[Autophagy 3:6, 610-613; November/December 2007]; ©2007 Landes Bioscience

Addendum

Role of Autophagy in Breast Cancer

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Original manuscript submitted: 08/03/07
Manuscript accepted: 08/14/07

Previously published online as an *Autophagy* E-publication:
http://www.landesbioscience.com/journals/autophagy/article/4867

KEY WORDS

autophagy, breast cancer, beclin1, DNA damage, genomic instability

Addendum to:

Autophagy Mitigates Metabolic Stress and Genome Damage in Mammary Tumorigenesis

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Genes Dev 2007; 21:1621-35

ABSTRACT

Autophagy is an evolutionarily conserved process of cytoplasm and cellular organelle degradation in lysosomes. Autophagy is a survival pathway required for cellular viability during starvation; however, if it proceeds to completion, autophagy can lead to cell death. In neurons, constitutive autophagy limits accumulation of polyubiquitinated proteins and prevents neuronal degeneration. Therefore, autophagy has emerged as a homeostatic mechanism regulating the turnover of long-lived or damaged proteins and organelles, and buffering metabolic stress under conditions of nutrient deprivation by recycling intracellular constituents. Autophagy also plays a role in tumorigenesis, as the essential autophagy regulator *beclin1* is monoallelically deleted in many human ovarian, breast, and prostate cancers, and *beclin1*$^{+/-}$ mice are tumor-prone. We found that allelic loss of *beclin1* renders immortalized mouse mammary epithelial cells susceptible to metabolic stress and accelerates lumen formation in mammary acini. Autophagy defects also activate the DNA damage response in vitro and in mammary tumors in vivo, promote gene amplification, and synergize with defective apoptosis to accelerate mammary tumorigenesis. Thus, loss of the prosurvival role of autophagy likely contributes to breast cancer progression by promoting genome damage and instability. Exploring the yet unknown relationship between defective autophagy and other breast cancer-promoting functions may provide valuable insight into the pathogenesis of breast cancer and may have significant prognostic and therapeutic implications for breast cancer patients.

Autophagy is an evolutionarily conserved catabolic process of self-digestion whereby double-membrane vesicles engulfing cellular organelles and cytoplasm form in the cytosol and fuse with lysosomes, where the sequestered contents are degraded and recycled for protein and ATP synthesis.[1] The essential components of the autophagic machinery were initially identified in yeast, but several of the corresponding proteins have homologues in higher eukaryotes.[2] During starvation or growth factor deprivation of normal cells, autophagy is a temporary survival mechanism, as it provides an alternative energy source.[3,4] Autophagy also promotes cancer cell survival under metabolic stress.[5] Autophagy is involved in both the recycling of normal cellular constituents and the removal of damaged proteins and organelles, as autophagy defects result in ubiquitinated protein aggregates and deformed organelles that may promote cellular degeneration.[6,7] Autophagy is also a form of cell death, when allowed to proceed to excessive levels and when apoptosis-defective cells are triggered to die. Under these circumstances, it is often unclear whether autophagy contributes to cell death actively or represents an exhausted attempt to preserve cell viability under stress. Recent studies indicate that autophagy may play an active role in programmed cell death, but the conditions under which autophagy promotes cell death versus cell survival are still unknown.[8]

ALLELIC LOSS OF *Beclin1* AND BREAST CANCER

beclin1 is the mammalian orthologue of the essential yeast ATG*6*/VPS*30* gene, which is required for autophagosome formation in a complex with the class III phosphatidylinositol-3-kinase (PIK3C3 or Vps34).[9] *beclin1* complements the autophagy defect present in *atg6*/*vps30*-disrupted yeast and in human MCF7 breast cancer cells.[10] Originally entered in GenBank as a gene of unknown function during the positional cloning of BRCA1,[11] *beclin1* was independently rediscovered in a yeast two-hybrid screen of an adult mouse brain library for proteins interacting with the anti-apoptotic protein Bcl-2.[12] The mouse

