The decreased expression of Beclin-1 correlates with progression to esophageal adenocarcinoma: The role of deoxycholic acid

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Abstract

Beclin-1 has a central role in the regulation of autophagy. Barrett’s esophagus (BE) is associated with a significantly increased risk for the development of esophageal adenocarcinoma (EAC). In the current study, we evaluated the role of Beclin-1 and autophagy in the esophageal carcinogenesis.

Biopsies obtained from patients with BE and EAC, tissues from a rat model of BE and EAC, and esophageal cell lines were evaluated for the expression of Beclin-1 by immunohistochemistry, immunoblotting, or RT-PCR. Since reflux of bile acids is important in esophageal carcinogenesis, we also evaluated the effect of exposure to deoxycholic acid (DCA) on autophagy and Beclin-1 expression.

Beclin-1 expression was high in squamous epithelium and nondysplastic BE, while its expression was low in dysplastic BE and EAC. The same pattern of expression was observed in rat tissues and in esophageal cell lines. Normal esophageal epithelium and HET-1A cells (derived from normal squamous epithelium) show high levels of Beclin-1, but lower levels of Beclin-1 were found in BE and EAC cell lines (CP-A, CP-C, OE33). Acute exposure to DCA led to increased Beclin-1 expression and increased autophagy as evaluated by electron microscopy and counting percentage of GFP-LC3 positive BE cells with punctate pattern. In contrast, chronic exposure to DCA did not result in the alteration of Beclin-1 levels or autophagy.

In summary, this data suggests that autophagy is initially activated in response to bile acids, but chronic exposure to bile acids leads to decreased Beclin-1 expression and autophagy resistance.
INTRODUCTION

Barrett’s esophagus (BE) is a condition where normal squamous epithelium is replaced by metaplastic columnar epithelium containing goblet cells. This condition is associated with a 40-fold increased risk for the development of esophageal adenocarcinoma (EAC) (14). Now, there is overwhelming evidence that BE arises as a consequence of chronic gastroesophageal reflux disease (GERD) (19, 20). Although the importance of GERD in the pathogenesis of BE is undisputed, it is not yet clear which are the key elements in the refluxate responsible for metaplastic change to intestinal epithelium (38). In addition to gastric acid, also bile acids (secreted into duodenum in response to a high-fat diet) are implicated in the development of BE and EAC (9). It was shown that 22.2% of patients with esophagitis, 54.5% of patients with BE and 78.6% of patients with EAC exhibit pathologic exposure to duodenal refluxate, indicating the importance of bile acids in EAC pathogenesis (42).

Autophagy is a major physiological lysosome-dependent mechanism for degrading and recycling cellular proteins and organelles (50). Basal levels of autophagy contribute to the maintenance of intracellular homeostasis and is required for cell cleansing and remodeling (12). Under conditions of starvation, the autophagic pathway supplies cells with metabolic substrates, and represents a pro-survival mechanism. In response to stress, autophagy is important mechanism to prevent the accumulation of damaged organelles and proteins. However, excessive autophagy induced by cellular stress leads to cell death characterized by the massive accumulation of autophagosomes (21). Experimental data and animal studies indicate that autophagy has an adaptive role to protect organisms against diverse pathologies, including cancer, neurodegeneration and aging (28). Tumor suppressor genes activate autophagy, whereas oncogenes usually inhibit autophagy (49). However, once cancer develops many cancer cells upregulate autophagy to survive hypoxia and nutrient limitation (33). Autophagy is, thus, considered a double-edged sword, since it is a tumor-suppression mechanism, but also it enables survival of tumor cell during stress (32).
Beclin-1 has a central role in the regulation of autophagy in mammals (28). Autophagic triggers upregulate Beclin-1, which in turn binds to class III phosphatidylinositol 3-kinase (PI3KC3) and activates autophagosome formation and maturation. Beclin-1 acts as a tumor suppressor in mammalian systems and the deletion of Beclin-1 was observed in various cancers, including prostate, ovarian, breast, brain and lung cancers (30, 36).

