The role of autophagy in cancer – characterization of crosstalk between apoptosis and autophagy; autophagy as a new therapeutic strategy in glioblastoma

ABSTRACT

The Noble Assembly decided to award the 2016 Nobel Prize in Physiology or Medicine to Yoshinori Ohsumi for his discoveries of mechanisms of autophagy, a fundamental process for degrading and recycling cellular components. His discoveries opened a path to understanding the fundamental importance of autophagy in many physiological processes, such as adaptation to starvation or response to infection. Mutations in autophagy genes can cause disease, and the autophagic process is involved in several conditions including cancer and neurological disease. It shows the importance of autophagy research, as many questions remain open. The main aim of our research presented here was to better understand the role of autophagy in cancer. Here, we present articles concerning correct monitoring autophagy in cancer cells, characterization of the molecular links between autophagy and apoptosis and analysis of autophagy as a new therapeutic strategy in glioma.

INTRODUCTION

The term *autophagy* derived from the Greek meaning "self-eating", was first coined by Christoph de Duve over 50 years ago, and was largely based on the observed degradation of intra-cellular structures within lysosomes. Research reveals that autophagy is a highly conserved process that occurs in yeast, plant, and mammalian cells. In mammalian cells, there are three primary types of autophagy: microautophagy, macroautophagy and chaperone-mediated autophagy. Of the three types of autophagy, macroautophagy has been studied the most exclusively. Macroautophagy (referred to here as autophagy) is an intracellular process, in which cytoplasmic components, including long-lived or malfunctioning proteins and obsolete or damaged organelles are sequestered within double-membraned vesicles called autophagosomes. The complete autophagosome matures by fusion with an endosome and/or a lysosome to form a single-membraned autolysosome, in which the contents are digested by hydrolases and recycled for biosynthesis in the cell [1,2] (Figure 1A). Sequestration of cytosolic components can be either nonspecific (the engulfment of bulk cytoplasm), or selective, targeting specific cargoes, such as organelles (mitochondria, endoplasmic reticulum) or invasive microbes.

Autophagy is important for a wide range of physiological processes. Autophagy occurs at a low basal level in most cells to perform homeostatic functions (cytoplasmic and organelle turnover) but is also a critical mechanism for the adaptation of cells to stress. It can be induced by numerous stimuli, including nutrient deprivation, accumulation of misfolded proteins, protein aggregates, hypoxia or toxic compounds [3,4]. Although autophagy is thought to be predominantly a cell-survival mechanism, some evidence points towards a role in cell death. In some context autophagy, as distinct from type I programmed cell death (apoptosis), is called as the type II programmed cell death or autophagic cell death. We need to be aware that this term simply describes cell death with autophagy rather than cell death by autophagy [5]. Perhaps the most fundamental point is that either too little or too much autophagy can be deleterious, that is exemplified by its dual role in cytoprotection and cell death. Various forms of cell death were for long time studied in isolation, as the prevailing model suggested that they represented mutually exclusive cellular states. However, over the last decade studies suggest that apoptosis, autophagy and necrosis are often regulated by similar pathways, engage common sub-cellular sites and organelles, and even share the same initiator and effector molecules [6,7]. Autophagy is also induced by many apoptotic stimuli [8]. The mechanisms by which apoptotic stimuli activate autophagosome formation and how autophagy machinery contributes to cell death were not well defined ten years ago. Therefore, our studies focused on the regulatory ties between autophagy and apoptosis [9,10].

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Abbreviations: ATG – autophagy-related genes; CsA – cyclosporine A; ER – endoplasmic reticulum; GFAP – glial fibrillary acidic protein; IRE – inositol-requiring enzyme 1; MAP1-LC3 – microtubule-associated protein 1 light chain 3; mTOR – mechanistic target of rapamycin; PARP – poly-ADP-ribose polymerase; PERK – PKR-like ER kinase; TMZ – temozolomide

