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Review article

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ROLE OF AUTOPHAGY IN MAMMARY GLAND DEVELOPMENT

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Autophagy is a highly conserved catabolic process responsible for degradation and recycling of long-lived proteins and organelles by lysosomes. This degradative pathway, together with proteasome system is particularly important during development and under certain environmental stress conditions. This review summarizes the latest achievements of studies aiming to explore the role of autophagy in development and differentiation of eukaryotic cells. It shows the importance of this process in the development of lower eukaryotic organisms such as *Dicyostelium discoideum*, and *Caenorhabditis elegans*, as well as functions of autophagy and autophagy related genes (Atg) in development and differentiation of higher eukaryotic organisms. The review is focused on the results of studies conducted on mammary gland, as it is a good model for studying the mechanisms controlling higher eukaryotic organisms' development. Studies have shown that autophagy is involved in the removal of epithelial cells during formation of alveolar structures, indicating its role in mammogenesis. There are also evidences of involvement of Atg's in epithelial tumors development. Context dependent manipulations of autophagic pathways may create more effective anticancer therapies in the future.

Key words: *autophagy, mammary gland, 3D cell cultures, mammogenesis, tumorigenesis*

INTRODUCTION

Normal cell growth and development requires well controlled balance between protein synthesis and organelle biogenesis versus protein degradation and organelle turnover. Autophagy is the major cellular pathway for the degradation of long-lived proteins and cytoplasmic organelles. This degradative pathway, together with the proteasome system is particularly important during

development and under certain environmental stress conditions (1). For example, cellular death and resorption are critical during processes that involve extensive cellular remodeling such as insect metamorphosis, remodeling of mammary gland during lactation cycle, postpartum luteal cell regression, differentiation and aging (2-4). Turnover also occurs on a subcellular scale, for example under starvation conditions cells need to scavenge nonessential proteins and organelles and to recycle the components for reuse in the cytosol (5).

Autophagy can be divided into three major forms: macroautophagy, microautophagy and chaperone-mediated autophagy, based on the way substrates reach the lysosomal lumen (6). In macroautophagy, the cytosolic elements that must be degraded are sequestrated by an isolating membrane of non-lysosomal origin that seals, creating an autophagic vacuole or autophagosome. Fusion of lysosomes with autophagosomes provides the enzymes required for the degradation of the sequestrated components. This isolating membrane is formed from small preformed membranous structures of unknown origin (7). The most common results suggest that the endoplasmic reticulum (ER) plays a significant role in supplying membrane during vesicle formation, although the mechanism which would be required to divert a portion of the ER away from its normal function, and into the process of isolating membrane formation is not clear (8). A similar process of sequestration occurs in microautophagy, but in this case the lysosomal membrane deforms to engulf the cytosolic substrates (6). In both macro- and microautophagy the membrane of vesicles that carry substrates to the lysosomal lumen is rapidly degraded, granting lysosomal hydrolases access to the internalized substrates. In the third form of autophagy, named chaperone mediated autophagy, soluble cytosolic proteins selectively bind to a receptor at the lysosomal membrane that mediates their translocation into the lysosomal lumen (9). Selectivity depends on the recognition of a targeting signal in the amino acid sequence of the substrate proteins by a cytosolic chaperone. The chaperone-substrate complex then binds to a lysosomal membrane receptor. A second chaperone located in the lysosomal lumen is required for substrate translocation. In this review, we will focus on macroautophagy (herein referred to as autophagy), as it is an evolutionarily conserved process that occurs in virtually all eukaryotic cells (from yeast to mammals) and plays a major role in development and differentiation.

