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Review

Cell death and autophagy: Cytokines, drugs, and nutritional factors

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ABSTRACT

Cells may use multiple pathways to commit suicide. In certain contexts, dying cells generate large amounts of autophagic vacuoles and clear large proportions of their cytoplasm, before they finally die, as exemplified by the treatment of human mammary carcinoma cells with the anti-estrogen tamoxifen (TAM, ≤1 μM). Protein analysis during *autophagic cell death* revealed distinct proteins of the nuclear fraction including GST-π and some proteasomal subunit constituents to be affected during autophagic cell death. Depending on the functional status of caspase-3, MCF-7 cells may switch between autophagic and apoptotic features of cell death [Fazi, B., Bursch, W., Fimia, G.M., Nardacci R., Piacentini, M., Di Sano, F., Piredda, L., 2008. Fenretinide induces autophagic cell death in caspase-defective breast cancer cells. Autophagy 4(4), 435–441]. Furthermore, the self-destruction of MCF-7 cells was found to be completed by phagocytosis of cell residues [Petrovski, G., Zahuczky, G., Katona, K., Vereb, G., Martinet, W., Nemes, Z., Bursch, W., Fésüs, L., 2007. Clearance of dying autophagic cells of different origin by professional and non-professional phagocytes. Cell Death Diff. 14 (6), 1117–1128].

Autophagy also constitutes a cell's strategy of *defense upon cell damage* by eliminating damaged bulk proteins/organelles. This biological condition may be exemplified by the treatment of MCF-7 cells with a necrogenic TAM-dose (10 μM), resulting in the lysis of almost all cells within 24 h. However, a transient (1 h) challenge of MCF-7 cells with the same dose allowed the recovery of cells involving autophagy. Enrichment of chaperones in the insoluble cytoplasmic protein fraction indicated the formation of aggresomes, a potential trigger for autophagy. In a further experimental model HL60 cells were treated with TAM, causing dose-dependent distinct responses: 1–5 μM TAM, autophagy predominant; 7–9 μM, apoptosis predominant; 15 μM, necrosis. These phenomena might be attributed to the degree of cell damage caused by tamoxifen, either by generating ROS, increasing membrane fluidity or forming DNA-adducts.

Finally, autophagy constitutes a cell's major adaptive (survival) strategy in response to *metabolic challenges* such as glucose or amino acid deprivation, or starvation in general. Notably, the role of autophagy appears not to be restricted to nutrient recycling in order to maintain energy supply of cells and to adapt *cell(organ) size* to given physiological needs. For instance, using a newly established hepatoma cell line HCC-1.2, amino acid and glucose deprivation revealed a pro-apoptotic activity, additive to TGF-β1. The pro-apoptotic action of glucose deprivation was antagonized by 2-deoxyglucose, possibly by stabilizing the mitochondrial membrane involving the action of hexokinase II. These observations suggest that signaling cascades steering autophagy appear to provide links to those regulating *cell number*.

Taken together, our data exemplify that a given cell may flexibly respond to type and degree of (micro)environmental changes or cell death stimuli; a cell's response may shift gradually from the elimination of damaged proteins by autophagy and the recovery to autophagic or apoptotic pathways of cell death, the failure of which eventually may result in necrosis.

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Contents

1. Introduction

Autophagy, in eukaryotic cells, constitutes a degradative mechanism for the removal and the turnover of bulk cytoplasmic constituents via the endosomal–lysosomal system. Early studies revealed autophagy as an adaptive response of cells to nutrient deprivation, i.e. to ensure minimal housekeeping functions (nutrient recycling). In the last decade, a tremendous gain of knowledge on autophagy has been achieved, showing that the function of autophagy, in multicellular organisms, is much more complex as it is involved in physiological processes as diverse as biosynthesis (cvt pathway), regulation of metabolism through the elimination of specific enzymes, morphogenesis, cellular differentiation, tissue remodeling, aging and cellular defense, among others (Dann et al., 2007; Maiuri et al., 2007; Scherz-Shouval and Elazar, 2007; Rubinsztein et al., 2007; Chiang and Abraham, 2007; Kopelovich et al., 2007; Levine and Kroemer, 2008; Mizushima et al., 2008). Briefly, in instances of cell injury or accumulation of damaged organelles/membranes, intracellular inclusions may be transferred to the autophagic pathway, serving as homeostatic mechanism at subcellular scale. Overall, autophagy constitutes a fundamental survival strategy of cells. On the other hand, autophagy has also been linked to programmed cell death (PCD), initiating a controversial discussion on how a suggested role of autophagy in cell suicide might meet with its survival function.

Disturbance of autophagy contributes to the pathogenesis of cancer, liver and immune disease, pathogen infection, myopathies as well as neurodegenerative disorders such as amyotrophic lateral sclerosis, Parkinson's, Huntington's and Alzheimer's disease (Chiang and Abraham, 2007; Kopelovich et al., 2007; Levine and Kroemer, 2008; Rubinsztein et al., 2007; Mizushima et al., 2008).

The present paper addresses autophagy as a means for cells to flexibly respond to environmental changes, ranging from adaptation to shortage in nutrient supply, to defense (survival) against cell injury and, in case adaptive mechanisms are beggared, the manifestation of cell death.

2. Autophagy: a basic mechanism to maintain cell homeostasis

Tightly controlled degradation of surplus cellular components is essential as their biosynthesis for sustaining cell homeostasis and cells own various catabolic pathways to cover a broad range of demands as elimination of small molecules up to complete organelles may become necessary. The *ubiquitin–proteasome* system plays an essential role in the controlled degradation of most short- and long-lived intracellular proteins in eukaryotic cells (Hung et al., 2006; Levine and Kroemer, 2008). *Autophagy* is responsible for the elimination and recycling of bulk cytoplasmic constituents including whole organelles, for instance in case misfolded proteins are sterically hindered to enter the proteasomal pathway. Based on the mechanism of autophagic vacuole formation and delivery of material to this compartment, three types of autophagy can be discriminated: chaperone-mediated autophagy, macro- and microautophagy. *In chaperone-mediated autophagy*, proteins containing a pentapeptide motif related to KFERQ are transported across the lysosomal membrane. The most prominent morphological manifestation of *macro autophagy* is double (or multiple)-membrane bounded vacuoles, the formation of which is highly conserved from yeast to humans (Klionsky et al., 2007, 2008). Briefly, the macroautophagic pathway (for the sake of simplicity herein subsequently referred to as "autophagy") in mammalian cells starts with the sequestration of cytoplasmic material to form an early autophagosome. Current concepts suggest that the membrane of the early autophagosomes derive from specialized membrane cisternae of not yet clarified origin, named "phagophore"; recruitment of membranes from the endoplasmic reticulum and trans-golgi-network may contribute as well (Codogno and Meijer, 2004; Fengsrud et al., 2004). Autophagic vacuoles (autolysosomes) result from the fusion of late autophagosomes with lysosomes; thereby, the final degradation of the sequestered cytoplasmic material is triggered. Cytoskeletal proteins are an integral part of this pathway; the sequestration requires intermediate filaments (cytokeratin and vimentin), the movement and fusion of lysosomes with the late autophagosomes require the microtubular system (Fengsrud et al., 2004). All steps including the final degradation of sequestered cytoplasmic material in autolysosomes are ATP-dependent (Codogno and Meijer, 2004; Fengsrud et al., 2004). *Microautophagy* means that the lysosome itself takes up cytosolic components (including. macromolecules such as glycogen) and organelles by invagination; it appears not to be subjected to metabolic regulation (Wang and Klionsky, 2004).

