrient stress (Maiuri et al., 2007a) .

Autophagy works as a cellular housekeeper

Autophagy can eliminate defective proteins and organelles, prevent the accumulation of abnormal protein aggregates and remove the intracellular pathogens. Such functions are likely critical for autophagy-mediated protection against aging, cancer, neurodegenerative disease and infection. Although some of these functions overlap with those of the ubiquitin-proteosome system, the other major cellular proteolytic system, the autophagy pathway is capable of degrading entire organelles such as mitochondria, peroxisomes and ER as well as intact intracellular microorganisms. Unlike proteosomal degradation, the autophagic breakdown of substrates is not limited by steric considerations and therefore autophagy can sequester and degrade entire organelles.

Tissue-specific disruption of ATG genes has revealed a critical role for basal autophagy in protein quality control in murine post-mitotic cells. Atg7 deletion in hepatocytes (REF), atg5 and atg7 deletion in neurons (REF), and atg5 deletion in cardiomyocytes(REF) result in the accumulation of ubiquitin-positive protein aggregates in inclusion bodies that are associates with cellular degeneration.

Autophagy may be a guardian of the genome

Recent studies in ATG gene-deficient immortalized epithelial cells indicate that the autophagy machinery can limit DNA damage and chromosomal instability (Mathew et al., 2007).

Because theses studies used cells with simultaneous defects in DNA checkpoints and apoptosis pathways, it is not yet known whether autophagy plays a primary function in preventing genomic instability in normal cells. However, in view of known functions of autophagy in energy homeostasis and in protein and organelle quality control, this seems likely. Failure to control the damage of checkpoint or repair

proteins, deregulated turnover of centrosomes, insufficient energy for proper DNA replication, repair and excessive generation of reactive oxygen species due to inefficient removal of damaged mitochondria are possible alterations that may contribute to genomic instability in autophagy-defective cells (Jin and White, 2007; Mathew et al., 2007).

Autophagy in life and death decisions of the cell

Under most circumstances, autophagy constituts a stress adaption pathway that promotes cell survival. An apparent paradox is that autophagy is also considered a form of nonapoptotic programmed cell death calle d “type II” or autophagic cell death. The knockdown of ATG genes has shed some lights on the involvement of autophagy in the execution of cell death in different settings (Maiuri et al., 2007a). It is not yet understood what factors determine if autophagy is cytoprotective or cytotoxic and if cytoxicity occurs as the result of self-cannibalism, the specific degradation of cytoprotective factors or other as yet undefined mechanisms.

However, cells subjected to prolonged growth factor deprivation or shortage of glucose and oxygen can loose the majority of their mass via autophagy and fully recover when placed in optimal culture conditions (Degenhardt et al., 2006) (Lum et al., 2005), suggesting that cell death via autophagy may not be simply a matter of crossing a quantitative threshold of selfdigestion.

AUTOPHAGY IN DISEASES

Autophagy and Neurodegenerative diseases

Early reports demonstrating that autophagosomes accumulate in the brains of patients with diverse neurodegenerative diseases, including Alzheimer's disease, transmissible spongiform encephalopathies, Parkinson's disease and Huntington's disease (Rubinsztein et al., 2007) (Williams et al., 2006), led to the initial hypothesis that autophagy contributed to the pathogenesis of these disorders. In mice with cerebellar degeneration due to mutations in glutamate receptor, autophagy was also postulated to be a mechanism of nonapoptotic cell death (Yue et al., 2002).

In contrast, more recent studies provide compelling evidence that at least in model organism autophagy protects against diverse neurodegenerative diseases and that the accumulation of autophagosomes represents the activation of autophagy as a beneficial physiological response or, in the case of Alzheimer's disease, the consequence of a defect in autophagosomal maturation (Martinez-Vicente and Cuervo, 2007) (Rubinsztein et al., 2007) (Williams et al., 2006).

Beyond its role in the clearance of misfolded proteins spontaneously generated during routine protein turnover, autophagy plays an important role in the clearance of aggregate-prone mutant proteins associated with several different neurodegenerative diseases. These include proteins with polyglutamine (polyQ) expansion tracts such as those seen in Huntington's disease and spinocerebellar ataxia, mutant α-synucleins that cause familial Parkinson's disease and different forms of tau including mutations causing frontotemporal dementia (Williams et al., 2006).

Pharmacological activation of autophagy reduces the levels of soluble and aggregated forms of mutant huntingtin protein, protein mutated in spinocerebellar ataxia, mutant forms of α-synucleins and mutant tau.

The role of autophagy in protection against neurodegenerative diseases is estabilished in animal models but not yet in patients. Preclinical animal data provide a strong rationale for proceeding with clinical trials with autophagy-stimulatory agents; this is especially true as agents shown to be beneficial in reducing neurotoxicity of mutant aggregate-prone proteins are already in clinical use to treat other diseases. Rapamycin analogs, which are approved for the use of preventing organ transplant rejection and postangioplasty coronary artery restenosis and are in phase II oncology trials, protect against neurodegeneration seen in *Drosophila* and mouse polyQ disease models (Rubinsztein et al., 2007). Another group of new agents, small molecule enhancers or rapamycin (SMERs), enhance the clerance of mutant huntingtin and α-synuclein and protect against neurodegeneration in a *Drosophila* Huntington's disease model (Sarkar et al., 2007). Since lithium and SMERs both act independently of TOR, it is possible that they may be used therapeutically in combination with rapamycin analogs.

Recently it has been shown the protective effects of lithium, infact for the first time this drug can stimulate the biogenesis of mitochondria in the central nervous system and, uniquely in the spinal cord, induces neuronogenesis and neuronal differentiation (Fornai et al. 2008).

Autophagy and Liver disease

Tissue-specific knockout studies in mice indicate an important role for basal hepatocyte autophagy in intracellular protein and organelle quality control. The protein quality-control function may be important in the pathogenesis of the most common genetic cause of human liver

disease, α1-antitrypsin deficiency, which is associated with chronic inflammation and carcinogenesis (Perlmutter, 2006).

Perhaps, similar to neurodegenerative disorders caused by aggregate-prone proteins, pharmacological activation of autophagy my be helpful in this setting.

Autophagy and Muscle disease

Similar to neurodegenerative diseases, the pathogenesis of myodegenerative disorders may involve either the failure of autophagosomes to fuse with lysosomes or the aggregation of misfolded proteins that exceed the autophagic clearance capacity of the myocite. Danon disease, a genetic disease characterized by cardiomyopathy , myopathy and variable mental retardation, results from a mutation in the lysosomal protein LAMP-2 and is associated with extensive accumulation of autophagosomes in the muscles of LAMP-2-deficient mice and patients. There are several other histologically related diseases, such as X-linked myopathy with excessive autophagy, infantile autophagic vacuolar myopathy, adult-onset vacuolar myopathy with multiorgan involvement and X-linked congenital autophagic vacuolar myopathy that have unclear molecular defects (Nishino, 2006); however, the prediction is that these disorders may be due, at least in part, to an impairment in autophagosome-lysosome fusion.

Muscle diseases in which autophagy may promote the clearance of inclusion bodies, limb girdle muscular dystrophy type 2B and Miyoshi myopathy. In sporadic inclusion body myositis, the most common acquired muscle disease in patients above 50 years of age, overexpression of amyloid precursor protein (APP) and accumulation of its proteolytic fragment β-amyloid in vacuolated cells is thought to be a central pathogenetic mechanism (Askanas and Engel, 2006).

Both APP and β-amyloid colocalize with LC3 in cultured human muscle cells and in degenerating muscle fibers in human tissue biopsies, suggesting that these proteins are cleared by autophagy (Lunemann et al., 2007).

Autophagy and Cardiac disease

Defective autophagy may play a role in relatively rare forms of inherited diseases of the heart (Dannon disease, Pompe disease). Of greater medical significance is the possibility that autophagy constitute an important physiological or pathophysiological response to cardiac stresses such as ischemia or pressure overload, which are frequently encountered in patients with coronary artery disease, hypertension, aortic valvular disease and congestive heart failure. The accumulation of autophagosomes has been noted in cardiac tissues biopsies of patients with these disorders, rodent models of these cardiac diseases and isolated stressed cardiomyocytes (Terman and Brunk, 2005). Alternatively the cytoprotective effects of autophagy (either via ATP production, protein and organelle quality control or other mechanisms) may predominate and the disease rather reflects an insufficience of such mechanisms.

CANCER

Cancer cells are defined by two heritable properties: they and their progeny reproduce in defiance of the normal restraints on cell division and invade and colonize territories normally reserved for other cells. It is the combination of these actions that makes cancers dangerous. Infact, if the proliferation is out of control, it will give rise to a tumour, or neoplasm.

As long as the neoplastic cells remain clustered together in a single mass, the tumour is said to be *benign*. At this stage, a complete cure can usually be achieved by removing the mass surgically. A tumour is considered a cancer only if it is *malignant* when its cells have acquired the ability to invade surrounding tissue. Invasiveness usually implies an ability to break loose, enter the bloodstream or lymphatic vessels with *metastases* and form secondary tumours at other sites of body.

Cancers are classified according to the tissue and cell type from which they arise. Cancers arising from epithelial cells are termed *carcinomas;* those arising from connective tissue or muscle cells are termed *sarcomas*. Cancers that do not fit in either of these two broad categories include the various *leukemias,* derived from hamopietic cells and cancers derived from cells of the nervous system.

There are good reasons to think that the vast majority of cancers are initiated by genetic changes. First, cells of a variety of cancers can be shown to a have a shared abnormality in their DNA sequence that distinguishd them from the normal cells surrounding the tumour. Second, many of the agents known to give rise to cancer also cause genetic changes. Thus carcinogenesis (the generation of cancer) appears to be

linked with mutagenesis (the production of a change in the DNA sequence). This correlation is clear for three classes of agents:

-chemical carcinogenesis, which tipically cause simple local changes in the nucleotide sequence.

-ionizing radiations, such as X-rays, which cause chromosome break and translocations.

-viruses, which introduce foreign DNA into te cell.

It' is important to know that not all substances that favor the development of cancer are mutagens. Some of the clearest evidence comes from studies done long time ago on the effects of cancer-causing chemicals on mouse skin, where it is easy to observe the stages of tumour progression.

In fact a single application of the carcinogen, usually does not by itself give rise to a tumour or any other obvious lasting abnormality. It can cause only latent genetic damage. This type of carcinogen is said to act as a *tumour initiator*. Only repeated exposure over a long period to certain substances known as *tumour promoters*, which are not themselves mutagenic, can cause cancer selectively in skin previously exposed to a tumour initiator.

The cancer-critical genes

They are grouped into two broad classes, according to whether the cancer risk arises from too much activity of the gene product. The two classes are:

-proto-oncogenes and their mutant over-active forms are called oncogenes

-tumor suppressor genes.

Mutation of a single copy of a proto-oncogene can have a dominant, growth-promoting effect on a cell. Thus the oncogene can be detected by

its effect when it is added- by DNA transfection, for example, or through infection with a viral vector- to the genome of a suitable type of tester cells. In the case of the tumor suppressor gene, the cancer-causing mutations are generally recessive: both copies of the normal gene must be removed or inactivated in the doploid somatic cell before an effect is seen.

Colorectal cancer

Colon carcinoma is the second largest cause of cancer-related mortality in the United States and both prevention and treatment of this disease are the focus of intensive research efforts. Diet has been recognized as an important modifiable risk factor for colon cancer for decades (Ellington and Bull, 2005). Specifically, a consistent inverse association between diets rich in plant foods and colon cancer incidence ha been identified through epidemiologic studies.

Colorectal cancer arise from the epithelium lining the colon and rectum . It is common, currently cusing over 60000 deaths a year in the United States, or about 11% of total deaths from cancer. Routine examination of normal adults with a colonscope often reveals a small benign tumor, or adenoma, of the gut epithelium in the form of a protruding mass of tissue called a polyp. The progression of the disease is usually very slow; there is typically a period of 10-35 years in which the slowly growing tumor is detectable but has not yet turned malignant.

Gut epithelium is constantly regenerated from progenitor cells located near the bottom of intestinal and colonic crypts which produce transit amplifying cells that in turn give rise to the cell lineages required for gut function (Cheng and Leblond, 1974).

The most abundant differentiated cells are absorptive enterocytes, which have a highly organized cytoskeletal arrangement with an apical actin-rich brush border and cortical, lateral actin-rich junctions and highly ordered parallel arrays of microtubules (Cheng H and Leblond CP 1974).

Maintenance of the gut epithelium depends on a combination of cell proliferation balanced by cell death, coupled with differentiation and active cell migration, while adhering to neighbouring cells and the basement membrane. It is therefore likely that changes that cause an imbalance between these processes can contribute to the initiation of tumors in this tissue.

Many genes have been to be involved in colorectal cancer: K-Ras, a member of the Ras gene family, p53 and APC. Other critical genes remain to be identified.

Some mutations inactivating the APC (adenomatous polyposis coli) gene appears to be the first or at least a very early step in most cases. They can be detected already in small benign polyps at the same high frequency as in large malignant tumors. Loss of APC seems to increase the rate of cell proliferation in the colonic epithelium relative to the rate of cell loss, without affecting the way the cells differentiate or the details of the histological pattern they form. Mutations activating the K-Ras oncogene appear to take place a little later than those APC; they are rare in small polyps but common in larger ones that show disturbances of cell differentiations and histological pattern. When malignant colorectal carcinoma cells containing such Ras mutations are grown in culture, they show typical features of transformed cells, such as the ability to proliferate without anchorage to a substratum. Loss of cancer-critical genes on chromosome 18 and mutations in p53 may come still later. They are rare in polyps but common in carcinomas, suggesting that they may often occur late in the sequence. Loss of p53 function is thought to allow

the abnormal cells not only to avoid apoptosis and to divide, but also to accumulate additional mutations at a rapid rate by progressing through the cell cycle thus favouring the formation of abnormal chromosomes.

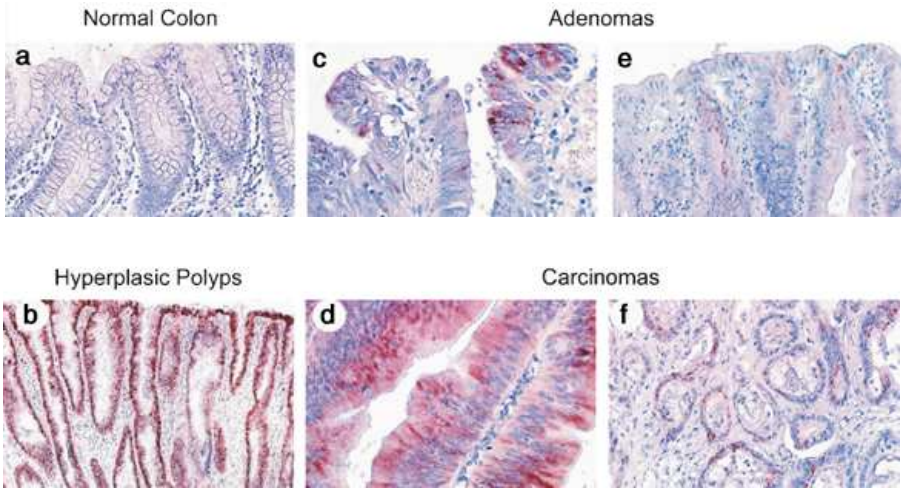


Fig 6: Histochemistry of tissues from normal colon to Carcinomas

APOPTOTIC CELL DEATH AND CARCINOGENESIS

Apoptosis can be induced by various stimuli, and radiation or chemicals in particular have been used in cancer therapy (Johnstone et al., 2002) (Fesik, 2005). Two major signalling pathways that lead to apoptosis in mammalian cells are known: the intrinsic pathway and the extrinsic pathway. The intrinsic pathway is controlled by antiapoptotic Bcl2 family proteins at the mitochondria and has a substantial role in chemotherapy and radiationin duced cell death. By contrast, the extrinsic death pathway is initiated through apoptotic signal transduction cascades mediated by members of the tumour necrosis factor (TNF) receptor superfamily.

In cancer cells, apoptosis induced by the extrinsic pathway complements that induced by the intrinsic pathway, so targeting death receptors is considered a useful new therapeutic approach.

TNF-related proteins

The pathway involving TNF-related apoptosis inducing ligand (TRAIL, also known as APO2L and TNFSF10) and TRAIL receptors (TRAILRs) is most promising, as preclinical models suggest that apoptosis of tumour cells is achievable *in vivo* without lethal toxicities (Ashkenazi, 2002). In addition to triggering a pro-apoptotic signal through activation of caspases, TRAIL can activate diverse intracellular signalling pathways involving NFκB (Russo et al., 2004), phosphoinositoide 3-kinase (Aggarwal and Shishodia, 2006) and mitogen activated protein kinase (Li et al., 2007) family proteins that can stimulate cell survival and proliferation.

TRAIL is an important immune effector molecule in the surveillance and elimination of developing tumours. Moreover, genetic lesions in various

components of the TRAIL pathway have been found in human tumour samples, suggesting that inactivation of the TRAIL pathway and/or escape from TRAIL-mediated immunosurveillance might have an important role in tumour onset and progression.

In preclinical trials, recombinant forms of TRAIL and agonistic anti-TRAIL receptor antibodies can have single-agent activity against TRAIL-sensitive tumour cells *in vitro* and *in vivo*. These agents can synergize with chemotherapeutic drugs and novel molecular therapeutic agents to more effectively kill TRAIL-sensitive tumour cells and TRAIL-resistant tumours.

Early-phase clinical trials using recombinant TRAIL and agonistic anti-TRAIL receptor antibodies indicate that these agents can be delivered safely and are generally well-tollerated. Although some objective anti-tumour responses have been reported with these agents as monotherapies, they probably hold greater promise for further clinical development when used in combination with other cancer treatments (Buchsbaum et al., 2007).

FLIP as an anti-cancer therapeutic target

Antiapoptotic proteins overexpressed in tumor cells have been recognized as targeting points for anti-cancer therapeutic interventions, and their inhibitors at the levels of mRNA and protein have been developed, which are mostly antisense oligonucleotides and small molecule inhibitors (Fesik, 2005) (Debatin and Krammer, 2004).

These drug candidate compounds are now mostly in the preclinical and early clinical stages.

FLIP is an another important antiapoptotic protein overexpressed in various types of tumor cells (Kataoka, 2005), but the agents directly targeting it have not yet been reportedly developed.

Elevated expression of c-FLIP has been found in various types of tumor cells which are often resistant (Kataoka, 2005) to death-receptor-mediated apoptosis.

Those tumors include colorectal carcinoma (Ullenhag et al., 2002) pancreatic carcinomas (Elnemr et al., 2001), non-Hodgkin's lymphomas (Valente et al., 2006), B cell chronic lymphocytic leukemias (Olsson et al., 2001), ovarian carcinoma (Abedini et al., 2004).

The expression of c-FLIP has been proven to be one of the major determinants of the resistance to death ligands such as FasL and TRAIL, and numerous reports have shown that down-regulation of c-FLIP results in sensitizing a various types of resistant tumor cells. Conversely, forced expression of c-FLIP renders cells resistant to Fas and/or TRAIL. These observations collectively imply that c-FLIP may be an attractive therapeutic target against at least the above mentioned kinds of tumors of which malignancy and resistance.

p53-based cancer therapies

The tumor suppressor protein p53 is activated in response to stressful stimuli such as DNA damage, hypoxia and oncogene activation. p53 is often referred to as the 'guardian of the genome' because it plays a key role in determining a cell's fate following DNA damage. When DNA is damaged, p53 can trigger cell cycle arrest, senescence (permanent arrest) or apoptosis to eliminate the damaged cell. Cell cycle arrest provides time for repair of the damage thereby allowing the cell to recover and survive. The mechanisms governing whether a cell will arrest or die are not well understood, but these functions are essential to protecting an organism from the effects of aberrant cell divisions, and defects can lead to cancer development and progression.

The fact that p53 is defective in >50% of tumors supports the assertion that p53 is an important player in the prevention of tumor development. The pivotal role of p53 in regulating the decision of a cell to live or to die makes it an attractive target for cancer therapeutics. However, there are many conflicting ideas and approaches as to which would be the best therapeutic strategy to pursue. Initial attempts were based on the premise that reintroduction or activation of p53 would induce apoptosis in the tumor. Other approaches are based on the observation that cells with defective p53 are more sensitive to certain drugs and combinations.

Understanding the factors influencing the decision of a cell to undergo cell cycle arrest or die in response to DNA damage is essential to developing cancer therapies that specifically target tumor cells without damaging normal cells. There is a huge literature addressing both cell cycle arrest and cell death, but in the context of therapeutic strategies, the widely accepted model is that p53 is required for DNA damage-induced apoptosis.

There are numerous cases where p53-defective cells undergo apoptosis, and many cases in which the loss of p53 even sensitizes cells to DNA damage.

So pervasive has p53 become that it sometimes appears there are only two pathways of apoptosis, either p53-dependent or p53-independent, and this extends far beyond any role in response to DNA damage. It has frequently been implied that p53 is needed if cells are to succumb to anticancer drugs, yet many years of research have resulted in the discovery of drugs while using p53-defective tumor models, and the drugs have been used successfully to treat many patients with p53-defective tumors. Furthermore, radiation therapy is routinely administered to patients independent of their p53 status as it does not predict the outcome.

Numerous studies have focused on activation of p53 as a strategy for cancer therapy. These studies have generally not considered the particular oncogenic context of each tumour and whether arrest or apoptosis would be the likely outcome.

However, re-introduction of p53 into p53-defective tumor cells has frequently been shown to increase apoptosis (Willis and Chen, 2002).

The consequence of over-expression of exogenous p53 may have little bearing on the pathways activated by DNA damage in the presence of a relatively low level of endogenous protein.

For example, overexpression of p53 may overwhelm any selective activation of growth arrest genes versus proapoptotic genes. Difficulties in this therapeutic strategy lie in controlling the level of p53 expression and in delivering the p53 gene effectively to tumor cells.

While much of the drug-induced phosphorylation of p53 is required to dissociate p53 from HDM2, the human homologue of murine mdm2, other modifications of p53 that may not occur in the absence of DNA damage may be required for its full activity. An alternate explanation provided for this tumor selectivity is that tumor

cells are sensitized to apoptosis because of oncogene addiction (Issaeva et al., 2004). This is an important concept for which there is considerable support. For example, tumors almost invariably have elevated levels of Myc which is a known repressor of $p21^{waf1}$ (Seoane et al., 2002). Accordingly, p53 wild-type tumors (or those in which p53 has been reintroduced or reactivated) may still be defective in their ability to arrest compared to normal cells, and therefore more sensitive to DNA damage. Mice engineered to express reduced levels of MDM2, negative regulator of the p53, and as a consequence elevated p53, showed an increase in apoptosis of the thymus and small intestine when irradiated, although there was little toxicity in most tissues (Mendrysa et al., 2003).

