Autophagy Regulates Keratin 8 Homeostasis in Mammary Epithelial Cells and in Breast Tumors

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Abstract

Autophagy is activated in response to cellular stressors and mediates lysosomal degradation and recycling of cytoplasmic material and organelles as a temporary cell survival mechanism. Defective autophagy is implicated in human pathology, as disruption of protein and organelle homeostasis enables disease-promoting mechanisms such as toxic protein aggregation, oxidative stress, genomic damage, and inflammation. We previously showed that autophagy-defective immortalized mouse mammary epithelial cells are susceptible to metabolic stress, DNA damage, and genomic instability. We now report that autophagy deficiency is associated with endoplasmic reticulum (ER) and oxidative stress, and with deregulation of p62-mediated keratin homeostasis in mammary cells, allograft tumors, and mammary tissues from genetically engineered mice. In human breast tumors, high phospho(Ser73)-K8 levels are inversely correlated with Beclin 1 expression. Thus, autophagy preserves cellular fitness by limiting ER and oxidative stress, a function potentially important in autophagy-mediated suppression of mammary tumorigenesis. Furthermore, autophagy regulates keratin homeostasis in the mammary gland via a p62-dependent mechanism. High phospho(Ser73)-K8 expression may be a marker of autophagy functional status in breast tumors and, as such, could have therapeutic implications for breast cancer patients. Mol Cancer Res; 8(6); 873–84. ©2010 AACR.

Introduction

Macroautophagy (hereafter referred to as autophagy) is a cellular self-consumption process whereby cytoplasm, long-lived proteins, and organelles are engulfed in double-membrane vesicles and delivered to lysosomes for degradation. Basal autophagy maintains cellular homeostasis by eliminating damaged proteins and organelles. Autophagy defects have been implicated in the pathogenesis of diseases such as myopathy, neuronal degeneration, microbial infection, inflammatory bowel disease, aging, and cancer. In addition to its basal function, autophagy is induced by nutrient deprivation, metabolic stress, endoplasmic reticulum (ER) stress, radiation, and chemotherapy, mostly functioning as a temporary survival mechanism (1).

Apoptosis inactivation occurs frequently in tumors, indicating that aberrant cell survival contributes to cancer progression. Loss of a survival pathway, such as autophagy, might have been expected to undermine tumorigenesis; however, the essential autophagy regulator beclin 1 is a haploinsufficient tumor suppressor (2, 3) arguing against this simplistic scenario. Recent studies described non-cell-autonomous and cell-autonomous mechanisms for autophagy-mediated tumor suppression (4, 5). Autophagy may suppress tumorigenesis by limiting necrosis-associated inflammation (6) and by preserving genome integrity and cellular fitness, as autophagy defects result in accumulation of ubiquitin-positive protein aggregates in neurons and liver (7), deformed mitochondria (8) and peroxisomes (9), DNA damage and genomic instability in tumor cells (4, 10), accumulation of oxidative and ER stress–sensing proteins, and reactive oxygen species (ROS) in kidney epithelial cells (5).

Because autophagy mediates protein degradation, we used a proteomic approach to investigate how beclin 1+/− and beclin 1−/− iMMECs respond to metabolic stress. We found that ER chaperones, oxidative stress–mitigating mitochondrial proteins, enzymes involved in glucose metabolism, and cytoskeletal proteins were upregulated in mammary cells under stress, preferentially in beclin 1−/− iMMECs. Defective autophagy was also associated with accumulation of p62, a scaffolding protein involved in cell signaling, receptor internalization, and protein turnover.
(11) during metabolic stress and recovery. Furthermore, autophagy defects deregulated keratin homeostasis in mammary cells, and phospho(Ser73)-K8, which is involved in stress-induced keratin remodeling, accumulated in metabolically stressed iMMECs in a p62-dependent manner upon autophagy inhibition. Higher levels of p62, keratins, and ER chaperones were also observed in autophagy-deficient mammary tissues and beclin 1−/− immortalized mouse mammary epithelial cell (iMMEC)–generated tumors. Evaluation of a human breast cancer tissue microarray (TMA) revealed that ER chaperone, p62, and keratin upregulation reliably discriminated tumors from normal adjacent tissue (NAT); however, only high phospho-K8 levels inversely correlated with Beclin 1 expression. Thus, elevated phospho-K8 may be an epithelial cell marker of autophagy deficiency in breast tumors.