Typically, cells exhibit a low basal rate of autophagy to maintain homeostasis (kinetic aspects were reviewed by Codogno and Meijer, 2004). Autophagy can be upregulated, for instance, to replenish amino acids and glucose pools for protein synthesis in response to nutrient/growth factor deprivation (nutrient recycling). In recent years, a tremendous progress has been achieved in elucidating the underlying molecular/biochemical events and the reader is referred to recent reviews addressing the transcriptional and translational control of autophagy (Dann et al., 2007; Maiuri et al., 2007; Scherz-Shouval and Elazar, 2007; Rubinsztein et al., 2007; Chiang and Abraham, 2007; Kopelovich et al., 2007; Levine and Kroemer, 2008; Mizushima et al., 2008). Briefly, mammalian target of rapamycin (mTOR) is a major integration site for nutrient responses in eukaryotic cells. For instance, upstream

of mTOR class I PI3K/Akt signaling molecules link receptor tyrosine kinases to mTOR activation, thereby inhibiting autophagy in response to insulin-like and other growth factor signals. A class III PI3K complex including Beclin 1/Atg6/hVps34 controls autophagosome formation. Downstream of mTOR kinase, more than a dozen gene products, referred to as ATG genes, have been found to tightly control initiation and execution of autophagy in yeast and eukaryotic cells (Dann et al., 2007; Maiuri et al., 2007; Scherz-Shouval and Elazar, 2007; Rubinsztein et al., 2007; Chiang and Abraham, 2007; Kopelovich et al., 2007; Levine and Kroemer, 2008; Mizushima et al., 2008). Notably, guidelines for the use and interpretation of autophagocytosis assays taking into account the broad spectrum of morphological and molecular features of autophagy are now available (Klionsky et al., 2008).

3. Autophagy: role in cell death

Cells may use multiple pathways to commit suicide. *Apoptosis* (often and in a broader sense called programmed cell death) means an orchestrated collapse of a cell, staging membrane blebbing, cell shrinkage, chromatin condensation, DNA and protein degradation, accomplished by phagocytosis of corpses by neighbouring cells (Kerr et al., 1972; Wyllie et al., 1980). *Necrosis*, according to the early concept of "apoptosis–necrosis dichotomy", results from violent environmental perturbation leading to the collapse of internal homeostasis, cell swelling and eventually cell lysis; necrosis usually provokes an inflammatory response (Kerr et al., 1972; Wyllie et al., 1980).

In a number of biological settings apoptosis involves the action of caspases as major players. For instance, the typical morphology of apoptosis largely is the end result of caspase-mediated destruction of the cellular architecture (Logue and Martin, 2008). Caspases belong to a large family of highly conserved proteins that have been found in hydra, insects, nematodes and mammals; a number of them constitute a set of sequentially acting "initiator" and "executioner" caspases mediating a wide range of physiological and non-physiological pro-apoptotic signals down to a final coordinated self-destruction of the cell. Mitochondria constitutes a major site for the integration of diverse pro-apoptotic signals ["intrinsic pathway" via caspase-9 activation (apoptosome)] as opposed to "extrinsic pathway", triggered by the activation of caspase-8 via death receptors of the TNF/NGF-family; both pathways join at the level of caspase-3 (Logue and Martin, 2008), but the endoplasmic reticulum (Pizzo and Pozzan, 2007), lysosomes (Stoka et al., 2007) and the trans-golgi-network (Nakagomi et al., 2008) play important roles as well. Thus, each organelle possesses sensors that detect specific alterations, locally activate signal transduction pathways and emit signals that ensure inter-organellar crosstalk.

However, morphological, biochemical and molecular observations revealed that active self-destruction of cells is not confined to apoptosis but cells may use different pathways to commit suicide, thereby severely challenging the initial apoptosis–necrosis dichotomy (Clarke, 1990; Zakeri et al., 1995; Bursch, 2001; Lockshin and Zakeri, 2004a,b; Edinger and Thompson, 2004; Shintani and Klionsky, 2004; Levine and Yuan, 2005; Tolkovsky, 2004). For instance, cell death induced by apoptotic stimuli such as CD95-L or TNF exhibits hallmarks of necrosis under conditions of caspase-inhibition ("programmed necrosis"; Edinger and Thompson, 2004; Festjens et al., 2006). Moreover, caspase-independent cell death may also ensue with the morphology of apoptosis (Lockshin and Zakeri, 2004a,b). Notably, early morphological and histochemical studies revealed no evidence for autophagic or lysosomal events in apoptotic cells *in vivo* (Kerr et al., 1972; Wyllie et al., 1980; Bursch et al., 1985). However, more recently the autophagic–lysosomal compartment has been implicated in the initiation of programmed cell death, either upstream or independent of caspase cascades, often denoted "type II programmed cell death" or "autophagic cell death" (Clarke, 1990; Zakeri et al., 1995; Bursch, 2001; Baehrecke, 2003; Gozuacik and Kimchi, 2004; Lockshin and Zakeri, 2004a,b).

3.1. Autophagic cell death

Since several decades human mammary carcinoma (MCF-7) cells have been used as a biological test system in drug development, namely to select drugs with a strong anti-proliferative potency for the treatment of human mammary tumors (Levenson and Jordan, 1997). In view of cell death it is important to note that the "classical" MCF-7 cells turned out to lack functional caspase-3 (MCF-7/7.0.3 cells; 47 bp deletion in exon 3 of caspase-3 gene (Jänicke et al., 1998; Kurokawa et al., 1999; Yang et al., 2001). An MCF-7 cell subline reconstituted with caspase-3 has been provided some 10 years ago by Jänicke et al. (1998; MCF-7/7.3.28). Others and we have used MCF-7 cells as a model to study the anti-survival effect of anti-estrogens such as tamoxifen, ICI 164384 and toremifene (England and Jordan, 1997; Wakeling et al., 2001). Thus, tamoxifen at high doses (10 μM) caused lysis (necrosis) of almost all cells within 24 h that cannot be prevented by estradiol (Bursch et al., 1996). The cytotoxic action of tamoxifen may result from perturbations in membrane fluidity (Kazanci and Severcan, 2007), formation of reactive oxygen species (Nazarewicz et al., 2007), DNA damage by DNA-adducts or chromosomal aberrations, which have been found to occur in kidney and liver (Han and Liehr, 1992; Sargent et al., 1994). On the other hand, lower concentrations of tamoxifen (1 μM and below) induced a gradual appearance of cell death starting to occur approximately 2–3 days after treatment (Bursch et al., 1996, 2000). This type of cell death is considered to be a receptor-mediated, active cell suicide because it can be inhibited by estradiol (Bursch et al., 1996).[1]