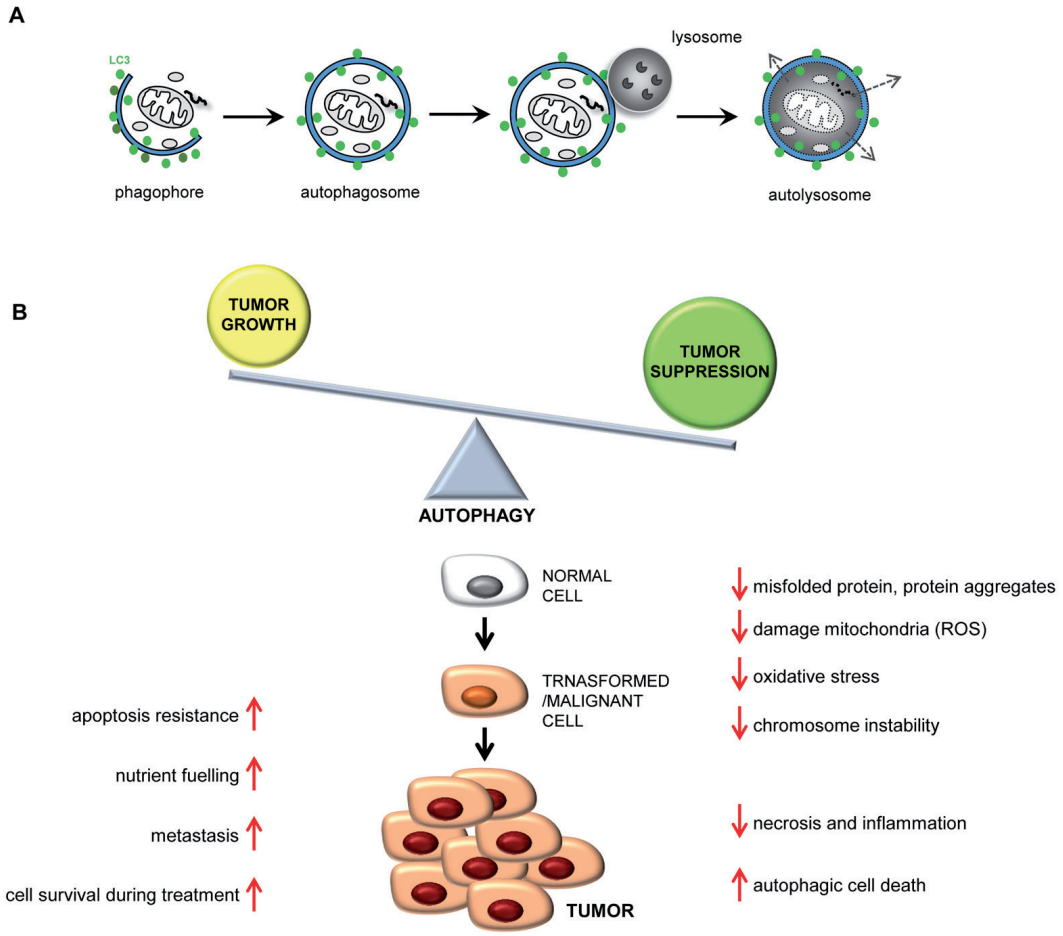

Figure 1. Autophagy and its paradoxical role in cancer.

(A) Autophagy is a highly conserved cellular degradation process in which portions of cytosol and organelles are sequestered into a double-membrane vesicle, an autophagosome, before delivered to lysosome for breakdown and eventual recycling of the resulting macromolecules. (B) At the early stages of tumor, autophagy acts as a tumor suppressor through preventing accumulation of toxic cellular waste (misfolded protein, protein aggregates, defective organelles, source of oxidative stress), reducing chromosome instability, inhibiting necrosis and inflammation. Additionally, induction of autophagy by treatments may lead to autophagic cell death. On the other hand, during tumor progression, autophagy can contribute to tumor growth by increasing resistance to apoptosis, metastasis and survival during therapy. Autophagy also provides metabolic substrates to support cellular metabolism.

Autophagy has been implicated in several human diseases, including cancer [11-13]. For tumor cells, autophagy is a double-edged sword. On one hand autophagy can be tumor suppressive through the elimination of oncogenic protein substrates, toxic unfolded proteins, damaged organelles and prevention of chromosomal instability. But on the other hand, it can be tumor promoting in established cancers through autophagy-mediated intracellular recycling that provides substrates for metabolism, and maintains the functional pool of mitochondria [14] (Figure 1B). Some data indicate that the role of autophagy in tumorigenesis is tissue and genetic context-dependent. Recently, autophagy has become one of the most attractive targets in designing novel anticancer treatments, since most chemotherapeutics induce cellular damage that triggers autophagy, but the impact of autophagy on tumor cell death or survival is unclear. Therefore in our studies, we try to understand the paradoxical role of the autophagy in cancer, in particularly in brain tumors [15-18].

Autophagy is regulated by a complex network that consists of different signaling pathways and a number of highly conserved autophagy-related genes (*ATGs*). Recent studies have described that besides transcriptional factors and microRNAs, also histone modifications are involved in the nuclear regulation of autophagy [19,20]. *ATGs* have been originally identified in yeasts, but many of their homologues have been discovered and characterized in mammalian cells [2,21,22]. Some of them are required for autophagosome formation, and thus are termed the core autophagy machinery. The core Atg proteins can be divided into different functional subgroups. Atg1/ULK complex is the autophagy initiation complex that regulates the induction of autophagosome formation and integrates signal from mTOR serine-threonine kinase (mechanistic target of rapamycin) [23,24]. mTOR kinase negatively regulates autophagy. Expansion of phagophore, a small cup-shaped membrane precursor formed upon induction of autophagy, is poorly defined events, in which ATG9 and its cycling system may have a role in membrane delivery to the expanding phagophore. Nucleation and elongation of the pre-autophagosomal membrane are controlled by class-III phosphoinositide 3-kinase (PI3KC3)/hVps34, which is es-

sential for generating phosphatidylinositol (3)-phosphate (PtdIns(3)P). PI3KC3 is a part of a large macromolecular complex that contains Beclin 1 (a homologue of Atg6), a central scaffold protein [25]. The elongation and expansion of phagophore membrane is mediated by two ubiquitin-like (ATG12-ATG5 and Atg8/LC3-PE) conjugation systems [26], which regulate each other. ATG12 is conjugated to ATG5 in a reaction that requires ATG7 and ATG10 (E1 and E2-like enzymes, respectively) [27]. The second ubiquitination-like reaction involves the conjugation of microtubule-associated protein 1 light chain 3 (MAP1-LC3, also known as Atg8 and LC3) to the phosphatidylethanolamine (PE) [28]. This reaction requires the E1 protein ATG7 and the E2 protein ATG3. Lipidation of LC3 converts the soluble LC3-I into LC3-II, the autophagosome-associated form of LC3, which is attached to the membranes of autophagosomes. LC3-II levels correlate with the number of autophagic vacuoles, which can be assessed by scoring LC3-positve cytoplasmic vesicles [29]. Therefore, expression of LC3 is now in widespread use to visualize autophagy in cultured cells also in cancer cells. However, there are some cautions, and proper monitoring of autophagy in cells is very important [30,31].