Materials and Methods

Stable cell line generation and culture conditions
Primary mouse mammary epithelial cells from beclin 1−/− and beclin 1−/− mice were immortalized to generate iMMEC cell lines, which were engineered to stably express Bcl-2 as previously described (10, 12). Metabolic stress was induced as previously described (12). N-acetylcysteine (NAC) (Sigma-Aldrich) was used at 1 nmol/L concentration.

Western blotting, immunofluorescence, and immunohistochemistry
Antibodies against the following antigens were used: GRp78 (Stressgen); ATF6α, beclin 1 (BECN1 H-300), calnexin, superoxide dismutase 2 (SOD2; Santa Cruz Biotechnology); aconitase (Atlas); K19 (GeneTex); protein disulfide isomerase (PDI; Sigma); p62 (Biomol); K8/18, K17, peroxiredoxin 3, PGAM1, phospho(Ser73)-K8 (Abcam); actin (Oncogene); β-catenin (Zymed); K8 (University of Iowa Developmental Studies Hybridoma Bank); and LC3B (Cell Signaling).

Three-dimensional morphogenesis
Three-dimensional culture of iMMECs and immunofluorescence on mammary acini generated by Bcl-2 expressing beclin 1−/− and beclin 1−/− iMMECs were done as previously described (10, 12).

Generation of mammary gland–specific

Proteins upregulated under stress included ER chaperones [glucose regulated protein (Grp) 170, Grp94, Grp78, and PDI], mitochondrial proteins with antioxidant activity [peroxiredoxin (PRDX) 3, SOD2], enzymes involved in glucose metabolism (pyruvate kinase, enolase, phosphoglycerate mutase, triose phosphate isomerase), and cytoskeletal proteins, including keratins and annexins. Up-regulation was higher in beclin 1−/− iMMECs for all above protein families (Supplementary Table S1), indicating that defective autophagy is associated with increased ER and oxidative stress, elevated metabolic demands, and cytoskeletal alterations in mammary cells under stress. Similarly to iMMECs, autophagy-deficient immortalized baby mouse mammary cells under metabolic stress and in recovery

Elevated ER stress in autophagy-deficient mammary cells under metabolic stress and in recovery
beclin 1 monoallelic loss renders iMMECs susceptible to metabolic stress, DNA damage, and genomic instability, and enhances mammary tumorigenesis (10). Because autophagy is a lysosomal protein degradation pathway, we used proteomics to investigate the role of autophagy in mammary cell response to metabolic stress and assess possible mechanisms of stress management failure. iMMECs were engineered to express Bcl-2, as autophagy assessment is facilitated in an apoptosis-defective background and Bcl-2 expression is functionally equivalent to Bax and Bak deficiency regarding autophagy modulation and tumorigenesis (5, 6, 10, 13). Apoptosis-defective autophagy-competent beclin 1−/− and autophagy-defective beclin 1−/− iMMECs (10) were exposed to metabolic stress (glucose and oxygen limitation), and differential protein expression after 4 and 7 days of stress was determined by two-dimensional fluorescence difference gel electrophoresis (2D-DIGE). One hundred sixty differentially regulated proteins were identified by mass spectroscopy. Representative images of differential protein expression (red, upregulated; green, downregulated) for beclin 1−/− and beclin 1−/− iMMECs after 7 days of stress are presented in Fig. 1A and quantified in Supplementary Table S1.

Immunohistochemical staining quantification and statistical analysis

Beclin 1, GRp78, GRp170, p62, K8, K17, and phospho(Ser73)-K8 levels were evaluated by immunohistochemistry in a human breast cancer TMA. Protein expression (i.e., staining intensity) in epithelial cells was manually quantified by two study investigators (N.B. and V.K.). The following scale was used: 0-1+, 2+, and 3+ staining intensity corresponded to low, moderate, and high protein levels, respectively. A two-sided exact Wilcoxon test was used to compare Beclin 1 levels in human breast tumors to NAT. A two-sided test was used to compare high levels (3+ staining) of p62, GRp170, GRp78, or phospho(Ser73)-K8 in breast tumors to NAT. Correlation between absolute Beclin 1 levels and high expression (3+ staining) of p62, GRp170, GRp78, or phospho(Ser73)-K8 in breast tumors were examined using logistic regression.