Closer electron microscopy studies revealed that the active self-destruction of MCF-7/7.0.3 cells induced by tamoxifen is characterized by the autophagic degradation of cytoplasmic components including organelles preceding the nuclear collapse, but preservation of cytoskeletal elements until late stages (Bursch et al., 2000). Notably, cytoskeletal elements are necessary for the autophagic process to ensue (Fengsrud et al., 2004). Furthermore, in cells exhibiting a highly condensed nucleus, structures required for protein synthesis such as polyribosomes, ER and golgi have disappeared, whereas a few clusters of intact mitochondria persist in close vicinity to autophagic vacuoles and the nuclear envelope. These observations suggest a certain level of specificity and/or feedback mechanism controlling autophagic degradation and thereby, allowing a coordinated completion of the overall cell death process (Bursch et al., 2004). This hypothesis is in line with the accumulating evidence suggesting that macroautophagy in yeast and mammals eliminates cell constituents not always at random, but may include selection of either ER, or mitochondria, or peroxisomes, or pathogens or protein aggregates for degradation; the underlying mechanisms are just emerging (Codgono et al., 2004;

[1] Historically, denoting cell death as autophagic cell death was based upon electron microscopical demonstration of autophagic vacuoles in dying cells. In addition, dying cells can be characterized by an increasing number of histo- and biochemical criteria indicating a role of the autophagosomal–lysosomal compartment (e.g. involvement of mTOR pathway; occurrence of LC3-II; Klionsky et al., 2008). However, it should be emphasized that referring to the morphological/histochemical features should not imply a causative relationship between autophagocytosis and eventual manifestation of a cell's suicide; this will require either an established functional link between these phenomena and/or elucidation of specifically related genetic/epigenetic events.

Kim et al., 2007; Van der Vaart et al., 2008). The electron microscopy studies were confirmed and extended by histochemical studies with monodansylcadaverine (MDC), which has been described to accumulate in autophagic vacuoles (Biederbick et al., 1995; Klionsky et al., 2008). MDC was used to visualize AVs in MCF-7/7.0.3 cells and to compare the kinetics of AV formation with those of nuclear condensation at the light (fluorescence) microscopy level: AV formation preceded nuclear collapse which is considered to reflect the irreversible stage of cell death (Bursch et al., 1996, 2000). Petrovski et al. (2007) confirmed the kinetics of the occurrence of autophagic vacuoles by light chain-3 (LC3) expression, along with quantification of nuclear collapse. Furthermore, by fluorescence microscopy more than 95% of the dying cells were found to be MDC positive (day 4 after TAM); inhibition of autophagy at day 4 by 3-methyladenine almost completely abolished the MDC-positive staining and, as demonstrated by FACS-analysis, led to a significant drop in the number of annexin-V-positive and annexin-V-positive/PI-positive cells (Petrovski et al., 2007).

However, morphological features as described above are not sufficient to imply a causative relationship between autophagocytosis and eventual manifestation of a cell's suicide. Consequently, a crucial question to be answered is whether autophagy might be just a side effect of the stress imposed on the cells or whether a functional link exists between autophagocytosis and execution of the final death program. Indeed, such a functional link between autophagocytosis and cell suicide was suggested by a number of inhibition experiments with 3-methyladenine, wortmannin and LY294002 (PI3-Kinase inhibitors) (reviewed by Codogno and Meijer, 2004; Bursch et al., 2004). These compounds have been found to prevent both, the formation of autophagic vacuoles and eventual cell death (indicated by nuclear destruction) induced by cytokines, gene(over)expression, drugs, bacterial toxins in a variety of different cell types (reviewed by Bursch et al., 2004). As to 3-MA, it has to be taken into account that the action of 3-MA is not limited to the autophagic but also may affect apoptosis signaling [JNK and p38 kinases; mitochondrial permeability transition pore opening (Tolkovsky, 2004)]. However, studies with autophagy inhibitors other than 3-MA such as wortmannin and LY294002 strongly support functional links between signal transduction pathways for autophagy and those for cell death. Additional support is provided by crosstalk between autophagy signal cascades and those of cell death, as exemplified by cells with deregulated apoptosis machinery (Buytaert et al., 2007; Lefranc et al., 2007; Fazi et al., 2008; Hu and Xuan, 2008). Notably, Yousefi et al. (2006) showed that calpain-mediated cleavage of Atg5 switches autophagy to apoptosis.

3.2. Phagocytosis

The final clearance of apoptotic cell residues by phagocytosis is well understood (Savill and Gregory, 2007; Tanaka and Miyake, 2007), but relatively little is known about the final fate and clearance mechanism of cells dying through autophagic cell death (Petrovski et al., 2007). Therefore, Petrovski et al. (2007) initiated a series of experiments, among which the engulfment of autophagic MCF-7/7.0.3 cells by macrophages ("professional phagocytes") and vital MCF-7/7.0.3 ("non-professional phagocytes") was studied. As demonstrated by FACS-analysis and confocal microscopy, MCF-7/7.0.3 cells undergoing autophagic cell death were engulfed by human macrophages as well as vital MCF-7/7.0.3 cells (Petrovski et al., 2007). MCF-7 cells used mainly the phosphatidylserine (PS) recognition and signaling pathway for phagocytosis of dying cells. Inhibition of autophagy by 3-MA, applied 2 days before the peaking of autophagy, and harvesting these cells for a phagocytosis assay, almost completely abolished the engulfment of autophagic MCF-7/7.0.3 cells by both, macrophages and vital MCF-7 cells. Furthermore, blocking phosphatidylserine by recombinant annexin-V inhibited phagocytosis by vital MCF-7/7.0.3 cells, but not by macrophages (Petrovski et al., 2007). Gene expression profiling of dying MCF-7/.7.0.3 cells upon tamoxifen (autophagic cell death) revealed, for instance, that the expression of the phagocytosis (tethering/tickling) receptors ASGR1 (asialoglycoprotein receptor 1), OLR1 (oxidized low-density lipoprotein 1 receptor; scavenger receptor) and ITGAX (integrin) were increased approximately 10 times above untreated controls (Petrovski et al., 2007).

In conclusion, the final phagocytosis of autophagic MCF-7 cells by professional and non-professional phagocytes adds support to the concept on a concerted action between regulatory elements of autophagy and preparation for final steps in cell death such as the expression of "eat me" signals.

3.3. Protein analyses during tamoxifen-induced autophagic cell death in MCF-7/7.0.3 cells

In addition to studies aimed at establishing functional links between autophagy and cell death, we initiated analyses at protein level to achieve a broader insight into the molecular events associated with autophagic cell death. To investigate the protein synthesis profile, control and tamoxifen-treated MCF-7/7.0.3 cells were metabolically labeled with a mixture of ^{35}S-methionine and ^{35}S-cysteine, *cytoplasmic proteins* were separated by 2D gel electrophoresis and visualized by autoradiography. Fig. 1A and B shows a representative protein synthesis profile at 36 h upon tamoxifen, i.e. shortly before the steep increase in the number of autophagic vacuoles (Bursch et al., 2000; Petrovski et al., 2007). We experienced rather subtle alterations such as proteins modified (marked by rectangles: calreticulin, eif-3B, PCNA, myosin light chain, stathmin), downregulated (marked by hexagons: tubulin alpha, beta and hsp27) or upregulated (marked by circles: hsc70, PDI and ER-ATPase) upon tamoxifen treatment. Notably, the synthesis of lysosomal proteases as exemplified by cathepsin B, D (Fig. 1A and B), providing the "tools" for the eventual degradation of cytoplasmic constituents, is not found to be upregulated. As to caspase-cleavage products, even in late stages after tamoxifen treatment, when 15–25% of cells exhibited chromatin condensation/fragmentation, still no previously identified proteolytic products as typically generated by caspases (Gerner et al., 2000, 2002) became detectable by silver-staining, for instance, no cleavage of cytokeratin in tamoxifen-treated MCF-7/7.0.3 cells (Bursch et al., 2000, 2004), in contrast to caspase-3 reconstituted MCF-7/7.3.28 (data not shown).