ASSESSMENT AND OPTIMIZATION OF AUTOPHAGY MONITORING METHOD: ANALYSIS OF GFP-LC3 DOTS

LC3 or the protein tagged at its N terminus with a fluorescent protein such as GFP, GFP-LC3, has been used to monitor/vizualize autophagy through indirect immunofluorescence or direct fluorescence microscopy, measured as an increase in punctate LC3 or GFP-LC3. However, Kuma et al. [32] highlighted some limitations using GFP-LC3, demonstrating that GFP-LC3 is easily incorporated into protein aggregates independently of autophagy, and that it is difficult to distinguish these aggregates from the true autophagosomes. The difficulty in scoring GFP-LC3 puncta per cell (or the percentage of cells with GFP-LC3 puncta) is compounded by the fact that GFP-LC3 is diffusely located in the cytosol and in the nucleus, giving rise to high background fluorescence. Therefore, the detergent saponin has been used to improve detection of membrane-bound proteins, such as disabled-2 [33], Rab7 [34], and LC3-II [35], as a low amount of saponin removes soluble cellular proteins, including the soluble nuclear and cytosolic fluorescence of GFP-LC3 [35].

Surprisingly, we have found [31] that treatment with saponin and other detergents can provoke artifactual GFP-LC3 puncta formation in different cell lines such as Cos 7 (fibroblast-like cell line derived from monkey kidney tissue), human embryonic kidney 293 cells (HEK 293A), and also cervical cancer HeLa cells. We observed aggregation of GFP-LC3 into autophagosome look-alike structures when cells were permeabilized with saponin for as short as 1 minute before cell fixation. Treatment with saponin reduced diffused cytosolic and nuclear GFP-LC3, but caused an increase in the number and intensity of fluorescent puncta per cell regardless of whether the cells were induced to undergo autophagy. Saponin also induced GFP-LC3 puncta in autophagy-deficient cells (*Atg5-/-* MEF cells) transfected with GFP-LC3, where no LC3-II is produced, demonstrating that the puncta are autophagosome-independent. The increase in GFP-LC3 puncta was not matched by an increase in endogenous LC3-II or GFP-LC3-II detected by immunoblotting when protein samples were normalized to cell number. Our results also indicated a qualitatively similar effect when cells were treated with other detergents, commonly used for membrane permeabilization, such as CHAPS, Triton X-100, or digitonin. We also noted that α-tubulin could not be used to normalize for protein loading on blots after applying saponin, as it was selectively extracted from untreated cells but not from cells treated with vinblastine, a drug that perturbs microtubule networks and stimulates autophagosome formation. When using mild detergents to remove background fluorescence, we recommend using a membrane-associated protein such as ATP synthase β for normalization. Detergents used prior to fixation may precipitate GFP-LC3 aggregation into structures that appear autophagosomal and so should be used with caution. These results suggest that use of the saponin extraction method has its limitation and caution should be taken when it is performed before the cell fixation. The controls need to be included in such experiments in light of these findings. In general, it is preferable to include additional assays that measure autophagy, rather than relying solely on monitoring GFP-LC3.

CHARACTERIZATION OF MOLECULAR LINKS BETWEEN AUTOPHAGY AND APOPTOSIS: BECLIN 1 AT THE NEXUS OF AUTOPHAGY AND APOPTOSIS

How autophagic machinery contributes to cell death was not clear ten years ago, therefore we investigated the regulatory ties between autophagy and apoptosis, focusing on whether the interaction of Beclin 1 with Bcl-2 alters the ability of Bcl-2 to protect cells from apoptosis.

Beclin 1, a protein essential for autophagy and a haploinsufficient tumor suppressor, was first identified as a protein that interacts with the anti-apoptotic proteins Bcl-2 and Bcl-xL, but not with Bax/Bak [36]. The interaction between endogenous Beclin 1 and Bcl-2 was found to inhibit Beclin 1-dependent autophagy in yeast and mammalian cells [37]. Interestingly, the binding of Beclin 1 to Bcl-2, specifically endoplasmic reticulum (ER)-localized Bcl-2 (but not mitochondrion-targeted Bcl-2) reduces starvation induced autophagy [38]. Additionally, it was shown that Beclin 1 possesses a BH3 domain (amino acids 114–123) [39,40] that enables its binding to anti-apoptotic members of the Bcl-2 family (e.g., Bcl-2, Bcl-xL). BH3-only proteins are pro-apoptotic regulators of apoptosis, which binding to the anti-apoptotic Bcl-2 members is one mechanism of facilitating the activation of Bax/Bak, crucial mediators of the mitochondrial pathway of apoptosis [41-43]. Indeed, the Beclin 1-derived BH3 peptide inhibits Bcl-2 anti-apoptotic function [39, 40, 44]. Hence Beclin 1, like other BH3-only proteins, may be expected to be pro-apoptotic. However, no experiments using intact/full-length Beclin 1 have been described that evaluate its role in modulation of apoptosis.