Results

Elevated ER stress in autophagy-deficient mammary cells...
FIGURE 1. Metabolic stress causes preferential upregulation of ER chaperones, mitochondrial enzymes, metabolism-related proteins, and keratins in autophagy-deficient mammary cells. A, 2D-DIGE gels showing differential regulation of ER chaperones (GRP170, orange arrows), mitochondrial enzymes (PRDX3, yellow arrows), metabolism-related proteins (glyceraldehyde-3-phosphate dehydrogenase, magenta arrows), and keratins (K8, blue arrows) in Bcl-2–expressing beclin 1+/+ (left) and beclin 1+/− (right) iMMECs in response to metabolic stress (7 days). Total protein from unstressed or metabolically stressed iMMECs were labeled with Cy3 (unstressed) or Cy5 (stressed) and analyzed by 2D-DIGE. Images show 2D-DIGE gels with proteins that are induced (red), repressed (green), or unchanged (yellow) under stress. One hundred six differentially expressed protein spots were identified by mass spectroscopy. B, Western blots showing levels of ER chaperones (GRP170, GRP78, calnexin), ATF6, mitochondrial enzymes (PRDX3, SOD2, aconitase), glycolytic enzyme (PGAM), and keratins (K8/18, K17, and K19) in Bcl-2–expressing beclin 1+/+ (left) and beclin 1+/− (right) iMMECs under metabolic stress for 0 to 5 days. One hundred six differentially expressed protein spots were identified by mass spectroscopy. B, Western blots showing levels of ER chaperones (GRP170, GRP78, calnexin), ATF6, mitochondrial enzymes (PRDX3, SOD2, aconitase), glycolytic enzyme (PGAM), and keratins (K8/18, K17, and K19) in Bcl-2–expressing beclin 1+/+ (left) and beclin 1+/− (right) iMMECs under metabolic stress for 0 to 5 days. One hundred six differentially expressed protein spots were identified by mass spectroscopy. C, PDI (red) and K8 (green) immunofluorescence in Bcl-2–expressing beclin 1+/+ and beclin 1+/− iMMECs following 5 days metabolic stress and 1 day of recovery. D, SOD2 and PDI immunofluorescence (red) in mammary acini generated by Bcl-2–expressing beclin 1+/+ and beclin 1+/− iMMECs. β-Catenin (green) was used as an epithelial cell marker and 4′,6-diamidino-2-phenylindole (DAPI; blue) for counterstaining nuclei. E, ROS (H2O2) measurements in Bcl-2–expressing beclin 1+/+ and beclin 1+/− iMMECs using beclin 1 versus scramble (control) siRNA.
kidney (iBMK) cells showed preferential induction of ER chaperones under metabolic stress (5) and the microsomal fractions of atg7-deficient livers had high PDI, GRP78, and GRP94 levels (14), indicating that defective autophagy is associated with elevated ER stress, independently of tissue type and mode of autophagy defect. Because protein level estimation by spot volume ratios is complicated by representation of individual proteins by multiple spots in 2D-DIGE, results were validated by Western blotting. ER chaperones (GRP170, calnexin), the unfolded protein response (UPR) mediator (ATF6), mitochondrial enzymes (PRDX3, SOD2, aconitase), glycolytic enzymes (PGAM), and keratins (K8/18, K19, K17) were preferentially upregulated in beclin 1+/− iMMECs under stress (Fig. 1B), whereas stress-induced processing of LC3-I to LC3-II was attenuated in these cells, consistently with their previously reported decreased autophagy potential (10). Other markers of UPR were also examined (Supplementary Fig. S1A), and elevated ER stress in autophagy-deficient iMMECs was confirmed by accumulation of stress-induced phospho-elf2a and by increased XBP-1 splicing under normal growth conditions and upon metabolic stress induction (Supplementary Fig. S1B).

PDI and K8 expression in beclin 1+/− and beclin 1−/− iMMECs under stress and recovery was also examined by immunofluorescence. PDI expression dramatically increased in both autophagy-competent and autophagy-defective iMMECs under stress (Fig. 1C). Upon recovery (in normoxia and regular growth medium), PDI expression decreased to near-baseline levels in beclin 1+/− iMMECs, but remained high in beclin 1−/− iMMECs, indicating that autophagy-defective mammary cells have persistent need for PDI function during recovery due to the remaining unfolded protein load, and thus revealing that autophagy mediates misfolded protein clearance during recovery from metabolic stress. Expression of K18 and K19 increased under stress and remained elevated in recovery from metabolic stress. Expression of K18 and K19 increased upon stress in beclin 1+/− iMMECs and to a higher degree in beclin 1−/− iMMECs (Supplementary Table S1; Fig. 1B), whereas K17 was significantly upregulated only in stressed autophagy-defective iMMECs (Supplementary Table S1). In autophagy-competent iMMECs, K8 levels remained stable (Fig. 1B) or decreased (Fig. 1C) with stress and slowly recovered after 1 day of normal growth conditions, whereas in autophagy-defective iMMECs, K8 levels greatly increased under stress and remained elevated in recovery (Fig. 1C).