We also tackled the question concerning the nuclear destruction in MCF-7/7.0.3 cells lacking functional caspase-3. As demonstrated by the alterations of proteins visualized by silver stain, no significant changes of nuclear proteins were observed during the initial formation of autophagic vacuoles (data not shown). Fig. 1C and D depicts silver-stained 2D gels of proteins of the nuclear fraction of control cells and cells treated with 1 μM tamoxifen for 5 days. Expectedly, we observed a substantial decrease of proteins indicative for an active proliferation status, such as PCNA, hnRNP-U and numatrin B23 (Fig. 1C and D), reflecting a cell cycle arrest induced by tamoxifen. A group of chromatin-associated proteins, including SUPT6H, HA95 and PWP-1, was found likewise decreased (Fig. 2C and D). Intriguingly, the same subset of chromatin-associated proteins has been found decreased at the onset of apoptotic chromatin condensation (Gerner et al., 2000, 2002). The only new spots appearing during autophagic cell death in the nuclear protein fraction corresponded to GST-π and to a group of proteasomal subunit constituents (Fig. 1D).

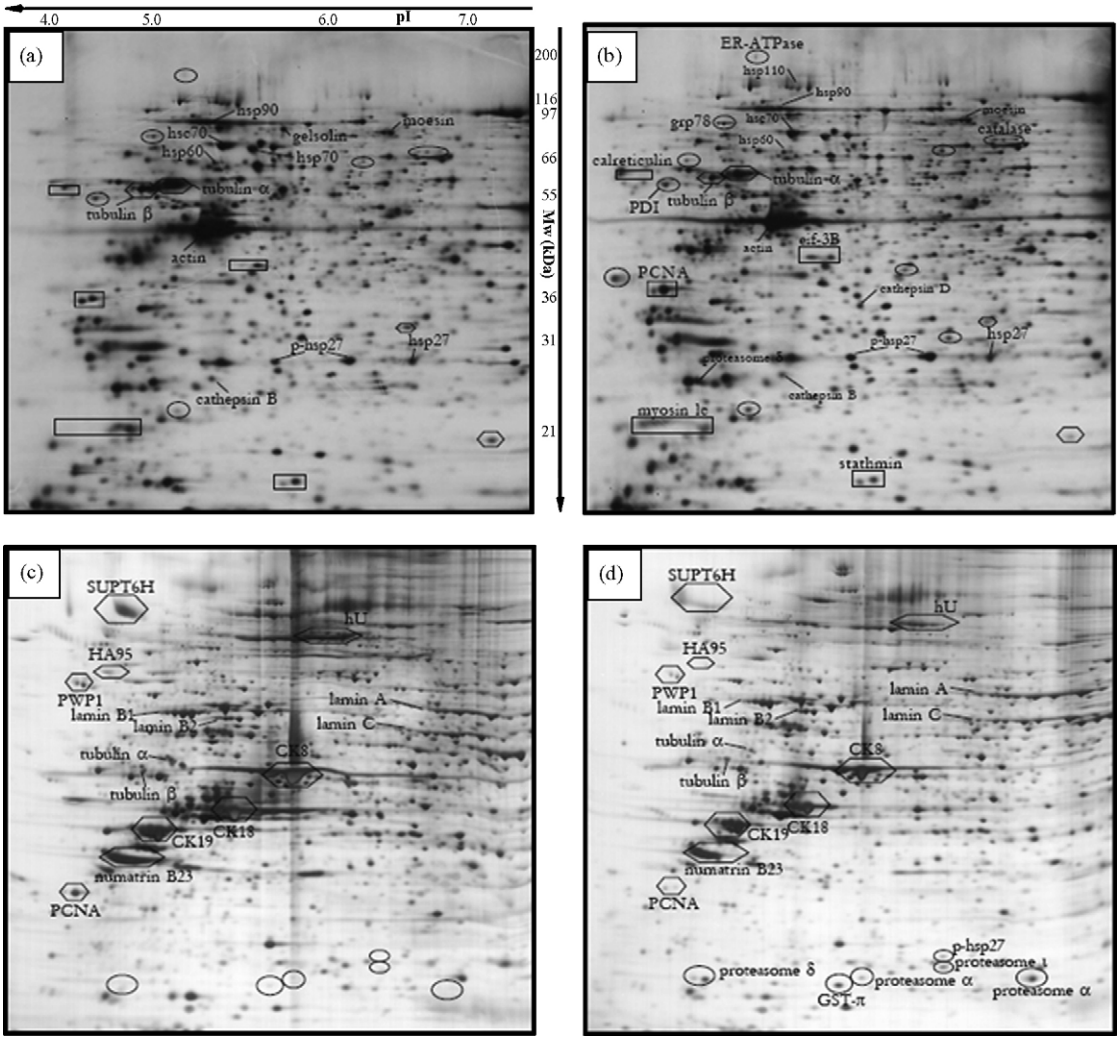

Fig. 1. Protein synthesis profile and nuclear protein alterations of MCF-7/7.0.3 cells upon tamoxifen treatment. Treatment of MCF-7 cells: see Bursch et al., 1996, 2000; (A, B): 2D-autoradiograph[2] of cytosol prepared from 35S-labeled MCF-7 cells untreated (A) or treated (B) with 1 μM tamoxifen for 36 h. Annotation: circles, proteins displaying induced synthesis; hexagons, proteins displaying repressed synthesis; rectangles, proteins modified upon tamoxifen treatment; see text for further explanation. (C, D): Silver-stained 2D gels of the nuclear matrix – intermediate filament fraction of MCF-7 cells untreated (C) or treated (D) with 1 μM tamoxifen for 5 days. Annotation: circles, protein spots displaying higher intensities; hexagons; proteins displaying lower intensities upon tamoxifen treatment; see text for further explanation.

In summary, the analysis of the cytoplasmic fraction revealed only subtle changes in protein synthesis before the onset of the formation of autophagic vacuoles. As to proteins of the nuclear fraction, some are cleaved exclusively during apoptosis (of Jurkat cells) but not during autophagic cell death, for example lamin B and scaffold attachment factor; other nuclear proteins are cleaved during apoptosis as well as autophagic cell death such as SUPT6H, HA95 and PWP-1. Likewise, autophagic and apoptotic PCD seem to share the cell's "stress response" as indicated by the translocation of heat shock protein-90. Overall, these data suggest the coincident occurrence of proteins attributable to autophagic as well as apoptotic pathways. From a teleological point of view, it is tempting to speculate that such an equipement allows a given cell to flexibly respond to environmental changes, either physiologically or pathologically.