There are only limited studies on esophageal cancer and autophagy. It was shown that increased expression of Beclin-1 is an important determinant of survival in patients with esophageal squamous cell carcinoma (ESCC) (7). Though, no studies have been yet reported on Beclin-1 expression and EAC and it is not clear what role autophagy plays in esophageal adenocarcinoma. In this study, we tested the hypothesis that exposure to an environment that induces cellular stress (such as bile acids present in refluxate) first leads to increased Beclin-1 expression and autophagy activation. However, long-term repeated exposures lead to decreased Beclin-1 expression, autophagy inhibition and cancer progression.
METHODS:

Cell lines

HET1A cells were provided by Dr. Curtis C. Harris (National Cancer Institute, Bethesda, MD). HET1A is a normal human esophageal epithelial cell line immortalized by transfection of the SV40 T antigen early region gene (43). The cells were cultured in BRFF-EPM2 medium (Athena Environmental Sciences, Baltimore, MD) supplemented with 50 μg/ml gentamicin and 0.25 μg/ml fungizone. BE derived CP-A and CP-C cells were kindly provided by Dr. Rabinovitch (Fred Hutchinson Cancer Research Center, University of Washington). The CP-A cells are derived from patient with non-dysplastic BE and CP-C cells are derived from patient with dysplastic BE. The cells were maintained in MCDB 153 medium as described previously (22). JH-EsoAd1 esophageal adenocarcinoma cells were a kind gift from Dr. James R. Eshleman (Johns Hopkins University, Baltimore, MD) (2). The cells were cultured in RPMI medium containing 10% FBS. All experiments were performed in cells passaged less than 13 times. The cells were exposed to control medium (pH7.4) or medium containing with 0.2mM deoxycholic acid (DCA).

CP-AR cells resistant to cell death induced by DCA were developed from CP-A cells. Initially, CP-A cells were initially grown in medium containing 0.02mM DCA for at least two passages. As the cells became adapted, the concentration of DCA was increased by 0.02mM. This procedure was repeated until the cells were able to survive and proliferate in medium with 0.2mM DCA. It took 15 weeks to achieve this resistance.

Patients

Sixty-two patients with known BE or EAC were included in the present study. All patients gave written informed consent with the approval of the University of Arizona Human Subjects Committee. Biopsies of BE, squamous mucosa, EAC and colon were taken from patients
undergoing regular surveillance procedures and fixed in formalin. Biopsies for microarray analysis were immediately stored in RNAlater solution (Ambion Inc., Austin, TX). Adjacent biopsies were stained with hematoxylin and eosin (H&E) and Alcian blue (pH2.5) for histological evaluation and assessment of intestinal metaplasia. BE was defined as the presence of intestinal-like metaplastic epithelium containing goblet cells (IM) from the sites above the gastro-esophageal (GE) junction.

**Experimental model of BE and EAC**

The study protocol was approved by the Animal Care and Use Committee at the North Carolina Central University (Durham, NC). Eight-week-old rats were administered anesthetics pre-mixed in normal saline (80 mg/kg ketamine and 12 mg/kg xylazine, *i.p.*). Esophagogastroduodenal anastomosis was performed through an upper midline incision as described previously (6). Iron dextran (12mg/kg/week, *i.p.*) was administered to promote carcinogenesis. Animals were sacrificed at 40 weeks after surgery and tissues harvested for histopathological analysis in the future. Twelve animals were examined in total.

**Electron Microscopy**

Transmission electron microscopy was used to detect ultrastructural changes in patient biopsies and CP-A cells. Tissue and cells were fixed with 3% glutaraldehyde in 0.1mM cacodylate buffer. Samples were postfixed in 1% osmium tetroxide, dehydrated in a graded series of ethanols, and embedded in epoxy resin. Ultrathin sections were evaluated for morphological changes using a Phillips CM12 transmission electron microscope (Eidenhoven, Netherlands) (3).
**Cell proliferation**

Cell proliferation was analyzed using CellTiter 96® AQ®ueous Non-Radioactive Cell Proliferation Assay[^3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay] from Promega (Madison, WI) according to the manufacturer's instructions as described previously (22). The MTS assay was performed following the manufacturer's protocol. Blank control values were subtracted from experimental and control samples and the percentage viable cells was calculated as follows: % viable cells = \[\frac{(A_{480\text{ Experiment}} - A_{480\text{ Blank control}})}{(A_{480\text{ Control}} - A_{480\text{ Blank control}})}\] x 100.