The response of beclin 1+/− and beclin 1−/− iMMECs to stress was also examined by three-dimensional morphogenesis. SOD2 and PDI levels were upregulated in the acinar center, particularly in beclin 1−/− iMMECs (Fig. 1D), indicating that central acinar cells, which are under increased metabolic stress (10), are also under oxidative stress. Higher oxidative stress levels in autophagy-defective iMMECs were confirmed by ROS measurements in beclin 1+/− and beclin 1−/− iMMECs and upon beclin 1 knockdown (Fig. 1E).

Metabolic stress-induced morphologic changes in beclin 1+/− and beclin 1−/− iMMECs were examined by transmission electron microscopy (Fig. 2A). Autophagosomes were apparent in beclin 1+/− iMMECs after 1 day of metabolic stress, whereas beclin 1−/− iMMECs were defective in autophagosome formation and progressively filled with electron-dense material suggestive of “cellular garbage,” due to decreased clearance and/or increased protein aggregate formation, similarly to autophagy-deficient iBMK cells (5).

Elevated ER stress in autophagy-deficient mammary tumors

Mammary tumors generated by orthotopic implantation of Bcl-2–expressing beclin 1+/− and beclin 1−/− iMMECs in nude mice were also examined by transmission electron microscopy. Autophagosomes were present only in mammary tumors generated by apoptosis-defective beclin 1+/− iMMECs, whereas beclin 1−/− iMMEC-generated tumors displayed prominent ER expansion indicative of ER stress (Fig. 2B). Thus, autophagy induction is likely a frequent occurrence in breast cancer, whereas ER proliferation and chaperone upregulation are characteristics of autophagy-defective mammary tumors.

iMMEC-generated tumors were also examined for ER stress by immunohistochemistry. GRP170 levels were higher in mammary tumors generated by beclin 1+/− iMMECs (Fig. 2C), indicating that defective autophagy is associated with increased ER stress in mammary tumors in vivo. Similarly, tumor allografts generated by autophagy-deficient iBMK cells displayed ER chaperone upregulation, and spontaneous lung and liver tumors from beclin 1−/− mice showed high GRP170 expression (5), indicating that autophagy defects lead to ER stress in epithelial tumors, independently of tissue type.

Autophagy-defective mammary tumor cells are sensitized to proteasome inhibitors and ER stress inducers

A prediction based on the induction of ER stress in beclin 1−/− iMMECs under metabolic stress is that autophagy-defective mammary cells may be sensitive to ER stress–inducing agents and to proteasome inhibitors. To test this hypothesis, beclin 1+/− and beclin 1−/− iMMECs were treated with the ER stress inducer tunicamycin and the proteasome inhibitor epoxomycin under normal growth conditions and upon recovery from metabolic stress, when the difference in ER stress levels between autophagy-competent and autophagy-defective mammary cells is maximal (Fig. 1C). Similarly to autophagy-defective iBMK cells (5), beclin 1−/− iMMECs were more sensitive than beclin 1+/− iMMECs to both agents (Fig. 3), suggesting that induction of ER stress and/or proteasome inhibition may be useful in autophagy-defective tumor treatment.

Autophagy regulates keratin homeostasis in mammary epithelial cells in a p62-dependent manner