The aim of our research was to determine whether Beclin 1 neutralizes the anti-apoptotic function of Bcl-2 and thereby sensitizes cells to apoptosis [9]. We used several apoptotic stimuli that initiate ER and/or mitochondrial signaling pathways (UV radiation, TNFα and cycloheximide,

staurosporine, thapsigargin and tunicamycin). To investigate protection against a wide range of insults, we targeted Bcl-2 to mitochondria (mit) or endoplasmic reticulum (ER). We show that overexpressed Beclin 1 interacts equally well with Bcl-2 variants that are exclusively targeted to the mitochondrial or ER membranes (mit-Bcl-2 and ER-Bcl-2). Beclin 1 followed to the appropriate organelle with complete or near-complete overlapping. These interactions were observed in cells in which autophagy is active (HeLa cells), as well as in autophagy deficient cells due to *Atg5* gene deletion (*Atg5*-/- MEF cells). The interaction between Beclin 1 and Bcl-2 was verified by immunoprecipitation, and a membrane-proximate localization of Beclin 1 was shown by immunoelectron microscopy. By contrast, mutated Beclin 1 lacking the Bcl-2 binding domain was not colocalized with Bcl-2, and no Bcl-2 was immunoprecipitated. This indicated that the redistribution of Beclin 1 is due to its binding to Bcl-2 and that the strength of binding to Beclin 1 is not altered by subcellular targeting of Bcl-2. Moreover, the interaction between overexpressed Bcl-2 and Beclin 1 was functional in that all Bcl-2 variants inhibited Beclin 1-induced autophagy, whereas ER-Bcl-2 was most effective during amino acid starvation, in keeping with previous reports. To examine whether Beclin 1 can modify Bcl-2 anti-apoptotic activity, cells were exposed to four different pro-apoptotic stimuli, because protection by Bcl-2 variants can be stimulus- and localization-specific. Following induction of apoptosis the changes in nuclear morphology, caspase-3 activity, poly-ADP-ribose polymerase (PARP) cleavage, or punctation of mRFP-Bax on mitochondria were analyzed. We found that protection of Bcl-2 against caspase-dependent apoptosis induced by each stimulus was not altered by coexpression of Beclin 1 even when the molar concentration of Bcl-2 was 3.6-fold lower than that of Beclin 1, or Beclin 1 was expressed in the absence of exogenous Bcl-2. Moreover, Beclin 1 did not disrupt the prevention by Bcl-2 of Bax-punctation, a key BH3 domain-dependent step in apoptosis commitment. The anti-apoptotic action of Bcl-2 coexpressed with Beclin 1 was maintained in *Atg5*-/- MEF cells, arguing against compensation for the loss of protection by Bcl-2 by autophagy-mediated survival induced by Beclin 1. Hence, binding of Beclin 1 to Bcl-2 does not modify apoptosis irrespective to Bcl-2 concentration, location, apoptotic stimulus, and whether the autophagy is active or inactive [9]. Our results suggest that although Beclin 1 contains a BH3-only motif typical for pro-apoptotic proteins, is at best a weak modulator of Bcl-2 anti-apoptotic function, and that suppression of tumorigenesis in mammals by Beclin 1 is not related to its capacity to bind to Bcl-2.

The importance of our finding was noticed by the scientific community. Firstly, editorial commentary was published together with our original article. P. Boya *et al*. discussed the far-reaching implications for the comprehension of the cross-talk between apoptosis and autophagy [45]. Moreover, we were invited to write Autophagic Punctum review in the prestigious journal Autophagy. In this punctum [10] we discussed possible reasons why Beclin 1 fails to behave like other BH3-only proteins and induce apoptosis. One explanation is that the binding of Beclin 1 to Bcl-2 is weak relative to that of other BH3-only proteins, so Beclin 1 would be easily displaced by BH3-only proteins (or by Bax) when their activity increases specifically in response to apoptotic stimuli. Other possibility is that Beclin 1 proapoptotic potential requires a special stimulus that mobilizes the BH3-only function of Beclin 1 preferentially. It is also possible, that due to the weak binding of Beclin 1 to Bcl-2, there is always ample free Bcl-2 to mediate full anti-apoptotic function. Moreover, we have to remember about the complexity of Beclin 1 partnerships in cells, which is just beginning to be appreciated. At least three complexes were discovered, which promote or inhibit autophagy. Additionally, post-translational modification of both proteins, such as phosphorylation of Bcl-2 and/or Beclin 1, and caspase cleavage of Beclin 1, can also undermine their interaction. It remains to be determined which complexes predominate when Bcl-2 is overexpressed, and whether these are modified posttranslationally during apoptosis. To better understand mutual relationship between both proteins, we performed dynamic interplay between Beclin 1 and Bcl-2 in the modulation of autophagy and apoptosis on the schematic diagram [10]. We believe that our studies contributed to understanding of the molecular interdependencies that exist between apoptosis and autophagy. Recent studies have shown that the crosstalk between autophagy and apoptosis occurs at several levels that can be classified into distinct paradigms on the basis of mechanistic features. Nowadays, we can distinguish three paradigms by which autophagy regulates apoptosis: (1) regulation of apoptosis by specific autophagy proteins; (2) activation of caspases on autophagosomal membranes depends on autophagosome formation, but not on lysosomal activity; (3) regulation of apoptosis by autophagic degradation requires both autophagosome formation and lysosomal activity. The two mechanistic paradigms by which apoptosis regulates autophagy are: (1) direct regulation of autophagy by specific apoptotic proteins; (2) regulation of autophagy by activated caspases, which necessitates the activation of the apoptotic process as a whole [46].

AUTOPHAGY AS A NEW THERAPEUTIC STRATEGY IN GLIOBLASTOMA

Glioblastoma (GBM), grade IV astrocytoma, is the most frequent and malignant brain tumor in adults, characterized by aggressive cell proliferation, diffuse infiltration, and resistance to conventional therapy. Current treatment modalities have only a modest effect on patient outcomes. The median survival times of patients is around 15 months. Probably due to many genetic alterations and intratumoral heterogeneity, gliomas are resistant to radiation or/and chemotherapy. Recently, autophagy emerged as a promising therapeutic concept in glioma treatment [47], as autophagy rather than apoptosis accompanies cytotoxicity induced in glioma cells by conventional anti-cancer drugs, irradiation, and other compounds [48-53]. More information concerning therapeutic potential of autophagy in glioma was described in book chapter "*Autophagy in Glioma Cells*" [18]. Although autophagy has received increasing scientific attention as a promising therapeutic target, there are some data indicating that role of autophagy in tumor cells may depend on the type of tumor, the stage of tumorigenesis, or the nature and extent of the insult. It is important to determine the role of autophagy in each circumstances, therefore, our studies focus on the role of autophagy in glioma cells.