**Western blot analysis**

Western blot analysis was performed as previously described (18). Briefly, the cells exposed to different treatments were lysed using lysis buffer (50 mM Tris, pH 8, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40) supplemented with Halt protease inhibitor cocktail (Thermo Scinetific, Rockford, IL). Protein aliquots (30 μg/lane) were loaded on 10-15% SDS-polyacrylamide gels for size fractionation by electrophoresis. The proteins were blotted onto Immobilon-P PVDF® transfer membrane (Millipore, Bedford, MA). The membranes were immunostained with antibodies against Beclin-1 (1:100, Santa Cruz, Santa Cruz, CA) and then incubated with goat anti-mouse secondary antibody conjugated to horseradish peroxidase (1:20,000; Pierce, Rockford, IL). Antibody complexes were detected using the Supersignal West Pico chemiluminescence detection system (Pierce, Rockford, IL). After this, the membranes were stripped using Stripping buffer (2% SDS, 100 mM BME, 62.5 mM Tris at pH 6.8) and immunostained with β-actin antibody (1:10,000, Calbiochem. Gibbstown, NJ), or the membranes were stained for 20 minutes with Bio-Safe Coomassie Stain (Bio-Rad, Hercules, CA) to confirm equal protein loading. The densities of individual bands were determined using QuantiScan software (Biosoft, Cambridge, UK).
Autophagy evaluation

During autophagy, the cytoplasmic form of microtubule-associated protein light chain 3 (LC3) is processed by addition of a phosphatidylethanolamine to form LC3-II. This modified form of LC3 translocates rapidly in a punctuate pattern to the autophagosome. The change in LC3 localization (from diffuse, cytoplasmic to punctuate, membrane-associated) is used as a specific marker to monitor autophagy. cDNA encoding human LC3 was inserted into the EcoRI site of pEGFP-C2 (Addgene, Cambridge, MA). pEGFP-LC3 was transfected into the CP-A cells using Lipofectamine™2000 (Invitrogen) according to the manufacturer’s protocol. Briefly, 2 μl of Lipofectamine and 0.8 μg of pEGFP-LC3 DNA were incubated separately in 50 μl of serum-free and antibiotic-free medium. After 5 minutes of incubation, DNA and Lipofectamine were mixed and incubated for another 20 minutes. This transfection medium (100 μl) was then added to cells growing in a 500 μl 4-chamber slide. After four hours, the media was replaced with fresh antibiotic-free media and allowed to grow for 24 hours. CP-AR cells were transfected in a similar way, however, during transfection DCA was not added into the media. The cells were then exposed to control medium, medium supplemented with 0.2mM DCA and HBSS. Immediately following treatments, cells were washed with PBS, fixed in ice-cold methanol for 6 minutes, mounted with Prolong Gold Anti-fade Reagent with DAPI, and analyzed by fluorescent microscopy. The percentage of cells displaying punctate distribution of GFP-LC3 was determined for at least 200 cells per sample. The experiment was repeated at least three times.

Immunohistochemistry

The expression of Beclin-1 was determined in BE tissues with no dysplasia (BE), low grade dysplasia (LGD), high grade dysplasia (HGD) EAC and in squamous epithelium by immunohistochemistry as described previously (15). The Beclin-1 antibody was from ProSci (2μg/ml, Poway, CA) A simple grading system (0-4) was employed to grade the level of Beclin-1
expression. Immunocontrol slides were prepared by replacing the primary antibody with rabbit IgG at the same protein concentration as the primary antibody (2μg/ml). Staining was evaluated independently by two experienced investigators. Beclin-1 in rat tissues was evaluated by fluorescent microscopy as described previously (34).

For confocal microscopy, the cells were grown on 4-chamber slides, fixed with formaldehyde and permeabilized with methanol as described previously (15). After blocking with 5% bovine serum albumin the cells were incubated overnight with antibodies against Beclin-1 (1:100, ProSci, Poway, CA). Next, Alexa Fluor®488 secondary antibodies (1:100, Molecular Probes Inc., Eugene, OR) were applied for 60 minutes. The slides were counterstained with propidium iodide (PI) and coverslipped using VectaShield HardSet medium (Vector Laboratories, Burlingame, CA).