and human *beclin1* genes share 93% identity at the nucleotide level and 98% identity at the amino acid level.[12] The human *beclin1* gene is on chromosome 17q21 within a region commonly allelically deleted in ovarian, breast and prostate cancers.[13] Many breast carcinoma cell lines, although polyploid for chromosome 17, exhibit deletions of one or more *beclin1* alleles[13] and human breast tumors show decreased Beclin1 levels compared to normal adjacent tissue.[10] Restoration of Beclin1 and autophagy in MCF-7 cells is associated with inhibition of MCF7-induced tumorigenesis in nude mice.[10] *beclin1*$^{-/-}$ mice die early in embryogenesis, likely due to loss of the essential role of autophagy during development. Mammary tissue from aging *beclin1*$^{+/-}$ mice shows hyperproliferative, preneoplastic changes,[14] indicative of a correlation between low Beclin1 levels and mammary tumorigenesis. *beclin1*$^{+/-}$ mice do not have increased incidence of mammary tumors, but rather are susceptible to lymphomas and carcinomas of the lung and liver after a long latency.[14,15] Tumors forming in *beclin1*$^{+/-}$ mice express wild-type *beclin1* mRNA and protein, indicating that *beclin1* is a haploinsufficient tumor suppressor.[15] Clearly other mutations are required to cooperate with allelic loss of *beclin1* for tumorigenesis, which has yet to be modeled in mice.

THE TWO FACES OF AUTOPHAGY: CELLULAR SURVIVAL AND TUMOR SUPPRESSION

Although the studies summarized above clearly implicate allelic loss of *beclin1*, and thus defective autophagy, in breast cancer pathogenesis, how loss of a survival pathway such as autophagy promotes breast tumorigenesis was initially an enigma. Allelic loss of *beclin1* in immortalized mouse mammary epithelial cells (iMMECs) compromises the autophagy potential of these cells and results in decreased iMMEC viability under metabolic stress in two-dimensional (2D) culture, and in accelerated lumen formation in three-dimensional (3D) morphogenesis assays (Fig. 1). For apoptosis-competent *beclin1*$^{+/-}$ iMMECs, metabolically stressed central acinar cells undergo apoptosis more readily than *beclin1*$^{+/+}$ cells due to failure of autophagy, whereas in the case of Bcl-2-expressing *beclin1*$^{+/-}$ iMMECs, accelerated lumen formation involves induction of necrotic cell death due to concurrent autophagy and apoptosis defects.[16]

In contrast to the prediction that autophagy-deficient cancer cells would similarly exhibit decreased survival in tumors in vivo, autophagy defects in iMMECs enhance tumor progression in orthotopic growth in the mammary fat pad.[16] Similarly, allelic loss of *beclin1* in immortalized baby mouse kidney (iBMK) cells increases susceptibility of iBMK cells to metabolic stress in vitro, yet promotes tumorigenesis in vivo.[5] An explanation reconciling the two intuitively contradictory roles of autophagy resides with the finding that deficient autophagy, in the form of either *beclin1* heterozygosity or *atg5* deficiency, leads to DNA damage and genomic instability. This genetic instability likely requires inactivation of the p53 and Rb pathways, and thus non-functional checkpoints,[16,17] and is most prominently manifested when apoptosis is also disabled, thus leading to an increased mutation rate and accelerated tumorigenesis.