THE ROLE OF ENDOPLASMIC RETICULUM STRESS AND AUTOPHAGY IN MALIGNANT GLIOMA CELLS UNDERGOING CYCLOSPORINE A-INDUCED CELL DEATH

We have previously demonstrated that cyclosporine A (CsA), which revolutionized transplantology due to its ability to block the activation of lymphocytes and other immune system cells, induces cell death in rat C6 glioma cells with some apoptotic features, and in human malignant glioma cells either growth arrest or non-apoptotic programmed cell death [54-57]. Interestingly, CsA-induced death in glioma cells was accompanied by formation of numerous cytoplasmic vacuoles [56], suggesting that also other mechanisms are involved in cell death. Moreover, CsA binds and inhibits the activity of the cyclophilin A (CyPA), a peptidyl-prolyl isomerase implicated in the maturation and folding of native proteins [58].

The aim of our study was to characterize a novel molecular pathway playing role in CsA-induced cell death of malignant glioma cells, which in the future could be used as a new therapeutic approach to enhance the anti-cancer efficacy of different drugs. We investigated whether CsA-induced cell death of malignant glioma cells was associated with autophagy and ER stress, and how blocking of these processes would affect glioma cell death [15]. At the beginning, we compared efficacy of CsA-induced cell death with common apoptosis inducers: UVC and adriamycin (Adr, doxorubicin). All insults resulted in similar reduction of cell viability, but gradual appearance of biochemical hallmarks of apoptosis: cleaved fragments of caspase 7, caspase 3 and PARP, were more prominent after UVC or Adr treatments. UVC and Adr induced a 'ladder-like' oligonucleosomal DNA fragmentation in glioma cells. Quantification of subdiploid cell population by flow cytometry confirmed an increase in DNA fragmentation after tested stimuli. However, the percentages of cells with fragmented DNA were lower after CsA exposure, when compared with UVC and Adr-treated cultures. We observed formation of large vacuoles in rat and human glioma cells treated with CsA, which did not appear in cells treated with Adr or irradiated with UVC. Vacuolization induced in human glioma LN18 and T98G cells by CsA was completely blocked by the inhibitors of transcription and translation, suggesting block of *de novo* protein synthesis, or attenuation of CsA-induced protein aggregate accumulation or both. Evidently, UVC irradiation and adriamycin induced apoptosis more potently than CsA, which triggers programmed cell death associated with vacuolation in studied glioma cells. We revealed that cytoplasmic vacuoles were immunostained for two specific ER markers: calreticulin and KDEL (retention sequence-marking proteins localized in ER). The induction of ER stress in glioma cells by CsA was evidenced by accumulation of ubiquitinated (unfolded) proteins and activation of the unfolded protein response (UPR). We found that CsA treatment triggers accumulation of IRE1α (inositol-requiring enzyme 1) and phosphorylation of PERK (PKR-like ER kinase) in human glioma cells. The functional activation of IRE1α and PERK was confirmed by the appearance of *Xbp1* mRNA splice variants and the increase of eIF2α phosphorylation upon CsA-treatment, respectively. Accumulation of BIP/Grp78 (immunoglobulin-binding protein) and CHOP (C/EBP homologous protein), followed by the activation of IRE1α and PERK kinases in CsA-treated glioma cells. Similar changes were observed in malignant human glioma cells treated with typical ER stress inducers, such as thapsigargin (Tg) and tunicamycin (Tm). The presented evidence indicate that CsA induces ER stress in malignant glioma cells, characterized by accumulation of ubiquitinated proteins, induction of an UPR and dilatation of ER cisternae.

To examine whether CsA induces autophagy in glioma cells, we evaluated the appearance of several autophagy features following CsA treatment. We demonstrated the formation of acidic vesicular organelles (AVOs), increase of autophagosomes (detected by an electron microscopy and GFP-LC3 dots formation); and finally LC3-II accumulation upon CsA treatment. The changes induced by CsA in glioma cells were partially blocked by autophagy inhibitor, 3-methyladenine. Additionally, we found that GFP-LC3 dots or autophagosomes localize close to cytoplasmic vacuoles/swollen ER cisternae. The increase of LC3 staining in a murine intracranial glioma model in CsA-treated animals demonstrates that autophagy also occurs *in vivo*. Additionally, CsA-induced autophagy was associated with inhibition of the mTOR pathway, which acts as a negative regulator of autophagy. Decrease of phosphorylation of 4E-BP1, and p70S6K (the two direct downstream effectors of mTORC1) was observed in human glioma cells. These results suggest that CsA-induced autophagy was associated with mTORC1 inhibition in human glioma cells. We also studied interdependence between ER stress, autophagy, and cell death. We found that an ER stress response develops prior to autophagy, as its pharmacological inhibition reduces autophagy. Use of salubrinal (inhibitor of eIF2α dephosporylation) or silencing of PERK or/and IRE1α partially blocked CsA-induced accumulation of LC3-II. These results suggest that ER stress is triggered prior to CsA-induced autophagy.