There is another important variable to mention which will impact a potential therapeutic strategy . Specifically, it has been shown that p53 can also induce apoptosis independent of any transcriptional activity by directly interacting with Bcl-2 family members at the mitochondria (Mihara et al., 2003)(Chipuk et al., 2005)(Chipuk and Green, 2005)(Leu et al., 2004).

An alternative therapeutic strategy is based on the belief that activation of p53 in normal cells will indeed be toxic to the patient. p53 inhibitors were developed with the intent to protect normal tissues from the adverse effects of chemotherapeutic agents. The p53 inhibitor pifithrin-α (PFT-α) rescued p53 wild-type cells from apoptosis induced by the DNA damaging agent etoposide and reduced lethality in mice following γ–radiation (Komarov et al., 1999).

PFT-α displays a number of chemo- and radiation-protective qualities, including neuroprotection, cardioprotection and renal protection (Gudkov and Komarova, 2005). These observations have led to the proposal that p53 inhibitors could be useful protective agents when used in combination with chemotherapy and radiation.

Targeting the IAPs for cancer therapy

The IAPs are the only known endogenous proteins that regulate the activity of both initiator and effector caspases. Controlled expression of the IAPs has been shown to influence cell death in a variety of contexts and is believed to have important consequences with respect to human cancer. The mechanism by which the IAPs inhibit apoptosis was first interrogated in the laboratory of John Reed (Deveraux et al., 1998).

In these early studies, XIAP was found to prevent caspase-3 processing in response to caspase-8 activation. Therefore, XIAP was suggested to inhibit the extrinsic apoptotic signaling by blocking the activity of the

downstream effector caspases, as opposed to interfering directly with caspase-8 activation (Deveraux et al., 1998). Supporting this concept, XIAP was shown to specifically bind to caspase-3 and -7, but not to caspase-1, -6, -8 or -10. *In vitro* assays confirmed that XIAP, as well as cIAP1 and cIAP2, could prevent downstream proteolytic processing of pro-caspase-3, -6 and -7 by blocking cytochrome *c*-induced activation of pro-caspase-9 in the intrinsic pathway.

The role of the IAPs in apoptosis suppression is continuously being evaluated. The Salvesen's laboratory (Eckelman et al., 2006) suggests that only XIAP is a direct inhibitor of caspases, and that other IAPs simply bind caspases but do not inhibit them. This suggestion is based on the fact that XIAP is relatively stable and exhibits the greatest potency for caspase inhibition compared with the other IAPs.

A variety of cancer cell lines and primary tumor biopsy samples show elevated IAP expression levels (Ambrosini et al., 1997)(Fong et al., 2000).

The most dramatic example of IAP over-expression in tumors is seen with survivin. Survivin expression is limited to embryonic tissues and many different tumor types, but is absent in most adult differentiated tissues (Ambrosini et al., 1997). Furthermore, the presence of survivin in patient tumor biopsy samples correlates with poor prognosis, increased rates of treatment failure and relapse. The prognostic significance of IAP overexpression is less clear for some of the other IAPs. For example, XIAP protein levels correlate with disease severity and prognosis in acute myelogenous leukemia (AML) (Tamm et al., 2000) and renal cell carcinoma (Ramp et al., 2004), but not in non-small cell lung carcinoma (Ferreira et al., 2001) or cervical carcinoma (Liu et al., 2001).

XIAP levels have also been shown to increase in leukocytes with the transformation of myelodysplastic syndromes to overt AML (Yamamoto

et al., 2004). High expression of XIAP or cIAP2 is associated with shorter overall survival, and lower complete response rates for AML (Hess et al., 2007). Other cross-validation and forward selection studies reveal a three-gene signature, consisting of cIAP2, Bax and BMF, to optimally predict overall survival in AML. Furthermore, an immunocytochemical survey of tumor tissue shows that XIAP immunostaining patterns allow for the ready distinction of malignant from benign populations in effusion washes (Wu et al., 2005). XIAP is also identified as part of a progression signature in prostate cancer using an immunoblot approach to characterize proteomic alterations in prostate tumors. XIAP protein is seen to increase in expression between benign prostatic tissue, and clinically localized prostate cancer, or metastatic prostate cancer (Varambally et al., 2005). In addition, XIAP protein expression is a strong predictor of prostate cancer recurrence (Seligson et al., 2007). Collectively, these results suggest that the expression levels of the IAPs could be expected to have a significant impact in the development and maintenance of cancer due to their central role in the regulation of apoptosis.

Furthermore, cIAP1 cooperates with the Myc oncogene in transformation, by acting as a ubiquitin ligase for Mad1, an antagonist of Myc. A recent report also suggests that cIAP1 and cIAP2 promote cancer cell survival by ubiquitinating RIP1, leading to constitutive RIP1 and NF-B activity.

Applications of single-stranded antisense oligodeoxynucleotides (AS ODNs) as selective inhibitors of gene expression are being studied for efficacy in treating particular genetic disorders. Hybridization of the AS ODNs to the mRNA prevents the target gene from being translated into protein, thereby blocking the action of the gene, and resulting in the

degradation of the target mRNA (Galderisi et al., 1999)(Gleave et al., 2002)(Jansen and Zangemeister-Wittke, 2002).

A multitude of candidate genes, involved in apoptosis regulation, represents potential targets for antisense-based therapies.

Additional proof-of-principle studies performed with newer double-stranded RNAi-based technologies show that downregulating XIAP gene expression through siRNA increases apoptosis in cultured MCF-7 breast cancer cells and subsequently enhances the killing effects of etoposide and doxorubicin (Lima et al., 2004). Another study using short-hairpin RNAs as an RNAi approach directed against XIAP shows that XIAP mRNA can be reduced by as much as 85% in some breast carcinoma cell lines. This reduction in XIAP dramatically sensitizes these cell lines to TRAIL- and to taxane-induced killing (McManus et al., 2004).

Survivin is expressed in fetal tissues, becomes restricted during development, and is absent in most healthy, differentiated adult tissues (Adida et al., 1998), with some notable exceptions, such as stem cells, thymus, testes, regenerating hepatocytes and endothelial cells (Kobayashi et al., 1999) (Fukuda and Pelus, 2006). Significantly, survivin is re-expressed during malignant transformation and is found in nearly all tumor types, including neuroblastomas, pancreatic, prostate, gastric, colorectal, hepatocellular and breast carcinomas, as well as lung and bladder cancers, melanomas, B-cell lymphomas and esophageal cancer (Altieri, 2008). The expression of survivin in cancer is a predictor of both poor prognosis and decreased survival time, and is implicated in conferring chemo- and radio-resistance phenotypes to tumor cells. Numerous studies have addressed the diagnostic and prognostic potential of survivin expression, as well as that of its nuclear versus cytoplasmic localization, phosphorylation status and the presence of splice isoforms

(Baur and Sinclair, 2006)(Dohi et al., 2007)(Pannone et al., 2007) (Brennan et al., 2008).

MDM2 is a protein that binds to the tumour suppressor p53 and negatively regulates its activity and stability (Momand et al., 2000) .

The MDM2-mediated loss of p53 impairs a cell's ability to block cell-cycle progression and induce apoptosis in response to DNA damage, which can contribute to the cancerous phenotype.

The observation that MDM2 is amplified or overexpressed in several human tumour types (Momand et al., 2000) provides further support for the involvement of MDM2 in cancer.

Therefore, strategies aimed at blocking the interaction between MDM2 and p53 to inhibit the degradation of p53 could be important for cancer drug discovery (Lane and Lain, 2002). The X-ray crystal structure of the p53–MDM2 complex showed how p53 interacts with MDM2 and aided in the design of p53 mimetics hat bind to MDM2 (Kussie et al., 1996).

In the first attempt to identify MDM2 inhibitors, peptide-based compounds were discovered that bind tightly to the MDM2 (Bottger et al., 1997).

However, these compounds lacked the ability to penetrate cells and did not possess drug-like properties. Although several small molecules such as polycyclic compounds, chlorofusin (Duncan et al., 2001), sulfonamides (Galatin and Abraham, 2004) and benzodiazepines (Parks et al., 2005) were also identified that bind to MDM2 and displace p53 peptides, these compounds only bind weakly to MDM2.

PI3K and apoptotic cell death in carcinogenesis

There are many other compounds that induce apoptosis.

However, this might not be their primary function, or the mechanism by which they induce programmed cell death has not yet been defined. For example, in addition to its other functions, the phosphatidylinositol 3-kinase PI3K–AKT pathway regulates both the intrinsic and extrinsic apoptotic pathways (Workman, 2004). Activated AKT decreases apoptosis by phosphorylating and inactivating BAD (Datta et al., 1997) (del Peso et al., 1997) and apoptosis signal-regulating kinase ASK. AKT also regulates FAS and IAP expression through the phosphorylation of the forkhead (FOXO) transcription factor family (Brunet et al., 1999) (Kops and Burgering, 1999) and nuclear factor NF-κB (Gelfanov et al., 2001).

The PI3K–AKT pathway is deregulated in many cancers, indicating that this signalling pathway might contain possible anticancer targets. Although inhibitors of the PI3K family (such as LY294002) have been shown to inhibit the growth of both cancer cells *in vitro* and tumours in animal models, these compounds lack selectivity and have mostly served as pharmacological tools to study the functions of this pathway (Mitsiades et al., 2004). AKT inhibitors have so been reported to inhibit tumour growth when used as a monotherapy or in combination with paclitaxel. Unfortunately, compounds within these series were found have a narrow therapeutic window and show significant metabolic toxicities. Another target in the PI3K–AKT pathway is the mammalian target of rapamycin mTOR. Rapamycin analogues that inhibit mTOR slow the growth of tumours in animal models without displaying significant toxicity. These compounds are currently in clinical trials for the treatment of breast, colon and lung cancers (Rowinsky, 2004).

The proteasome could also be considered as a target for inducing apoptosis, and proteasome inhibitors are being used to treat cancer. They have been shown to promote apoptosis in part by inducing endoplasmic reticulum stress and reactive oxygen species in head and neck squamous cell carcinoma cells (Fribley et al., 2004). The proteasome degrades ubiquitylated proteins, including inhibitor of κBα (NF-κBIα), which inhibits NF-κB and modulates several pathways. However, the proteasome is not part of the core apoptotic pathway and degrades many other proteins besides NF-κBIα. Other compounds that indirectly induce apoptosis include inhibitor of κB kinase (IKK) inhibitors (Burke, 2003), arsenic trioxide (Chen et al., 1996) and many chemotherapeutic agents that are widely used for cancer therapy.

ROLE OF AUTOPHAGY IN CANCER

Cancer results from the accumulation of mutations that deregulate cell growth, checkpoints, cell death and conditions in the tumor microenvironment in a manner that fovors tumor growth and progression. Cell death by apoptosis is an important means to purge abnormal, emerging cancer cells and thus is a prominent mechanism for tumor suppression (Cory et al., 2003)(Danial and Korsmeyer, 2004).

Apoptosis is also a common cellular response to stress and many of the molecular events that promote tumorigenesis create or amplify cellular stress. For these and other reasons, defects in apoptosis commonly evolve during tumorigenesis, which further promotes tumor growth and treatment resistance. Once apoptosis is inactivated, tumor cells clearly possess a survival advantage; however, it may be the survival of the damaged cells that promotes tumor progression through the manifestation

and preservation of genome damage and chromosome instability (Bianchi et al., 2004; Degenhardt et al., 2002).

An example of the induction of apoptosis in tumors is metabolic stress. It is a common feature of solid tumors caused by inadequate vascularization that results in nutrient, growth factor and oxygen deprivation. Once tumors exceed a diameter of 1-2 mm, angiogenesis must occur to ameliorate metabolic stress associated with the central and most metabolically deprived hypoxic tumor domain. Metabolic stress also occurs in mature tumors, where interruption in the blood supply is common due to vascular collapse because abnormal vascularization.

Solid tumors with defects in apoptosis survive this metabolic stress and autophagy localizes to these hypoxic tumor regions (Degenhardt et al., 2006).

Compromising autophagy in apoptosis-defective tumor cells substantially impairs survival in metabolic stress conditions *"in vitro"* and in tumors *"in vivo"*, establishing that autophagy is a survival pathway utilized to sustain viability during periods of nutrient limitation. Indeed, autophagy functions to sustain metabolism during periods of growth factor deprivation of hematopoietic cells (Lum et al., 2004) and upon nutrient deprivationin normal mouse development (Kuma et al., 2004).

Autopahgy, cell growth and proliferation

Several lines of evidence suggest that the downregulation of autophagy could play a role in the active proliferation of malignant cells, which is one of the major hallmarks of cancer. In proliferating cells, including cancer cells, cell size control ralies on the coordinated regulation of cell growth and cell division (Jorgensen and Tyers, 2004) so that on average each cell division is accompanied by a doubling in cell mass.

In many cell types, cell cycle progression depends on growth-factor deprivation results in cell cycle arrest. It was also shown recently that mitotic vells shut down their autophagic protein pathway while their chromosomes and organelles are dividing and that the atophagic response reemerges during the late telophase/G_1 phase. These observations have been supported by mechanistic studies showing that several regulatory proteins exerting pivotal functions in autophagy are also involved in regulating cell proliferation.

The first specific link between the autophagy machinery and human cancer was the implication of Beclin1 in tumor progression. This gene is monoallelically deleted in a high percentage of human cancers and tumor cell lines (Liang et al., 1999).

Stable transfection of Beclin1 into MCF-7 human breast carcinoma cells their tumorigenicity in nude mice and slowed their proliferation rate. Furthemore, Beclin1-haploinsufficient mice displayed an increased incidence of spontaneous tumors and the cell proliferative capacity was markedly increased in some of their tissues (Qu et al., 2003).

Frequent chromosomal aberrations of several other components of the autophagic machinery in human cancers are observed.

Atg5, a component of the ubiquitin-like protein conjugation system, has tumor suppressor effects in a mouse xenograft model (Yousefi et al., 2006), and knock-out of atg4, a cysteine protease involved in processing LC3, increases chemically induced fibrosarcomas in mice. Other proteins of the Beclin1/PI3K III complex required for autophagy activity may also play a role in cell growth control and tumor suppression. UVRAG, originally identified through its ability to complement UV-radiation sensitivity in tumor cells, is monoallelically deleted at a high frequency in human colon cancers and suppresses the proliferation and tumorigenicity of human colon cancer cell (Liang et al., 2006). Bif-1 interacts with

Beclin 1 through UVRAG and activates the Becln1/PI3K III complex, and its deletion in mice results in the development of spontaneous tumors. Another component of this complex that positively regulates autophagy is Ambra1 and its deficiency in mouse embryos leads to severe neural tube defects associated with autophagy impairment and uncontrolled cell proliferation, further supporting the concept that the beclin1/PI3K III complex regulates cell growth.

In mammalian cells mTOR integrates signals from nutrients and growth factors to produce coordinated regulation of cell growth and cell-cycle progression (Fingar and Blenis, 2004). mTOR regulates traslation via its downstream targets: p70S6 kinase (which phosphorylates the S6 ribosomal protein) and 4E-BP1 (a binding protein for the translational initiation factor eIF4E) (Gingras et al., 1998).

In addition to these functions, mTOR also acts as a rheostat, adjusting the rate of autophagy in response to the levels of amino acids and ATP (Meijer and Codogno, 2004).

The class PI3KI/Akt pathway is an upstream nutrient- and growth factor-responsive regulator of mTOR that plays an evolutionarily conserved role in the regulation of autophagy (Rusten et al., 2004; Scott et al., 2004).

Akt activation phosphorylates mTOR and this in turn inhibits autophagy. PTEN, a phosphatase that counteracts the lipid kinase activity of PI3KI has been shown to promote autophagy in HT-29 colon cancer cells (Arico et al., 2001). Since many cancers display aberrantly high class PI3KI-dependent signalling, either via the constitutive activation of class PI3KI or Akt or via inactivation of PTEN, the decreased autophagic activity of tumor cells observed in these cases may result from activation of the mTOR pathway.

Several other tumor suppressors and oncogenes that were initially identified as regulators of cell growth and proliferation were recently shown to be capable of modulating autophagy. One of the most intriguing examples is p53, a tumor suppressor that plays an important role in preserving the integrity of the genome. Genotoxic stresses activate p53 which in turn initiaes tumor suppressor process such as growth arrest (Feng et al., 2005). Although p53 can affect cell growth and proliferation by activating cyclin-dependent kinase p21, recent observations indicate that once activated, p53 also inhibits mTOR activity and upregulates autophagy. These findings indicate that p53 and mTor pathways can crosstalk, regulate cell growth, proliferation and autophagy and suggest that in cancers in which p53 is mutated, mTOR activation resulting from abnormal p53 activity may contribute to the lower autophagic capacities of the malignant cells.

A recent study has shownthat c-myc, a proto-oncogene that controls cell division and cell growth, increases autophagic activity when overexpressed in rat 3Y1 fibroblast (Tsuneoka et al., 2003).

Ras is another oncogene involved in regulating cell proliferation and oncogenesis that could modulate autophagy. Ras activates pathways that produce conflicting effects on autophagy.

The possibility of interplay between autophagy and cell proliferation has been further supported by a recent report showing that p27, which belongs to the family of cyclin-dependent kinase inhibitors, is a potential regulator of autophagy. Overexpression of p27, by recombinant adenoviral vector, induced autophagic cell death in human glioma cell lines, whereas the same treatment did not affect the viability of non-malignant cultured astocytes or induce autophagy (Komata et al., 2003).

Autophagy and growth factors

It has recently been reported that growth factors are involved in the suppression of autophagy. These results are consistent with previous observations that:

-growth factors enhance cell growth and proliferation; this is closely linked to the concomitant stimulation of anabolism and the suppression of catabolism

-growth factors regulate the uptake of nutrients, such as glucose and aminoacids, which are downregulators of autophagy (Edinger and Thompson, 2002)

-growth factors regulate the activity of PI3KI/Akt/mTOR pathway that coordinates the regulation of both autophagy and growth factor signalling (Broker et al., 2005).

Inhibition of the autophagy genes atg5 and atg7 and the pharmacological blockade of autophagy both accelerate cell death, even in the presence of an abundant supply of extracellular nutrients. Cells can be rescued from death by the bioenergetic substrate, methylpyruvate, suggesting that in the absence of growth factors cells use autophagy-induced cell catabolism to maintain a sufficient level of ATP production and ensure cell survival.

It is known that autophagy is necessary to remove damaged macromolecules and organelles and inhibition in cancer cells by activating the growth factor signalling pathway would lead to an accumulation of such damage, and thereby contribute to the development of cancer.

Another possibility is that autophagy plays a more direct role in negative growth control, perhaps by degrading specific organelles or proteins essential for cell growth regulation.

It’s important to point that the genetic links between deficiencies in the autophagy machinery and tumor susceptibility highlight the likely importance of autophagy in tumor suppression. However, the molecular mechanisms by which autophagy functions in tumor suppression are poorly defined. Increasing evidence suggests that the tumor suppressor functions of autophagy may be independent of both potential and prosurvival effects (Mathew et al., 2007).

The association between increased cell death and increased tumorigenic potential in the setting of ATG gene deficiency suggests that autophagy-dependent survival, at least in certain experimental models, does not promote tumorigenesis. When tumor cells cannot die by apoptosis upon exposure to metabolic stress, autophagy may prevent death from necrosis, a process that might exacerbate local inflammation and increase tumor growth rate (Degenhardt et al., 2006). ATG gene deletion may promote genomic instability in metabolically stressed cells, leading to oncogene activation and tumor progression. Indeed, immortalized mouse epithelial cells with ATG gene deficiency display increased DNA damage, centrosome abnormalities and gene amplification, especially during ischemic stress that is associated with increased tumorigenicity.

Another possibility is that autophagy plays a more direct role in negative growth control, perhaps by degrading specific organells or proteins essential for cell growth regulation. In support of this theory, enforced Beclin1 expression slows the proliferation of tumor cell lines and causes a decrease in expression of cyclin E and phosphorylated Rb (Koneri et al., 2007; Liang et al., 1999).

Also, mice with a monoallelic deletion of beclin1 display hyperproliferation of both mammary epithelial cells and splenic lymphosytes (Qu et al., 2003).

Although it is presently unclear if cell survival/cell death effects are relevant to the tumor suppressor role of autophagy, such effects are likely important in cancer therapeutics. A large series of clinically approved and experimental anticancer therapies induce the accumulation of autophagosomes in tumor cell lines in vitro (Maiuri et al., 2007a).

For many years, it was thought that these therapies kill cells through autophagy. However, specific inhibition of autophagy with siRNAs targeted against ATG genes usually accelerates, rather than prevents, indicating that autophagy activation represents a cellular attempt to cope with stress induced by cytotoxic agents. The inhibition of autophagy might be beneficial in cancer treatment. Indeed, in mice harbouring c-Myc-induced lymphomas, the drug chloroquine, an alkalinizing lysosomotropic drug that impairs autophagic degradation, enhanced the ability of p53 or a DNA alkylating agent to induce tumor cell death and tuor regression (Amaravadi and Thompson, 2007), indicating a potential prosurvival and pro-tumorigenic role for autophagy during cancer chemotherapy.

In vivo studies are needed with more specific inhibitors of autophagy to determine if the beneficial effects of blocking a tumor cell survival outweigh the potential detrimental effects of blocking a tumor suppressor pathway.

LYSOSOMES IN CELL DEATH CONTROL

Cancer cells characteristically provide their own growth signals, ignore growth-inhibitory signals, replicate without limit, sustain angiogenesis, invade through basal membranes and capillary walls, proliferate in unnatural locations, and avoid cell death. Although resistance to cell death is attributed to the inhibition of classic apoptosis and its hallmarks, particularly caspase activation and mitochondrial outer-membrane permeabilzation (MOMP) (Green and Kroemer, 2004), it is possible that alternative, non-apoptotic cell death pathways are also relevant to carcinogenesis (Jaattela, 2004). Therefore, changes in lysosomal trafficking and content that support invasion and angio-genesis could account for disorders in the regulation of apoptotic and non-apoptotic cell death, in particular through the aberrant cellular release of cathepsins “a class of proteases” usually sequestered within the lysosomal lumen.

The importance of lysosomes and lysosomal hydrolases in the clean-up phase of apoptosis (that is, the engulfment and digestion of dying and dead cells by neighbouring cells or phagocytes), and in cellular and tissue autolysis during uncontrolled necrosis is well established (de Duve, 1983; Savill and Fadok, 2000). However, it has taken a long time to recognize the role of lysosomes in the more immediate events of programmed cell death (PCD). One of the reasons for this delay is that methylketone peptide inhibitors (for example, zVAD-fluoromethylketone (fmk)) that are commonly used to assess the role of caspases in PCD also inhibit other cysteine proteases, including several lysosomal cathepsins (Schotte et al., 1999)(Foghsgaard et al., 2001). Additionally, lysosomal involvement in PCD has been overlooked because the lysosomal ultrastructure seems intact even when lysosomal hydrolases have leaked into the cytosol (Brunk et al., 2001).