p62 (SQSTM1) is an oligomerizing signaling adaptor protein (11, 15) that binds to ubiquitin (16) and LC3 (17), shuttles ubiquitinated proteins to proteasome (16)
FIGURE 2. Metabolic stress causes autophagy induction in beclin 1+/+ iMMECs, preferential accumulation of cytoplasmic “garbage” in beclin 1+/- iMMECs, and elevated ER stress in autophagy-deficient mammary tumors. A, representative electron micrographs of Bcl-2-expressing beclin 1+/+ (left column) and beclin 1+/- (right column) iMMECs following 5 days of metabolic stress. Arrows point to autophagosomes (red) in beclin 1+/+ iMMECs and to electron-dense cytoplasmic material (yellow) preferentially accumulating in beclin 1+/- iMMECs. B, representative electron micrographs of apoptosis-defective beclin 1+/+ (top) and beclin 1+/- (bottom) iMMEC-generated mammary tumors. An autophagosome is magnified in the red inset (top), whereas prominent ER expansion is presented in the green inset (bottom). C, GRp170 immunohistochemistry in mammary tumors generated by beclin 1+/+ (top) and beclin 1+/- (bottom) iMMECs.
and autophagosomes for degradation (18), and is induced by oxidative stress (19). beclin 1+/+ and beclin 1+/− iMMECs were examined for p62 expression under metabolic stress and recovery (Fig. 4A). Similarly to iBMK cells (5), p62 levels increased in stressed iMMECs, more so in autophagy-defective cells, whereas they decreased during recovery, especially in beclin 1+/+ iMMECs, indicating that oxygen and nutrient limitation is a potent stimulus for p62 expression. The ROS scavenger NAC suppressed stress-induced p62 upregulation, particularly in autophagy-competent iMMECs. Higher p62 levels under metabolic stress and recovery in beclin 1+/− iMMECs, both in the absence and presence of NAC at a concentration adequate to reduce p62 accumulation in beclin 1+/+ iMMECs, indicated that autophagy-deficient mammary cells exhibit increased oxidative stress upon hypoxia and glucose deprivation, as well as during recovery. These results complement recently reported higher ROS levels in autophagy-defective iBMK cells (5). p62 accumulation in beclin 1+/− iMMECs was also observed in metabolically stressed central acinar cells in three-dimensional morphogenesis (Fig. 4A).

In beclin 1+/+ iMMECs under stress, p62 colocalized with K8 in perinuclear puncta reminiscent of autophagosomes, whereas p62 masked keratin epitopes in beclin 1+/− iMMECs, possibly due to higher expression levels and oligomerization around aggregated keratin structures (Fig. 4B). K8 accumulation in autophagy-defective cells became apparent in recovery when p62 levels decreased.

Intermediate filament reorganization under stress is known to involve keratin phosphorylation (20), particularly K8 phosphorylation at Ser73 (21). Phospho(Ser73)-K8 basal levels were similarly low in beclin 1+/+ and beclin 1+/− iMMECs (Fig. 4C, left column), but increased in autophagy-defective iMMECs under stress (Fig. 4C, middle column) and even further upon pharmacologic inhibition of autophagy with bafilomycin, more so in beclin 1+/− iMMECs (Fig. 4C, right column), suggesting that K8 phosphorylation is an intermediate step in autophagy-mediated keratin remodeling in mammary epithelial cells under stress. After 5 days of metabolic stress, autophagosomes could not be visualized as they were degraded in lysosomes (Fig. 4C, middle column), unless lysosomal function was inhibited with bafilomycin (Fig. 4C, right column). p62 knockdown

FIGURE 3. Defective autophagy sensitizes mammary tumor cells to ER stressors and proteasome inhibitors. MTT assays showing sensitivity of Bcl-2–expressing beclin 1+/+ (blue) and beclin 1+/− (red) iMMECs to increasing concentrations of epoxomycin (left column) and tunicamycin (right column) after 2 days of metabolic stress (A), and after a 2-day recovery following metabolic stress for 3 days (B) and 5 days (C). Data are presented as mean ± SD. P values were calculated by two-way ANOVA. ***, P < 0.001; **, P < 0.01; *, P < 0.05.
FIGURE 4. p62 and keratin accumulation in autophagy-deficient iMMECs under metabolic stress and recovery. A, left top, Western blot showing p62 levels in Bcl-2–expressing beclin 1+/+ and beclin 1+/− iMMECs following 7 days of metabolic stress (D7i) and 2 days of recovery (R2) in the absence and presence of the ROS-scavenger NAC (N); left bottom, p62 immunofluorescence (red) in mammary acini generated by Bcl-2–expressing beclin 1+/+ and beclin 1+/− iMMECs. β-Catenin (green) was used as an epithelial cell marker and 4′,6-diamidino-2-phenylindole (blue) for counterstaining nuclei; right, p62 immunofluorescence (red) in Bcl-2–expressing beclin 1+/+ and beclin 1+/− iMMECs following 5 days of metabolic stress (D5i) and 1 day of recovery (R1) in the absence and presence of NAC. B, p62 (red) and K8 (green) immunofluorescence in Bcl-2–expressing beclin 1+/+ and beclin 1+/− iMMECs following 5 days of metabolic stress (D5i) and 1 day of recovery (R1) in the absence and presence of NAC. C, phospho(Ser73)-K8 (red) immunofluorescence in Bcl-2–expressing beclin 1+/+ and beclin 1+/− iMMECs stably expressing eGFP-LC3 (green) following 5 days of metabolic stress (D5i) in the absence and presence of bafilomycin. D, Western blots showing levels of p62, phospho(Ser73)-K8, and actin in Bcl-2–expressing beclin 1+/+ and beclin 1+/− iMMECs following metabolic stress for 1 to 3 days after transfection with control or p62 siRNA.
by siRNA (Dharmacon) also increased phospho(Ser73)-K8 levels in iMMECs under stress (Fig. 4D), particularly in beclin 1−/− cells, indicating that p62 is involved in this process. Thus, both p62 deficiency and abnormal p62 accumulation due to defective autophagy result in phospho(Ser73)-K8 accumulation, a finding that at first glance seems paradoxical. However, in both cases, autophagy-mediated keratin homeostasis is impaired, either due to failure of keratin delivery to autophagosomes in the absence of its carrier (p62) or due to a defect in the autophagic process, which also leads to abnormally increased p62 levels.