ROLE OF AUTOPHAGY IN DEVELOPMENT OF EUKARYOTIC ORGANISMS

For many years, it has been presumed that autophagy is involved in cellular architectural changes that occur during differentiation and development. However until recently, studies on the role of autophagy in these processes have been confined largely to morphologic correlations. With landmark discovery of the autophagy-related (ATG) genes, and their protein products in yeast, it has become

possible to use genetic approaches in model organisms to discover the events in the cellular autophagic machinery. Many of the yeast *ATG* genes have candidate orthologs in higher eukaryotes, and a number of these genes have now proven roles in autophagy in nematodes, flies, mammals and even plants. Furthermore, inactivation of theses orthologs in higher eukaryotes has revealed not only conservation of autophagy function, but also their important roles in different aspects of development, such as normal reproductive growth, stress-induced adaptations, aging, growth control and cell death (10-13). Atg6, known in higher eukaryotes as Beclin 1, is an important component of the type III phosphatidylinositol 3-kinase (PI3-K) complex, which is involved in vesicle nucleation, and plays a role in development of *C. elegans*, *Dictyostelum* and mice. It has also been shown that that beclin $1^{-/-}$ mutant mice die early in embryogenesis (12). Death occurs on approximately day E7.5, and is postulated to result from a defect in the visceral endoderm, an exchange system responsible for nutrition and waste product detoxification in the developing embryo. Interestingly in *Beclin 1* null mouse embryos, a widespread cell death is observed, indicating a possible pro-survival role of autophagy during early development. Moreover, the biochemical properties of Beclin 1 suggest its role in the regulation of apoptosis. The work of Liang *et al*. (14) showed that Beclin 1 interacts with Bcl-2 a protein involved in the control of apoptosis. Several other Atg proteins (Atg3, Atg4, Atg5, Atg7, Atg8, Atg10, Atg12, Atg16) engaged in autophagosome formation and progress of autophagy, have been shown to function in development of *C. elegans* and/or *Dictyostelium* (15, 16, 10). One of the mentioned proteins – Atg8, in mammals named microtubule-associated protein light chain 3 (MAP1 LC3 or LC3), is considered as a specific autophagy marker. Upon induction of autophagy, LC3 is conjugated to phosphatidylethanolamine and targeted to autophagic membranes. Therefore, changes in LC3 localization have been used to measure autophagy (17).

In both yeast and mammalian cells, nutrients limitation, high population density, and increased temperature are potent inducers of autophagy (18, 19). Differentiation and developmental events, in which autophagy contributes, are triggered by some of these forms of environmental stress. One of the examples seen in lower eukaryotic organisms is the development of *Dictyostelium discoideum.* Upon nutrient deprivation this soil amoeba undergoes a complex developmental cycle to produce a multicellular organism (20). Starving amoeba aggregate using a cyclic AMP signaling system to form mounds of about 100,000 cells, and each mound undergoes morphogenesis to produce a mature fruiting body composed of a sphere of spores supported by a cellular stalk. Insertional mutagenesis of the *Dictyostelium* orthologs of yeast *ATG5, ATG6, ATG7* and *ATG8* genes has minimal effects on cellular viability and growth in the presence of nutrients; however, it results in loss of cellular viability and aberrant multicellular development during starvation (15, 16). Autophagy genes were also proven to be essential for another stress-induced developmental process – the formation of dauer larvae in the nematode, *Caenorhabditis elegans*. Under

conditions of limited food, high population density, or increased temperature, *C. elegans* reversibly arrest in an alternative third larval stage, the dauer diapause that is metabolically and morphologically specialized to survive in an unfavorable environment (21). Melendez *et al*. (10) have shown that autophagy is enhanced during dauer development in lateral hypodermal seam cells, a cell type which is responsible for formation of the specialized dauer cuticle and radial construction of the body. Furthermore the group has shown that inactivation of C. elegans *Atg* genes (*e.g. Atg1, Atg6, Atg7, Atg8* and *Atg18*) does not affect dauer initiation, but blocks morphogenic and physiological features of dauer development, inhibits seam cell autophagy, and prevents dauer survival.

During development, autophagy functions to remodel cells, but also is believed to represent a mechanism by which cells undergo self destruction (22). Autophagosomal structures were first noted during developmental cell death in regressing insect salivary glands (23) and have been observed in dying cells of developing animals of diverse taxa (24, 25). Nowadays, the consensus view is that autophagic cell death (characterized by early degradation of cytoplasmic organelles, but preservation of cytoskeletal elements until late stages) is activated primarily when the developmental programs of homeostatic processes in adulthood require massive cell elimination (22). In such cases, autophagy has been proposed not only to contribute to cell death, but also to function as a mechanism for "self-disposal" during large scale tissue histolysis, when a number of professional phagocytic or engulfment cells are insufficient to remove dead cell corpse (26).