RT real-time PCR and microarray studies

To evaluate Beclin-1 mRNA expression in patient samples, total RNA from BE (N=18) and EAC (N=6) was isolated using the Qiagen RNeasy Mini Kit (Valencia, CA) according to the manufacturer's instructions. The isolated total RNAs were used to produce a labeled target, hybridized to Affymetrix U133A GeneChips, and read using the Agilent/Affymetrix 2500A scanner according to manufacturer's protocols as described previously (47).

Total RNA was isolated from different cell lines using the RNeasy Plus Mini Kit (Qiagen, Santa Clarita, CA) as described in the manufacturer's protocol. RNA concentration and purity was evaluated by NanoDrop (ThermoScientific, Wilmington, DE) at 260nm/280nm. RT real-time PCR assays were performed to quantify mRNA levels of Beclin-1 as described previously (15). Primers were obtained from Real Time Primers, LLC (Elkins Park, PA). The following sequences were used for each of the primers: Beclin-1: forward primer; reverse primer 5'- 3'; SCLA92: β-actin: forward primer 5'- AGA-GGG-AAA-TCG-TGC-GTC-AC-3' reverse primer 5'-
CAA-TAG-TGA-CCT-GGC-CGT-3'). Beclin-1 mRNA Cp values were normalized to β-actin Cp values for each cell line. The relative mRNA expression of Beclin-1 in different cells was compared to HET1A cells.

Beclin-1 siRNA experiments

CP-A were co-transfected with pEGFP-LC3 DNA and Ambion® Silencer® Select predesigned Beclin-1 siRNA (Ambion, Carlsbad, CA) or control siRNA (scrambled siRNA, Ambion) using Lipofectamine™ 2000 according to manufacturer’s instructions. After transfection, the cells were exposed to control medium, medium supplemented with 0.2mM DCA or HBSS for 4 hours. The slides were fixed with formaldehyde and immunostained using Beclin-1 antibody as described above. AlexaFlour®594 (1:100, Molecular Probes Inc, Eugene, OR) was used as a secondary antibody. The percentage of GFP-LC3 positive cells with punctuate pattern was counted in at least three independent experiments for each treatment.

Statistical analysis

Statistical significance was determined by the Student t-test or by Mann-Whitney test at the 95% confidence level.
RESULTS:

Beclin-1 is decreased in patients biopsies from dysplastic BE and esophageal adenocarcinoma

First, we wanted to know if Beclin-1 expression changes in the progression from normal squamous epithelium to esophageal adenocarcinoma. Normal colon was used as a control. The expression of Beclin-1 was evaluated altogether in 62 patients’ samples by microarray analysis or by immunohistochemistry.

Microarray analysis revealed that Beclin-1 mRNA is significantly decreased in EAC (N=6) compared to BE (N=18, p<0.05, Figure 1A). Next, we evaluated Beclin-1 expression by immunohistochemistry. In agreement with microarray studies, low signal of Beclin-1 was detected in EAC samples (N=9). Beclin-1 was expressed in nondysplastic BE (N=12), while a lower signal of Beclin-1 was detected in BE with low grade dysplasia (LGD, N=8) and in BE with high grade dysplasia (HGD, N=9, Figure 1B). As a control we used normal colonic tissue and squamous epithelium (SQ, N=5) where high Beclin-1 signal was found. No signal of Beclin-1 was detected in immunocontrols (negative control, Figure 1B).

Two independent investigators used simple grading system (0-4) to evaluate the intensity of Beclin-1 staining in different tissues. Mann-Whitney test was used to determine statistical significance. We found a significantly lower signal of Beclin-1 in dysplastic BE and EAC compared to non-dysplastic BE tissue (Figure 1C, p<0.05).

The expression of Beclin-1 is reduced in rat model of BE/EAC and esophageal cancer cells

Beclin-1 expression was evaluated in tissues from twelve rats that underwent esophagojejunostomy (Figure 2). Similarly to human biopsies, we found a high signal of Beclin-1 in squamous epithelium, while Beclin-1 expression was lower in BE and adenocarcinoma (Figure 2).
In the next experiment Beclin-1 expression was evaluated in the esophageal cell lines derived from normal esophagus (HET-1A), non-dysplastic BE (CP-A), dysplastic BE (CP-C) and esophageal adenocarcinoma (JH-EsoAD1). Real time RT-PCR and western blot analysis were used to detect changes in Beclin-1 levels. High levels of Beclin-1 mRNA were detected in normal HET1A cells, while lower Beclin-1 levels were found in BE derived cells (CP-A, CP-C). Lower levels of Beclin-1 were also detected in JH-EsoAD1 (Figure 3).