Depending on the cell type and the extent of(:::::::☺) LMP, the resulting release of lysosomal hydrolases (coloured circles in the figure — different colours represent different hydrolases) and reactive oxygen species (ROS), as well as cytosolic acidification, then leads to the release of cytochrome c. This results in classical apoptosis, caspase-independent apoptosis-like or necrosis-like programmed cell death (PCD).

Lysosomes in invasion and angiogenesis

Tumour invasion and metastasis are associated with altered lysosomal trafficking and increased expression of cathepsins, especially the cysteine cathepsins — cathepsin B and cathepsin L — as well as the aspartate cathepsin, cathepsin D (Joyce and Hanahan, 2004)(Roshy et al., 2003).

In cancer cells, particularly those at the invasive edges of tumours, the localization of lysosomes often shifts from a perinuclear to a peripheral pattern and the lysosomal contents can be secreted into the extracellular space.

Autophagy and cell death

Autophagy begins when a flat membrane cistern wraps around cytoplasmic organelles and/or a portion of the cytosol, forming a closed double-membrane bound vacuole — the autophagosome — containing cytoplasmic material destined for degradation. Autophagosomes mature in a stepwise process that involves fusion events with endosomal and/or lysosomal vesicles that generate amphysomes or autolysosomes.

The final degradation step only takes place within autolysosomes when lysosomal hydrolases digest the luminal content of the autophagic vacuols.

It is important to note that the mere presence of autophagosomes in cells is not proof of increased autophagy because a reduced turnover of

autophagic vacuoles — for instance, owing to a decreased fusion of autophagic vacuoles with lysosomes — suffices to increase the number of autophagic vacuoles.

The fusion autophagic vacuole with lysosomes can be inhibited , for instance, by addition of lysosomotropic agents (hydroxychloroquine or monensin), by inhibition of the vacuolar proton pump (V-H^+-ATPase) with bafilomycin A1 (Boya et al., 2005) or by knock-down of the lysosome-associated membrane protein gene LAMP2 (Gonzalez-Polo et al., 2005). Under such conditions, nutrient-starved cells manifest high levels of cytoplasmic vacuolization with accumulation of autophagic vacuoles, as determined by electron microscopy or by following the autophagic vacuole marker LC3 fused to green fluorescent protein (GFP) (Kabeya et al., 2000). Protein turnover is also inhibited. So, autophagic vacuole accumulation will occur when biochemical signs of autophagy are inhibited. In spite of their textbook appearance of autophagic cell death, these cells can still be rescued by re-addition of nutrients and removal of the inhibitor, provided their nucleus appears normal and their mitochondria are energized. However, following prolonged nutrient starvation and autophagolysosome inhibition, vacuolated cells manifest hallmarks of apoptosis, including MOMP and caspase activation, indicating that the point-of-no-return has been reached (Boya et al., 2005).

Therapeutic opportunities

The study of lysosomal changes in tumour progression and treatment is still very young, but the recent advances and increasing interest in the field promise rapid progress in the near future. A more defined role for each of the cathepsins by dissecting their non-redundant functions in tumour progression and cell death is awaited. Important lessons might

also be learned from wound-healing models where changes in lysosomal trafficking, similar to those seen in invasive tumours, give rise to cell migration and subsequent wound closure. The therapeutic exploitation of the differences between normal cell lysosomes and cancer cell lysosomes requires a better knowledge of the molecular changes that occur during tumori-genesis. A thorough mass-spectrometric analysis of the cancer-associated changes in lysosomal composition combined with systematic functional studies (employing, for example, RNAi technology) will probably reveal new and exciting targets for cancer therapy. A key question that needs to be answered is whether there are cancer-specific subpopulations of lysosomes and whether such populations account for lysosomal secretion and/or leakage in cancer cells.

Targeting endocytosis and/or lysosomal secretion

Lysosomes in advanced tumours are abnormal in their content, subcellular localization and function. This offers an exciting possibility to target them for the specific eradication of tumours. The emerging data imply that cysteine cathepsins have a dual role in tumour progression as they increase malignancy by promoting invasion when outside the cell, but counteract it through their pro-apoptotic features when released into the cytosol. Therefore, inhibiting the secretion of cathepsins could give rise to populations of lysosomes that are more prone to membrane rupture, and thereby inhibit invasion and sensitize cells to the lysosomal cell death pathways. Indeed, genetic disorders that interfere with lysosomal trafficking and secretion — for example, Chediak Higashi Syndrome in humans and beige mice, both of which are caused by mutations in *LYST* (lysosomal trafficking regulator) — produce cells with enlarged lysosomes that are more susceptible to LMP91, (Ono et al., 2003). Interestingly, mutations in *RAB27A* (which encodes a Rab GTPase

involved in lysosomal secretion that causes Griscelli Syndrome Type when mutated in humans, and is also the gene that is disrupted in ashen mice) and in *RABGGT* are associated with disorders and cellular changes that resemble Chediak–Higashi syndrome (Dell'Angelica et al., 2000). *RABGGT* encodes RAB geranylgeranyl transferase, also known as GGT II — an enzyme that is essential and specific for the prenylation and activity of Rab GTPases — and is also the gene that is disrupted in gunmetal mice. Therefore, pharmaceutical inhibitors of RABGGT or inhibition of the other components of the secretory pathway might provide the means to give the tumour cells the double hit proposed above. The therapeutic potential of RABGGT inhibitors has been indicated by a recent study demonstrating an increased expression of RABGGT in tumours of various origins, and the potent p53-independent pro-apoptotic effects of RABGGT inhibition in various cancer cell lines.

Targeting HSP70

As described above, immortalization and oncogene-driven transformation initially sensitize cells to lysosomal cell death pathways, and many commonly used chemotherapeutic agents trigger LMP in cancer cells. However, it remains to be shown whether lysosomes in advanced tumours are actually more fragile than those in normal cells or whether additional changes during tumorigenesis overcome the initial destabilization of lysosomes. The membrane localization of HSP70, found in 80% of human cancer biopsies, provides tumour lysosomes with increased resistance to LMP23 (Hantschel et al., 2000). As this subcellular localization of HSP70 seems to be cancer-specific, targeting HSP70 might have selective therapeutic effects. Specific inhibition of HSP70 can be facilitated by decoy interactors such as apoptosis inducing-factor (AIF)-derived decoy of HSP70 (ADD70) (Schmitt et al., 2003), or

by reducing the cellular concentrations of HSP70 with specific antisense constructs (Nylandsted et al., 2000), RNAi (Rohde et al., 2005) or U73122 (a pharmaceutical inhibitor of phospholipase C that increases the secretion of HSP70 from the cell). The therapeutic potential of such approaches has been demonstrated by the ability of an adenovirus expressing antisense *HSPA1A* (the gene that encodes HSP70-1) to induce LMP and to attenuate the growth of xenografted human tumours in immunodeficient mice (Nylandsted et al., 2004; Nylandsted et al., 2002). Similar to many novel experimental cancer therapies, the lack of effective methods for delivering macromolecules to the cancerous tissue poses the biggest limitation for the clinical success of DNA-, RNA- and polypeptide-based anti-HSP70 therapies. However, the cancer-specific localization of HSP70 to the inner leaflet of the lysosomal membrane offers a possible opportunity to interfere with its pro-survival function by introducing HSP70 inhibitors or molecules that interfere with the membrane-binding of HSP70 by the endocytic route.

Targeting lysosomal pH

The low pH of the lysosomal compartment of tumour cells could be exploited by generating acid-labile pro-drugs that would be activated in the lysosomal compartment. This effect can be achieved by the incorporation of acid-sensitive spacers between the drug and carrier that are specifically cleaved by lysosomal proteases (Ulbrich and Subr, 2004). This approach could also be used to deliver compounds with direct membrane toxicity safely into the lysosomal lumen. On the other hand, raising the lysosomal pH by pharmacological inhibition of the vacuolar proton pump increases the cytoplasmic retention of cisplatin, 5-fluorouracil and vinblastine, and ameliorates the nuclear targeting of doxorubicin, thereby sensitizing tumour cells to these treatments (Luciani

et al., 2004). The neutralization of lysosomes can also sensitize cancer cells to other treatments, possibly by inhibiting autophagy-mediated cytoprotection (Kanzawa et al., 2003) (Kanzawa et al., 2004) (Paglin et al., 2001) or by exerting direct cytotoxic effects through the induction of lysosomal dysfunction, LMP and cathepsin-mediated apoptosis-like cell death.

RESVERATROL

The polyphenolic compound Resveratrol (3,5,4'-trihydroxystilbene) is a phytoalexin found in a wide variety of dietary sources including grapes, plums and peanuts.

Numerous studies have reported interesting properties of this compound as a preventive agent against several important pathologies.

Resveratrol has been shown to have anti-inflammatory, antioxidant, antitumor, neuroprotective and immunomodulatory activities (Cos et al., 2003; Inoue et al., 2003; Jang and Pezzuto, 1999). The compound has also been examined in several model system for its potential effect against cancer (Agarwal et al., 2000; Hsieh and Wu, 2000; Mitchell and El-Deiry, 1999). Its anticancer effects include its role as a chemopreventive agent, its ability to inhibit cell proliferation, its direct effect in cytotoxicity by induction of apoptosis and on its potential therapeutics effect in preclinical studies (Bhat and Pezzuto, 2002).

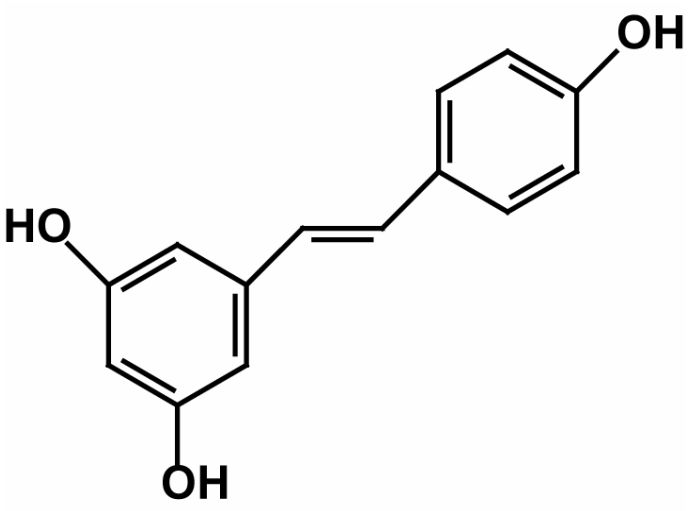


Fig 7: Resveratrol formula

Resveratrol is produced by plants in response to infection by the pathogen Botrytis cinerea (Delmas et al., 2006). It is also induced in response to a variety of stress conditions, such as vicissitudes in climate, exposure to ozone, sunlight and heavy metals (Bavaresco, 2003). It exists in both cis and trans isomeric forms. In plants, it mostly exists in glycosylated piceid forms (3-O-B-D-glucosides). Glycosylation is known to protect resveratrol from oxidative degradation, and glycosylated resveratrol is more stable and more soluble and readily absorbed in the human gastrointestinal tract (Regev-Shoshani et al., 2003). Although the biological positive effects of resveratrol are largely admitted, little is known about the transport and the distribution of the resveratrol through the body. Due to its low water solubility (Belguendouz et al., 1997), it must be bound to proteins and/or conjugated to remain at a high concentration in serum. Moreover, the efficiency of a therapeutic substance is related to its capacity (selectivity and affinity) to bind protein transporters (Khan et al., 2008). It is known that resveratrol is conjugated in intestine as glucoronide and sulfate derivates, but aglycone is also bioavailable.

Recently it has been shown that resveratrol uptake involves both passive diffusion and a carrier-mediated process. Moreover, an increasing lowering of resveratrol uptake was observed by addition of increasing concentration of BSA to a serum-free medium (Jannin et al., 2004).

MECHANISM OF ACTION

Regulation of Bcl-2 family members.

Overexpression of Bcl-2 and Bcl-X_L protein protects a wide variety of cells from many death-inducing stimul(Hickman et al., 1994; Srivastava et al., 1999).

Resveratrol inhibits the expression of antiapoptotic proteins such as Bcl-2 and Bcl-$X_{L,}$ and induces the expression of Bax, Bak, Bad, PUMA, Noxa and Bim (Aggarwal and Shishodia, 2006; Fulda and Debatin, 2004; Shankar and Srivastava, 2007) (Bhardwaj et al., 2007). These data suggest that the regulation of Bcl-2 family members plays a major role in resveratrol-induced apoptosis.

Further insights into the signaling network and interaction points modulated by resveratrol may provide the basis for novel drug discovery programs to exploit resveratrol for the prevention and treatment of human diseases.

Regulation of Cell cycle

Resveratrol has been reported to modulate cell cycle and to induce apoptosis. Several authors have studied the effect of resveratrol on cell cycle-control. In colon cancr cells, a down-regulation of the cyclin D1/Cdk4 complex has been reported (Kotha et al., 2006), while in transplantable liver cancer H22 resveratrol decreased cyclin B1 and Cdc2

protein, although no alteration of cyclin D1 was observed, G_2 arrest was itself been reported to be linked with the inhibition of Cdk7 and Cdc2. Moreover it has been shown that resveratrol is also involved in the modulation of $p21^{/Cip/WAF1}$ and $p27^{/Kip1}$ levels. However, the observed accumulation of cells in G1 could also imply the Rb or the p53 pathway.

In melanoma (Bruder et al., 2007; Ferrer et al., 2005), endothelial and fibroblastic cell line, the treatment with this compound led to an activation of p53 activity, which correlated with suppression of cell progression through the S and G2 phases of the cell cycle and apoptosis. The resveratrol effect on the G2-phase of cell cycle could be due to the action of resveratrol on the cytoskeleton (Bruder et al., 2001).

Regulation of Mitogen Activated Protein Kinase (MAPK)

MAPK pathways in mammalian include p38, c-Jun N-terminal protein kinase (JNK) and extra cellular signal-regulated kinase (ERK). These MAP kinase pathways consist of several other kinases, which activate each other via phopshorylation cascades and thus activate several transcription factors (Bruder et al., 2001; Shih et al., 2002). The interaction between the MAPK pathways and resveratrol could provide future beneficial therapies against cancer. It has been shawn that resveratrol has its effects on upregulating p53 protein thereby downregulating the expression of NF-kB and AP-1 simultaneously (Huang et al., 1999).

In the last years it has found to induce apoptosis in mouse fibroblast cells with wild-type p53, but could not work out in absence of p53-protein in mouse fibroblast cells (Huang et al., 1999).

Resveratrol-induced apoptosis though of p53 expression (via phopshorylation) mediated through ERK and p38 pathways (She et al.,

2001; Shih et al., 2002). Similarly it downregulates the expression of AP-1 which is thought to be the key in inducing melanoma in humans.

Generation of Reactive Oxygen Species (ROS)

Reactive oxygen species (ROS) include free oxygen ions, free radicals and both inorganic as well as organic peroxides. ROS are formed during natural matabolism as a byproduct and have important roles in cell signalling (Kannan and Jain, 2000).

During of environmental stress ROS levels can dramatically increase and couse significant damage to cell structures due to oxidation of nascent molecules. These aggregates into a situation known as oxidative stress. Cells are normally able to defend themselves against ROS damage through the use of enzymes such as superoxide dismutases (SOD) and catalases. ROS have been implicated as a key factor in the activation of p53 by many chemotherapeutic drugs. Apoptosis triggered by p53 has been reported to be dependent on an increase in ROS and the release of apoptotic factors from mitochondrial damages. These studies suggest that ROS are downstream mediators in p53-dependent apoptosis in transcription-dependent or transcription-independent pathways. When cells are exposed to oxidative stress, p53 is expressed at high levels by posttranslational modifications, including phosphorylation, acetylation and glycosylation (Buccellato et al., 2004). These findings suggest the novel functions of ROS as p53 activators or p53 downstream effectors.

Moreover resveratrol has been reported to increase as well as decrease NO production (Chander and Chopra, 2006; Holian et al., 2002). The involvement of NOS ha already been reported in cultured pulmonary (Holian et al., 2002), leading to inhibition of NOS activity reverted resveratrol action, indicating a direct relationship of increased NO

production and inhibition of cell growth. In addition, resveratrol has been shown to modify iNOS expression (Das et al., 2005; Madar et al., 2005).

Regulation of PI3K/AKT pathway

Recent work has indicated that loss of PTEN (phosphatase and tensin homolog) unction occurs 10-20% of organ-confined and over 50% of advanced prostate cancer. Knockout mice lacking PTEN develop multiple cancers, including prostatic hyperplasia and prostatic intraepithelial neoplasia. This is supported by recent observation showing a correlation between the status of PTEN and activation of the serine/threonine kinase Akt. PTEN functions as a negative regulator of the PI3-kinase/Akt-signalling pathway.

In tumors, inactivating mutations in PTEN led to increase activity of Akt/PKB , one of the most well characterized downstream effectors of PI3K (Dahia et al., 1999). Akt/PKB exerts antiapoptotic effects through phosphorylation of substrates such as Bad (Datta et al., 1997) or caspase-9 (Cardone et al., 1997) that directly regulate the apoptotic machinery or substrates such as the human telomerase reverse transcriptase subunit. Moreover Akt plays an important role in the stimulation of cell proliferation, cell survival and it contributes to tumor growth and progression by promoting cell invasiveness and angiogenesis (Dudek et al., 1997; Kennedy et al., 1997). These data suggest that targeting a specific kinase that promotes survival such as Akt could change the apoptosis-inducing potential of resveratrol.

In the last years it has been shown that resveratrol induced the phopshorylation of Akt/PKB activating AMP-activated protein kinase (AMPK) and preventing cardiac myocyte hypertrophy via these two kinase system (Chan et al., 2008). It has been shown ROS as an upstream regulator of AMPK in colon-carcinoma cell line HT29 (Hwang et al.,

2007). Finally it has been demonstrated for the first time that resveratrol inhibits Ang II-induced hypertrophy of vascular smooth muscle cells (VSMC) by interfering mainly with the PI3K/Akt and $p70^{S6K}$ (ribosomal protein) phosphorylation (Haider et al., 2002).

Regulation of Transcription Factors

NFkB family is a group of structurally related and evolutionary conserved proteins subunits that have been identified and cloned in mammalian cells (Le Beau et al., 1992). The NFkB subunits form homo- or hetero-dimers through their rel hology domain (RHD), which is also responsible for the DNA binding of NFkB and interaction of NFkB with IkB, the family of inhibitory proteins of NFkB (Baldwin, 1996). NFkB usually exists in a latent state in the cytoplasm and its activation requires extracellular stimuli leading to the phpsphorylation and subsequent proteasome-mediated degradation of inhibitory IkB proteins (Karin and Ben-Neriah, 2000). In mammalian cells, NFkB also regulates expression of more than 150 genes and some of them were linked to cancer initiation, proliferation, angiogenesis, sirvival and metastasis (Pahl, 1999). Mitotic cellular division is a crucil feature in the growth of normal as well as cancer cells. The cellular division can be characterized as a cycling process where cells are proceeding from the resting stage (G_0) to DNA synthesis (S) and mitosis (M) stages of cell cycle. The check-points are controlled by a group of D cyclins and cyclins E and A. These check-points of the cell cycle are usually deregulated in oncogenesis and amplification or overexpression of cyclin D1 have been identified in the development of a subset of human cancers including a lot of kinds of cancer. It has been shown that the constitutive activation of NFkB is probably the major effector responsible for the overexpression of cyclin D1 in tumorigenesis .

For this reason many studies suggest that resveratrol may have a potential in the treatment and prevention of cancer, being involved in the regulation of cell cycle and in the inhibition of the nuclear transcription factor NFkB (Cheeke et al., 2006).

Regulation of TRAIL-Death Receptor Pathway

TRAIL, belonging to the TNF cytokine family, is a type II membrane protein that induces apoptosis in a wide variety of transformed cells (Bodmer et al., 2000; Walczak et al., 1999).

Histological analysis of TRAIL-treated tumors revealed an increase in apoptotic cells and confirmed the ability of TRAIL to induce apoptosis in animal models without toxicity toward normal tissues. TRAIL is considered to be a tumor-selective, apoptosis-inducing cytokine and a promising new candidate for cancer prevention and treatment.

TRAIL binds several receptors with DR4 and DR5, the intracellular death domain, essential for the induction of apoptosis following receptor ligation (Golstein, 1997). The binding of TRAIL to DR4 and DR5 leads to the activation of caspase-8 or caspase-10 (Marsters et al., 1998), that in turn activates downstream effector caspases such as caspase-3 and caspase-7 (Muzio et al., 1998). After the activation of the caspase cascades, BID is cleaved and it triggers mitochondrial depolarization and subsequent release of mitochondrial proteins (cytochrome c, AIF, Smac/DIABLO) to the cytosol, and activation of of the effectors caspases after formation of apoptosome (Green and Amarante-Mendes, 1998).

Recently some TRAIL resistant cancecr cell lines have been discovered but the molecular mechanisms responsible for the TRAIL resistance is still not very clear. It has been recently demonstrated that downregulation of Akt/PKB or NFkB sensitizes breast, prostate and lung cancer cells to TRAIL in vitro (Chen et al., 2003). These studies suggest that combined

chemotherapy or radiotherapy with TRAIL could be an effective treatment of epithelial cell-derived cancers. Study of the intracellular mechanisms that control TRAIL sensitivity may enhance the knowledge of death receptor-mediated signalling and help to develop resveratrol and/or TRAIL-based approaches to cancer prevention/treatment.

CLINICAL SIGNIFICANCE OF RESVERATROL

Several studies indicate the importance of resveratrol in many diseases:
it is involved in angiogenesis, in cardiovascular disease, in diabetes;
it has antiviral effects and shows the ability to stop the metastatic process in cancer .

Angiogenesis is the process of formation of new blood capillaries required to support the growth of solid tumours. In various studies it has been observed that resveratrol inhibits tumour-induced formation of new blood capillaries. Its inhibition of vascularization in the corneal micropocket assay in mice has been clearly demonstrated (Brakenhielm et al., 2001)(Brakenhielm E et al. 2001).

Moreover, several studies suggest that resveratrol is an effective antioxidant (Chanvitayapongs et al., 1997). It inhibits lipid peroxidation of low-density lipoprotein (LDL), prevents the cytotoxicity of oxidized LDL and protects cells against lipid peroxidation. It is thought that because it contains highly hydrophilic and lipophilic properties, it can provide more effective protection than other well known antioxidants such as Vitamin C and E (Chanvitayapongs et al., 1997).

The cardioprotective effects of resveratrol may also be due, in part, to its vasorelaxation properties. This activity has ben seen due to its ability to

stimulate Ca^{2+}-activated K^{+} channels and to enhance NO signalling in the endothelium.