ROLE OF AUTOPHAGY IN MAMMARY GLAND DEVELOPMENT

Most of the knowledge about the role of autophagy in development, presented so far in this review, is based on the results of studies carried out on the models of lower eukaryotes. The reason for this is their short life cycle and the ease of conducting the experiments in laboratory conditions. However, not every conclusion based on the developmental processes occurring in amoebae, nematodes or insects can be directly extrapolated on higher eukaryotes, such as mammals. Therefore there are also accumulating studies on the role of autophagy in development and differentiation of many mammalian cell types like: white blood cells, neurons, cardiac myocytes, epithelium (27-31). While most tissues and organs undergo massive growth in early stages of development, i.e. during embryogenesis and in the early period of postnatal life, mammary gland expresses its maximum growth potential after the animal has reached maturity (following the onset of pregnancy and during lactation). The cycle of proliferation - differentiation – regression is repeated at each gestation and can be reproduced in culture systems *in vitro*. That is why mammary gland has been used by many researchers as a very good model for studying processes involved in development and differentiation. A deeper understanding of how growth and differentiation of the mammary tissue are

regulated can complement the knowledge of developmental process as well as treatment and prevention of mammary cancers (32).

During the last few years our group has been interested in the involvement of autophagy in remodeling of ruminants' mammary gland. Bovine mammary gland undergoes intensive remodeling during lactation cycle and the escalation of this process is observed during drying off. The dry period in cattle lasts 6 - 8 weeks, during which mammary gland involution is observed, together with the growth of new secretory tissue. This type of involution is known as regenerative involution, because of the characteristic overlap in time between mammogenesis controlled by pregnancy hormones, and involution of the senescent tissue, during which the intensive death of secretory cells is observed (30). Our *in vitro* and *in vivo* studies of bovine mammary gland physiology have revealed that the enhanced process of autophagy is observed at the end of lactation and during dry periods (4, 30). It is manifested by increased expression of Beclin 1 and the highest number of cells with typical morphological features of autophagy (autophagosomes and autophagolysosomes). Our *in vitro* studies on bovine mammary epithelial cell line BME-UV1 showed also the regulation of autophagy by TGF-β1, a growth factor with apoptogenic activity, which is considered as an important local regulator of mammary tissue involution. Treatment of BME-UV1 cells with TGF-β1 caused a significant increase in protein levels of MAP1 LC3 and Beclin1, which proved the ability of this growth factor to induce autophagy (33). The process of autophagy is probably the manifestation of cellular defense against apoptogenic TGF-β1 action. When this defense is failed the secondary apoptosis is induced. In this case cells share morphological features typical of apoptosis: cell shrinkage, plasma membrane blebbing, margination and condensation of chromatin, and autophagy: autophagic vacuoles.

The *in vitro* systems of studying morphogenesis, growth and differentiation of the mammary gland have also shed light on the role of autophagy during mammogenesis. Introduction of the non-plastic substrates, such as collagen gels and extracellular matrix proteins to the cell culture systems enabled to recapitulate certain essential structural features of glandular epithelium *in vivo*. In physiological conditions mammary gland is a complex organ, comprised of multiple cell types and surrounded by a proteinaceous extracellular matrix (ECM). Mammary epithelial cells (MECs) represent the fundamental functional unit of the gland and comprise a polarized secretory network of bilayered hollow ducts and alveolar units. The epithelial tree is embedded in a collagen-rich matrix and develops specialized attachments to the surrounding basement membrane. Integration of the array of signals mediated by both cell-cell and cell-matrix interactions is required to regulate many aspects of cell behavior, including cell polarity, proliferation, adhesion, differentiation, and survival. Cultured MECs respond most appropriately to matrix proteins that mimic the composition of normal basement membrane, including among other components a high proportion of laminin and collagen. One commonly used mixture is the extracellular matrix derived from the Engelbreth-Holm-Swarm