Inhibition of different stages of autophagy may result in different outcomes in glioma cells [48,59]. Therefore, we silenced ULK1, implicated in the initiation of autophagy, and ATG5 and ATG7, required for autophagosome formation. We observed effective silencing of ULK1, ATG5, ATG7 at the mRNA and protein levels. To test whether autophagy controls the extent of CsA-induced death, we performed clonogenic survival assay and analysis of biochemical hallmarks of apoptosis after knockdown of autophagy regulators followed by treatment with CsA. Inhibition of autophagy increased the efficacy of CsA in reduction of colony formation, suggesting a protective role of autophagy. Silencing of autophagy effectors *ULK1*, *ATG5* or *ATG7*, increased the level of active caspases 3, 7 and PARP degradation, as well as enhanced caspase 3 activity in CsA-treated cells. Interestingly, the strongest effect was observed in *ATG5* siRNA-transfected cells. Our findings show that genetic inhibition of autophagy at different levels enhances the CsA-induced cell death, suggesting a mainly protective role of autophagy in those cells. These observations lead us to conclude that CsA induces both apoptosis and autophagy in malignant glioma cells via induction of ER stress and inhibition of the mTORC1/p70S6K pathway; however, autophagy is cytoprotective in this context. As the cytoprotective role of au-

tophagy could be the main obstacle in anticancer therapy, its inhibition should be a new strategy to explore. So far, different inhibitors of autophagy such as hydroxychloroquine, 3-methyladenine and bafilomycin A1 effectively sensitized cultured cancer cells to therapeutic agents [60]. It is noteworthy, that some autophagy inhibitors are tested in clinical trials in GBM patients [18].

In the review [16], we described complex molecular mechanism of CsA action in malignant glioma cells, which was discovered by Prof. Bozena Kaminska's group. We have previously found that rat glioma cells express NFAT (nuclear factor of activated T cells) proteins, and CsA inhibits calcineurin-NFAT signalling; however, blocking this pathway was not sufficient to induce death of glioma cells. CsA induces cell death with apoptotic features in rat C6 glioma cells that is mediated by activation of p53 and its transcriptional target gene *Bax* resulting in stimulation of the intrinsic mitochondrial death pathway. Interestingly, in human malignant glioma cells, defective in p53 and/or PTEN tumor suppressors, CsA induces either senescence-like growth arrest (U87MG cells) or non-apoptotic programmed cell death (U373MG/U251MG, T89G cells) [54,56,57]. In p53-deficient cells CsA induces Erk1/2-dependent activation of cell cycle inhibitor p21 that results in cell cycle arrest. Moreover, inhibition of the prosurvival pathway (Akt1) results in the activation of FoxO1 transcription factor, that cooperates with the AP-1 transcription factor in the transcriptional upregulation of Fas ligand (that belongs to TNF (tumor necrosis factor) family). CsA, likely due to inhibition of cyclophilins, induces accumulation of unfolded proteins in the endoplasmic reticulum that triggers an unfolded protein response. Activation of PERK/eIF2α and the IRE1a/XBP1/JNK pathways upregulates the expression of CHOP and BIP. Induction of ER stress and/or decreased activity of the Akt1/mTORC1 pathway contribute to initiation of autophagy. Autophagy inhibits apoptotic cell death induced by CsA. These observations lead us to conclude that CsA induces both apoptosis and autophagy (which is cytoprotective) via induction of ER stress, but also CsA induces cell cycle arrest in malignant glioma cells.

THE ROLE OF AUTOPHAGY IN DIFFERENTIATION OF GLIOMA STEM-LIKE CELLS

Stem-like cell populations have been identified in a number of malignancies including glioblastoma. These rare stem cells (called also glioma-initiating cells) are believed to be responsible not only for tumor initiation and progression but also resistance to therapeutic agents and tumor recurrence. Recently, the population of cells within glioblastoma with stem-like properties has gained increasing attention as a target to refine treatment strategies. In the review [61] we summarize the recent data regarding isolation, biology and mechanisms of chemotherapy resistance of glioma stem-like cells (GSCs). GSCs are characterized by the ability (i) to self-renew, (ii) to initiate brain tumors upon orthotopic implantation in immunodeficient mice, (iii) to express neural stem cell markers, and (iv) to differentiate into cells with a neuronal, astrocytic, or oligodendroglial phenotype. The differentiated cells loose long-term self-renewal potential *in vitro* and fail to propagate tumors *in vivo* [62], suggesting that induction of GSC differentiation may be a good strategy to eliminate GSCs in GBM [63]. Autophagy is highly active during mammalian development and differentiation, maintains the quality control and cellular homeostasis of terminally differentiated cells [64], as well as participates in self-renewal, pluripotency and differentiation of stem cells [65, 66]. Recently, it was shown that activation of the autophagic process may promote GSC differentiation and increase radio- and chemosensitivity [67-70].

Epigenetic-based drugs targeting epigenetic enzymes in cancer have been shown to affect GSC differentiation and induce autophagy [69,71]. BIX01294, a specific inhibitor of G9a histone methyltransferase (introducing H3K9me2 and H3K27me3 repressive marks) [72], induced autophagy or autophagy-associated cell death in many tumor cell lines [73-77], but underlying mechanisms have not been explored in glioma cells. Additionally, BIX01294 is a potent modulator of stem cell differentiation. BIX01294 pre-treatment improved differentiation of stem cells [78-81].