3.4. Caspase-3 constitutes a switch between autophagic or apoptotic cell death pathway

As outlined above, MCF-7/7.0.3 cells are deficient of functional caspase-3 and accumulating evidence suggests that this is responsible for the absence of "classical" apoptotic features during tamoxifen-induced cell death (Jänicke et al., 1998; Fazi et al., 2008). Indeed, tamoxifen treatment of caspase-3 expressing MCF-7/7.3.28 cells resulted in cell death displaying essentially all classical features of apoptosis (see above). In a closer series of experiments with defective caspase-3 (MCF-7/7.0.3) as well as functional caspase-3 (MCF7/7.3.28) cells, Fazi et al. (2008) evaluated the ability of fenretinide to induce cell death in tumor cells. Fenretinide, a synthetic derivative of retinoic acid, promotes growth inhibition and induces apoptosis in a wide range of tumor cell types (Fazi et al., 2008). Fenretinide was found to trigger an autophagic cell death pathway in cells with non-functional caspase-3 (MCF-7/7.0.3) as demonstrated by an increase in Beclin 1 expression, the conversion of the soluble form of LC3 to the autophagic vesicle-associated form LC3-II and its shift from diffuse to punctuate staining and finally, an increase in lysosomes/autophagosomes. Moreover, the

[2] Cell fractionation, metabolic labeling with ^{35}S-methionine/^{35}S-cysteine, two-dimensional gel electrophoresis and autoradiography, evaluation of 2D data including protein identification were described in detail previously (Gerner et al., 2000, 2002).

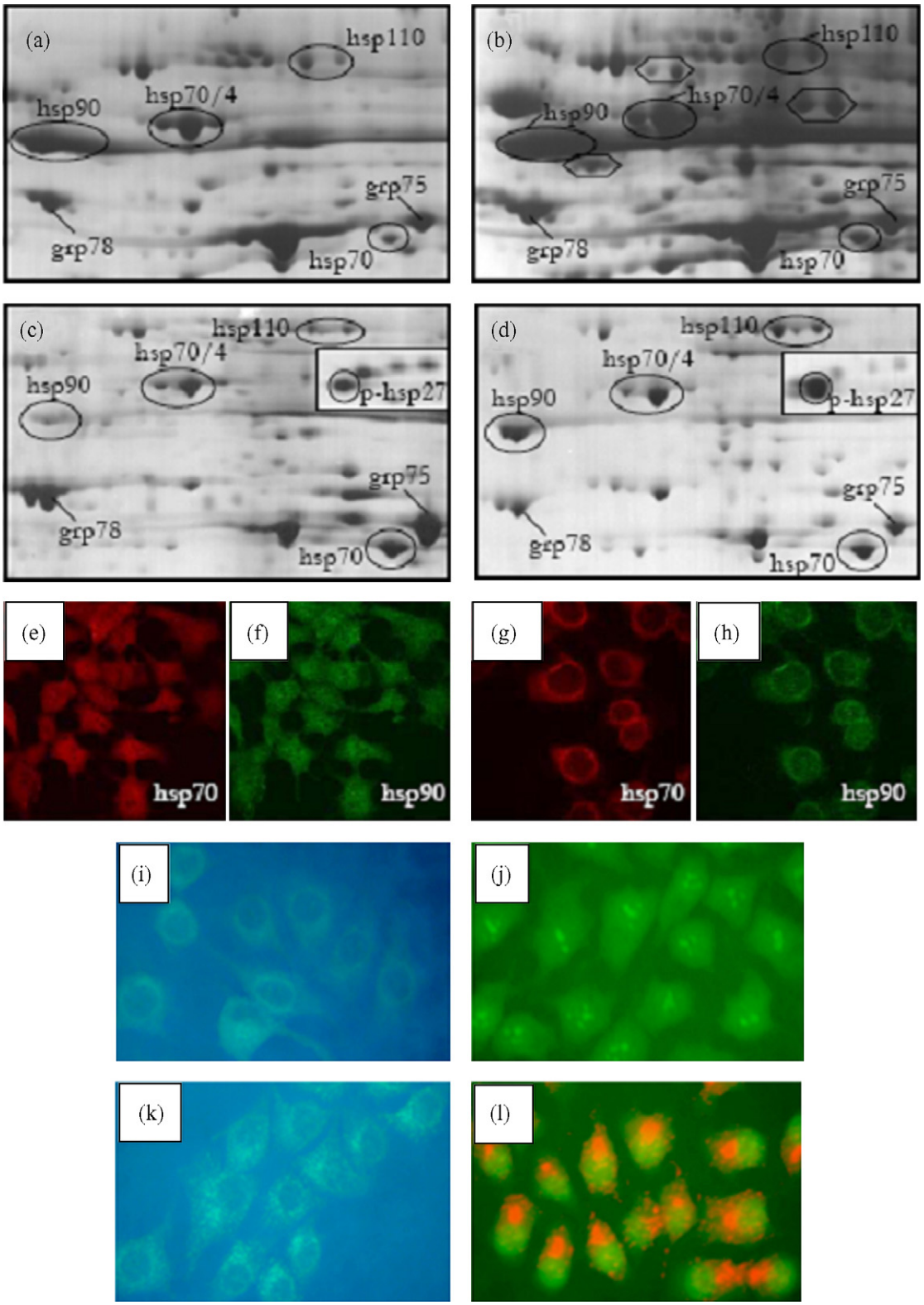

Fig. 2. Formation of protein complexes enriched in chaperones and autophagic vacuoles in apoptotic Jurkat cells as well as after toxic injury of MCF-7/7.0.3 and MCF-7/7.3.28 cells. *Jurkat cells* (A, B) were treated with 50 ng/ml anti-Fas antibody to induce apoptosis as described previously (Gerner et al., 2000). After 5 h, cells were lysed and the insoluble cytoplasmic protein fraction analyzed by 2DE. The insoluble protein fraction was obtained by the centrifugation of cell lysates at 100 g and subsequently, the pellets were dissolved in 10 M urea, 4% CHAPS, 0.5% SDS, 100 mM dithiothreitol supplemented with 2% (v/v) ampholyte, pH 7–9. Isoelectric focusing of 70 μg protein samples was performed at 15.500 V–h in a stepwise fashion (2 h at 200 V; 3 h at 500 V; 17 h at 800 V) in 4% acrylamide (Gerbu, Gaiberg, Germany), 0.1% piperazine diacrylamide (Bio-Rad) 1.5 mm × 16-cm tube gels. The gel buffer contained 1.55% CHAPS and 0.52% Nonidet P-40 and 2% ampholytes (Merck) (1 volume pH 3.5–10; 1 volume pH 4–8; 2 volumes pH 5–7). Degassed 20 mM NaOH served as catholyte, and 6 mM H_3PO_4 served as anolyte. For SDS–polyacrylamide gel electrophoresis the extruded tube gels were placed on top of 1.5-mm thick 12% polyacrylamide slab gels. After a 3-min equilibration with 2.9% SDS, 70 mM Tris–HCl, 0.003% bromphenol blue, the gels were run at 15 °C in electrode buffer (0.1% SDS, 25 mM Tris–HCl, pH 8.3, 200 mM glycine) (Gerner et al., 2000). 2DE gel sections of control (A), anti-Fas antibody (B). Note the enrichment of the

effect of fenretinide was found to be specific since treatment with other chemotherapeutic agents (cisplatin and etoposide) does not result in the induction of autophagic cell death program (Fazi et al., 2008). By contrast, caspase-3 reconstituted MCF-7 cells showed apoptotic cell death features in response to fenretinide treatment (Fazi et al., 2008). These data strongly suggest that, depending on the functional status of caspase-3, MCF-7 cells may switch between autophagic and apoptotic features of cell death. In view of cancer therapy it should be emphasized that fenretinide does not invariably elicit an apoptotic response, but it is able to induce autophagy when apoptotic pathway is deregulated.