This compound was found to increase the potency of some antiretroviral drugs synergistically against HIV and herpes simplex viruses or can downregulate the expression of NFkB and suppress the activation of this transcription- and apoptosis-related protein (Aggarwal and Shishodia, 2006; Docherty et al., 2006).

In another study, the replication of severe acute respiratory syndrome (SARS) was totally inhibited by resveratrol derivatives in vitro (Li et al., 2006).

The inflammation process is mediated by prostaglandins (PGs), and their inhibition may be partially responsible for the chemopreventive and cardioprotective effects of resveratrol. Resveratrol decreases the expression of cyclooxygenase-2 (COX-2), an enzyme that catalyzes PG synthesis by inhibiting its expression via signal transduction pathways (Subbaramaiah et al., 1998). NFkB is also involved in inflammation and tumourigenesis. Inhibition of NFkB activity is a possible mechanism by which resveratrol exerts its anti-inflammatory activity. Inhibition of TNF-induced NFkB activation by resveratrol has been observed in several cell lines (Holmes-McNary and Baldwin, 2000).

In other studies, this compound has was shown to lower plasma glucose level in normal and diabetic rats including the animal model of streptozotocin (STZ)-induced and nicotinamide-STZ-induced (NA-STZ) and insuilin-resistant diabetic rats. It was observed that resveratrol produced a hypoglycaemic effect in a dose-dependent manner in normal as well as in diabetic rats, and it was also found that in both cases the insulin level was increased following resveratrol treatment. The results indicated that the mechanisms contributing to the hypoglycemic effect of resveratrol include insulin-dependent and insulin-independent pathway,

along with PI3K-Akt-signalling pathways to enhance glucose uptake in skeletal muscle . Resveratrol has been suggested in treatment of diabetic neuropathy and its protective effect may be mediate through reduction in oxidative stress and DNA fragmentation (Sharma et al., 2006).

Finally resveratrol has been shown to have analgesic properties to protect against hearing loss and enhance lipopolysaccharide-induced anorexia in rats although it has no anorexic effect when given alone (Granados-Soto et al., 2002).

AIM OF THE STUDY

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene, RV), a polyphenol enriched in red wine, grapes, peanuts and other food products, has recently attracted the interest of researchers owing to its protective effects in carcinogenesis. These properties support the potential utilisation of RV as a chemotherapy and chemopreventive drug for cancer treatment.

To fully exploit such a use, it is mandatory to understand in detail its mechanism of cytotoxicity and the pathway(s) activated, which might be useful to design strategies to avoid negative side effects and/or implement its antituomor activity.

The aim of the present work was to examine the (possible) involvement of autophagy response and activation of the endosomal-lysosomal death pathway in the cytotoxic mechanism of RV in human colorectal cancer cells. Based on our studies, we propose a model of RV cytotoxicity in which autophagy initially represents an adaptive response with pro-survival function, but on chronic intoxication autophagy is hyper-stimulated and the (autophago)lysosomal membrane becomes permeable, thus allowing the cytosolic relocation of pro-apoptotic cathepsins (namely Cathepsin D) which triggers the bax-mediated intrinsic death pathway.

SUMMARY

Here we present data on the mechanism of cytotoxicity exerted by RV in cultured human colorectal cancer cells.

In the first work (published in Carcinogenesis 2007 by Trincheri et al.) we identify the lysosome as a novel target of resveratrol activity and demonstrate a hierarchy of the proteolytic pathways involved in its cytotoxic mechanism in which the lysosomal cathepsin D acts upstream the cytosolic caspase activation. Our data indicate imply that metabolic or pharmacologic or genetic conditions affecting cathepsin D expression and/or activity could reflect on the sensitivity of cancer cells to RV.

In the second work (published in Carcinogenesis 2008 by Trincheri et al.) we focused our attention on autophagy, a lysosomal-mediated degradative pathway, that plays a major role in cell and tissue homeostasis. How and to what extent autophagy contributes to cancer development and progression/regression is still a matter of investigation. We show that the pro-apoptotic activity of RV in DLD1 colorectal cancer cells is mediated by proteins involved in the regulation and execution of autophagy. The present results indicate that genetic or epigenetic inactivation of proteins involved in the regulation and/or execution of autophagy in cancer cells confer resistance to RV-mediated killing. These data emphasize the role of autophagy in the response to chemotherapy drugs and the strict relationship between autophagy and apoptosis in the execution of the death program.

FIRST WORK

In human colorectal cancer cells the polyphenol resveratrol activated the caspase-dependent intrinsic pathway of apoptosis. This effect was not mediated via estrogen receptors. Pepstatin A, an inhibitor of lysosomal cathepsin D, not E-64d, an inhibitor of cathepsins B and L, prevented resveratrol cytotoxicity. Similar protection was attained by small interference RNA-mediated knock-down of cathepsin D protein expression. Resveratrol promoted the accumulation of mature cathepsin D, induced lysosome leakage and increased cytosolic immunoreactivity of cathepsin D. Inhibition of cathepsin D or its post-transcriptional down-regulation precluded Bax oligomerization, permeabilization of mitochondrial membrane, cytosolic translocation of cytochrome c, caspase 3 activation and TUNEL positivity occurring in resveratrol-treated cells. The present study identifies the lysosome as a novel target of resveratrol activity and demonstrates a hierarchy of the proteolytic pathways involved in its cytotoxic mechanism in which the lysosomal cathepsin D acts upstream the cytosolic caspase activation. Our data indicate that metabolic or pharmacologic or genetic conditions affecting cathepsin D expression and/or activity could reflect on the sensitivity of cancer cells to resveratrol.

RESVERATROL INDUCES CELL DEATH IN COLORECTAL CANCER CELLS BY A NOVEL PATHWAY INVOLVING LYSOSOMAL CATHEPSIN D

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INTRODUCTION

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene, RV), a polyphenol enriched in red wine, grapes, peanuts and other food products, has recently attracted the interest of researchers owing to its protective effects in carcinogenesis (Bhat and Pezzuto, 2002). RV was shown to suppress cancer initiation and promotion (Jang et al., 1997), to reduce ascites tumor growth (Carbo et al., 1999) to prevent chemical carcinogen-induced epithelial cell transformation (Li et al., 2002) (Banerjee et al., 2002), and to inhibit neo-angiogenesis (Tseng et al., 2004) (Cao et al., 2004). In *in vitro* models, RV inhibited the growth of tumor cell lines derived from various human cancers (Hsieh et al., 1999) (Joe et al., 2002) (Pozo-Guisado et al., 2002) (Opipari et al., 2004). This effect has been associated with the ability of RV to arrest cell cycle progression (Hsieh and Wu, 1999) (Wolter et al., 2001), to promote cell differentiation (Wolter and Stein, 2002) and to induce programmed cell death by caspase-independent or caspase-dependent apoptosis (Park et al., 2001) (Huang et al., 1999) (Tinhofer et al., 2001) or by autophagocytosis (Opipari et al., 2004). These properties make RV an attractive

chemotherapy and chemopreventive drug for cancer treatment (Aggarwal et al., 2004). In this respect, it is interesting to note that RV exhibits estrogenic and anti-estrogenic activities (Bowers et al., 2000) (Bhat and Pezzuto, 2001), a property that might influence the dynamic of estrogen receptor (ER)-positive cancers, such as breast (Lu and Serrero, 1999) (Levenson et al., 2003) and colorectal (Schneider et al., 2000) (Qiu et al., 2002) (Chen and Donovan, 2004) cancers. To fully exploit its potential as anticarcinogenic drug it is mandatory to elucidate the cytotoxic pathways activated by and the molecules and organelles targeted by RV in cancer cells.

Two principal pathways of apoptosis have been described: in the 'intrinsic' pathway the cytotoxic stress affects primarily the mitochondrion inducing the release of molecules that promote the activation of caspases, while in the 'extrinsic' pathway the activation of caspases follows the stimulation of so-called 'death receptors' on the plasma membrane (Danial and Korsmeyer, 2004). It is now clear that programmed cell death pathways independent of caspases also exist (Broker et al., 2005). In various circumstances the initial trigger of the death machinery was shown to be a protease normally resident within the endosomal-lysosomal compartment. Lysosomal cathepsins B and D (CB, CD) have been shown to mediate apoptotic cell death induced by TNFα (Deiss et al., 1996) (Guicciardi et al., 2000) (Foghsgaard et al., 2001) (Demoz et al., 2002) and cytotoxic drugs (Broker et al., 2004) (Bidere et al., 2003) (Emert-Sedlak et al., 2005). The type of lysosomal protease recruited in the death pathway seems to vary depending on the cell model and the trigger, and likely on the availability of specific substrates and other unknown metabolic factors. The aim of the present work was to examine the possible involvement of lysosomal cathepsins in the cytotoxic mechanism of RV in human colorectal cancer cells. Our data

demonstrate that RV activates a lysosome-dependent cytotoxic pathway ending in caspase-dependent cell death. We provide evidences that CD, not CB or cathepsin L (CL), mediates RV cytotoxicity. The present data indicate the lysosome as a novel and primary target organelle of the cytotoxic activity of RV.

MATERIALS AND METHODS

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

Cell cultures, treatments and evaluation of cytotoxicity

Human colorectal cancer cell lines (DLD1 and HT29) were cultivated in standard culture conditions (37°C; 95% air: 5% CO_2) in Dulbecco's modified Minimal Essential Medium supplemented with 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA, USA), 2 mM L-glutamine and 1% penicillin-streptomycin solution. Cells were seeded and let adhere on sterile plastic dishes for 24 h prior to start any treatment. Treatments included 1 to 100 μM RV, 100 μM Pepstatin A (Pst), 10 μM E-64d (Bachem AG, Bubendorf, Switzerland) and 30 μM ZVAD-fmk (Alexis Laboratories, San Diego USA). Inhibitors were added to the culture medium 12 h (Pst) or 1 h (E-64d, ZVAD-fmk) before the incubation with RV. In some experiments, the estrogen receptor antagonist fulvestrant (ICI 182,780 from Tocris, Bristol, UK) also was used at 1 μM concentration. Culture medium was changed and substances were re-added daily. At designated time-points, adherent and suspended cells were collected, diluted in a solution containing trypan blue and counted. Cell death was also assessed by cytofluorometer analysis. To this end, 10^5 cells were washed in PBS and incubated for 15 min at room

temperature with 2 μl annexin V-FITC (fluoroscein isothiocyanate, Alexis Laboratories, S. Diego, USA), 5 μl propidium iodide (PI, stock solution 50 μg/ml) and 98 μl of buffer (10mM Hepes/NaOH pH 7.4, 140mM NaCl, 2.5mM $CaCl_2$). Alternatively, 10^6 cells were 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 (0.1 mg/ml) for 15 min in the dark, at room temperature. Cells with hypodiploid content of DNA (subG1 peak) were assumed as apoptotic. Cells (at least 10,000 per sample) were analyzed in a FacScan flow cytometer (Becton Dickinson, Mountain View, Ca, USA) equipped with a 488 nm argon laser. Data were interpreted with the winMDI software.

siRNA transfection

Post-transcriptional 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 GAACAUCUUCUCCUUCUAC, corresponding to the positions 724-742 relative to the start codon of the CD mRNA (28). 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.). Afterward, the transfection mixture was removed and cells were incubated for further 24 h in fresh medium prior to any treatment.

Protein expression analysis

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). The filter was probed in two rounds with specific monoclonal antibodies against CD (EMD Biosciences, Calbiochem, San Diego, CA, USA) or Actin following standard Western blotting procedure (Faust et al., 1985). Immunocomplexes were revealed by incubation with peroxidase-conjugated goat-anti-mouse antibody and subsequent peroxidase-induced chemiluminescence reaction (Biorad). Intensity of the bands was estimated by densitometry analysis (Quantity one software).

Assessment of caspases activity

Caspase-3 activity was assayed with the fluorogenic substrate DEVD-AFC following manufacturer's protocol (MBL, Naka-Ku, Nagoya, Japan). Total caspases activity was measured with the Caspases Detection kit (Merck Biosciences Ltd, Nottingham, UK) using FITC-VAD-fmk (Val-Ala-Asp-fluoromethylketone) as a substrate following the manufacturer's protocol. Stained cells (at least 10,000 per sample) were analyzed by flow cytometry and data were interpreted with the winMDI software.

Immunofluorescence studies

Cells grown and treated on coverslips were fixed with methanol for 20 min and permeabilized with 0.2% Triton X-100 in PBS for 15 min. The following primary antibodies were used: a monoclonal (EMD Biosciences) or a rabbit polyclonal anti-human CD (Demoz et al., 2006) (36); a rabbit polyclonal anti-Bax (Cell Signaling Technology, Danver,

MA, USA); a mix of mouse monoclonal antibodies against alfa- and beta-tubulin; and a monoclonal anti-cytochrome c (Alexis). FITC- or TRITC (tetramethylrhodamine isothiocyanate)-conjugated secondary antibodies against mouse or rabbit IgG were used as appropriate. As negative control, the primary antibody was omitted or substituted with pre-immune antiserum.

TUNEL and DAPI staining

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. Apoptosis-associated chromatin alterations were detected by staining the cells with the DNA-labeling fluorescent dye 4-6-diamidino 2-phenylindol-dihydrochloride (DAPI, 1:100 in PBS/0,1% Triton X-100/4% fetal bovine serum).

Lysosomes and mitochondria integrity assessment

Lysosomal membrane integrity was assessed by the Acridine Orange (AO) retention test. AO is an acidotropic fluorochrome that emits red fluorescence when accumulates in its protonated form within lysosomes, while it emits a green fluorescence when localizes in the cytosol and in the nucleus. Cells adherent on coverslips were loaded with AO (1:200 from 0,5 mg/ml in distilled water for 10 min at 37°C) and immediately observed and photographed under the confocal fluorescence microscope. Lysosome leakage was quantified by cytofluorometer analysis of cells labeled with AO in suspension. The integrity of mitochondrial membrane was tested by using either the fluorescent dye Mitotracker (Invitrogen Corp.) or Rhodamine-123. Briefly, cells on cover-slips were incubated

with 0.2 μl of mitotracker solution for 15 min at 37°C, then fixed in 3.7% paraformaldehyde for 15 min and permeabilized with 0.2% Triton X-100 for 15 min for further fluorescence staining. Alternatively, cells on coverslips were incubated with Rhodamine 123 (50 nM in culture medium; 10 min) and immediately observed under the fluorescence microscope. Loss of mitochondrial membrane integrity was also assessed by cytofluorometer analysis of rhodamine-labeled cells.

Fluorescence microscope imaging

Coverslips 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. Three coverslips were prepared for each experimental condition. Representative images, selected by two independent investigators, are shown.

Statistical analysis

All experiments were independently replicated at least three times. Data are presented as means ± SD. The Instat-3 Statistical software (Graphpad Software Inc, San Diego, CA, USA) was used. Significance was calculated by the test of Mann-Whitney.

RESULTS

Resveratrol cytotoxicity in colorectal cancer cells is concentration- and time-dependent, is not mediated via estrogen receptors and is prevented by caspase inhibition

The present study was conducted in DLD1 and HT29 cells, two cell lines derived from human coloncarcinomas that express ERβ, not ERα, isoforms (Qiu et al., 2002) (Fiorelli et al., 1999) (Campbell-Thompson et

al., 2001). RV has been shown to bind to both α and β isoforms of ER (Bowers et al., 2000) (Bhat and Pezzuto, 2001) and to exert estrogenic activity when used at very low doses (i.e., 1 to 10 µM) (Levenson et al., 2003). We tested the concentration- and time-dependency of inhibitory or stimulating effects of RV on the growth of human colorectal cancer cells. To assess whether such effects were mediated through ER, cells were treated with concentrations of RV ranging from 1 to 100 µM in the absence or the presence of 1 µM fulvestrant (ICI 182,780), which has been shown to inhibits the nuclear translocation of ERs and to promote their cytoplasmic degradation at a concentration of 100 nM (Dauvois et al., 1992). A treatment for 24 to 48 h with 1 µM or 10 µM RV did not affect cell vitality or final cell density in DLD1 and HT29 cultures (Fig. 1A and not shown). At 24 h treatment with 100 µM RV some 35% reduction of cell culture growth was observed (not shown and Fig. 3C), while cell death, as assessed by cell counting and cytofluorometer evaluation of annexin V positivity, was apparent only at 48 h (Fig. 1A and not shown). Based on the results obtained with low doses of RV and on the lack of effects of ICI 182,780, we conclude that RV cytotoxic effects were not mediated via ER (Fig. 1A). In the following experiments RV was used at 100 µM. With this concentration, on average, annexin V-positive apoptotic cells in DLD1 cultures amounted to about 50% and 75 % after 48 h and 96 h of treatment, respectively (Fig. 1B). In parallel cultures in which the potent and broad-range caspase inhibitor ZVAD-fmk was added daily along with RV cell death was practically absent (Fig. 1B). HT29 cells showed more sensitive to RV than DLD1, the apoptotic cells amounting to 50-70% and 90% at 48 h and 72 h of treatment, respectively. Also in the case of HT29 cells ZVAD-fmk effectively prevented RV-induced cell death starting at 48 h (Fig. 1B). It is to note that ZVAD-fmk, while inhibiting occurrence of cell death at 48

h, could not prevent cell growth inhibition induced by RV in the first 24 h of treatment both in DLD1 and in HT29 cultures (not shown).

Resveratrol cytotoxicity is associated with mitochondrial permeabilization and caspases activation

To better assess the significance of ZVAD-fmk protective effects, we monitored the time-dependent activation of caspases during RV treatment in DLD1 cells. The cleavage of a fluorogenic peptide substrate of different caspases remained at basal levels in the first 24 h of RV treatment and increased by a factor of approximately 2.2 by 48 h (Fig. 2A). By this time the percentage of cells in which caspase activation occurred roughly corresponded to that of cells positive for annexin V, a biomarker of apoptotic-like programmed cell death (Fig. 2B). In apoptotic cells (identified on the basis of chromatin alteration) mitochondrial integrity was lost, indicating that RV activated the intrinsic pathway of cell death (Fig. 2C). The time-dependency of mitochondrial permeabilization was assessed by cytofluorometry in DLD1 and HT29 cells treated with RV for up to 48 h and labeled with rhodamine. No changes in the cytofluorometry profiles were observed during the first 24 h of treatment. Mitochondrial permeabilization, as shown by loss of rhodamine retention, was observed starting at 36 h of treatment (Fig. 2D). The microscope images of these cells shown in Fig. 2E well reflect the phenomenon as quantified by cytofluorometry. It is to note that mitochondrial permeabilization occurred more rapidly and more extensively in HT29 than in DLD1 cells, accordingly with the highest sensitivity of these cells toward RV.

Cathepsin D is an essential mediator of RV cytotoxicity in colorectal cancer cells

CB, CD and CL, the most abundant lysosomal proteases, have recently been involved in the activation of the intrinsic pathway of programmed cell death (Bidere et al., 2003) (Emert-Sedlak et al., 2005) (Stoka et al., 2005) (Guicciardi et al., 2004), and therefore were considered possible mediators of RV cytotoxicity. To determine their involvement we employed the inhibitors E64d, which is specific for the cysteine-type proteases CB and CL, and Pst, which is specific for the aspartic-type protease CD. In preliminary experiments in which cathepsins activity was assayed with specific fluorogenic substrates 10 μM E64d and 100 μM Pst were shown to effectively inhibit (by approximately 85%) the activity of CB and CL and of CD, respectively, in the cells. DLD1 cells exposed to RV for up to 48 h in the absence or the presence of Pst or E64d were tested for annexin V positivity and PI labeling. Cytofluorometer analysis of these samples indicated that Pst, not E64d, protected DLD1 cells from RV cytotoxicity (Fig. 3A). We then checked whether the CD-dependent pathway of RV cytotoxicity was also operative in the other human colorectal cancer cell line. HT29 cells were treated for up to 48 h with RV in the absence or the presence of Pst or E64d and cytotoxicity was assessed by cytofluorometer estimation of the hypodiploid (not shown) and annexin V-positive cell population (Fig. 3B). In the first 24 h of RV treatment cell death was negligible (not shown), but by 48 h the treatment was toxic for >60 % of the culture. At this time, inhibition of CB and CL by E64d revealed itself toxic and did not protect from RV; by contrast, Pst protected a large fraction of the cell population from RV cytotoxicity. In parallel samples cell survival was assessed by counting viable (trypan blue excluding) adherent cells. Data shown in Fig. 3C indicate that: i) RV reduced the rate of cell proliferation in the first 24 h. This effect

could not be prevented by Pst or E64d; ii) at 48 h RV induced a dramatic cell loss in the monolayer. This effect was largely prevented by Pst, not by E64d; iii) Pst and E64d were not toxic on itself in DLD1 cells, while E64d only revealed toxic to HT29 cells after 48 h incubation. It is to note that Pst, while unable to prevent the growth inhibitory effect of RV in the first 24 h, showed very effective in preventing RV-induced cell death in the following 24 h both in DLD1 and HT29 cells. In this respect, Pst behaved much alike ZVAD-fmk. Not only Pst prevented RV-induced cell death, but it also allowed to rescue cell proliferation. Pst inhibits not only CD, but also cathepsin E, another aspartic protease resident in endosomes and lysosomes. To definitely prove the active role of CD in the death pathway activated by RV we specifically down-regulated the expression of this protease by transient transfection with a siRNA 21-mer duplex. As controls, parallel cultures were not transfected or transfected with an inefficient duplex oligonucleotide not targeting CD mRNA (sham transfected). Proper conditions were assessed to achieve optimal down-regulation of CD expression, which was monitored by assaying the proteolytic activity at acid pH on fluorogenic substrate (not shown) and by immunoblotting determination of CD protein level (Fig. 3D, upper panel). siRNA transfection successfully down-regulated CD expression in not treated as well as in RV-treated cells. By densitometry, in siRNA-transfected cells CD protein level, normalized against actin protein level, was down-regulated by >95% (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 untransfected cultures (Fig. 3D, lower panel). After exposure to RV, viable cells recovered from untransfected or sham-transfected cultures amounted to approximately 50% and those recovered from CD-siRNA-transfected

culture amounted to almost 90% of those initially present in the control culture, indicating that no cell loss occurred when CD expression was down-regulated (Fig. 3C, lower panel). Cytofluorometry analysis of annexin V-labeled cells confirmed the protection by CD-siRNA against RV cytotoxicity (not shown). The fact that Pst and siRNA elicited the same protective effect rules out the involvement of any aspartic protease other than CD and permits to exclude any role of CD polypeptides other than the enzymatically-active one in RV cytotoxicity.