Defective autophagy leads to ER chaperone, p62, oxidative stress marker, and keratin accumulation in vivo

Higher p62 levels were also observed in mammary tumors generated by orthotopic implantation of beclin 1−−/− iMMECs (Fig. 5A), similarly to autophagy-deficient iBMK cell allograft tumors, and lung and liver tumors arising spontaneously in beclin 1−−/− mice (5), indicating that p62 accumulates in autophagy-deficient epithelial tumors, independently of tissue type and nature of autophagy defect. Mammary tumors generated by autophagy-defective iMMECs also showed higher K8, K17, K19, and phospho-K8 levels (Fig. 5A), similarly to in vitro results (Fig. 1B and C; 4B-D). Mammary tissues from beclin 1−/− and beclin 1−−/− 9-month-old retired breeders also exhibited increased expression of GRp78, GRp170, p62, SOD2, K8, K17, and phospho-K8 (Fig. 5B and C), suggesting that concurrent upregulation of these proteins may constitute an autophagy deficiency signature in vivo. Elevated ER chaperone and oxidative stress marker (p62, SOD2) levels were also observed in atg7−/− null mammary tissues (Supplementary Fig. S2). Furthermore, phospho-K8 levels were higher in atg7−/− deficient mammary glands (Fig. 5C), indicating that autophagy-defective mammary epithelial cells accumulate keratin independently of autophagy defect type. Metabolically stressed liver tissue (near portal vein) from beclin 1−/− mice also showed elevated phospho (Ser73)-K8 expression, which further increased in tumors (Fig. 5C), suggesting that phospho(Ser73)-K8 accumulation is not mammary gland specific but may be a general characteristic of autophagy-defective epithelial cells and tumors.

p62, ER chaperone, and keratin accumulation in human breast tumors

To examine whether concurrent ER chaperone, p62, and keratin accumulation constitutes an autophagy deficiency signature in human breast cancer, a breast cancer TMA (BR804, US Biomax), consisting of 40 pairs of matched tumor (T) and NAT, was examined for Beclin 1, GRp170, GRp78, p62, K8, K17, and phospho(Ser73)-K8 expression (Fig. 5D; Table 1). Beclin 1 levels were higher in T than in NAT in 13 of 25 evaluable T-NAT pairs, indicating that induction of autophagy regulators, and thus autophagy, is likely a frequent occurrence in breast tumors. Seven of 25 evaluable pairs had comparable, and 5 of 25 evaluable pairs had lower, Beclin 1 expression in T compared with NAT. Given that Beclin 1 was upregulated in 52% of breast tumors examined, lower and equal Beclin 1 levels may together constitute relative Beclin 1 deficiency in tumors compared with NAT, similarly to an earlier study that reported relative Beclin 1 loss in 40% of breast tumors using a different Beclin 1 antibody (22). K8 and K17 levels varied in tumors and NAT in a nonspecific pattern, whereas ER chaperone and p62 expression was consistently higher in T than NAT (Table 1) but did not correlate with beclin 1 levels. High (3+ staining) phospho (Ser73)-K8 levels were also more common in tumors than NAT and inversely correlated with Beclin 1 absolute levels, indicating that phospho(Ser73)-K8 accumulation may be a marker of Beclin 1, and thus autophagy deficiency in breast tumors. Elevated ER chaperone and p62 expression, although not significantly correlating with Beclin 1 expression in this breast cancer TMA, may still be part of an autophagy deficiency signature in breast cancer, as the functional level of autophagy in tumors is likely determined by additional parameters beyond Beclin 1 levels, such as phosphoinositide 3-kinase/AKT/mammalian target of rapamycin pathway activation (23).