4. Autophagy: defense (survival) mechanism upon cell injury

Autophagy also constitutes a cell's strategy of defense upon cell damage. Protein analysis of "classical" apoptosis, namely anti-Fas treated Jurkat cells, revealed the accumulation of some chaperones together with proteolytic products generated by caspases (Fig. 2A and B). Protein aggregates enriched in chaperones are reminiscent to aggresomes, which may activate the autophagic clearance mechanism (Grune et al., 2004; Terman et al., 2007). The similar accumulation of chaperones in aggregates during autophagic cell death (cf. Fig. 1) prompted us to investigate whether a *short-time high-dosage* tamoxifen treatment would trigger the formation of AVs but allow the cells to survive. Previous studies have shown that exposure of MCF-7/7.0.3 cells to dose of 10 μM tamoxifen resulted in massive necrosis within less than 24 h (Bursch et al., 1996). Therefore, in a first approach MCF-7/7.0.3 cells were challenged with 10 μM tamoxifen for 1 h. Indeed, the transient challenge allowed MCF-7/7.0.3 cells to recover involving an upsurge of autophagic vacuoles (data not shown). Analysis of the *insoluble cytoplasmic protein fraction* after 1-h treatment with 10 μM tamoxifen revealed a massive accumulation of chaperones (Fig. 2D), phosphorylation of hsp27 (Fig. 2D) and perinuclear accumulation of hsp70 and hsp90 (Fig. 2G and H). These data strongly suggest that tamoxifen treatment triggered the formation of aggresomes. Notably, MCF-7/7.3.28 cells responded in the same way as MCF-7/7.0.3 to transient exposure to the necrogenic tamoxifen dose as based upon evidence for aggresome formation (not shown). The formation of autophagic vacuoles was indicated by the punctuated pattern upon MDC- or acridine orange staining (Fig. 2I and J). Note the absence of necrosis (cell lysis) 23 h after the transient toxic challenge (Fig. 2K and L), strongly suggesting recovery from cell damage. Furthermore, the occurrence of aggresomes and autophagic vacuoles appeared to enuse independent of caspase-3 function.

In a further experimental model HL60 cells were treated with tamoxifen, causing dose-dependent distinct responses. In part, these may be attributed to the degree of cell damage caused by tamoxifen, either by generating ROS, increasing membrane fluidity or forming DNA-adducts (see above). Thus, 1 μM tamoxifen-induced autophagy within 4 h, as monitored by MDC-staining (Fig. 3A). Application of higher tamoxifen concentrations (3–9 μM) resulted in less prominent vacuolization, but instead favoured apoptotic chromatin condensation within the same period of time (Fig. 3A). Further elevation of tamoxifen concentrations resulted in cell lysis (necrosis) of HL60 cells, as detected by trypan blue exclusion assay (Fig. 3A). In addition, cell lysates of tamoxifen-treated HL60 cells were investigated for the formation of the 85 kDa PARP cleavage product, a sensitive marker for apoptotic caspase activity (Duriez and Shah, 1997). Caspase-mediated PARP processing was undetectable in untreated cells as well as in cells incubated with 1 μM tamoxifen. Application of higher tamoxifen concentrations (3–9 μM) resulted in an increased formation of the 85 kDa PARP cleavage product. At 15 μM, no more PARP cleavage product was detectable indicating rapid lytic cell death without the activation of caspases (Fig. 3B). Finally, assays for (oligo)nucleosomal DNA degradation paralleled these findings; cells treated with 15 μM tamoxifen barely displayed (oligo)nucleosomal DNA fragments (Fig. 3C). In conclusion, time-limited application of low tamoxifen concentrations resulted in the formation of autophagic vacuoles. Higher concentrations of tamoxifen shifted autophagy to apoptosis and subsequently, to necrosis.

Taken together, our findings on MCF-7 cells pulse-challenged with a necrogenic dose as well as the qualitatively different responses of HL60 cells to increasing tamoxifen doses suggests a rather smooth transition between autophagic and apoptotic cell death, in line with our conclusion on the protein data. Thus, an intriguing observation was the accumulation of molecular chaperones including phosphorylated hsp27 in the insoluble cytoplasmic protein fraction, indicative of the generation of aggresomes. Molecular chaperones are designed to efficiently bind to non-native proteins (Hohfeld et al., 2001; Calderwood and Ciocca, 2008; Lanneau et al., 2008). It may be assumed if the amount of denatured protein generated reaches a certain threshold level exceeding the capacity of proteasomal destruction of the protein-waste, the non-native proteins may assemble with the chaperones to larger complexes, the aggresomes (Grune et al., 2004; Terman et al., 2007; Pandey et al., 2007). As to MCF-7 cells, our observations suggest that these events may ensue independent of caspase-3 activity. In a cell's attempt to clear accumulated non-native proteins, the generation of autophagic vacuoles might be triggered; this interpretation implies a rescuing function of autophagy. This conclusion is in line with a large body of evidence showing that autophagy may serve as defense mechanism in clearing cells from damaged constiutents and for instance, to bypass cancer drug resistance due to apoptosis deregulation (Hetz, 2007; Moretti et al., 2007; Lefranc et al., 2007; Hu and Xuan, 2008; Buytaert et al., 2008; Moore, 2008). On the other hand, inhibition of autophagic defense may accelerate the apoptotic cell death pathway (González-Polo et al., 2007). Finally, fenretinide-induced cell death may ensue by either the autophagic or the apoptotic pathway, depending on the functional availability of caspase-3 (Fazi et al., 2008). Notably, fenretinide treatment is associated with ER-stress response (Corazzari et al., 2007; Fazi et al., 2008), most probably triggering autophagy, which eventually may result in either survival or cell death. Overall, the present data underline the significance of autophagy in a cell's response to toxic injury. Based on the biological conditions studied in our experiments, two key elements steering the final fate of cells could be identified, namely the degree of cell damage as well as the functional availability of caspase-3; it may be anticipated that there are much more.

chaperones hsp70, hsp70/4, hsp90 and hsp110 in the apoptotic sample (B). Hexagons indicate caspase-generated proteolytic products of myosin heavy chain. *MCF-7/7.0.3 cells* (C–H) were treated with 10 μM tamoxifen for 1 h; the insoluble cytoplasmic protein fraction was analyzed by 2DE subsequently. 2DE gel sections of controls (C) and 10 μM tamoxifen (D) are shown. Note the enrichment of the chaperones hsp70, hsp70/4, hsp90 and hsp110 and phosphorylated hsp27 (inlet) in the sample induced to undergo autophagy (D). Immunofluorescence analysis of tamoxifen-treated MCF-7 cells exhibit accumulation of the chaperones hsp70 and hsp90 in the perinuclear region (G, H) in contrast to controls (E, F). *MCF-7.3.28 cells* (I–L) were treated with solvent (I, J) or 10 μM tamoxifen (K, L) for 1 h and were examined 23 h thereafter. Cells were stained with monodansylcadaverin (I, K; mainly labeling later stages in the degradation process; Klionsky et al., 2008), or stained with acridine orange (J, L; primarily detecting lysosomes; Klionsky et al., 2008).