Cathepsin D mediates Resveratrol-induced caspase 3 activation, Bax oligomerization on mitochondria and cytosolic release of cytochrome c

Data so far obtained demonstrate that both CD-mediated and caspase-mediated pathways are involved in the cytotoxic mechanism of RV. Whether the two proteolytic death pathways are activated independently or are recruited in series remains to be determined. Caspase 3 is a key effector protease of the apoptotic machinery. To address the link between the cathepsin-dependent and caspase-dependent proteolytic pathways we determined the level of caspase 3 activity in cells that had been incubated or not with RV in the presence or the absence of Pst for 48 h. As shown in Fig. 4A inhibition of CD largely precluded the activation of caspase 3 in RV-treated cells. To definitely prove the involvement of CD in caspase 3 activation we further checked for the presence of TUNEL-positive cells in cultures exposed to RV along with Pst. The TUNEL technique evidences the presence of nicked DNA in apoptotic cells, which results from the caspase 3-mediated cleavage of poly(ADP-ribose) polymerase, an enzyme involved in DNA repair. As shown in Fig. 4B, Pst prevented the occurrence of TUNEL-positive cells in cultures treated with RV for 48 h. These data confirm that in RV-treated cells activation of the CD–

mediated proteolytic pathway precedes that of the caspase cascade. One of the main pathways leading to chemotherapy-induced activation of the caspase cascade relies on the permeabilization of mitochondria outer membrane and cytosolic translocation of cytochrome c. Bax, a protein belonging to the Bcl-2 family, has been involved in mitochondrial permeabilization and cytosolic relocation of cytochrome c and other pro-apoptotic proteins (Broker et al., 2004). In colon cancer cells RV was shown to up-regulate Bax expression, to promote the exposure of its occluded N-terminus and to trigger its translocation into mitochondria (Dauvois et al., 1992) (Stoka et al., 2005). CD has been shown capable to induce conformational changes and oligomerization on mitochondrial membrane of Bax in apoptotic lymphocytes (Bidere et al., 2003). Therefore, it seemed of obvious interest to look at Bax localization in RV-treated colon cancer cells. Both in DLD1 and HT29 cells, a 48 h exposure to RV resulted in increased immunoreactivity and changes in cellular distribution of Bax (Fig. 4C and not shown). To clearly assess the involvement of CD in Bax-mediated permeabilization of mitochondria induced by RV we performed a fluorescent double-staining for mitotracker and Bax in cells pre-treated with Pst and in cells in which CD expression had been silenced by siRNA technology. In most cells treated with RV only (Fig. 4C, upper panels) mitotracker staining is weak and diffuse, while Bax immunoreactivity is increased and appears as spots that in some cases co-localizes with the mitotracker stain. This pattern is compatible with oligomerization of Bax on mitochondrial membrane. By contrast, in cells pre-treated with Pst (middle panels) and in CD-siRNA transfected cells (lower panels) mitochondria appear well preserved (as shown by mitotracker staining) and Bax immunostaining is diffuse in the cytoplasm, whether or not treated with RV for 48 h. We finally investigated whether RV-induced mitochondrial permeabilization was

followed by cytosolic translocation of cytochrome c. The fluorescence staining of cytochrome c appears confined in mitochondria in control cells, while it is diffused throughout the entire cytoplasm after 48 h treatment with RV (Fig. 4D). The cytosolic relocation of cytochrome c is not observed in cultures treated with RV in the presence of Pst (Fig. 4D).

RV induces the cellular accumulation and the cytosolic release of mature CD in colorectal cancer cells

How to explain the mitochondrial and cytosolic events depending on the activity of a lysosome resident protease ? We reasoned that RV, like other chemotherapy drugs, could alter the permeability of lysosomal membrane without inducing the indiscriminate release of necrogenic hydrolases. We thus tested the lysosome integrity by looking at lysosomal retention of acridine orange (AO), an acidophilic fluorochrome that upon protonation is retained within lysosomes and becomes intensively red fluorescent. DLD1 and HT29 cells on coverslip were exposed to RV and observed under the fluorescence microscope at intervals of 6-8 h after AO staining. In control cells (time 0) AO fluorescence appeared as intense red spots, indicating that it was confined within acid organelles (Fig. 5A). No changes were observed in cells treated with RV for a period of time up to 36 h, beside an increase in size and number of red fluorescent organelles. At this time-point of RV treatment, however, some cells showed a diffuse cytosolic staining of AO fluorescence, which appeared yellow-colored and less intense (Fig. 5A). The proportion of cells showing such staining features was increased at 48 h of RV treatment (Fig. 5A). We quantified this phenomenon by cytofluorometry. Data shown in Fig. 5B confirm the occurrence of lysosome leakage in cells treated with RV for 36 h or longer. We then ascertained that CD indeed re-located into the cytosol under treatment with RV. Immunofluorescence studies showed that this was in fact the case in injured DLD1 and HT29 cells exposed for 48 h to

RV (Fig. 5C). Finally, considering the involvement of CD in RV cytotoxicity, we wondered if RV could affect its expression and maturation. DLD1 and HT29 cells were thus exposed for up to 72 h to RV and the expression of CD polypeptides in cell homogenate was analyzed at designated time-points by immunoblotting. As shown in Fig. 5D, RV induced a time-dependent accumulation of the mature double-chain (31 + 13 kDa) form of CD in both the colorectal cancer cell lines. Cell-associated mature CD greatly accumulated between 24 and 48 h of RV treatment: by this time CD increased of about 2.5-fold in DLD1 and of about 3.5-fold in HT29 cells. In separate experiments we also checked the expression of CD in DLD1 cells exposed for up to 48 h to 1 µM and 10 µM RV, doses that have been shown to induce CD synthesis in ER-positive breast cancer cells (Vyas et al., 2006). However, no changes in CD expression level were observed under these conditions (not shown). It is interesting to note that in our study cell death by (100 µM) RV became evident in cultures after 48 h of treatment and that HT29 showed more sensitive to RV cytotoxicity than DLD1 cells. The data suggest a time-dependent correlation between the induction of CD accumulation and the toxic effect by RV.

DISCUSSION

The naturally occurring polyphenol RV possesses potential chemopreventive and chemotherapy activities in colorectal cancer (Bodmer et al., 2000) (Tessitore et al., 2000) (Mahyar-Roemer et al., 2002). Its mechanism of cytotoxicity is not fully understood, as yet. RV has been categorized as a phytoestrogen, because of its ability to act as an agonist or antagonist of ERs (Bowers et al., 2000) (Bhat and Pezzuto, 2001). Thus, its effects could be mediated via ER, as it has been shown to occur for genistein, another alimentary phytoestrogen (Chen and

Donovan, 2004). Here we show that in human colorectal cancer DLD1 and HT29 cells, which are known to express ERβ isoforms, the death pathway activated by RV is not mediated through ERs, rather it involves primarily the lysosome. The findings of the present study lead to the proposed mechanism of RV toxicity reported in Fig. 6. Based on the effects of E64d, any involvement of lysosomal CB and CL in RV cytotoxic activity was excluded. By contrast inhibition of CD by Pst or post-transcriptional silencing of CD by specific siRNA completely suppressed RV-induced cell death. In the first 24 h of treatment RV slowed down the growth of cell cultures, and this effect could not be reverted by inhibitors of caspases or cathepsins. Consistently, during this period caspases activity and the cellular content of CD did not change. RV-induced cell death became apparent at 48 h of treatment, and it was paralleled by induction of caspases activity and cellular accumulation of CD. These observations indicated that, while not playing an active role in the growth inhibitory effect, both caspases and CD were involved in the death effect of RV. Since Pst prevented caspase activation in RV-treated cells, we argued that CD was the lethal trigger and therefore we investigated on its downstream targets. Cytotoxicity by chemotherapy drugs is associated with the intrinsic apoptotic pathway (Wei et al., 2001) (Castino et al., 2003) (Hu and Kavanagh, 2003). This death pathway is initiated by loss of mitochondrial transmembrane potential followed by cytosolic release of pro-apoptotic molecules such as cytochrome c, SMAC/DIABLO (second mitochondria-derived activator of caspase/direct IAP-binding protein with low PI) and apoptosis-inducing factor, which eventually results in activation of the caspase proteolytic cascade. CD was shown to play an active role in such mitochondrial events during cell death induced by staurosporine and chemotherapy drugs (Bidere et al., 2003) (Emert-Sedlak et al., 2005). The question still

unanswered concerns the molecular targets of CD proteolysis that link this lysosomal protease with the mitochondrial release of pro-apoptotic mediators and subsequent activation of cytosolic caspase. Two candidates have been proposed for such a role: Bax (Bidere et al., 2003) and Bid (Heinrich et al., 2004). Several experimental evidences favor Bax as the likely target of CD in RV cytotoxicity. In an animal model of colon carcinogenesis RV chemopreventive effect was associated with Bax hyper-expression (Tessitore et al., 2000) and in colorectal cancer cell lines RV-induced activation of the apoptotic intrinsic pathway relied on Bax conformational activation (Mahyar-Roemer et al., 2002). Moreover, Bax gene appears frequently mutated in human colorectal cancers, and it has been suggested that this may affect their responsiveness to chemotherapy drugs (Zhang et al., 2000). In the present study RV caused the oligomerization and relocation onto mitochondrial membranes of Bax, the permeabilization of mitochondria and the consequent release of cytochrome c; all these events were associated with the permeabilization of lysosomes and the cytosolic relocation of CD and could be prevented by Pst or siRNA-mediated down-regulation of CD. In conclusion we have identified a novel pathway of RV cytotoxic mechanism in which the lysosomes act as death signal integrators. In colorectal cancer cells RV cytotoxicity was associated with up-regulation of CD expression and, conversely, siRNA-mediated down-regulation of CD expression abolished the cytotoxic effect of RV, indicating that this protease was the master trigger of RV lethal activity (Fig. 6). The present findings might therefore be of relevance when assessing the responsiveness or the resistance of tumors to RV-based therapy. In this respect, we notice that RV is fast and extremely metabolized in the body, so that predicted serum concentrations achieved when RV is injected for therapeutic purposes (at 100 mg per kg body weight) are much lower (probably 10 times) than

those utilized in the ‘in vitro’ experiments here described (Baur and Sinclair, 2006).Yet, in intestinal mucosa a 30-fold enrichment of RV over serum concentration has been reported (Sale et al., 2004). These observations emphasize the need to develop RV analogues with improved bioavailability, as well as to engineer drug delivery systems that allow the efficient accumulation of RV within the target diseased tissue.
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FIGURE LEGENDS

Figure 1. RV-induced cell death in colorectal cancer cells is not mediated by estrogen receptors and is prevented by the pan-caspases inhibitor ZVAD-fmk.

A) DLD1 cells exposed to increasing concentrations of RV for 48 h in the absence or the presence of the ER disruptor ICI 182,780. Substances were added at time zero and re-added in fresh medium at 24 h. Cytofluorograms of annexin V-labeled cells and histograms of adherent viable cell counts are shown (upper and lower panels, respectively). Cell counting data show that initial cell density (at day 0) is almost halved after a 48 h exposure to 100 μM RV. This effect was not prevented by ICI 182,780. No effects on cell growth were elicited by 1 and 10 μM RV. B) DLD1 and HT29 cells were incubated with 100 μM RV in the absence or the presence of 30 μM ZVAD-fmk for up to 96 h. Substances were added at time zero and re-added in fresh medium every 24 h. Typical

cytofluorograms of cells double-labeled with annexin V-FITC and PI. In the cytogram apoptotic cells (positive for annexin V) are distributed in the lower and upper right panels (values in percent are given). ZVAD-fmk prevented cell death by RV in both DLD1 and HT29 cell cultures.

Figure 2. Time-dependent permeabilization of mitochondria and activation of caspases by RV.

A) Cleavage of fluorogenic peptide substrate of different caspases at 0 to 48 h of RV treatment in DLD1 cells. B) Caspases fluorescence assay (upper panels) and annexinV-FITC staining (lower panels) in DLD1 cells treated or not with RV for 48 h. Representative cytofluorograms are shown. C) Control (Co) and 48 h RV-treated DLD1 cells double-stained for chromatin with DAPI (blue fluorescence) and for mitochondria with mitotracker (red fluorescence). D) cytofluorograms of DLD1 and HT29 cells incubated for up to 48 h with 100 μM RV and labeled with Rhodamine-123. Loss of mitochondrial retention of the fluorescent dye was apparent at 36 h of treatment. E) Images at the fluorescence confocal microscope of cells treated for 48 h with RV and labeled with Rhodamine.

Figure 3. CD mediates RV cytotoxicity in colorectal cancer cells. A) Cells were incubated with RV in the absence or the presence of Pst or E64d for 48 h. Medium was changed and substances re-added every 24 h. At the end of treatments cells were double-labeled with annexin V-FITC and PI and analyzed by cytofluorometry. Typical cytofluorograms are presented. Apoptotic cells (in lower and upper right panels of the cytofluorogram) amounted to approximately 40-48 % in RV- and RV plus E64d-treated cultures, and to approximately 10% in RV plus Pst-treated cultures. B) HT29 cells were incubated with 100 μM RV in the absence or the presence of 10 μM E64d or 100 μM Pst for 48 h, then adherent and suspended cells were labeled with annexin V-FITC and

analyzed by cytofluorometry. Medium was changed and substances re-added every 24 h. Representative cytofluorograms (of four experiments) are shown. At 48 h >60 0% of the cell population in RV-treated culture shows apoptotic features. RV-induced apoptosis was largely prevented by Pst, not by E64d. The latter inhibitor revealed itself cytotoxic to HT29 cells. C) Histograms of cell counting in DLD1 and HT29 cultures treated or not for 24 h and 48 h with RV in the absence or the presence of Pst or E64d. Medium was changed and substances re-added every 24 h. Control un-treated cells roughly doubled every 24 h. RV slowed down the rate of cell proliferation in the first 24 h. Compared to the initial cell density (day 0), final cell density (48 h) in RV-treated cultures was nearly halved. Cell loss from the monolayer did not occur when RV treatment was performed in the presence of Pst. Not only, in Pst pre-treated cultures cells also recovered from growth arrest despite the presence of RV. D) Silencing of CD by siRNA prevents RV cytotoxicity. Cells were plated, un-transfected (UT) or transfected with CD-siRNA or with control-duplex oligonucleotides as indicated and incubated or not with RV for 48 h. In the upper panel a representative (of three) western blotting of CD is shown (symbols: P, precursor; I, intermediate; LM, large chain of mature form). The filter was stripped and re-probed for actin to prove equal loading of protein homogenates; in the lower panel, data on adherent viable cells in cultures exposed to RV for 48 h are given as percentage of cell number in the control culture prior to the incubation (assumed as 100 %).

Figure 4. Cathepsin D triggers caspase 3 activation, Bax oligomerization on mitochondria and cytosolic release of cytochrome c induced by Resveratrol.

A) Histogram of caspase-3 activity in DLD1 cells exposed for 48 h to RV with or without Pst. B) TUNEL staining of DLD1 cells plated on

coverslips and incubated for 48 h with RV in the absence or the presence of Pst (representative images are shown). C) Control, Pst-pretreated and CD-siRNA transfected DLD1 cells plated on coverslips were exposed to RV for 48 h. Cells were then double-stained for mitotracker and Bax and photographed under the fluorescence confocal microscope. Compared to un-treated cells, RV-treated cells show a weak and diffuse staining of mitotracker. In these cells Bax immunoreactivity is increased and appears as spots that in some cases co-localizes with the mitotracker stain. This pattern, indicative of Bax oligomerization on mitochondrial membrane, is not observed in cells pre-treated with Pst (middle panels) and in CD-siRNA transfected cells (lower panels). In the latter cases mitochondria appear well preserved and Bax immunostaining is diffuse in the cytoplasm also in the presence of RV. Cells transfected with CD-unrelated siRNA duplexes behaved as control un-transfected cells. D) DLD1 cells on coverslips incubated for 48 h with RV in the absence or the presence of Pst and labeled for immunofluorescence detection of cytochrome c. The experiments demonstrate that Pst prevents mitochondrial permeabilization and cytosolic release of cytochrome c induced by RV. Images in C and D are representative of four independent experiments.

Figure 5. Resveratrol cytotoxicity in colorectal cancer cells is preceded by lysosome leakage and up-regulation of the expression of and by cytosolic relocation of CD.

A) DLD1 and HT29 cells plated on coverslips were incubated for increasing time with RV and then stained with the acidotropic AO fluorochrome and immediately observed under the fluorescence microscope (representative images are shown). Intense red fluorescent spots are indicative of intralysosomal retention of AO. At 36 and 48 h of RV treatment a large proportion of cells show a weak yellow-green

fluorescence indicative of cytosolic diffusion of the fluorochrome. B) Cells treated and labeled with AO as above and analyzed by cytofluorometry. C) DLD1 and HT29 cells plated on coverslips were incubated for 48 h with RV and double-labeled for immunofluorescence detection of CD (red fluorescence) and of tubulin (green fluorescence). D) Western blotting analysis of mature CD (LM, large chain of the double-chain) in a time-course treatment with RV of DLD1 and HT29 cells and relative densitometry (representative of three experiments). Filters were stripped and re-probed for actin to quantify protein loading in the lanes.

Figure 6. Interpretative scheme of the results. RV induces the up-regulation of CD expression and the cytosolic relocation of mature active CD from lysosomes. CD was shown essential to induce mitochondria permeabilization (associated with Bax oligomerization onto mitochondrial membrane), cytochrome c release, caspase 3 activation and appearance of annexin V and TUNEL positive cell death.

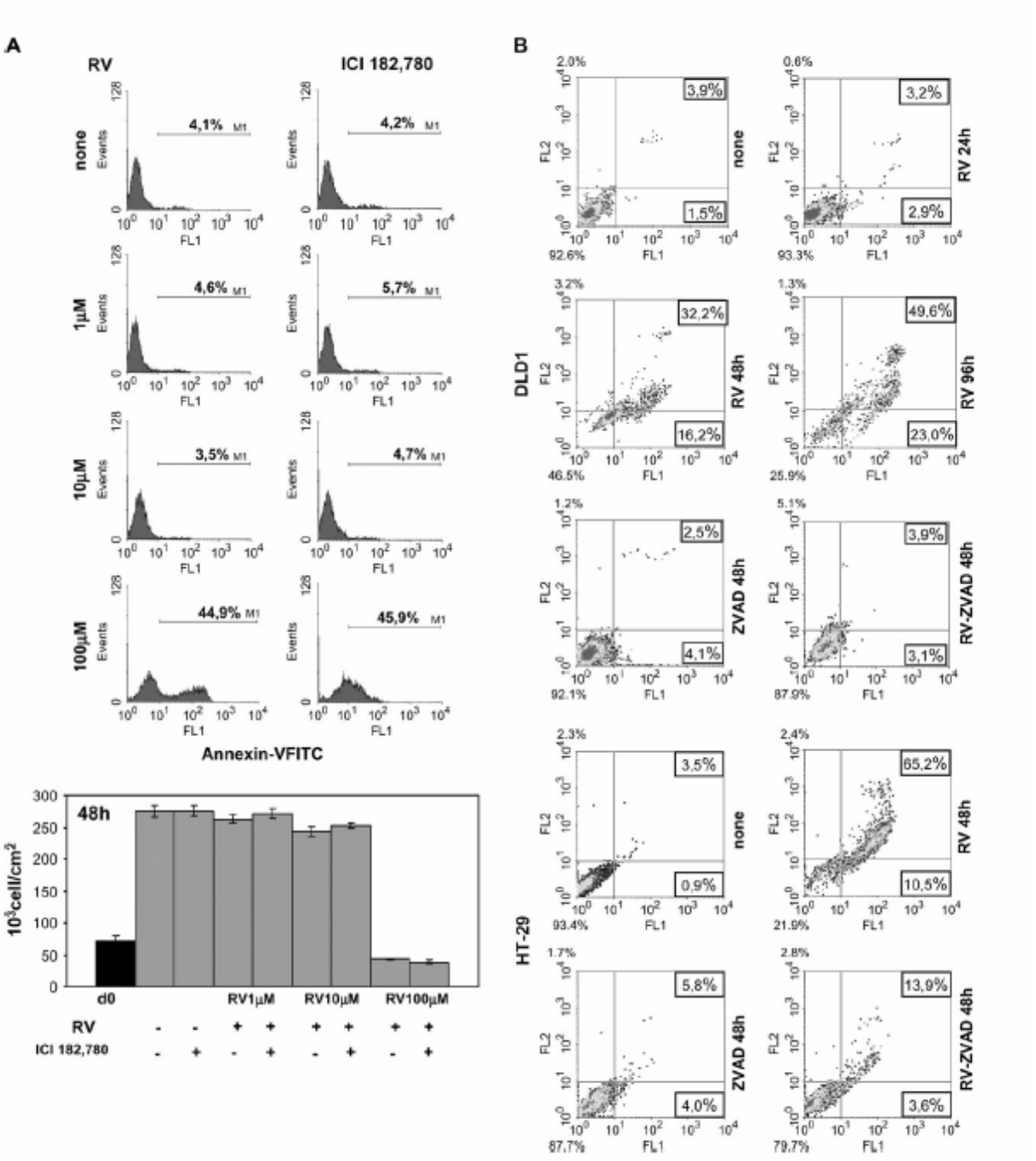
A
RV
ICI 182,780
none
1µM
10µM
100µM
4,1%
4,2%
4,6%
5,7%
3,5%
4,7%
44,9%
45,9%
Events
FL1
Annexin-VFITC
48h
10³cell/cm²
d0
RV1µM
RV10µM
RV100µM
RV
ICI 182,780
B
DLD1
HT-29
none
RV 24h
RV 48h
RV 96h
ZVAD 48h
RV-ZVAD 48h
FL2
FL1
3,9%
1,5%
3,2%
2,9%
32,2%
16,2%
49,6%
23,0%
2,5%
4,1%
3,9%
3,1%
3,5%
0,9%
65,2%
10,5%
5,8%
4,0%
13,9%
3,6%