**BE is resistant to autophagy induced by amino acid deprivation**

Since Beclin-1 is a critical regulator of autophagy, we tested whether autophagy can be induced in BE tissues in an *ex vivo* assay. In this experiment, we evaluated samples of dysplastic BE and duodenum because we wanted to compare the response in normal intestinal tissue and dysplastic BE. The tissues were incubated *ex vivo* with Hank’s balanced salt solution (HBSS, positive control) for 4 hours to induce autophagy and then evaluated by TEM for the presence of autophagic vacuoles. In BE and duodenal tissues that were incubated in normal medium, no changes typical for autophagy were found (Figure 4). However, many autophagic vacuoles were found in duodenum incubated in HBSS, while no such changes were detected in BE incubated with HBSS (Figure 4).

**Beclin-1 expression and autophagy are induced in BE cells by acute exposure to DCA**

Next we evaluated whether bile acids modulate the expression of Beclin-1 and autophagy. For these experiments we used CP-A cells that were exposed to 0.2mM DCA or HBSS for 4 hours. DCA is a model hydrophobic bile acid that also was shown to activate autophagy in normal colonic cells, colon or hepatocytes (39, 51).

Confocal microscopy and Western blot analysis indicated that Beclin-1 expression was increased after the exposure to DCA (Figure 5A, 6B). Beclin-1 expression was also increased
after the exposure to HBSS (positive control, Figure 5A).

Next we assessed autophagy by evaluating the punctate pattern in cells transfected with GFP-LC3 and transmission electron microscopy (TEM). DCA induces a significant increase in the percentage of cells with punctate pattern, as does HBSS (positive control, Figure 5B). While we found 14.9 ± 1.9% cells with punctuate pattern in untreated cells, after exposure to DCA or HBSS, the percentage of GFP-LC3 positive cells with punctate increased to 41.8 ± 4.0% and 27.2 ± 1.8%, respectively (p<0.05). In addition typical autophagic vacuoles were detected in the cells treated with DCA or HBSS by TEM (Figure 5C).

**Chronic exposure to deoxycholic acid does not induce upregulation of Beclin-1**

Since esophageal tissue is exposed to bile acids during reflux episodes we speculated that bile acids may be responsible for activation of autophagy after acute exposure. However, after long-term repeated exposure to bile acids, the tissues develop resistance to autophagic stimuli. To study chronic effects of bile acids we developed CP-AR cells that are resistant to cytotoxic effects of DCA. These cells are able to grow in medium containing 0.2mM DCA. Our studies show that these cells express increased levels of two antiapoptotic proteins, Mcl-1 and Bcl-xL compared to parental CP-A cells (data not shown). Using confocal microscopy and immunoblotting we found that Beclin-1 expression in DCA resistant cells is not increased after chronic exposure to DCA (Figure 6A,B). Furthermore, we counted the percentage of cells with punctate pattern after transfection with GFP-LC3 to evaluate autophagy. During transfection DCA was not present in the medium. Overall, the number of cells with GFP-LC3 punctate pattern was elevated (31.6 ± 5.3%) in CP-AR cells compared to parental CP-A (14.9 ± 1.9 %). However, treatment with 0.2mM DCA or HBSS for four hours did not result in any significant increase of the GFP-LC3 positive cells with punctate pattern (26.0 ± 3.0% for DCA and 25.4 ± 5.8 % for HBSS, p>0.05, Figure 6C). Altogether, this data suggests chronic exposure to bile acids leads to decreased Beclin-1 expression and reduced autophagic response.
Beclin-1 is necessary for autophagy induced by DCA

To study if Beclin-1 is truly important for DCA-induced autophagy, we co-transfected CP-A cells with pGFP-LC3 and Beclin-1 siRNA or control (scrambled) siRNA. First, we tested if Beclin-1 expression is decreased after treatment with Beclin-1 siRNA compared to control siRNA. Western blot as and immunofluorescence showed that siRNA reduced Beclin-1 expression (Figure 7A,B). Next, the autophagy was evaluated after exposure to DCA and HBSS. The percentage of GFP-LC3 positive cells with punctuate pattern was significantly increased after exposure to DCA and HBSS in the cells transfected with control siRNA (p<0.05), while no significant increase was detected in the cells transfected with Beclin-1 siRNA (Figure 7C, p>0.05).
DISCUSSION