The aim of our study was to understand the role of BIX01294-induced autophagy in differentiation of glioma stem-like cells, as a potential target for cancer therapy [17]. First, we examined whether BIX01294 induces autophagy in human glioma cell line LN18 and patient-derived glioma stem-like cells (L0125) without affecting cell viability. We found that 2 μM BIX01294 was sufficient to decrease H3K9me2 and H3K27me3 levels without reducing cell viability, treatment with higher doses of BIX01294 (3 and 10 μM) resulted in accumulation of the cleaved caspase 3, caspase 7 and PARP, the biochemical hallmark of apoptosis. At a non-toxic dose, BIX01294 stimulated LC3-II accumulation and autophagosome formation (detected by accumulation and GFP-LC3 punctation) in established cell line and primary human glioma cells that evidenced induction of autophagy. Moreover, BIX01294-induced autophagy was partially blocked by 3-methyladenine and by selective silencing of crucial autophagy genes *ATG5*, *ATG7*, *ULK1* and *BECN1*. Here, we demonstrated for the first time, that BIX01294 reduces H3K9me2 and H3K27me3 levels and induces autophagy in glioma cells, as well as in glioma stem-like cells.

Then using "selection by growth requirement", one of the method for enrichment in GSCs [61,82], we expanded GSCs from established glioma LN18 cells and culture derived from GBM specimen (WG4). We confirmed that GSCs enriched spheres expressed higher levels of pluripotency markers: NANOG, OCT4 (encoded by the *POU5F1*), SOX2 and CD133 (encoded by the *PROM1*) as compared to the adherent tumor cells growing in the presence of serum. Interestingly, tumor spheres expressed a significantly lower levels of LC3-II and autophagy related (*ATG)* genes than the parental glioma cell cultures. These data suggest reduced autophagy and increased stemness capacity of the spheres.

To explore the role of autophagy in GSCs differentiation, we used L0125 and L0627 cell lines (originating from human GBMs), previously characterized as GSCs possessing self-renewal and tumor-initiating ability, and multilineage differentiation potential [83,84]. These cell lines are characterized by different expression of epidermal growth factor receptor (EGFR), which is a known diagnostic and

prognostic marker of human GBM. L0125 cells are EGFR negative, while L0627 cells are EGFR positive. We revealed that typical differentiation inducers: serum and all-trans retinoid acid (ATRA), upregulated astrocytic (GFAP, glial fibrillary acidic protein) and neuronal (β-Tubulin III) markers, and concomitant decreased in OLIG2 and SOX2 expression at mRNA and protein levels. Untreated spheres did not express differentiation markers, while high levels of OLIG2, SOX2 and NESTIN (neural stem/progenitor markers) were detected. Transcriptomic analysis, using Affymetrix microarrays, also confirmed these changes. These results verify that serum and ATRA induce differentiation of GBM spheres along astrocytic and neuronal lineages. Next, we analyzed the level of autophagy using typical differentiation inducers. We found significantly higher levels of *ATG* genes (such as *ATG5*, *ATG7*, *BECN1, ULK1* and *LC3B*) in cells upon serum- and ATRA-induced differentiation. Western blot analysis confirmed accumulation of LC3-II and increases in the levels of ATG7 and ULK1 in differentiated cells, compared to untreated GSCs. Similar changes were demonstrated using the transcriptomic analysis. This indicates restoring of autophagic machinery and increased autophagy throughout GSC differentiation independently of EGFR level. Additionally, we found that serum induced changes in cell morphology. Under control conditions 80% of spheres were floating or semi-attached, maintaining their spherical shapes. In contrast, in serum-treated cultures, 93% of spheres were flattened and cells branched out. Co-treatment with autophagy inhibitors (3MA, BafA1) significantly decreased the number of flattened spheres, and increased the amount of spheres maintaining their spherical shapes. Moreover, GFAP and β-Tubulin III expression became less abundant in serum-differentiated spheres upon inhibition of autophagy with 3MA, suggesting causative relationship between autophagy and differentiation.

Next, we verified the hypothesis that there is causative relationship between BIX01294-induced autophagy and differentiation process. First, we assessed if spheres undergo morphological alterations (attachment to the cell culture plates) upon BIX0124 treatment and how autophagy inhibitors have an influence on this process. Although the changes were less potent than after serum administration, we found that spheres treated with BIX01294 were more attached to the culture plates and began to lose their shapes when compared to untreated spheres. These effects were slowed down when the cells were co-treated with autophagy inhibitors, BafA1 or 3MA. Additionally, BIX01294 induced the expression of *ATG* genes such as *LC3B* and *WIPI1*, along with differentiation markers *GFAP* and *TUBB3* in spheres, however, less potently than serum. Increased levels of astrocytic (GFAP) and neuronal (β-Tubulin III) markers in BIX01294-treated tumor spheres were confirmed at protein levels. It suggests that BIX01294 is a strong inducer of autophagy, as well as a potent modulator of GSC differentiation. We tested whether alleviating autophagy could influence BIX01294-induced differentiation process of GSCs. Autophagy inhibitor partially abolished the increase of GFAP and β-Tubulin III expression induced in sphere cultures by BIX01294 at both mRNA and protein levels. Similar changes were observed in GSCs-enriched spheres originating from established glioma cells and GBM patient-derived cultures. These data indicate that induction of GSC differentiation by BIX01294 depends on transcriptional activation of autophagy.

To get insights into molecular mechanisms of BIX01294-induced differentiation and autophagy processes, we performed a chromatin immunoprecipitation (ChIP)-qPCR analysis for selected histone modifications and G9a binding to the promoters of the genes involved in these two processes. We demonstrated that histone methyltransferase G9a binds to the promoters of autophagy-related (*LC3B*, *WIPI1*) and differentiation-related genes (*GFAP*, *TUBB3*) in spheres. Higher levels of H3K4me3 (an active chromatin mark) and lower levels of H3K9me2 (a repressive mark) at the promoters of studied genes were observed in serum-differentiated cells in comparison to spheres. BIX01294 increased H3K4me3 levels and reduced H3K9me2 levels, which was accompanied by upregulation of RNA polymerase II binding to the promoters of studied genes. This was consistent with prominent reduction of H3K9me2 in BIX01294-treated spheres. Based on our findings, we propose a model in which BIX01294 inhibits G9a histone methyltransferase in GSCs, which results in reduction of the repressive H3K9me2 mark and the increase in the activation H3K4me3 mark at the promoters of autophagy- and differentiation-related genes. These changes trigger restoration of autophagy components and activation of this process in GSCs, which triggers cell differentiation.