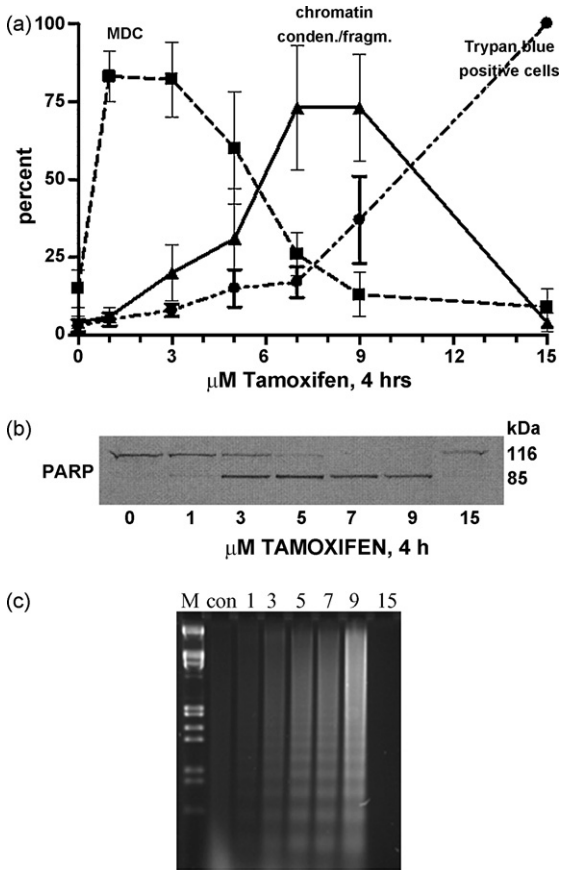

Fig. 3. Effect of increasing tamoxifen concentrations on HL60 cells. HL60 cells were treated with 0, 1, 3, 5, 7, 9 and 15 μM tamoxifen and incubated for 4 h in RPMI medium supplemented with 1% FCS[3]. (a) Percent cells exhibiting signs of autophagy as determined by MDC-staining, of apoptosis as determined by Hoechst 33258-staining and of necrosis as determined by the trypan blue assay. (b) Anti-PARP Western blot of whole cell lysates of HL60 cells as described elsewhere; (c) (oligo)nucleosomal DNA-fragmentation of HL60 cells treated with tamoxifen as indicated, M = marker; con = control; (b) and (c) according to standard protocols. S.E.M values given were determined from three independent experiments.

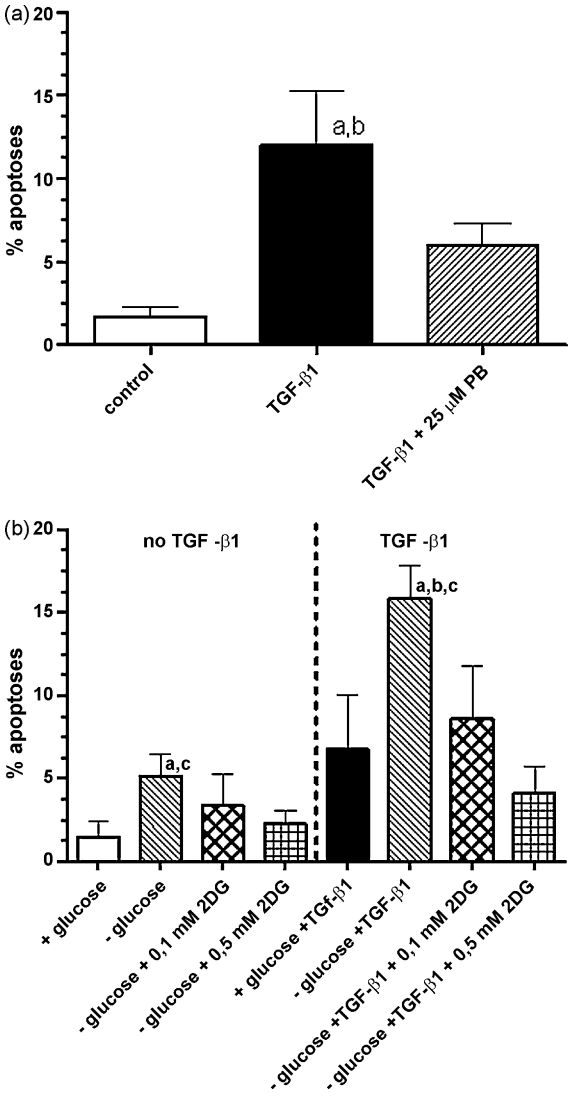

Fig. 4. Apoptosis of human hepatoma cells (HCC-1.2): effect of TGF-β1, phenobarbital and glucose withdrawal.[4] (a) Induction of apoptosis in human hepatoma cell culture (HCC-1.2) by TGF-β1 (10 ng/ml) and its inhibition by phenbarbital (25 μM), 48 h after treatment; (b) effect of glucose deprivation on apoptotic activity in the absence or presence of TGF-β1 and antagonistic action of 2-deoxyglucose (2-DG), 24 h after treatment. Mean ± S.D. of 3–6 experiments are given, statistical comparisons were performed using Student's *t*-test. $p < 0.05$, upper panel: a, TGF-β1 vs. control; b, TGF-β1 +Pb vs. TGF-β1. Lower panel: a, −glucose vs. +glucose; b, −glucose vs. −glucose + 0.1 mM 2DG; c, −glucose vs. +0.5 mM 2DG, either without or with TGF-β1.

5. Autophagy: response to metabolic challenges. Link to nutritional control of apoptosis?

Autophagy also constitutes a cell's major adaptive (survival) strategy in response to *metabolic challenges* such as glucose or amino acid (AA) deprivation, or starvation in general. Mammalian cells respond to nutrient deprivation by downregulating the energy consuming processes, such as proliferation and protein synthesis, and in turn, by stimulating catabolic processes such as autophagy; class III PI3-kinase (hVps34), mTOR and p70 S6 kinase (S6K1) constitute central components of the nutrient sensing apparatus (Wullschleger et al., 2006; Levine and Kroemer, 2008; Mizushima et al., 2008). Autophagic death upon glucose withdrawal involving IGF-I/PI3-kinase/mTOR/S6K has been

[3] HL60 cells were routinely cultivated in RPMI-1640 supplemented with 10% fetal calf serum (FCS). HL60 cells were washed in serum-free medium, seeded at a density of 10^5 cells/ml and treated with the respective drugs in medium containing 1% FCS. MDC- and nuclear condensation/fragmentation-assay was performed as described previously (Bursch et al., 2000). Trypan-Blue (Sigma, St. Louis, MO, USA) exclusion assay was performed to determine cellular viability. For Western analysis, cell lysates were prepared at the indicated times and aliquots separated using 7.5% acrylamide gels under standard SDS–PAGE conditions. Subsequent to protein transfer to a nitrocellulose membrane, immunoblotting was performed with mouse monoclonal anti-PARP (1:2000, Zymed Laboratories). For the detection of DNA fragments, HL60 cells were lysed and the supernatant precipitated. After treatment with RNAse and Proteinase K, DNA was extracted, separated by 1.5% agarose gel electrophoresis visualized under UV light after staining with 0.5 μg/ml of ethidium bromide (Stratagene).