In this paper, we show for the first time that during Barrett’s carcinogenesis, the expression of Beclin-1, a major autophagic protein, is decreased as the disease progresses from nondysplastic BE to adenocarcinoma. Our studies were focused on Barrett’s esophagus, a premalignant lesion, and EAC, because BE is an ideal model to study early events in neoplastic progression. In the majority of other cancers, premalignant conditions cannot be studied in detail because they are either not detected early enough or are removed before cancer develops, such as in the case of colonic polyps. Furthermore, we evaluated the effect of bile acids on autophagy and Beclin-1 expression since bile acids play an important role in EAC development. The data suggests that acute exposure to DCA increases Beclin-1 expression and activates autophagy, while chronic exposure to DCA leads to decreased expression of Beclin-1 and inhibition of autophagy.

Autophagy is a conserved process involving self-digestion of whole organelles and macromolecules, which promotes the survival of starved and stressed cells including cancer cells, but autophagy may also lead to so-called type II programmed cell death (8). Autophagy plays an important role in many physiological functions and defects in this process have been linked to many cancers suggesting that it may function as a barrier for cellular transformation (8, 28, 32).

Beclin-1, a tumor suppressor protein, acts as an initiator of autophagy. Previous studies demonstrated that Beclin-1 plays an important role in tumor biology (26). It is speculated that Beclin-1 protects cells against chromosomal instability and decreases the frequency of additional mutations (31). For example, transgenic mouse models have shown that monoallelic deletion of beclin 1 promotes tumor development (41). The growth of colorectal cancer cells that overexpress Beclin-1 is reduced compared to the mock transfected cells (26). Beclin-1 deficiency is also associated with increased angiogenesis (27).
The pattern of Beclin-1 expression in gastrointestinal carcinogenesis is not consistent. Studies show that Beclin-1 and autophagy were suppressed in hepatocellular carcinoma (HCC) and pancreatic cancer (13) but were elevated in colon and gastric cancers compared to adjacent normal tissue (1, 29). However, nearly all studies suggest that high Beclin-1 expression is associated with better prognosis in gastrointestinal cancers. For example, Kim et al. evaluated the expression of Beclin-1 in human pancreatic ductal adenocarcinoma (25) and found that increased Beclin-1 expression was associated with a significantly lower rate of distant metastasis (25). Similarly, a high expression of Beclin-1 was associated with a favorable prognosis in colon and gastric cancers (1, 29). We found that Beclin-1 is decreased in biopsies obtained from patients with dysplastic BE and EAC and is high in normal tissues such as colon and squamous epithelium and nondysplastic BE. In addition, we also observed significantly decreased Beclin-1 mRNA in EAC compared to BE tissues (Figure 1A). A similar trend of Beclin-1 expression was detected in animal model of EAC and BE (Figure 2) and esophageal cell lines derived from normal esophagus, nondysplastic BE, dysplastic BE and EAC (Figure 3).

In agreement with our studies conducted in human biopsies and in animal tissues, studies on pancreatic cancer in rats showed that cells from pancreatic adenocarcinoma had decreased autophagic activity compared to cells from premalignant nodules (45). These observations suggest that autophagy is initially increased during premalignant stages of carcinogenesis, and then decreased during the adenocarcinoma transition.

Because Beclin-1 expression was low in dysplastic BE, we wanted to determine if BE tissue is sensitive/resistant to autophagic stimuli. Our data suggests that BE is resistant to autophagy compared to duodenum. Typical changes associated with autophagy (the presence of large autophagic vacuoles containing cellular debris) were observed in the duodenum after incubation for 4 hours in HBSS by TEM (Figure 3A). No autophagic vacuoles were seen in BE incubated in media with HBSS (Figure 3B) or in duodenum or BE incubated with control medium (Figure 3).
The mechanism by which autophagy defects lead to accelerated tumorigenesis is not readily apparent. However, it was suggested that autophagy can inhibit tumorigenesis by several different mechanisms. An elegant study showed that the autophagic machinery can limit DNA damage and chromosomal instability (32). Failure to clear damaged mitochondria that produce reactive oxygen species (ROS) leads to nuclear DNA mutations and, consequently, to cancer. Also, impairment of both apoptosis and autophagy promotes necrosis and thus inflammation (48). Importantly, inflammatory response leads to recruitment of the proinflammatory cytokines such as TNF-α, IL-1β, or IL-6 that are associated with cancer development.