The animal studies mentioned above point toward a possible correlation between allelic loss of *beclin1* and inactivation of p53 and pRb in human breast cancer. This intriguing possibility is worthy of further investigation, particularly given the high frequency of

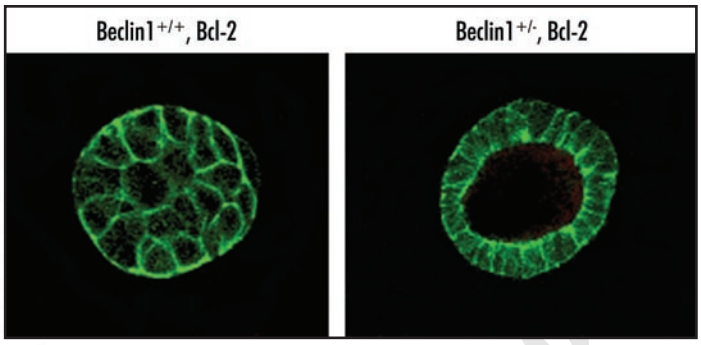

Figure 1. Deficient autophagy accelerates lumen formation in mammary acini. In 3D morphogenesis assays, metabolic stress and autophagy localize in the central acinar cells.[16] If apoptosis-deficient and autophagy-competent, the central acinar cells are capable of coping with metabolic stress and/or anoikis[33] and thus survive, resulting in mammary acini with filled lumens (left panel). Allelic loss of *beclin1*, and thus deficient autophagy, abrogates the survival advantage conferred to the central acinar cells by disabled apoptosis, resulting in induction of necrotic cell death and accelerated lumen formation (right panel).

beclin1 loss, *p53* mutant status and Rb pathway inactivation in breast tumors. *p53* mutations are present in 30% of sporadic human breast carcinomas and Li-Fraumeni syndrome patients carrying germ line *p53* mutations develop breast cancer at an early age.[18] Furthermore, *p53* mutations are associated with more aggressive and therapeutically refractory disease.[19] Similarly, Rb expression is aberrant in 20–30% of breast tumors, as demonstrated by lack of detectable Rb levels or loss-of-heterozygosity, and Rb function is further compromised by *cyclin D1* amplification and *p16ink4a* loss, both commonly observed in breast tumors.[20] Also, deregulation of the Rb pathway has therapeutic and prognostic implications for breast cancer patients.[21]

Investigation of any functional interaction between autophagy defects and mutant p53 status in mammary tumorigenesis can be performed by using two recently developed mouse models for Li-Fraumeni syndrome. These models involve conditional knock-in of two different dominant negative *p53* mutants into the endogenous murine *p53* locus,[22] and mammary gland-specific expression of one of these *p53* mutants resulted in spontaneous and carcinogen-induced mammary tumors at high frequency.[18] Accelerated mammary tumorigenesis in *beclin1*$^{+/-}$ mice with mammary gland-specific expression of a dominant negative *p53* mutant will indicate synergy between deficient autophagy and *p53* mutations. Validation of this result by examination of human breast tumors for concurrent p53 mutations and autophagy defects may provide valuable insight in breast cancer pathogenesis.

DEFICIENT AUTOPHAGY AND GENE AMPLIFICATION

Deficient autophagy in the form of *beclin1* heterozygosity promotes gene amplification,[16,17] as demonstrated by a high frequency of PALA resistance and PALA-induced amplification of the CAD gene[23] in *beclin1*$^{+/-}$ iMMECs and iBMK cells. Gene amplification is common in solid tumors[24] and is likely initiated by DNA double strand breaks in cells lacking robust checkpoints.[25] Breast cancers frequently show genome copy number aberrations, including high-level amplifications that correlate with worse prognosis.[26] For example, *HER2/neu* amplification is found in about 30% of human breast cancers and is associated with aggressive disease, poor clinical

outcome and chemotherapy resistance,[26] whereas c-*myc* amplification is found in about 15% of breast cancers and is significantly associated with risk of relapse and death.[27] Whether autophagy defects segregate with *HER2/neu* or c-*myc* or other specific gene amplification in breast cancer is not known. It is possible that the relationship between deficient autophagy and gene amplification is complex, as a vicious cycle may exist whereby autophagy defects promote genome damage and instability in the form of amplification involving genes that positively regulate cellular growth and proliferation. The concomitant increased metabolic demands may in turn lead to higher levels of DNA damage in autophagy-deficient tumor cells and propagation of genomic instability.[28,29]