Figure 1

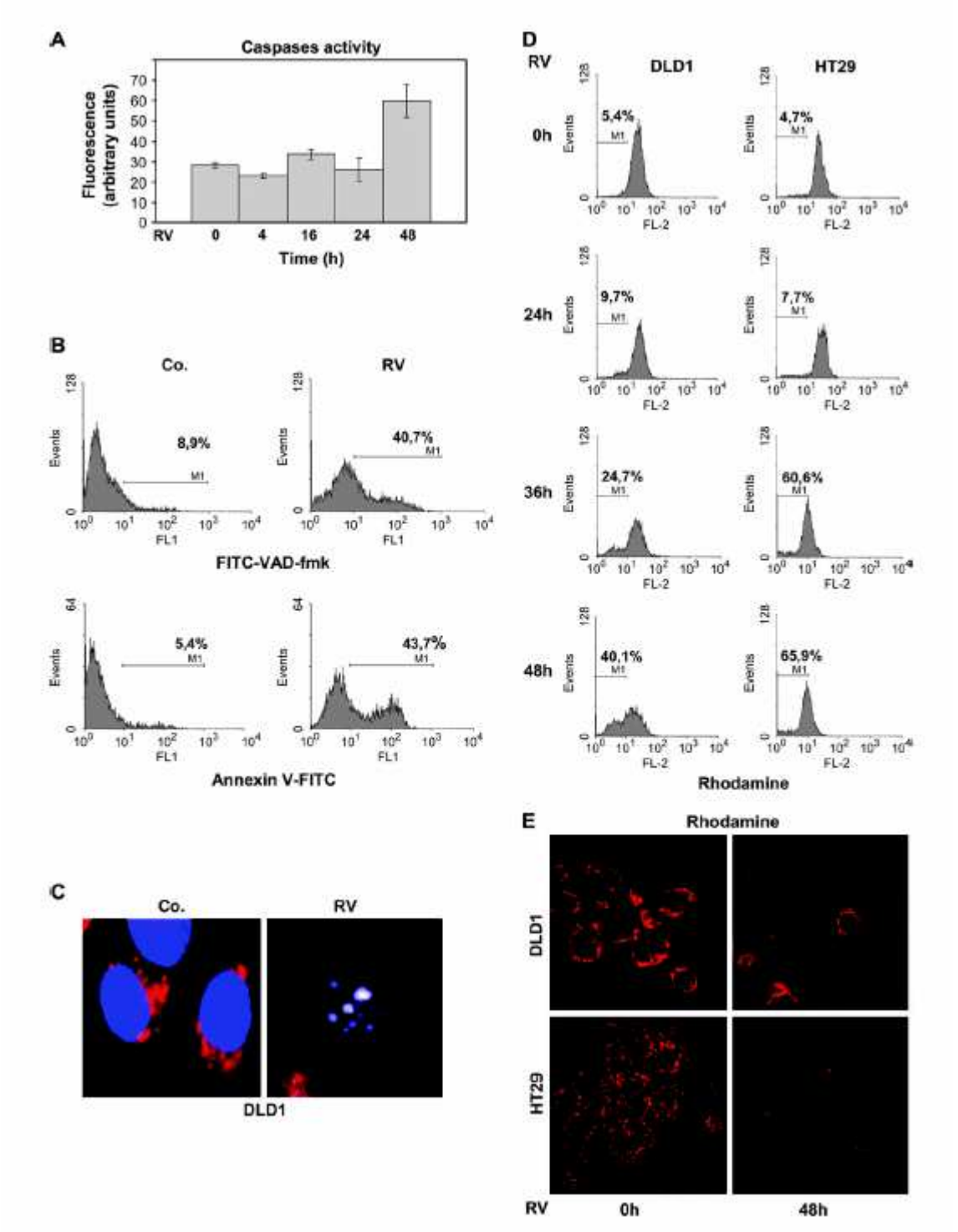
A
Caspases activity
Fluorescence (arbitrary units)
70
60
50
40
30
20
10
0
RV
0
4
16
24
48
Time (h)
B
Co.
RV
8,9%
40,7%
M1
Events
FL1
FITC-VAD-fmk
5,4%
43,7%
Annexin V-FITC
C
Co.
RV
DLD1
D
RV
DLD1
HT29
0h
5,4%
4,7%
24h
9,7%
7,7%
36h
24,7%
60,6%
48h
40,1%
65,9%
FL-2
Rhodamine
E
Rhodamine
DLD1
HT29
RV
0h
48h

Figure 2

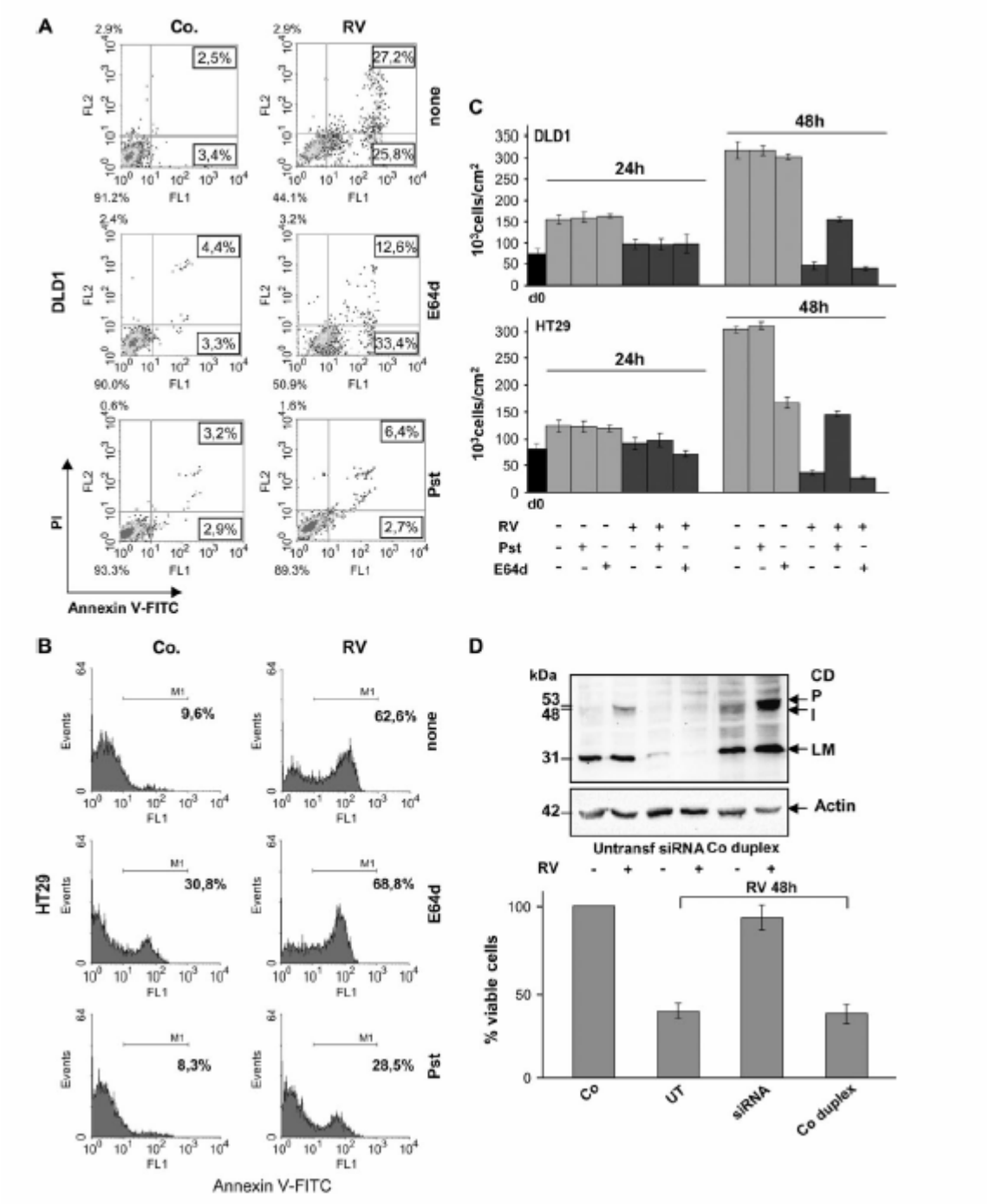
A
Co.
RV
2,5%
3,4%
27,2%
25,8%
none
DLD1
4,4%
3,3%
12,6%
33,4%
E64d
3,2%
2,9%
6,4%
2,7%
Pst
PI
Annexin V-FITC
FL1
FL2
B
Co.
RV
9,6%
62,6%
none
HT29
30,8%
68,8%
E64d
8,3%
28,5%
Pst
Events
M1
Annexin V-FITC
C
DLD1
HT29
24h
48h
10³cells/cm²
d0
RV
Pst
E64d
D
kDa
CD
P
I
LM
Actin
53
48
31
42
Untransf siRNA Co duplex
RV 48h
% viable cells
Co
UT
siRNA
Co duplex

Figure 3

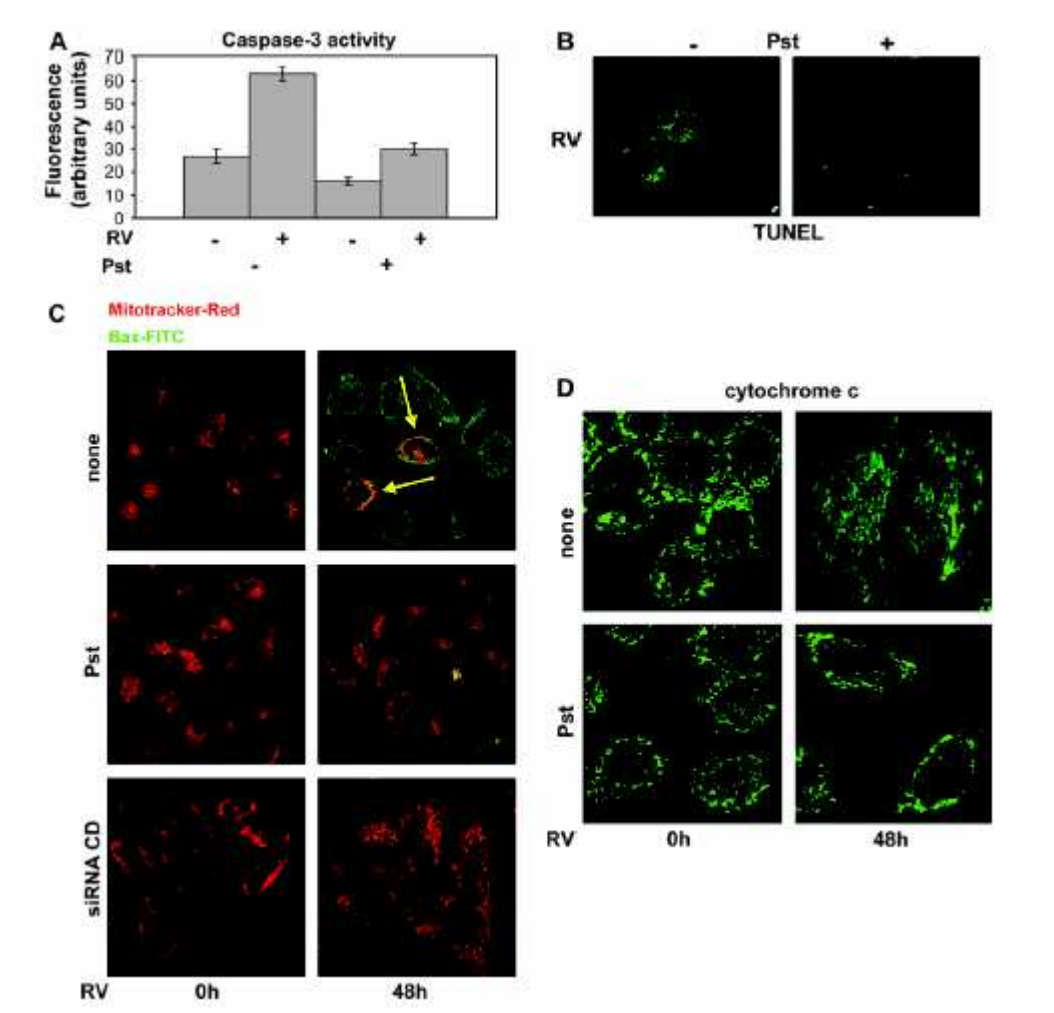
A
Caspase-3 activity
Fluorescence (arbitrary units)
70
60
50
40
30
20
10
0
RV - + - +
Pst - +
B
- Pst +
RV
TUNEL
C
Mitotracker-Red
Bax-FITC
none
Pst
siRNA CD
RV 0h 48h
D
cytochrome c
none
Pst
RV 0h 48h

Figure 4

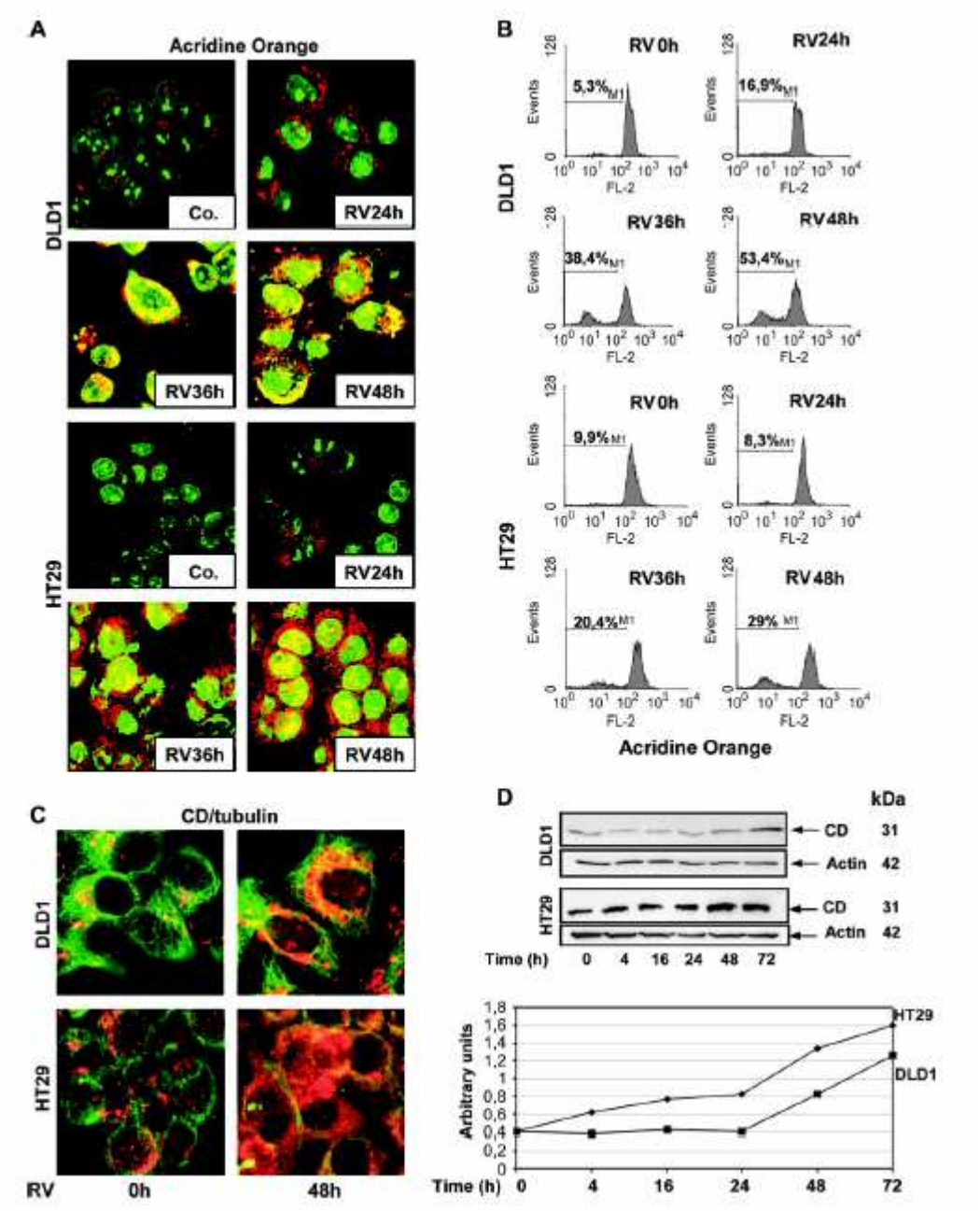
A
Acridine Orange
DLD1
Co.
RV24h
RV36h
RV48h
HT29
Co.
RV24h
RV36h
RV48h
B
DLD1
RV 0h
5,3%M1
RV24h
16,9%M1
RV36h
38,4%M1
RV48h
53,4%M1
HT29
RV 0h
9,9% M1
RV24h
8,3%M1
RV36h
20,4%M1
RV48h
29% M1
Events
FL-2
Acridine Orange
C
CD/tubulin
DLD1
HT29
RV
0h
48h
D
kDa
DLD1
CD 31
Actin 42
HT29
CD 31
Actin 42
Time (h) 0 4 16 24 48 72
Arbitrary units
HT29
DLD1
Time (h) 0 4 16 24 48 72

Figure 5

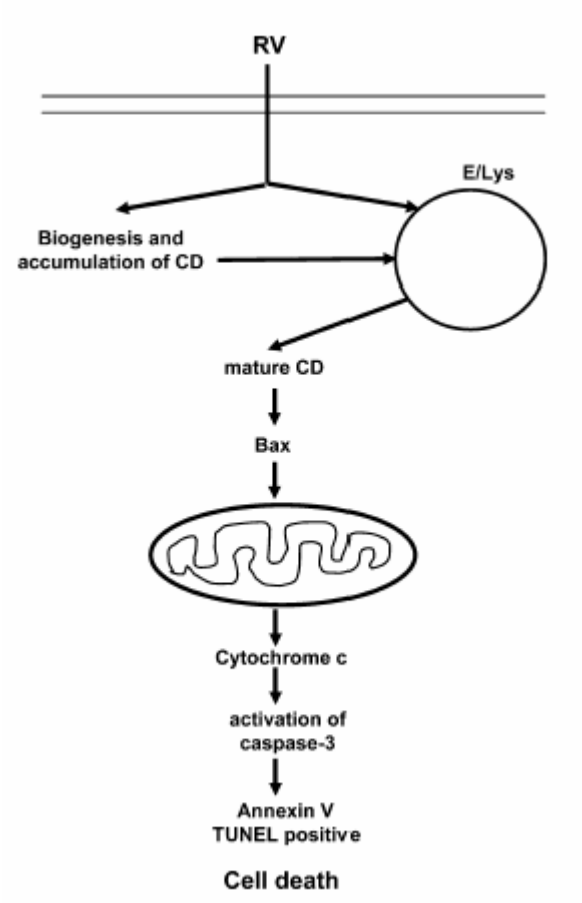
RV
E/Lys
Biogenesis and
accumulation of CD
mature CD
Bax
Cytochrome c
activation of
caspase-3
Annexin V
TUNEL positive
Cell death

Figure 6

SECOND WORK

In human colorectal DLD1 cancer cells, the dietary bioflavonoid Resveratrol (RV) rapidly induced autophagy. This effect was reversible (on removal of the drug), and was associated with increased expression and cytosolic redistribution of the proteins Beclin1 and LC3 II. Supplementing the cells with asparagine abrogated the Beclin-dependent autophagy. When applied acutely (2 h), RV was not toxic, however reiterate chronic (48 h) exposure to RV eventually led to annexin V- and TUNEL-positive cell death. This toxic effect was autophagy-dependent, as it was prevented either by asparagine, by expressing a dominant negative lipid kinase-deficient class III PI3k or by RNA-interference knock-down of Beclin1. Lamp2b silencing abolished the fusion of autophagosomes with lysosomes and preserved cell viability despite the ongoing formation of autophagosomes in cells chronically exposed to RV. The pan-caspase inhibitor ZVAD-fmk inhibited RV-induced cell death, but not autophagy. These results uncover a novel pathway of RV cytotoxicity in which autophagy plays a dual role: (i) at first, it acts as a pro-survival stress response and (ii) at a later time, it switches to a caspase-dependent apoptosis pathway. The present data also indicate that genetic or epigenetic inactivation of autophagy proteins in cancer cells may confer resistance to RV-mediated killing.

RESVERATROL-INDUCED APOPTOSIS DEPENDS ON THE LIPID KINASE ACTIVITY OF Vps34 AND ON THE FORMATION OF AUTOPHAGOLYSOSOMES

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INTRODUCTION

Resveratrol (3,4',5-trihydroxy-*trans*-stilbene, RV) is a polyphenolic antioxidant compound present in grapes, red wine, berries, peanuts and other alimentary products (Baur and Sinclair, 2006; Fremont, 2000). In 1997, Pezzuto and co-workers provided the first compendious evidence that RV possesses chemopreventive activity against the three steps of carcinogenesis (i.e., initiation, promotion and progression) in an animal model of skin cancer (Jang et al., 1997). The fact that about 70 to 90% of colorectal cancers seems associated to dietary habits stimulates the interest on dietary factors that can exert cancer chemopreventive action on the intestinal mucosa (Schatzkin and Kelloff, 1995). In this respect, RV is particularly appealing as preliminary *in vitro* and *in vivo* studies have shown no overt toxicity toward normal cells when administered at doses high enough to achieve a pharmacological effect (Boocock et al., 2007; Gusman et al., 2001). In addition, although it is fast and extensively metabolized in the body, in intestinal mucosa a 30-fold enrichment of RV over serum concentration can be reached (Sale et al., 2004). Several *in vivo* studies have shown that RV can actively contrast the development and/or progression of colorectal cancers (Schneider et al., 2001; Tessitore et al., 2000), and *in vitro* studies have confirmed the ability of RV (at concentration comparable to that found in some foods)

to halt cell proliferation and to induce enterocyte-like differentiation and cell death of human coloncarcinoma cells (Liang et al., 2003; Mahyar-Roemer et al., 2001; Schneider et al., 2000; Wolter et al., 2001; Wolter and Stein, 2002) (Kotha et al., 2006; Mahyar-Roemer et al., 2002; Mohan et al., 2006; Trincheri et al., 2007).

In ovarian cancer cells, RV was shown to induce both autophagocytosis and caspase-independent cell death (Opipari et al., 2004). Yet, it remained unexplained whether the induction of autophagy by RV represented an epiphenomenal stress response or it was actively involved in the toxic mechanism of RV. Autophagy preserves cell survival under unfavourable environmental conditions by ensuring the lysosomal degradation of aged or damaged proteins, membranes and cytoplasmic structures (Yorimitsu and Klionsky, 2005). Whether this pathway contributes to or counteracts the toxic outcome of chemotherapy drug treatments is still a matter of investigation. Autophagy might confer resistance to chemotherapy drugs in cancer cells by actively removing the proteins and the organelles that are damaged under antiblastic treatment (Abedin et al., 2007; Amaravadi and Thompson, 2007). Still, the toxic effects of some anticancer drugs has been associated with induction of autophagy (Bursch et al., 1996; Kessel and Reiners, 2007). In the present study we report the following findings: i) autophagy is rapidly and reversibly induced by an acute exposure to RV; ii) the prolonged exposure to RV eventually activates a caspase-mediated cell death pathway; iii) genetic inactivation of the autophagy proteins class III PI3k, Beclin 1 and Lamp2b abrogates RV toxicity. These data emphasize the role of autophagy in the response to chemotherapy drugs and the strict relationship between autophagy and apoptosis in the execution of the death program.