Bile acids are important in the etiology of gastrointestinal cancers including EAC, colon, or pancreatic cancer (4, 5, 42). During reflux episodes, the esophageal epithelium is exposed to gastric acid and hydrophobic bile acids (46). Evidence shows that the concentrations of bile acids are increased in the refluxate of patients with BE and are even higher in patients with EAC (42). Thus, progression from BE to adenocarcinoma is strongly influenced by bile acid exposure. Bile acid concentrations in the refluxate of BE are in the range of 0.03-0.82 mM (median 0.18 mM) (38). However, bile acid concentrations as high as 6.4 mM have been reported in some BE patients (23). In addition, BE patients also have a significantly greater duration of bile reflux compared to patients with esophagitis (24, 35). In our studies we used 0.2 mM DCA, which is in the physiological range.

Furthermore, the mainstream therapy for BE patients is treatment with proton pump inhibitors (PPIs). While the reflux of gastric acid is controlled by proton pump inhibitors (PPIs), this therapy does not suppress reflux of bile acids (44). Importantly, at normal pH of the stomach (pH ~2), the majority of bile acids present in the refluxate irreversibly precipitates. At higher pH (~5-7) bile acids are soluble, and they interact with esophageal mucosa. Thus, bile acids may cause cellular alteration especially in esophageal tissue of patients treated long-term with PPIs.
Bile acids induce oxidative stress (16), activation of STAT3 signaling (15), cytokine alteration (15, 17) and DNA damage (22) in BE. Hydrophobic bile acids, such as deoxycholic acid (DCA, model bile acid), induce apoptosis (11, 40). However, chronic exposure of cells to bile acids leads to the selection of clones with an apoptosis-resistant phenotype (10). Similarly, we show in this study that the short-term exposure to bile acids leads to a normal cellular response – the induction of autophagy and increased Beclin-1 expression. In contrast, chronic exposure to bile acids does not affect Beclin-1 expression and, consequently results in defective autophagy.

Beclin-1 expression and autophagy were evaluated in CP-A (nondysplastic BE) cells that were exposed acutely or chronically to DCA. For acute exposure experiments CP-A cells were treated for 4 hours with 0.2mM DCA. This concentration of DCA is relatively low and after 4 hours the cytotoxic effects of DCA are minimal. However, prolonged exposure to this concentration of DCA leads to cell death. After acute exposure to 0.2mM DCA, increased expression of Beclin-1 was detected by immunoblotting and immunocytochemical staining in conjunction with confocal microscopy. The cells were undergoing autophagy as indicated by increased GFP-LC3 punctate pattern and electron microscopy. In addition many autophagic vacuoles were detected in the DCA exposed cells by electron microscopy. In contrast, after chronic exposure to 0.2mM DCA Beclin-1 expression was low and autophagic response was not activated. In agreement with these results, we found that DCA-induced autophagy was low in CP-A cells when Beclin-1 expression was decreased by siRNA. This data confirm the importance of Beclin-1 for autophagy (Figure 7).

In summary, the decreased Beclin-1 expression and resulting autophagy decrease after chronic exposure to bile acids may lead to increased genomic instability and cancer progression. The incidence of esophageal adenocarcinoma (EAC) is rapidly rising for unknown reasons. Western style, high fat diet is probably one of the major contributing factors to this increase (37). This high-fat diet induces the release of bile acids into the gastrointestinal tract.
and consequently increases the concentration of bile acids in the refluxate. We suggest that the reflux of bile acids should be controlled in patients with BE to prevent cellular changes associated with chronic exposure to these toxic compounds.
REFERENCES:


FIGURE LEGEND

Figure 1: Beclin-1 expression in human tissues. Panel A represents relative mRNA levels of Beclin-1 in BE (N=18) and EAC (N=6). Panel B shows the representative images of immunohistochemical staining of Beclin-1 (brown signal) in squamous epithelium (SQ), colon, nondysplastic BE (BE), BE with low grade dysplasia (LGD), BE with high grade dysplasia (HGD), in esophageal adenocarcinoma (EAC) and immunocontrol (Neg. control, magnification 400x). Graph C shows the summary of immunohistochemical experiments for Beclin-1. We used a simple grading system 0-4. Overall staining was evaluated in SQ (N=5), BE (N=12), LGD (N=8), HGD (N=9) and EAC (N=9). Median values are shown as thick lines, asterisks indicate statistically significant difference (p<0.05), bar indicates 50μm.