FUNCTIONAL STATUS OF AUTOPHAGY AND BREAST CANCER TREATMENT

Autophagy-deficient iMMECs accumulate DNA damage in response to metabolic, and likely replication, stress,[16] raising the possibility that breast tumor cells with autophagy defects may be particularly sensitive to DNA damaging agents. In a similar situation, *BRCA1* and *BRCA2* mutant cells, which are defective in DNA repair, have been shown to be exquisitely sensitive to certain DNA damaging agents, such as cisplatin, and to agents interfering with DNA repair, such as poly(ADP-Ribose) polymerase (PARP) inhibitors.[30] It is therefore conceptually possible that autophagy-deficient breast tumor cells may also exhibit high sensitivity to agents causing DNA damage or those that interfere with DNA replication, as well as to drugs that inhibit DNA repair. The outcome of such treatment will likely depend on the functional status of the cell cycle checkpoints and the apoptotic cell death pathway.

For autophagy-deficient tumor cells with intact apoptotic response, high levels of DNA damage will likely lead to prompt activation of apoptosis and cell death. In the case of tumor cells with combined autophagy and apoptosis defects, but functional p53, DNA damage accumulation may result in senescence, which could be therapeutically beneficial, at least for as long as *p53* wild-type status is maintained. In the case of tumor cells triply deficient in apoptosis, autophagy and cell cycle checkpoints, and thus similar to Bcl-2-expressing *beclin1*$^{+/-}$ iMMECs, massive DNA damage may trigger cell death by mitotic catastrophe or necrosis. Potential problems with the later scenario include the possibilities that necrosis-associated inflammatory reaction may promote tumorigenesis[5] and that genomically unstable tumor cells may acquire mutations that enable them to survive, ultimately resulting in disease relapse. Apoptosis-defective *beclin1*$^{+/-}$ iMMECs are prone to gene amplification under selective pressure such as PALA treatment,[16] raising the concern that anticancer agents such as methotrexate, for which gene amplification mediates drug resistance, may be poor treatment choices for breast tumors with combined apoptosis, autophagy and cell cycle checkpoint defects.

Alternatively, treatment of breast carcinomas having normal Beclin1 levels and intact autophagic response with an autophagy inhibitor may sensitize tumor cells to a variety of anticancer agents, especially DNA damaging agents and other S-phase-specific drugs. This principle has already been demonstrated in a Myc-induced lymphoma model in which inhibition of autophagy with either chloroquine or ATG5 short hairpin RNA (shRNA) enhanced the ability of alkylating drug therapy to induce tumor cell death.[31] Similarly, autophagy inhibitors dramatically augmented the antineoplastic effects of the histone deacetylase (HDAC) inhibitor suberoylanilide hydroxamic acid (SAHA) in CML cell lines and primary CML cells expressing wild-type and imatinib-resistant mutant forms of Bcr-Abl, including T315I.[32] Taking it one step further, concurrent inhibition of autophagy and reactivation of apoptosis may provide an even more efficient way to augment the therapeutic benefit of several anticancer drugs, as metabolically susceptible autophagy-deficient cells will be diverted to cell death by apoptosis upon treatment with drugs that cause metabolic stress, such as angiogenesis inhibitors, or DNA damage. In support of this hypothesis, inhibition of autophagy enhanced the ability of p53 activation to induce tumor cell death by apoptosis in a Myc-induced lymphoma model.[31]

In conclusion, autophagy defects compromise the ability of mouse mammary, and very likely human breast, tumor cells to cope with metabolic stress resulting in genome damage and instability, which in turn may accelerate tumor progression. The functional interaction between defective autophagy and other breast cancer-promoting functions, in particular those involving gene amplification as a mechanism of tumorigenesis, remains to be investigated and could have significant prognostic and therapeutic implications for breast cancer patients. Furthermore, investigation of the impact that the functional status of autophagy and its pharmacological manipulation have on breast cancer treatment is of utmost interest and importance as a novel therapeutic target may be identified.

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