(EHS) murine tumor, commercially available as Matrigel™ (34). Upon seeding within an exogenous basement membrane, mammary epithelial cells proliferate and organize into spheroids, commonly called acini, forming a three dimensional (3D) culture system. These structures develop an axis of apicobasal polarity, illustrated by the basal secretion of matrix components (laminin 5), the apical orientation of the

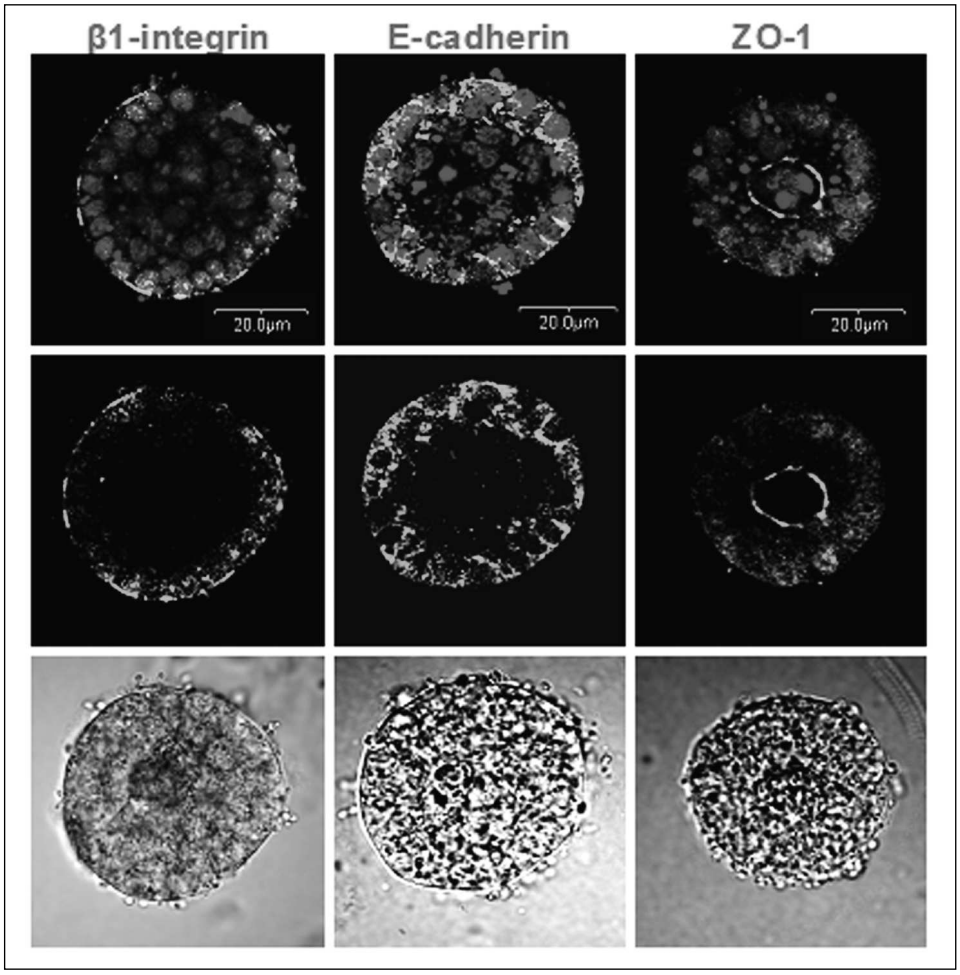

Fig. 1. Confocal images of BME-UV1 bovine mammary epithelial cells (MECs) cultured for 16 days in 3D system on Matrigel™; during the 16-day culture MECs develop an axis of apicobasal polarity, illustrated by the appropriate localization of junctional complexes: β1-integrin (belonging to a class of receptors localized on the basal membrane, contacting the cell with ECM), E-cadherin - localized on the lateral side of cell membrane, and ZO-1 - a tight junction protein, localized on the apicolateral surface of cell membrane. All markers were stained green with specific antibodies for immunofluorescence staining; nuclei were stained red with 7-AAD, and the lowest panels present the normal, white light image of stained acini.