Discussion

Defective autophagy, ER stress, and mammary tumorigenesis

ER is responsible for folding of secreted proteins and those destined to cell surface and intracellular organelles. Nutrient deprivation, hypoxia, deregulation of calcium homeostasis, and toxic chemicals disrupt ER protein folding and cause unfolded protein accumulation (ER stress), resulting in activation of the UPR, a tightly regulated cellular process that adjusts cell folding capacity, protein synthesis, and degradation. Recent studies showed that autophagy is induced by ER stress and is involved in polyubiquitinated protein aggregate removal (24-27). We provide evidence for another aspect in the relationship between autophagy and ER stress: ER stress induces autophagy and defective autophagy induces ER stress in mammary tumor cells in vitro and allograft mammary tumors in vivo. Prolonged ER stress and UPR activation have been linked to cancer progression, as downstream effects include NF-κB activation, G1 arrest, and p38 activation (28). A connection between elevated ER stress and mammary tumorigenesis has also been established, as the ER chaperone GRp78/BiP is overexpressed in aggressive breast tumors (29), whereas GRp78 heterozygosity prolongs mammary tumor latency and impedes tumor growth (30). Thus, ER stress may be a mechanism contributing to defective autophagy-associated mammary tumorigenesis in parallel with DNA damage and genomic instability (10). Indeed, ER stress and the resultant oxidative stress (31) may be major etiologies of genotoxic damage associated with autophagy defects (10, 13). In this case, antioxidants may rescue defective autophagy-induced genotoxic effects and tumorigenicity. Furthermore, chronic ER...
FIGURE 5. ER chaperone, p62, and keratin accumulation in beclin 1+/−/IMMEC-generated tumors, in autophagy-deficient mammary tissues and in human breast cancers with low Beclin 1 levels. A, p62 and keratin immunohistochemistry on mammary tumors generated by Bcl2-expressing beclin 1+/+ (top row) and beclin 1−/− (bottom row) IMMECs. Rectangular inset in the top left panel, p62 immunohistochemistry in normal mammary duct. B, ER chaperone (GRp70, GRp78), p62, and keratin immunofluorescence, and SOD2 immunohistochemistry on mammary tissues from 9-month-old beclin 1+/+ (top row) and beclin 1−/− (bottom row) female mice. C, phospho(Ser73)-K8 immunohistochemistry on (left) mammary tissues from 9-month-old beclin 1+/+ and beclin 1−/− mice; (middle) mammary tissues from atg7+/−;WAP-cre, atg7−/−;WAP-cre and atg7−/−;WAP-cre mice 3 weeks after completion of second pregnancy and lactation; and (right) liver tissues from 2-year-old beclin 1+/+ and beclin 1−/− mice. D, Beclin 1, phospho(Ser73)-K8, GRp78, GRp170, and p62 immunohistochemistry on human breast tumors. Top row, breast tumor with low (1+) Beclin 1 expression; bottom row, breast tumor with high (3+) Beclin 1 expression.
stress suppression by pharmacologic upregulation of autophagy may represent a cancer-preventive strategy for neoplastic lesions with autophagy defects.

The association between autophagy defects and elevated ER stress in tumors has treatment implications, as autophagy-defective mammary tumor cells are sensitized to ER stress–inducing agents and proteasome inhibitors. This finding indicates that the functional status of autophagy in tumors may determine the rational cancer treatment design. GRp170 upregulation in autophagy-defective mammary tumors may also be exploited for therapeutic benefit, as vaccination with tumor-derived GRp170 may have antitumor activity by inducing innate and adaptive immune responses (32).