Figure 2. Beclin-1 expression in rat tissues. The fluorescent microscopy images show Beclin-1 expression (green signal) in squamous epithelium, BE, EAC and negative control (NC). DAPI was used as a nuclear counterstain (blue signal). Inserts show the tissues stained with H&E. Bars indicate 50μm. The white arrows show typical BE glands.

Figure 3. Beclin-1 expression in esophageal cell lines. The relative mRNA amounts of Beclin-1 in different esophageal cell lines are shown in Panel A. Beclin-1 mRNA Cp values were normalized to β-actin Cp values for each cell line. Asterisk indicates significant difference compared to HET1A cells (p<0.05). The experiment was repeated four times. Panel B shows Beclin-1 and β-actin protein expression.

Figure 4: Transmission electron micrographs of duodenum and BE incubated ex vivo for 4 hours in control medium or in HBSS (inducer of autophagy). Typical autophagic vacuoles were found in duodenum incubated with HBSS (arrows). No changes consistent with autophagy were seen in BE incubated in control medium or in HBSS. The magnification was 7,100x (four top images) and 25,000x, respectively. Bars indicate 2 microns and 500nm.

Figure 5: The effect of acute exposure to DCA on Beclin-1 expression and autophagy. CP-A cells were treated for 4 hours with control medium, 0.2mM DCA or HBSS (positive control). Beclin-1 and β-actin immunoblots of CP-A are shown in Panel A. Graph B summarizes data from three different experiments evaluating percentage of GFP-LC3 positive cells with >3 puncta per cell. Asterisks indicate significant difference compared to untreated cells (p<0.05). Insert shows a fluorescent microscopy image of CP-A cell transfected with GFP-LC3 with typical puncta. Bar indicates 10μm. Transmission electron micrographs of CP-A cells are shown in Panel C (magnification 11,500X, insert 31,000x, bars represent 1000nm and 500nm respectively).

Figure 6: The effect of chronic exposure to DCA on Beclin-1 expression and autophagy. Panel A shows an immunoblot of Beclin-1 in CP-A cells treated with normal medium (CP-A), with 0.2mM DCA for 4 hours (CP-A+DCA) and CP-AR cells chronically exposed to 0.2mM DCA. Panel B shows confocal microscopy images of Beclin-1 immunostaining in these cells. The graph in panel C shows the percentage of GFP-LC3 positive cells with punctate pattern in CP-AR cells that were allowed to grow in DCA free medium for 24 hours after transfection and treated with either control medium or medium supplemented 0.2mM DCA or HBSS for four hours. Data are from three different experiments. Bar indicates 10μm.

Figure 7: The effect of Beclin-1 siRNA on autophagy. Panel A shows the fluorescent microscopy images of Beclin-1 in CP-A cells treated with Beclin-1 siRNA or control (scrambled)
siRNA (red signal). The nuclei are counterstained with DAPI (blue signal). Bar represents 25μm. Panel B shows an immunoblot of Beclin-1 in CP-A cells treated Beclin-1 siRNA and control siRNA. The graph in panel C shows the percentage of GFP-LC3 positive cells with punctate pattern in CP-A cells cotransfected with Beclin-1 siRNA or control siRNA that were allowed to grow in DCA free medium for 24 hours after transfection and treated with either control medium or medium supplemented 0.2mM DCA or HBSS for four hours. Data are from three different experiments. Asterisk indicate significant difference (*p<0.05 compared to control siRNA treated with DCA or HBSS, respectively).
Figure 4
Figure 5

A

Becn-1
β-actin

65kDa
42kDa

B

Cells with 3 GFP-LC3 puncta (%)

Control
0.2mM DCA
HBSS

* 

C

Control
DCA
HBSS
Figure 7

A

Beclin-1 siRNA  Control siRNA

B

Beclin-1  55kDa
β-actin  42kDa

C

GFP-LC3 positive cells (%) with >5 puncta

Control  DCA  HBSS