Golgi, and the appropriate localization of junctional complexes (lateral localization of desmosomes and gap junctions, and apicolateral localization of tight junction proteins, such as Zonula Occludens ZO-1) (35, 36) (*Fig. 1*). As the acini mature, two distinct populations of cells become evident within each acinus – an outer layer of cells in direct contact with the matrix and the inner subset of cells lacking matrix contact. Throughout morphogenesis, the outer cell layer remains polarized with respect to the acinus centre and the centrally located cells undergo cell death – apoptosis (*Fig. 2a*). This cell death contributes to the formation of a hollow lumen (37). However, apoptosis is not the only phenomenon that can be observed during lumen formation. Electron microscopic analysis of human mammary epithelial cell line MCF-10A revealed that numerous autophagic vacuoles are present in the central cells of developing acini (38). Additionally our group, which is presently working on the 3D model of bovine mammary epithelial cells grown on Matrigel™, has noted

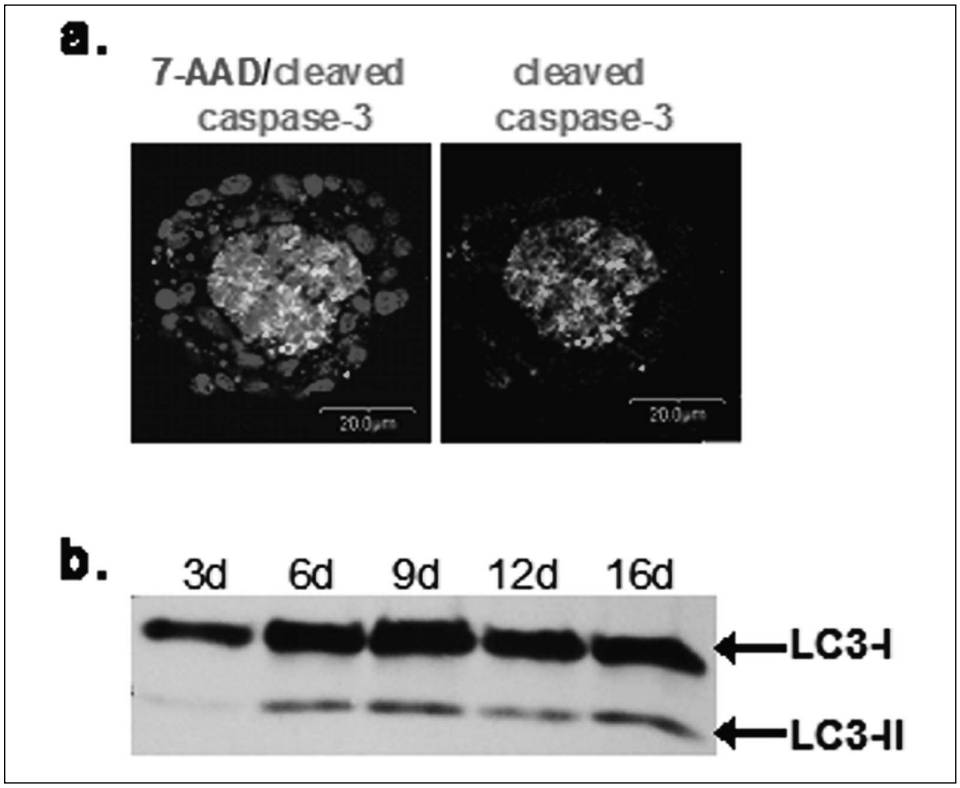

Fig. 2. (a) Confocal images of BME-UV1 cells from the 16th day of culture in 3D system, on Matrigel™, showing the process of lumen clearance by apoptosis. The green staining represents cleaved caspase-3, a marker of apoptosis localized in the centre of the acini, nuclei were stained red with 7-AAD; (b) Western-blot analysis of LC3 (marker of autophagy) levels in BME-UV1 cells cultured in 3D system for 3, 6, 9, 12 or 16 days.