MATERIALS AND METHODS

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

Cell cultures, treatments and evaluation of cytotoxicity

Human colorectal cancer DLD1 cells were cultivated in standard culture conditions (37°C; 95% air: 5% CO_2) in Dulbecco's modified Minimal Essential Medium supplemented with 10% fetal bovine serum (Invitrogen Corp., Carlsbad, CA, USA), 2 mM L-glutamine and 1% penicillin-streptomycin solution. Cells were seeded and let adhere on sterile plastic dishes for 24 h prior to start any treatment. Treatments included 100 µM RV, 50 mM Asparagine (Asn) and 30 µM ZVAD(OMe)-fmk (ZVAD) (Alexis Laboratories, San Diego USA). In experiments lasting 48 h, the culture medium was changed and the substances were re-added after the first 24 h of incubation. At designated time-points, adherent viable (trypan blue-excluding) cells were counted. Cell death was assessed by cytofluorometry analysis of cells labeled either with annexin V-FITC (Alexis Laboratories, S. Diego, USA) or propidium iodide (PI); for this purpose, adherent cells were trypsinized and mixed with the suspended cells recovered from the medium (Trincheri et al., 2007).

siRNA transfection

Post-transcriptional silencing of Beclin1 and Lamp2b expression was achieved by the small interference RNA (siRNA) technology. Duplexes of 27-nucleotide siRNA including two 3'-overhanging TT were synthesized by MWG Biotech AG (Washington, DC). An inefficient CD9 oligonucleotide corresponding to the AGGUAGUGUAAUCGCCUUG sequence was used as a negative control of transfection (referred to as 'sham'). The sense strands of siRNA targeting Beclin1 and Lamp2b

mRNAs were GGAACUCACAGCUCCAUUACUUACCAC and AAGAGUGUUCGCUGGAUGAUGACACCA, respectively. Transfection was performed with Lipofectamine 2000 (Invitrogen Corp.). Afterward, the transfection mixture was removed and cells were incubated for further 24 h in fresh medium prior to any treatment.

PI3K III dominant negative adenoviral vector

The recombinant adenoviral vector directing the synthesis of the dominant negative form of Vps34 (the yeast homolog of class III PI3k), which is devoid of lipid kinase activity, was kindly provided by Dr D. Murphy (University of Bristol). The vector also bears the coding sequence for the enhanced Green Fluorescent Protein, eGFP), thus allowing to monitor cell transfection. As a control, sham infection was performed with an empty paired adenoviral vector.

Western blotting analysis

Expression of proteins of interest was assessed by standard western blotting procedure *(Trincheri et al., 2007).* The filter was probed with the following antibodies: a rabbit polyclonal anti-Beclin1 (Santa Cruz Biotechnology, CA, USA); a polyclonal anti-MAPLC3 (Santa Cruz Biotechnology, CA, USA); a mouse monoclonal antibody specific for β-Actin. Immunocomplexes were revealed by incubation with peroxidase-conjugated goat-anti-rabbit or goat-anti-mouse antibody, as appropriate, and subsequent peroxidase-induced chemiluminescence reaction (Biorad, Hercules, CA, USA). Intensity of the bands was estimated by densitometry analysis (Quantity one software).

Immunofluorescence studies

Cells grown and treated on coverslips were fixed with methanol for 20 min and permeabilized with 0.2% Triton X-100 in PBS for 15 min. The following primary antibodies were used: a polyclonal anti-Lamp1 (BD Biosciences); a polyclonal anti-MAPLC3 (Santa Cruz Biotechnology, CA, USA). FITC- or TRITC-conjugated secondary antibodies against rabbit IgG were used. As negative control, the primary antibody was omitted.

TUNEL staining

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.

Fluorescence microscope imaging

Coverslips 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. Three coverslips were prepared for each experimental condition and were independently examined by two investigators. Representative images are shown.

Fluorescence assessment of autophagy

Autophagolysosomes were detected with the fluorescent dye monodansylcadaverine (MDC) (Munafo and Colombo, 2001). Living cells were incubated with 0.05 mM MDC in PBS at 37° C for 15 min.

After incubation, cells were washed twice with PBS and immediately analyzed by fluorescence microscopy (excitation: 380-420, barrier filter 450 nm). Induction of AV formation was directly monitored in living cells transiently transfected with a plasmid encoding the fluorescence chimeric protein GFP-LC3 or Beclin-GFP. Microtubule to vacuole translocation of LC3 is associated with limited proteolysis and lipidation of its C-terminus (Kabeya et al., 2000). Therefore, the fusion protein is made so that GFP (green fluorescent protein) is placed at the N-terminus of LC3. The DNA encoding human LC3 was cloned by PCR from the total cDNA of human OVCAR-3 cells (sense primer 5'-CAACAAGCTTCACCATGCCGTCGGAGAAGACC-3'; antisense primer 5'- AGATCTCGAGTTACACTGACAATTTCATCCCG-3'). The cDNA was subcloned into the expression vector pEGFPC2 (Clontech Laboratories, Mountain View, California, USA). The cDNA of Beclin1 was cloned by PCR from the total cDNA of human OVCAR-3 cells (sense primer 5'-GCCCGAATTCGGGATGGAAGGGTCTAAGA-3'; antisense primer 5'-GCCCGGGATCCTTTTCAGACTGCAGCAAATC-3') and it was subcloned into the expression vector pEGFPN1 (Clontech Laboratories). LC3 and Beclin1 DNAs were checked by automated sequencing.

Statistical analysis

All experiments were independently replicated at least three times. Data are presented as means ± SD. The Microsoft Excel XLStats software was used.

RESULTS

Autophagy is an early and reversible stress response to Resveratrol

To monitor the induction of autophagy by RV we employed DLD1 transfected cells that transiently express the chimeric fluorescent protein GFP-LC3 or Beclin1-GFP. LC3 is the mammalian equivalent of yeast atg 8 and is normally associated with the cytoskeleton; on induction of autophagy this protein translocates onto the membrane of the nascent AV (Kabeya et al., 2000). Images in Figure 1A show that both endogenous LC3 and transfected GFP-LC3 assume a punctate vacuolar-like localization in RV-treated cells. Cytosol to vacuole translocation of LC3 is associated with proteolytic processing and subsequent lipidation of the C-terminus of the precursor MAP-LC3, a process leading to the 16 kDa LC3 II isoform (Kabeya et al., 2000) (Mizushima et al., 2001). This process in fact occurred in RV-treated cells (Figure 1B). Beclin1, the homolog of yeast Vps30 (also known as atg 6), interacts with class III PI3-kinase (the homolog of yeast Vps34) and UVRAG to start the recruitment of autophagy proteins on the membrane of the pre-autophagosomal structure (Kihara et al., 2001) (Liang et al., 2006). We therefore also monitored the localization of Beclin1 in cells transiently expressing the Beclin-GFP fluorescent chimera. Beclin-positive aggregates were soon detectable after 15 min of exposure to RV (Figure 1C). To see whether the autophagy process triggered by a short exposure to RV lasted for long time, DLD1 cells were exposed to RV for 2 h, then the cells were washed and further incubated in fresh medium in the absence or the presence of RV and collected at 24 and 48 h. In parallel, cells were exposed to RV throughout the 24 or 48 h period of incubation. In the latter case, the medium was replaced and RV was re-added at 24 h, to avoid aspecific induction of autophagy due to nutrient consumption. The presence of autophagolysosomes was visualized by labeling the cells

with the auto-fluorescent dye monodansylcadaverine (MDC) (Kabeya et al., 2000; Munafo and Colombo, 2001) (Bampton et al., 2005; Kihara et al., 2001; Liang et al., 2006; Mizushima et al., 2001). A 2 h incubation with RV led to the accumulation of autophagolysosomes, as indicated by the increased MDC staining (Figure 1D). On removal of RV, the cell recovered from the toxic stress and down-regulated autophagy to (basal) control level within 24 h. By contrast, the chronic presence of RV sustained the autophagy process, as demonstrated by the intense MDC labeling of cells exposed to RV for 24 and 48 h (Figure 1D). Form these data we conclude that: i) autophagy induced by RV has the characteristics of a rapid and reversible stress response; ii) RV retains its stimulatory activity on autophagy for at least 24 h.

Asparagine prevents the hyper-regulation of Beclin-dependent autophagy induced by chronic exposure to Resveratrol

To confirm the induction of autophagy by RV we attempted to interfere with the process using the inhibitor 3-methyladenine (3MA) (Seglen and Gordon, 1982) This drug, at appropriate concentrations (5-10 mM), effectively inhibited the RV-induced formation of Beclin-positive macrocomplexes (not shown), yet it revealed itself toxic in prolonged incubation (Opipari et al., 2004), thus precluding its use in further experiments. Extra supplementation of certain aminoacids has been proved to down-regulate autophagy (Hoyvik et al., 1991; Seglen et al., 1980). 50 mM asparagine prevented the formation of Beclin-GFP macro-complexes (Figure 2A) and the vacuolar localization of GFP-LC3 (not shown) in cells exposed for 2 h to RV. Asparagine also prevented the accumulation of AVs positive for Beclin1, Rab24, and LC3 in cells chronically exposed (for 24 and 48 h) to RV (not shown). Further, the content of Beclin1 greatly increased in cells chronically exposed to RV,

yet this effect was not observed in cells co-treated with asparagine (Figure 2B). Thus, raising the intracellular concentration of asparagine efficiently impaired the induction of autophagy by RV.

Asparagine prevents apoptosis induced by Resveratrol

We have recently shown that chronic and reiterate administration of 100 µM RV induces caspase-dependent apoptosis of DLD1 cells through a pathway driven by lysosomal cathepsin D and bax (Trincheri et al., 2007). While a 24 h incubation with RV provoked cell growth arrest and no apparent cell death, the incubation with RV for further 24 h produced ~50% cell death in the monolayer (Trincheri et al., 2007). We asked whether the induction of autophagy by RV was finalized to protect the cells (thus accounting for the ~ 50% of cells that survived at the end of the treatment) or was part of the death pathway (thus accounting for the ~ 50% of cells that succumbed at the end of the treatment). Hyper-activation of autophagy is in fact *per se* deleterious for the cell, as excessive self-digestion ends up in autophagic cell death (ACD) (Bursch, 2001; Feng et al., 2005). To better assess the real contribution of apoptosis and autophagy in the cytotoxic mechanism of RV, DLD1 cells were exposed to 100 µM RV for up to 48 h in the absence or the presence of the pan-caspase inhibitor ZVAD-fmk and/or of the aminoacid asparagine. Cell death was assessed by counting the viable and necrotic cells and by cytofluorometry of cells labeled with annexin V-FITC, which is assumed as an early marker of apoptosis (Martin et al., 1995). In agreement with published data (Trincheri et al., 2007), cell counting data (not shown) and cytofluorometry quantification of annexin V-positive cells confirmed the occurrence of ~50 % cell loss (compared to day0 cell density) in the RV-exposed culture; cell death was completely abrogated by co-treating with ZVAD-fmk or asparagine or both the inhibitors

(Figure 3A). We then looked at the effect of these inhibitors on the regulation of autophagy. Fluorescence localization of LC3 in GFP-LC3 expressing cells (data not shown) and MDC staining of autophagolysosomes (Figure 3B) demonstrated the inability of ZVAD-fmk to halt RV-induced autophagy, whereas asparagine confirmed its ability to prevent the early formation of AVs.

Genetic down-regulation of Beclin1 abrogates the formation of autophagosomes and apoptosis induced by Resveratrol

The findings that asparagine was able to prevent the hyper-regulation of Beclin1 expression, autophagy and cytotoxicity by RV are suggestive of a functional link between Beclin-dependent autophagy and caspase-dependent apoptosis. We tested this hypothesis. The expression of Beclin1 protein was knocked-down through the expression of a specific siRNA (Figure 4A). MDC staining proved that under this condition the formation of autophagolysosomes was impaired in the cells exposed to RV for 48 h (Figure 4B). Cell counting (not shown) and cytofluorometry data (Figure 4C) demonstrated that while control (sham-transfected) cells were largely sensitive to a 48 h treatment with RV, Beclin1-siRNA-transfected DLD1 cells were not. To definitely implicate Beclin-dependent autophagy in RV-induced apoptosis we stained Beclin1-knocked-down cultures with the TUNEL technique, which evidences the presence of nicked DNA that accumulates in dying cells. On treatment with RV, TUNEL positive cells were detectable in sham-transfected, but not in Beclin-siRNA-transfected, cultures (Figure 4D).

Resveratrol toxicity depends on the lipid kinase activity of class III PI3 kinase

We further investigated the molecular pathways through which RV induces both the up-regulation of autophagy and cell death. The interaction of Vps34-Class III PI3k with Atg6-Beclin1 is a key step for membrane nucleation and formation of the AV at the level of the pre-autophagosomal structure (Kihara et al., 2001; Suzuki et al., 2001). We asked about the need of PI3k III activity for the formation of beclin-macrocomplexes under RV treatment. To overcome the unspecific toxic effects of 3MA, we interfered with the activation of PI3k III by ectopic over-expression of its dominant negative. To this end, we employed a recombinant adenoviral vector coding for a mutant Vps34 protein devoid of lipid kinase activity (Ad-Vps34dn). Adherent DLD1 cells were sham- or Ad-Vps34dn-infected and then incubated for 48 h with or without RV. On average, > 90% of cells were effectively infected, as estimated by GFP staining. Despite the chronic exposure to RV, the ectopic expression of Vps34dn completely prevented the induction of autophagy, as demonstrated by MDC staining (Figure 5A), and contemporary inhibited the occurrence of cell death, as demonstrated by the lack of annexin V-labeling (Figure 5B). Cell counting data confirmed the full protection attained by Vps34dn expression toward RV cytotoxicity (not shown).

Genetic down-regulation of Lamp2b abrogates the formation of autophagolysosomes and cell death induced by Resveratrol

An important point is to determine whether autophagy-dependent cell death associated with RV exposure is due to the excessive accumulation of AVs and/or of autophagolysosomes in the cell. To address this issue we interfered with the last step of autophagy, i.e. the formation of the autophagolysosome. Both Lamp1 and Lamp2 proteins are involved in the

process of reciprocal recognition and fusion of AVs and endosomal-lysosomal organelles (Eskelinen, 2006). We were able to inhibit the AV-lysosome fusion by siRNA-mediated knock-down of Lamp2b, as indicated by immunofluorescence co-staining of LC3 and Lamp1, which respectively identifies AVs and endosomal-lysosomal organelles (Figure 6A). MDC staining further proved that the formation of autohagolysosomes by RV was impaired in DLD1 cells transfected with an siRNA targeting Lamp2b (Figure 6B). We then assessed the toxic effect of RV under this condition. Cytofluorometry data (and cell counting data, not shown) demonstrated that silencing Lamp2b prevents the induction of annexin V-positive cell death by chronic and reiterate exposure to RV (Figure 6C).

DISCUSSION

Autophagy is a lysosomal-mediated degradative pathway that plays a major role in cell and tissue homeostasis. How and to what extent autophagy contributes to cancer development and progression/regression is still a matter of investigation (Ogier-Denis and Codogno, 2003). Defective autophagy favors tumorigenesis, and genes involved in the regulation of autophagy, such as BECLIN 1 and PTEN are frequently mutated in carcinomas and are therefore regarded as oncosuppressors (Di Cristofano and Pandolfi, 2000; Liang et al., 1999; Qu et al., 2003). On the other hand, functional autophagy helps the cancer cell to survive when the blood supply is reduced, thus preventing necrosis and inflammation-associated tumor progression (Degenhardt et al., 2006). The clarification of the real contribution of autophagy in the cellular response to anti-carcinogenic drugs is of obvious utility when designing chemotherapy and chemopreventive pharmacological strategies. Here we show that the pro-apoptotic activity of RV in DLD1 colorectal cancer cells is mediated

by proteins involved in the regulation and execution of autophagy. In DLD1 cells RV promptly induced autophagy, which was reversible and not harmful when the exposition to the drug was temporally limited. The reiterate and prolonged incubation with RV led to a cell death with the morphological and biochemical features of apoptosis (annexin V-positive, TUNEL-positive, caspase-mediated), yet it was dependent on the lipid kinase activity of class III PI3k. The signaling pathway linking RV and activation of class III PI3k definitely deserves further studies. Post-transcriptional down-regulation of either Beclin1 and Lamp2b, which respectively led to inhibition of AV and autophagolysosome formation, also protected the cells from RV toxicity. It is to note that the Lamp2b silencing condition inhibited the formation of autophagolysosomes and determined the accumulation of AVs in RV-treated cells, yet it did not precipitate an ACD as was shown to occur in starved cells (Gonzalez-Polo et al., 2005). Inhibition of autophagy prevented the occurrence of TUNEL-positive cell death, whereas inhibition of caspases, though saving the cells, did not impair autophagy in RV-treated cells. Again, in this latter condition ACD did not occur despite the chronic exposure to RV and ongoing autophagy. These observations suggest that the autophagy and apoptosis pathways were not independent, rather they were strictly linked and merged at the execution point, the caspases acting as death executioners downstream autophagy. We have previously shown that RV induces the cytosolic relocation of cathepsin D, which in turn activates the bax-mitochondrial intrinsic pathway of caspase-dependent cell death (Trincheri et al., 2007).

Taken together with the present findings, we propose a model of RV cytotoxicity in which initially autophagy represents an adaptive response with pro-survival function, but on chronic intoxication autophagy is hyper-stimulated and the lysosomal membrane becomes permeable, thus

allowing the cytosolic relocation of pro-apoptotic cathepsins. The present results indicate that genetic or epigenetic inactivation of proteins involved in the regulation and/or execution of autophagy in cancer cells confer resistance to RV-mediated killing.

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LEGENDS TO FIGURES

Figure 1. Resveratrol rapidly induces an autophagy response. A) Un-transfected and GFP-LC3 transfected DLD1 cells plated on coverslips were exposed to RV for 1 and 2 h. RV provokes the rapid changes in LC3 distribution from a diffuse cytoplasmic staining to a vacuolar-like staining. B) Western blotting analysis of MAP-LC3 processing in DLD1 cells. On RV treatment, the vacuolar-associated LC3 II isoform is rapidly generated. C) DLD1 cells were transfected with a plasmid encoding Beclin-GFP and exposed to RV for the time indicated. Vacuolar-like aggregates of Beclin1 could be detected after a 15 min exposure to RV. D) DLD1 cells plated on coverslips were exposed to RV for 24 h or 48 h (refreshing the medium and re-adding RV at 24 h) or for 2 h and then incubated in fresh medium (without RV) and collected at 24 h or 48 h. Cells were then stained with MDC and immediately imaged under the fluorescence microscope. Uptake and accumulation of MDC reflect the presence of autophagolysosomes. The experiment reveals that induction of autophagy is an early and reversible response to RV.

Figure 2. Asparagine prevents the induction of Beclin-dependent autophagy by Resveratrol. Beclin-GFP-transfected DLD1 cells were exposed for 2 h to RV in the absence or the presence of asparagine (Asn). Images show that the vacuolar-like localization of Beclin-GFP induced by RV does not occur in the presence of Asn. B) Western blotting analysis (one out of three is shown) of Beclin1 expression in cells exposed to RV for 24 or 48 h in the absence or the presence of Asn. RV induces the accumulation of Beclin1 protein (at 24 h this effect is higher than at 48 h). Inhibition of autophagy by Asn prevents the cellular accumulation of Beclin 1 induced by RV.

Figure 3. Asparagine prevents the toxic effect induced by chronic administration of Resveratrol. A) Annexin V-FITC flow cytometry

profiles of cells exposed to RV for 48 h in the absence (none) or the presence of ZVAD-fmk or Asn or the two inhibitors together, as indicated. Inhibition of caspases or of autophagy elicited complete protection against RV toxicity, and no synergistic protection was attained by co-treating with both inhibitors. B) MDC staining of DLD1 cells incubated for 2 to 48 h with RV and in the absence or the presence of Asn or ZVAD-fmk. Images confirm the inability of the pan-caspases inhibitor to prevent the formation and accumulation of autophagolysosomes induced by RV, while Asn maintains its inhibitory effect on autophagy induction in cells exposed to RV for as long as 48 h.

Figure 4. Ectopic expression of a PI3k III dominant negative protects from Resveratrol cytotoxicity. DLD1 cells (adherent either on coverslips or Petri dishes) were infected with the recombinant vector Ad-Vps34dn (which directs the expression of a lipid kinase-deficient mutant of class III PI3k) or with an empty adenoviral vector (sham) and then exposed to RV for 48 h. A) At the end, cells on coverslips were stained with MDC and immediately observed under the fluorescence microscope. The images show that expression of Vps34dn efficiently abolished the induction of autophagy by RV. B) At the end of the treatment, the cells were labeled with annexin V-FITC and analyzed by flow cytometry. The cytofluorometry profiles demonstrate that in Ad-Vps34dn-infected cultures RV did not induce annexin V-positive cell death. In a parallel set of cultures, adherent viable cells were counted. The data (not shown) confirmed the protection attained by Vps34dn against RV cytotoxicity.

Figure 5. siRNA-mediated silencing of Beclin 1 prevents Resveratrol-induced apoptosis. DLD1 cells were transfected with an inefficient duplex oligoRNA (sham) or with an siRNA specific for Beclin1. A) Western blotting analysis demonstrates the efficient knock-down of Beclin1 protein expression attained with the specific siRNA. B) The

cells, plated on coverslips, were exposed to RV for 48 h and stained with MDC. The images confirm that silencing the expression of Beclin1 prevents the accumulation of autophagolysosomes in RV-treated cells. C) The cells plated in Petri dishes were exposed to RV for 48 h and then trypsinized, conted and labeled with annexin V-FITC for flow cytometry evaluation of cell death. Data demonstrate that a complete protection against RV cytotoxicity was obtained by knocking-down the expression of Beclin 1. D) Cells plated on coverslips were exposed to RV for 48 h and then stained with the TUNEL technique. The images show that in sham-infected cultures, but not in Beclin1-silenced cultures, RV provokes the appearance of TUNEL-positive cells.

Figure 6. siRNA-mediated silencing of Lamp2b prevents autophagolysosome formation and cell death induced by Resveratrol. Sham- and Lamp2b-siRNA-transfected cells were incubated for 48 h with RV and at the end cells were assayed by immunofluorescence for the formation of AVs (LC3-positive) and of autophagolysosomes (LC3-Lamp1 positive) and for occurrence of annexin V-positive cell death. A) Images show that siRNA-mediated knock-down of Lamp2b abolishes the formation of vacuoles double-positive for both LC3 and Lamp1, which are seen in sham-infected cells. B) The cells were stained with MDC. The images confirm that silencing the expression of Lamp2b is sufficient to prevent the accumulation of autophagolysosomes in RV-treated cells. C). The cells were labeled with annexin V-FITC and analyzed by flow cytometry. Data demonstrate that the complete protection against RV cytotoxicity obtained by knocking-down the expression Lamp2b.