[4] Stock cultures: HCC-1.2 cells were grown in T75 bottles containing 10 ml RPMI/10% FCS medium at 37 °C. For the apoptosis assay, 1×10^5 cells were seeded in 3.5Ø dishes for 24 h in RPMI-medium containing 10% FCS to allow for recovery attachment; for controls and each experimental variable 2–3 dishes were used. Subsequently, FCS was removed for 48 h before commencing treatment with cytokines and/or nutritional modulation. TGF-β1 treatment and quantitative analysis of cell death as described previously (Hufnagl et al., 2001). Glucose deprived medium is equivalent to RPMI1640 without glucose addition; a stock solution of 2-deoxyglucose (500 mM, Sigma D8375) was prepared in RPIMI1640 medium without glucose and added directly to the culture dish to obtain the final concentrations as indicated in the figure.

reported to be induced in cardiomyocyte-derived H9c2 cells, human hepatoma cells (HepG2) and Chinese hamster-ovary (CHO)-derived cells (Aki et al., 2003; Byfield et al., 2005). Notably, death of CHO-cells triggered by glucose deprivation may result in autophagic as well as apoptotic pathways (Hwang and Lee, 2008).

In this context, the interaction of nutritional signals with the growth regulatory network of rat liver *in vivo* is of interest. For many years we have studied liver growth regulation, namely cell proliferation, and apoptosis (for review: Schulte-Hermann et al., 1995). One of our prominent *in vivo* findings was that the cytokine transforming growth factor β1 (TGF-β1) constitutes a major regulator of apoptosis in rat liver, acting in concert with liver tumor promoter as well as nutritional factors to maintain liver cell number homeostasis. As to hepatocarcinogenesis, liver tumor promoter (e.g. phenobarbital) shifts the ratio between cell birth and cell death in favour of cell birth, in particular in preneoplastic lesions of rat liver; thereby, cancer development is accelerated. The opposite applies to feed restriction resulting in cancer prevention (for review: Schulte-Hermann et al., 1995; Rodgarkia-Dara et al., 2006). In addition, the treatment of rats with TGF-β1 *in vivo* induced apoptosis in preneoplastic lesions of the liver, resulting in their rapid regression (Müllauer et al., 1996). These observations prompted us to tackle the underlying mode(s) of action (MOA), particularly in view of potential interactions between liver tumor promoter, TGF-β1 and nutrition (glucose and amino acid supply). Thus, signaling pathways steering autophagy came into the focus of our interest. In a first approach, along with the general need for alternative test models in toxicology, we searched for a liver cell culture system yielding a high concordance with our *in vivo* findings. Recently, Sagmeister et al. (2008) succeeded in establishing a new human hepatoma cell line denoted HCC-1.2 meeting this requirement. HCC-1.2 cells revealed to be sensitive towards the pro-apoptotic action of TGF-β1 (Fig. 4A). Apoptosis of HCC-1.2 cells was found to ensue via the intrinsic pathway as demonstrated by caspase analysis (Western blot analysis revealed cleavage of caspase-3 and 9, but not of caspase-8; data not shown). Furthermore, TGF-β1 induced apoptosis was inhibited by the liver tumor promoter phenobarbital (Fig. 4A). As to nutritional factors, glucose deprivation exerted a pro-apoptotic effect, additive to TGF-β1 (Fig. 4B). The pro-apoptotic action of glucose deprivation was antagonized by 2-deoxyglucose (2-DG; Fig. 4B), possibly by stabilizing the mitochondrial membrane involving the action of hexokinase II (Majewski et al., 2004a,b; Pastorino et al., 2005; Byfield et al., 2005; Robey and Hay, 2006). As the mitochondrial membrane stabilizing action of 2-DG has been reported to depend on its phosphorylation by hexokinase, we also tested the effect of the 5-thioglucose (5-TG). This substrate analogue is an even stronger inhibitor of glycolysis than 2-DG, but 5-TG practically is non-phosphorylatable by hexokinase (hexokinase-K_m 2-DG: 0.027 mM, 5-TG: 4 mM; Dills et al., 1981; Gottlob et al., 2001). Indeed, results of preliminary experiments suggest that 5-TG (0.5 mM) did not reduce apoptotic activity under glucose deprivation (data not shown). Taken together, these observations are in line with the concept suggested by Robey and Hay (2006). In addition to the potential role of mitochondria, evidence suggesting the involvement of the mTOR/S6K1 pathway in the induction of cell death upon glucose withdrawal has been reported (Hwang and Lee, 2008). Finally, deprivation of branched chain-amino acids (BCAA), but also of other amino acids (AA)), exerted a pro-apoptotic activity on HCC-1.2 cells; AA-deprivation and TGF-β1 appear to act additively (Mayer et al., 2007).

In summary, our observations suggest that HCC-1.2 cells constitute a valid test system as it meets well with prerequists for further studies tackling the MOA of liver tumor promoter, TGF-β1 and nutritional factors such as glucose and amino acid supply. Furthermore, our observations provided evidence for crossroads linking autophagy signaling in response to nutrient deprivation to apoptosis.

6. Conclusions

The present complication revealed a wide scope of biological functions of autophagy:

1. Autophagy constitutes a cell's major adaptive strategy in response to *metabolic challenges* such as glucose or amino acid deprivation, or starvation in general. Notably, the role of autophagy appears not to be restricted to nutrient recycling in order to maintain energy supply of cells and to adapt *cell(organ) size* to given physiological needs. Activation of autophagy signaling cascades also appears to provide links to the *regulation of cell number*.
2. Autophagy constitutes a cell's strategy of *defense* upon cell damage by eliminating damaged bulk proteins/organelles; a cell's repair capacity appears rate limiting for progression to cell death (see below). The generation of autophagic vacuoles may be therefore considered as a valuable parameter of toxic insult strength.
3. In certain contexts, dying cells generate large amounts of autophagic vacuoles and clear large proportions of their cytoplasm, before they finally die. It appears likely that the formation of autophagic vacuoles before the manifestation of cell death reflects the cells efforts to survive a life-threatening challenge. However, depending on the degree of cell damage this defense (repair) mechanism may become exhausted and cells proceed to commit autophagic cell death, including phagocytosis. Another key element in controlling autophagic and apoptotic cell death pathways turned out to be the functional availability of caspase-3. From a teleological point of view, it is tempting to speculate that caspase-driven apoptosis with its precise and selective proteolysis of relatively few but crucial proteins may be completed much more rapidly than a caspase-independent autophagic cell death. The advantage of the rapid completion of an apoptotic pathway might be facilitating the preservation of the tissue structure. On the other hand, the advantage of an autophagic pathway would be to contribute to the removal of large masses of cytoplasm/cells and thereby, to cope with the demands on tissue remodeling. As the intrinsic apoptosis pathway is frequently deregulated in human cancer cells, which eventually may give raise for the resistance of cancer to radiation and chemotherapy, pharmacological concurrent targeting of autophagy- and apoptosis-signaling pathways in anti-cancer therapy might greatly improve their efficiency.

Conflict of interest

None.

Acknowledgements

Because of the rapid progress in autophagy and cell death research with its large number of publications, it is not possible to cover all in detail. Therefore, we referred to review articles whenever possible and apologize to the authors whose original publications are not cited directly. Parts of the study were supported by Herzfelder'sche Familienstiftung.

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