that during the development and differentiation of acini the level of membrane-bound LC3-II is increasing (*Fig. 2b*). Conversion of LC3-I to LC3-II form indicates the involvement of this protein in the formation of autophagosomal membrane. The amount of LC3-II in the cell is correlated with the number of autophagosomes, and therefore with the induction of autophagy (39). Based on these initial results, autophagy has been proposed to promote luminal clearance by autophagic death (38, 40). Other studies have shown that a Tumor Necrosis Factor (TNF) family ligand - Tumor Necrosis Factor-related Apoptosis-Inducing Ligand (TRAIL) induces autophagy in epithelial cells and that TRAIL inhibition promotes luminal filling, when it is combined with Bcl-x_L- mediated inhibition of apoptosis (40). Epithelial cells depend on integrin-mediated cell adhesion to ECM for proper growth and survival, and they undergo apoptotic cell death (termed anoikis) upon detachment (41). It has been shown that this detachment induces autophagy in MCF-10A cell line and primary mammary epithelial cells. RNA interference-mediated depletion of autophagy regulators (*Atg5, Atg6 – Beclin 1*, and *Atg7*) results in both enhanced cleaved caspase-3 (apoptosis marker) during anoikis, and reduced clonogenic viability upon reattachment (42). At the same time the authors observed that matrix-detached cells still exhibit autophagy when apoptosis is blocked by Bcl-2 expression, and *Atg* depletion impairs the clonogenic survival of detached Bcl-2-expressing cells. They also noted that stable reduction of Atg5 or Atg7 in MCF-10A acini enhances luminal apoptosis during morphogenesis and fails to elicit long-term luminal filling. Thus these findings show that autophagy promotes epithelial cells survival during anoikis.

AUTOPHAGY IN MAMMARY TUMORIGENESIS

Early studies conducted on human breast tumor cell lines revealed that these cells do not form acini when grown in 3D culture, but they develop into non-polarized clusters with limited differentiation and inhibited lumen formation (43). These experiments have shown the stark behavioral contrast between normal and tumor cells in 3D culture, even though subtle phenotypic differences were evident when the same cells were grown as monolayers. Studies of cancer genes and activated receptor tyrosine kinases in MCF-10A cultures have illustrated some of the mechanisms and pathways that contribute to luminal filling in 3D culture. For example, when proliferation is increased within MCF-10A acini by the ectopic expression of cyclin D a hollow architecture is maintained by the increased apoptosis of excess cells that occupy the lumens of these structures (38). However, luminal filling does occur when the induction of increased proliferation is combined with the inhibition of apoptosis *via* the overexpression of anti-apoptotic factors such as: Epidermal Growth Factor Receptor 2 (ERBB2), proto-oncogenic nonreceptor tyrosine kinase Src, and Insulin-like Growth Factor Receptor 1 (IGF1R) (44-46). Decreased expression of pro-apoptotic protein BIM,

observed in MCF-10A cells expressing activated ERBB2 or Src, serves as one possible mechanism to promote survival in the lumen (44). Surprisingly, blocking classical apoptosis by overexpression of anti-apoptotic proteins, such as Bcl-2 only delays the development of hollow lumen, but cannot prevent this process. The central cells in Bcl-2-expressing acini also display autophagic vacuoles before clearance (38, 40), however, loss of function studies of known autophagy genes (*Atg*) are required to establish a functional role for autophagy in the regulation of lumen formation (37).

The involvement of autophagy in tumorigenesis has been also proven by studies of tumors and cancer cells, cultured in a classical 2D system. As previously mentioned, autophagy allows cells to respond to changing environmental conditions, such as nutrient deprivation. On starvation, autophagy is greatly increased and thereby provides cells with amino acids, fatty acids and nucleotides. During tumor formation, when limited angiogenesis leads to nutrient deprivation and hypoxia, autophagy is believed to protect tumor cells from dying. Moreover, when autophagy is prevented under these conditions, the cells undergo apoptosis (47, 48). Therefore one can conclude that increased autophagy would promote the growth of solid tumors.