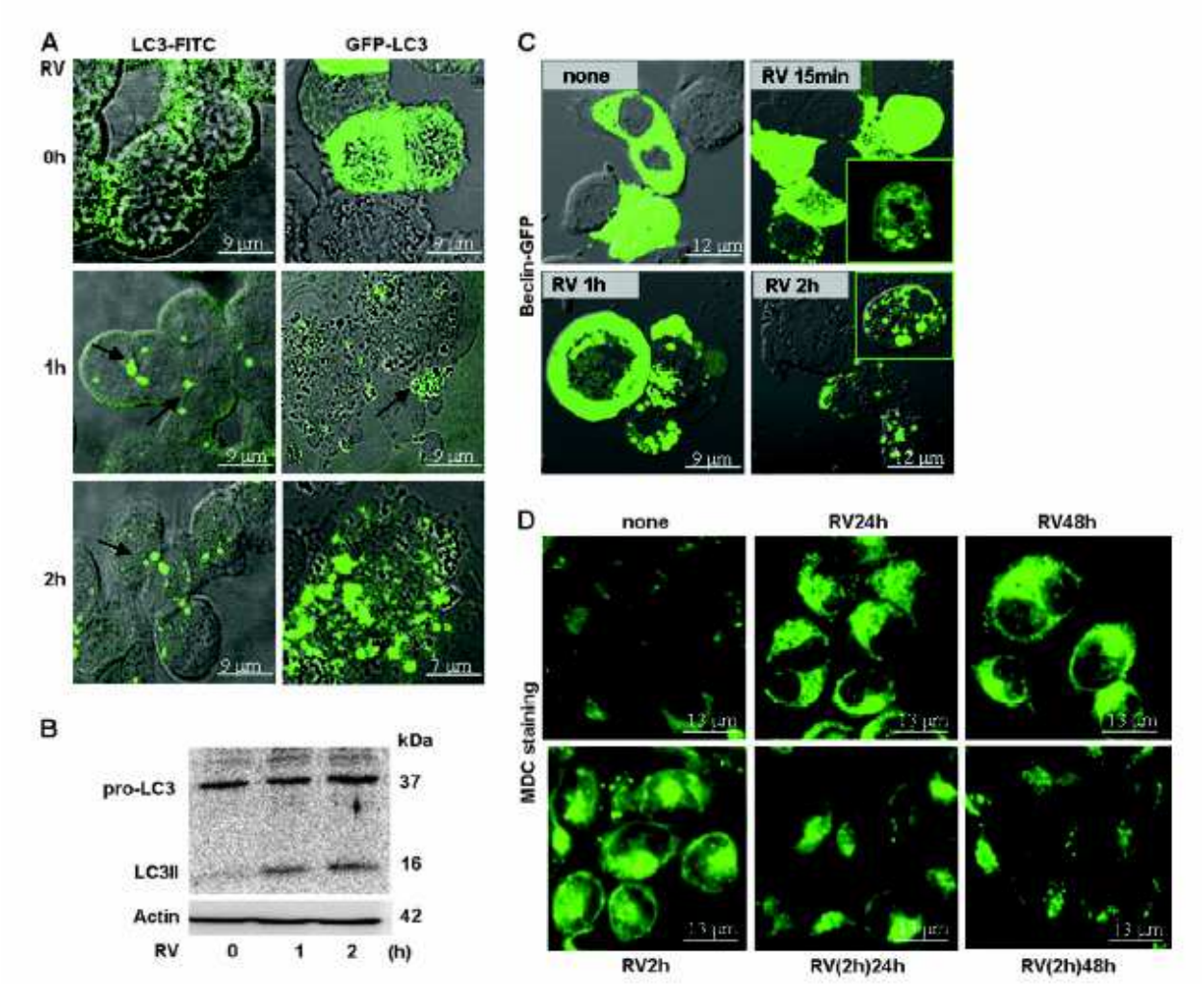
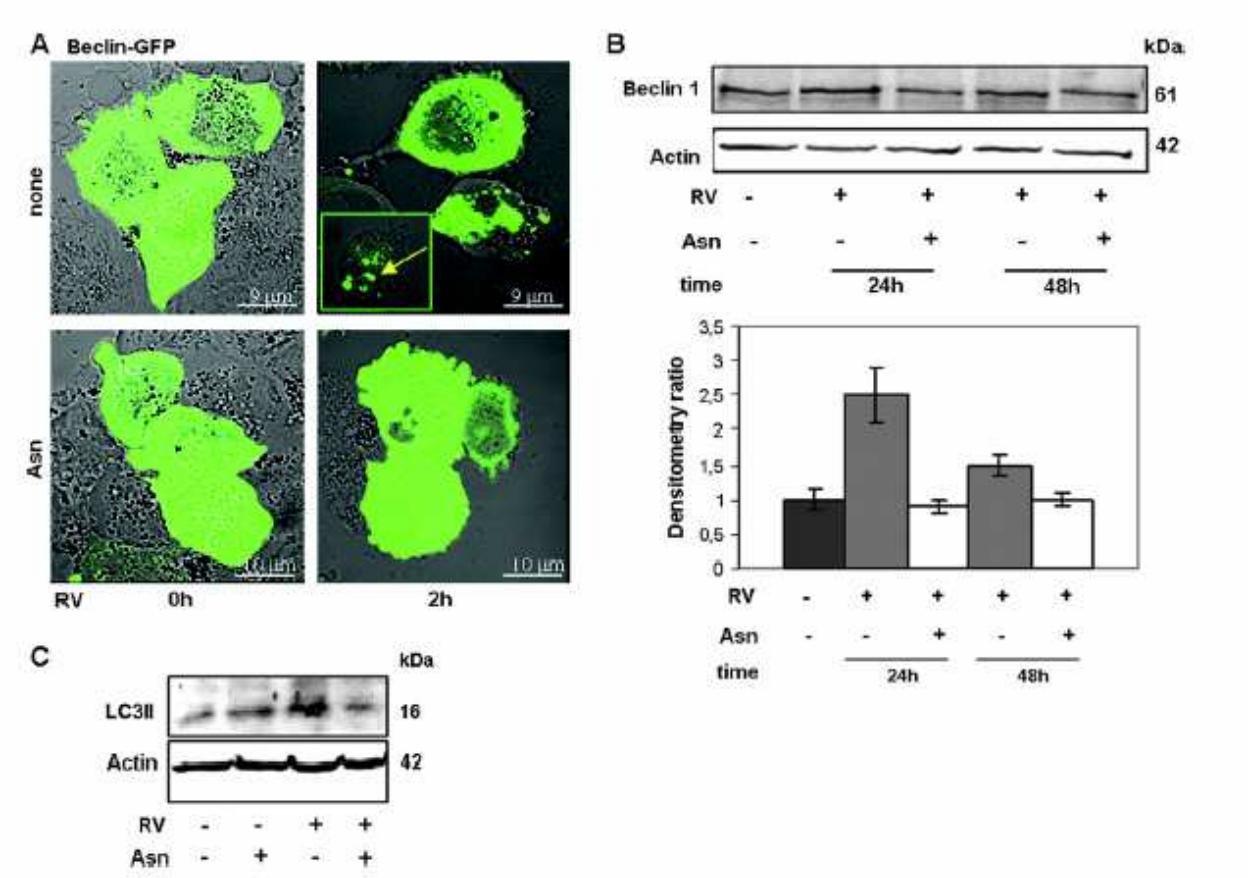

A
LC3-FITC
GFP-LC3
RV
0h
1h
2h
B
kDa
pro-LC3
37
LC3II
16
Actin
42
RV 0 1 2 (h)
C
none
RV 15min
RV 1h
RV 2h
Beclin-GFP
D
none
RV24h
RV48h
MDC staining
RV2h
RV(2h)24h
RV(2h)48h

Figure 1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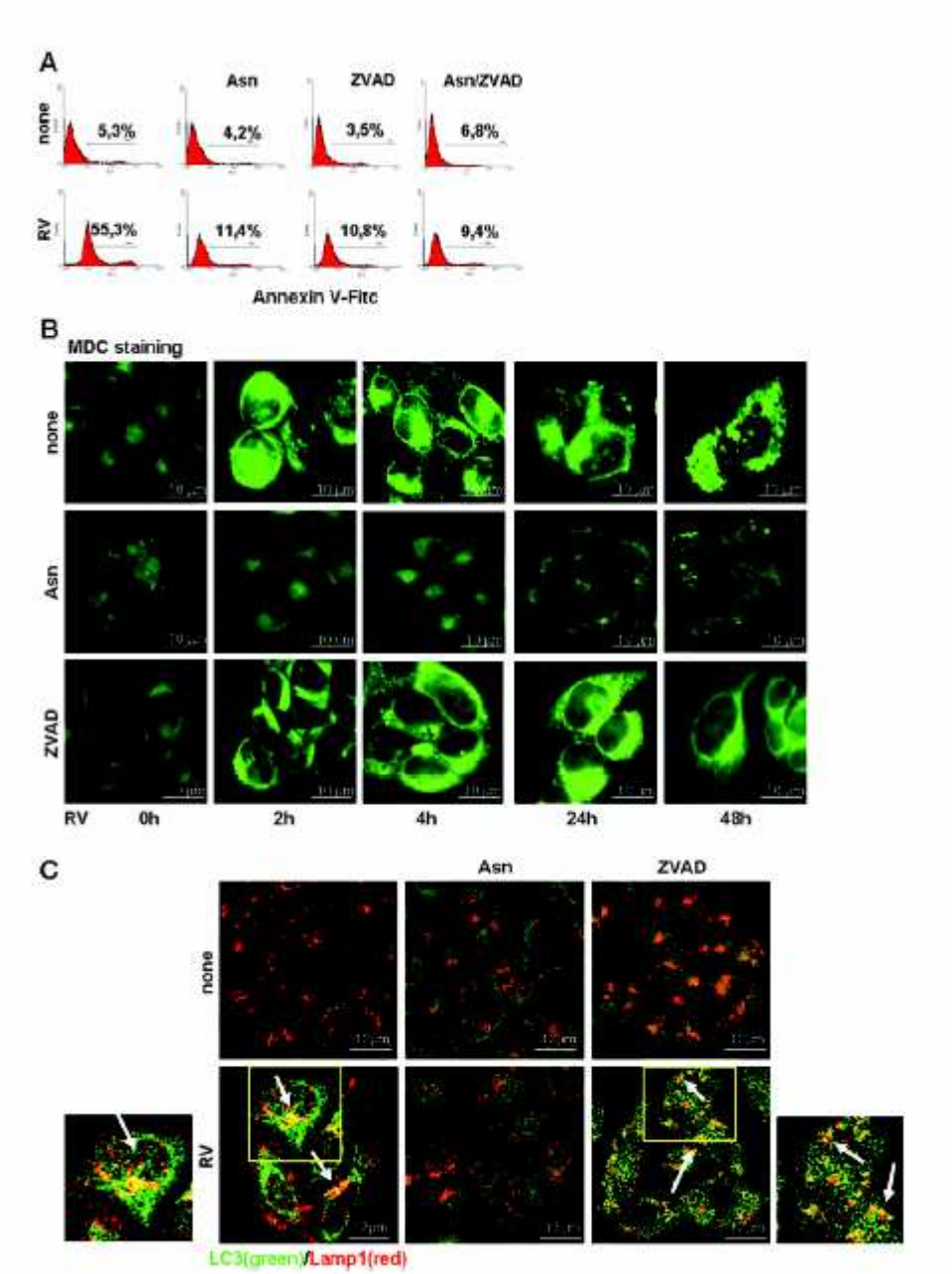
Beclin-GFP
none
Asn
9 µm
9 µm
10 µm
RV
0h
2h
B
kDa
Beclin 1
61
Actin
42
RV - + + + +
Asn - - + - +
time
24h
48h
Densitometry ratio
3,5
3
2,5
2
1,5
1
0,5
0
C
kDa
LC3II
16
Actin
42
RV - - + +
Asn - + - +

Figure 2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Asn
ZVAD
Asn/ZVAD
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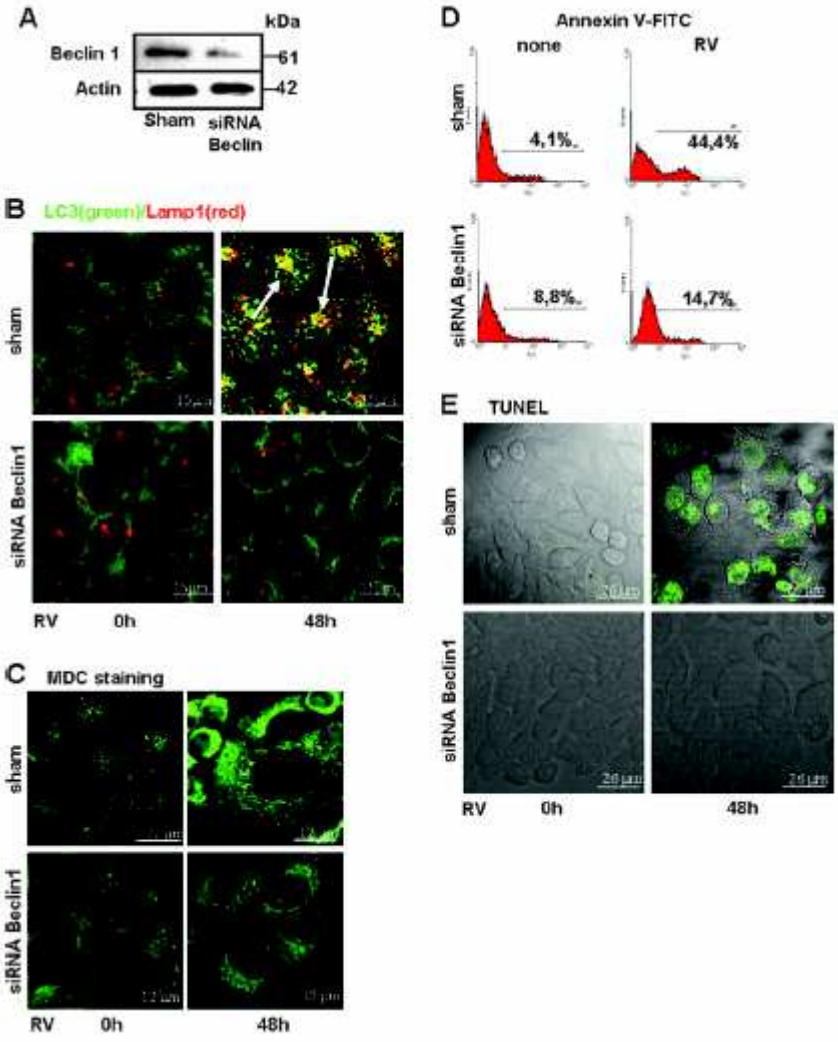
4,2%
3,5%
6,8%
RV
55,3%
11,4%
10,8%
9,4%
Annexin V-Fitc
B
MDC staining
none
Asn
ZVAD
RV
0h
2h
4h
24h
48h
C
Asn
ZVAD
none
RV
LC3(green)/Lamp1(red)

Figure 3


A
kDa
Beclin 1
Actin
61
42
Sham
siRNA Beclin
B
LC3(green)/Lamp1(red)
sham
siRNA Beclin1
RV
0h
48h
C
MDC staining
sham
siRNA Beclin1
RV
0h
48h
D
Annexin V-FITC
none
RV
sham
4,1%
44,4%
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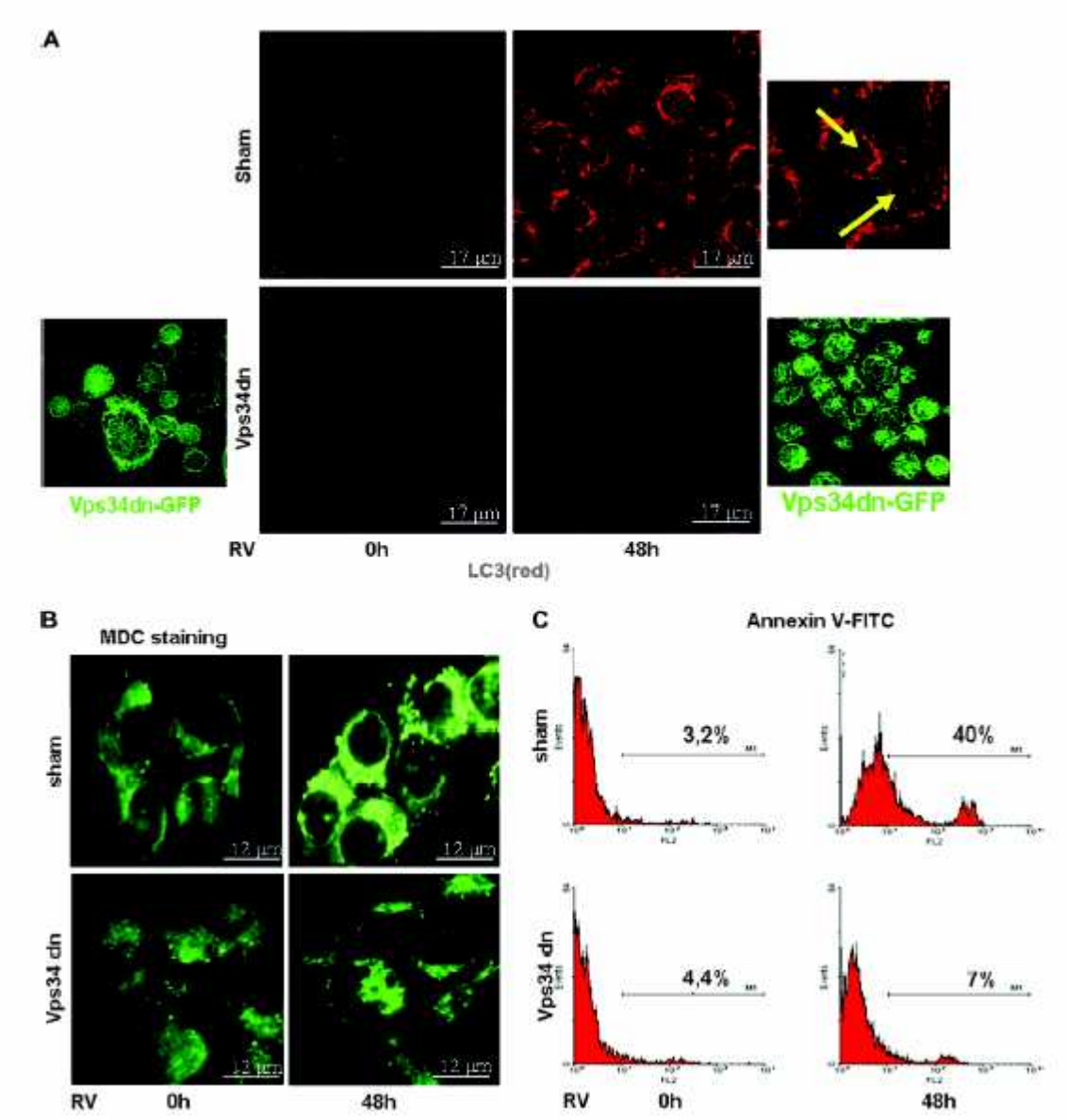
8,8%
14,7%
E
TUNEL
sham
siRNA Beclin1
RV
0h
48h

Figure 4


A
Sham
Vps34dn
Vps34dn-GFP
Vps34dn-GFP
RV
0h
48h
LC3(red)
B
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sham
Vps34 dn
RV
0h
48h
C
Annexin V-FITC
sham
3,2%
40%
Vps34 dn
4,4%
7%
RV
0h
48h

Figure 5

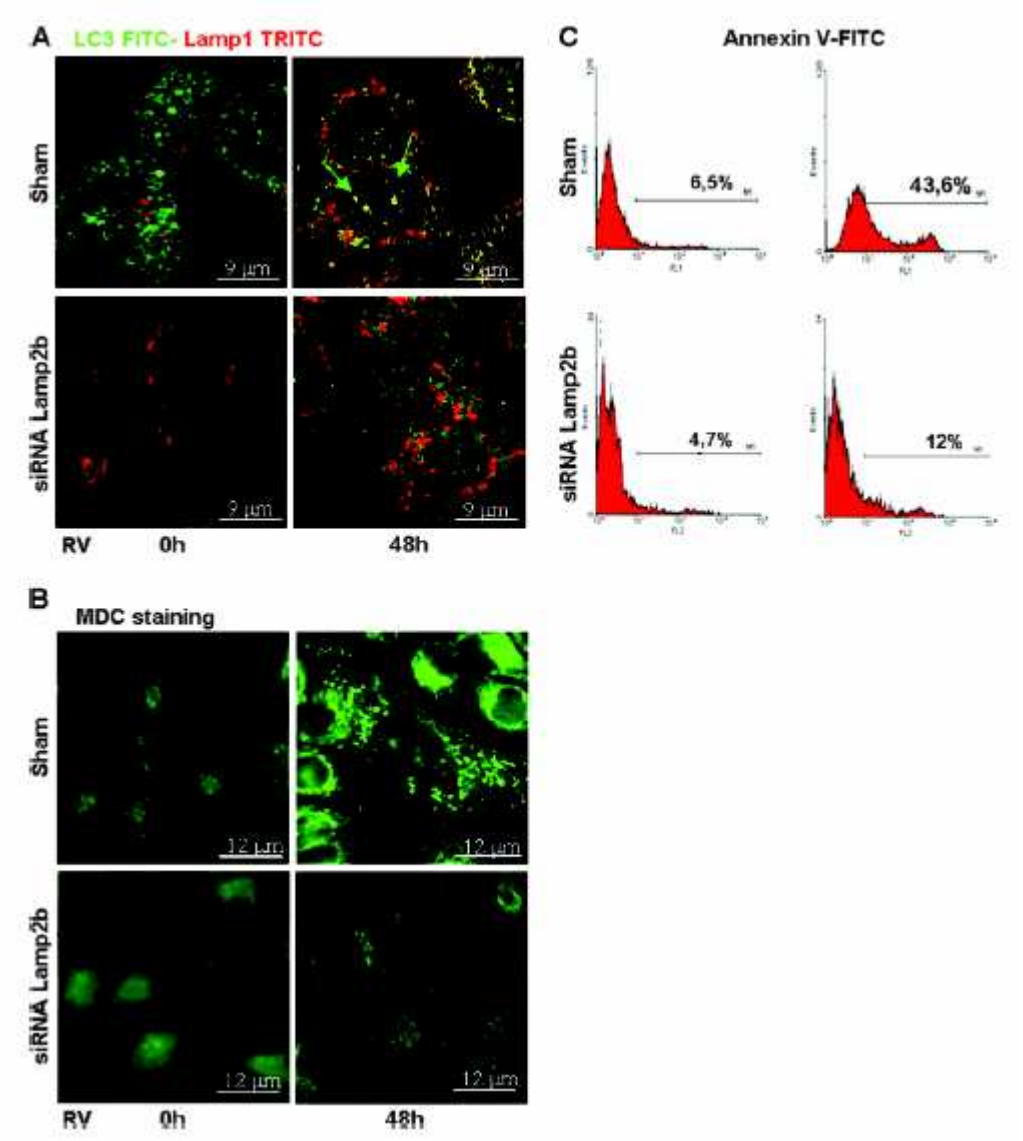
A
LC3 FITC- Lamp1 TRITC
Sham
siRNA Lamp2b
9 µm
RV
0h
48h
B
MDC staining
Sham
siRNA Lamp2b
12 µm
RV
0h
48h
C
Annexin V-FITC
Sham
siRNA Lamp2b
6,5%
43,6%
4,7%
12%

Figure 6

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