Finally, question arises regarding the role of epigenetic reprograming, restoration of autophagy and apoptosis in glioma cells in cancer therapy. A number of studies have shown, that adding inhibitors of histone modifying enzyme to conventional glioma therapy may increase its efficacy [71,85,86]. Temozolomide (TMZ) is a first line therapeutic agent in GBM patients, however median survival of such patients is poor (15 months). The resistance is due to high level of DNA repair protein, O^6-methylguanine-DNA-methyltransferase (MGMT) and/or occurrence of glioma stem-like cells contribute to GBM resistance to the drug. Recently, we have explored a possibility of epigenetic reprograming of glioma cells to increase sensitivity to TMZ and restore apoptosis/autophagy competence in glioma cells. We found that pre-treatment or post-treatment with non-toxic dose BIX01294 (the inhibitor of G9a) improve anti-tumor efficacy of TMZ in glioma cells and glioma stem-like cells (Ciechomska *et al*. 2018 submitted). Our results suggest that G9a is a potential therapeutic target in malignant glioma. A number of studies have shown an important role for G9a in the progression of solid tumors and metastasis [87]. The G9a inhibitor can be used alone or in combination with other standard therapies currently used in the clinic in order to develop better treatments for cancer patients. Nowadays, inhibitors of histone methyltransferases have not yet reached the clinical trial stage. Various h*istone deacetylase* inhibitors, for example valproic acid, have been approved by the FDA and are undergoing trials in different types of malignancies, including glioblastoma [88].

SUMMARY

In the series of the publications presented above we analyzed autophagy process in mammalian cells. We optimized the method for detection of autophagy, then we character-

ized the molecular link between apoptosis and autophagy, and finally we studied the role of autophagy in cancer cells.

We found accumulation of GFP-LC3 puncta upon saponin and other detergent extraction in transient and stable transfectants independently of autophagy. Therefore, detergents used prior to fixation should be used with caution. We have analyzed the bilateral links between apoptosis and autophagy. Our work focuses on the interaction between Beclin 1, a protein essential for autophagy, and anti-apoptotic protein Bcl-2. Our results suggest that, although Beclin 1 contains BH3-only motif typical of pro-apoptotic proteins, it is negligible modulator of Bcl-2 anti-apoptotic function. To understand the role of autophagy in cancer treatment we have used the most common and most aggressive cancer, glioblastoma, that is generally incurable. We found that immunosuppresant cyclosporine A, induces both apoptosis and autophagy in malignant glioma cells via induction of endoplasmic reticulum stress and inhibition of mTORC1/p70S6K pathway. Our results revealed that autophagy was cytoprotective in this context, suggesting that combining cyclosporine A with autophagy inhibitors may improve the anti-cancer efficacy of the drug, which is in agreement with the findings from other researchers. GBMs are characterized by extensive heterogeneity at the cellular and molecular levels. Therefore, we also analyzed the role of autophagy in subpopulation of cells called glioma stem-like cells, which are defined as undifferentiated cells responsible for tumor initiation and resistance to therapy. We found that BIX01294, the inhibitor that modulates the epigenetic status of chromatin (introducing H3K9me2 and H3K27me3 repressive marks) triggers autophagy in established cell line, as well as in the patient-derived glioblastoma stem-like cell cultures. Moreover, we found that BIX01294 induced differentiation of GSC by activating autophagy. Our recent data have shown that BIX01294 sensitized adherent and glioma stem-like cells to temozolomide (TMZ, a conventional chemotherapeutics). These results may contribute to designing novel therapeutic strategies.

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Rola autofagii w komórkach nowotworowych: charakterystyka wzajemnych zależności pomiędzy procesami autofagii i apoptozy; modulacja autofagii jako nowa strategia terapeutyczna w leczeniu glejaków

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Słowa kluczowe: autofagia, apoptoza, glejaki, cyclosporyna A, beklina 1, LC3

STRESZCZENIE

W 2016 roku nagrodę Nobla w dziedzinie medycyny i fizjologii otrzymał japoński naukowiec Yoshinori Ohsumi, za odkrycie molekularnych mechanizmów leżących u podstaw autofagii. Odkrycie to miało fundamentalne znaczenie w zrozumieniu w jaki sposób komórka poddaje recyklingowi swoją zawartość. Jego odkrycie utorowało drogę do zrozumienia wielu procesów fizjologicznych takich jak adaptacja do warunków niedoboru substancji odżywczych czy odpowiedz na infekcję patogenami. Ponadto mutacje genów regulujących proces autofagii mogą przyczyniać się do rozwoju wielu chorób, w tym nowotworów czy chorób neurodegeneracyjnych. Pokazuje to, jak ważne są badania dotyczące autofagii i jak istotną rolę odgrywa ten proces zarówno w stanach fizjologicznych i patologicznych. Wciąż wiele pytań pozostaje jednak bez odpowiedzi m.in. zagadnienie funkcji autofagii w komórkach nowotworowych. Celem omawianych prac było przybliżenie roli autofagii w komórkach nowotworowych poprzez ocenę niektórych metod detekcji autofagii, określenie molekularnych współzależności pomiędzy autofagią i apoptozą w komórkach nowotworowych, a także poszukiwanie nowych strategii leczenia najczęstszych nowotworów mózgu, glejaków.