**Defective autophagy and keratin homeostasis**

The keratin cytoskeleton acts as a signaling platform and provides cytoprotection in epithelial cells (33). K8- and K18-deficient mice exhibit susceptibility to toxic agents and Fas-induced apoptosis, whereas K8/K18 variants predispose humans to end-stage liver disease, acute liver failure, and liver fibrosis in patients with chronic hepatitis C (34). On the other hand, keratin overexpression correlates with extent of epithelial cell injury (35) and is a common finding even in nonepithelial tumor cells, such as malignant melanoma (36). In most cases, it is not known whether keratin accumulation is a cause or result of neoplastic transformation. In a mouse cancer model, transgenic K8 expression in the epidermis altered epidermal cell differentiation and favored transition to malignancy (37), indicating that keratin may be an active player in tumorigenesis. Keratin-containing aggregates have also been associated with liver disease, as Mallory bodies or Mallory-Denk bodies, consisting of ubiquitinated K8/K18, chaperones, and p62, are found in alcoholic and nonalcoholic steatohepatitis (38) and a subset of liver tumors (39). Recent studies implicated defective autophagy in Mallory-Denk body formation (40), as activation of autophagy by rapamycin was shown to eliminate mouse Mallory-Denk bodies and block their proteasome inhibitor–mediated formation (41). The role of Mallory-Denk bodies in liver disease pathogenesis remains unclear; however, their presence is a histologic and potential progression marker in liver disease (42).

Our studies define the important role of autophagy in keratin homeostasis in the mammary gland, similarly to what was already described for the liver (41), and indicate that keratins may be more than simple mammary epithelial state markers. Although Mallory-Denk bodies have not been described in the mammary gland or breast tumors, abnormal keratin accumulation in mammary tumors may be a histologic marker of defective autophagy status, elevated ER and oxidative stress, and possibly more aggressive disease. Whether defective autophagy-associated keratin upregulation in breast tumors has prognostic and/or treatment implications for breast cancer patients remains to be investigated in relevant animal models and human breast cancer specimens.

**Autophagy deficiency signature in breast cancer**

Our work indicates that high levels of ER chaperones, p62, phospho-K8, and K8, K17, and K19 may constitute an autophagy deficiency signature in mammary epithelial cells, as monoallelic beclin 1 deletion resulted in concurrent upregulation of these proteins in mammary tumor cells in vitro and in vivo, and in mammary tissues. The association between defective autophagy, ER stress, and breast cancer was discussed earlier. Higher p62 expression in beclin 1−/− mammary tumor cells is not surprising, given the well-established association between defective autophagy and abnormal p62 accumulation in neurons and hepatocytes (7), and kidney epithelial cells and tumors (5). Indeed, high p62 levels seem to be a reliable autophagy deficiency marker independently of tissue or autophagy defect type. In breast cancer, p62 expression also correlates with grade, distant metastasis, and epidermal growth factor receptor expression (43), suggesting that defective autophagy may be associated with more aggressive breast malignancies.

Accumulation of keratins, particularly posttranslationally modified keratin products, is relevant to breast cancer, as...
ubiquitin-immunoreactive K8/18 degradation products are detected in breast tumors and correlate with cancer aggressiveness (44). Furthermore, K17 is upregulated in wound-activated skin epithelial cells (45), which may explain why beclin 1−/− iMMECs, which are susceptible to metabolic stress and DNA damage (10), accumulate K17 under stress. K17 also regulates protein synthesis and epithelial cell growth through binding to the adaptor protein 14-3-3σ (46), indicating that it may be involved in defective autophagy-mediated mammary tumorigenesis. Finally, K19 is associated with aggressive breast cancer, as K19 mRNA-positive circulating tumor cell detection before adjuvant chemotherapy predicts poor clinical outcome in breast cancer patients (47).

K8 becomes phosphorylated at Ser73 by stress-activated kinases and participates in shear stress-mediated keratin filament disassembly (48) and ubiquitin-proteasome-mediated keratin degradation (49) in alveolar epithelial cells. Our studies revealed that phospho(Ser73)-K8 also plays a role in autophagy- and p62-mediated keratin remodeling in mammary epithelial cells under metabolic stress, and that strong (3+) phospho(Ser73)-K8 staining by immunohistochemistry inversely correlate with absolute Beclin 1 expression in breast cancer. Thus, although phosphoSer(Ser73)-K8 expression is a known general epithelial cell stress indicator, high (3+ staining) phospho(Ser73)-K8 levels may be further indicative of autophagy deficiency in breast tumors, and possibly in other human malignancies. Identification of an autophagy deficiency marker, and possibly an autophagy deficiency signature, in breast cancer may have prognostic and therapeutic implications for cancer patients, as the role of defective autophagy in breast cancer pathogenesis, prognosis, and treatment responsiveness becomes more precisely defined. Ultimately, successfully recognizing the functional status of autophagy in human tumors will lead to personalized cancer treatment and hopefully improved clinical outcomes (23).

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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