In contrast to this potential cancer-promoting effect of autophagy, numerous studies have shown an anticancer role of autophagy. The autophagy gene *Beclin 1* is a haploinsufficient tumor suppressor in mice (11, 12). Defective autophagy has been implicated in tumorigenesis, as the *Beclin 1* is found to be monoallelically deleted in human breast, ovarian, and prostate cancers (49). Mammary tissue from *Beclin1 +/-* mice shows hyperproliferative, preneoplastic changes (11). In addition, human breast carcinoma cell lines and tumor tissue show decreased Beclin 1 levels (50). On the other hand Beclin 1 expression in human MCF7 breast cancer cells suppresses tumorigenesis (50). Moreover, *p53* and *PTEN*, two of the most commonly mutated tumor suppressor genes, both induce autophagy (51, 52). Conversely, the oncogenic protein Bcl-2 directly interacts with Beclin 1 to inhibit autophagy (53). These observations suggest anticancer properties of autophagy process. However, the mechanism through which autophagy can inhibits tumor development is still unclear. Possibilities include limiting tumor cell growth or reducing mutagenesis or other damage caused by reactive oxygen species by removal of damaged mitochondria and other organelles (54).

Alternatively, autophagy may also lead to tumor cells death, as in certain conditions autophagic (type II) cell death is activated. Yu *et al*. (55) have recently shown that autophagic cell death caused by caspase inhibition is achieved through the selective autophagic degradation of catalase, which in turn leads to the generation of oxygen species that kills the cell. Many anticancer agents, such as: tamoxifen, rapamycin, arsenic trioxide, histone deacetylase inhibitors, camptotecin, vitamin D analogues, or etoposide have been reported to induce autophagy (56-60). However, it is still a matter of question whether autophagy is really a cell death mechanism, because it occurs in tumor cells before their

annihilation. In fact, autophagy may serve as a mechanism by which cells are trying to survive, but in long lasting stressful conditions it leads to death. So far most results only show that the examined anticancer drugs induce autophagy and then the cells died. Nevertheless, Ravikumar *et al*. (61) observed that rapamycin-induced autophagy can protect various tumor cell lines against apoptosis induced by general apoptotic stimuli, and might have a similar effect in the action of anticancer agents. Therefore one can conclude that manipulation of autophagy may provide a useful way to prevent cancer development, limit tumor progression, and increase the efficiency of cancer treatment, but the type of manipulations that are necessary have to be context dependent. Autophagy may need to be increased to prevent tumor formation in individuals at risk of cancer, but reduced if a tumor is already established and subjected to the environmental stresses associated with limited angiogenesis, nutrient deprivation, and hypoxia.

CONCLUSIONS

The progress which has been made in the last decade provided us with detailed information about molecular mechanisms of autophagy. As a consequence we have obtained data showing, that autophagy is not only an evolutionarily conserved cellular pathway for the degradation of long-lived proteins and cytoplasmic organelles, but it is involved in regulation of eukaryotic development, differentiation and cell death. Autophagy genes are essential for multicellular development in *Dicyostelium discoideum*, and the development of multicellular organisms, including *Drosophila*, *C. elegans* and mice. This suggests common requirements for efficient catabolic pathways, which rapidly recycle intracellular components in diverse aspects of development across a wide range of taxa. A good example of a model used in studies of the mechanisms controlling higher eukaryotic organisms' development is the model of mammary gland, as it is one of very few organs in which substantial development occurs after an animal is born. The model systems of mammary gland acini formation revealed that autophagy (together with apoptosis) is involved in the removal of epithelial cells to form luminal structures, showing its role in mammogenesis and suggesting its possible role in preventing early steps of epithelial tumor development. Context dependent manipulations of autophagic pathways done by enhancement of autophagy, that would promote autophagic cell death, or its inhibition that would lead to apoptosis, may be the way of creating more effective anticancer therapies in the future.

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