BILE ACIDS AND THEIR ROLE IN AUTOPHAGY RESISTANCE: THE LINK BETWEEN GASTRO-ESOPHAGEAL REFLUX DISEASE (GERD) AND DEVELOPMENT OF BARRETT'S ESOPHAGUS

By

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A Thesis Submitted to The Honors College

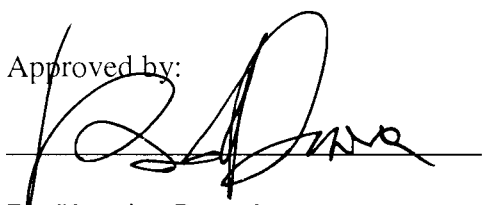
In Partial Fullfillment of the Bachelors degree
With Honors in

Molecular and Cellular Biology

THE UNIVERSITY OF ARIZONA

May 2011

Approved by:



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Degree title (eg BA, BS, BSE, BSB, BFA): BS	
Honors area (eg Molecular and Cellular Biology, English, Studio Art): MCB	
Date thesis submitted to Honors College: May 4, 2011	
Title of Honors thesis: Bile Acids and Their Role in Autophagy Resistance: The link between Gasto-esophageal reflux disease (GERD) and development of Barrett's Esophagus	
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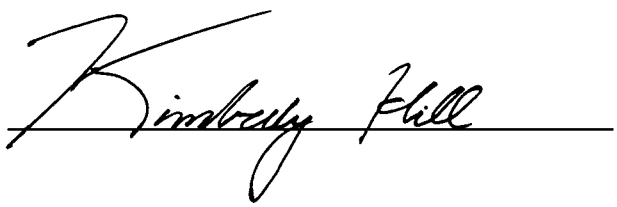
Signed: 

Bile acids and their role in autophagy resistance: the link between gastro-esophageal reflux disease (GERD) and development of Barrett's Esophagus

By: Kimberly A. Hill

ABSTRACT:

Barrett's esophagus is a premalignant condition where normal squamous epithelium is replaced by intestinal epithelium. BE is associated with an increased risk of developing esophageal adenocarcionoma (EAC). However, the BE cell of origin is not clear. We hypothesize that BE tissue originates from esophageal squamous cells, which can differentiate to columnar cells as a result of repeated exposure to gastric acid and bile acids, two components of refluxate which is implicated in BE pathology. The goal of our study was to explore the potential link between chronic exposure to bile acids and autophagy in cells taken from Barrett's esophagus patients. Our studies show that chronic exposure to bile acids is a crucial factor in the development of autophagy resistance in BE. Such alterations allow for a consequential increase and accumulation of DNA damage in the esophageal cells due to inhibition of the normal processes meant to hinder the proliferation of cells with damaged DNA.

INTRODUCTION:

BARRETT'S ESOPHAGUS:

Barrett's esophagus (BE) is a premalignant lesion of the distal esophagus that is associated with a 40-fold increased risk of esophageal adenocarcinoma (EAC) (1). Chronic gastro-esophageal reflux disease (GERD) has been implicated in BE development, suggesting that bile acids play a critical role in cancer development (2, 3). Approximately 10-15% of patients undergoing endoscopy for symptomatic GERD have BE, representing 1-2 million people in the U.S. alone with the disease.

Over the last 30 years, the incidence of EAC has increased by 600%, faster than any other malignancy in the Western world (4). The cancer has a poor prognosis with a median survival rate of less than one year (Cancer Facts and Figures 2008). However, the mechanisms and factors contributing to EAC development are not well understood. Epidemiological risk factors include aging, male gender, obesity, reflux symptoms, smoking, and a high-fat diet (5, 6).

Histopathological steps in the progression of BE to EAC include: (a) metaplasia of the normal esophageal squamous epithelium to a specialized intestinal glandular epithelium, (b) low-grade dysplasia (LGD), (c) high-grade dysplasia (HGD) and (d) esophageal adenocarcinoma (EAC) with invasive and metastatic potential (7) (Figure 1). Little is known concerning the signaling pathways that promote the development of metaplasia and dysplasia. It also remains unclear as to which patients will progress from metaplasia to dysplasia and EAC. Reliable biomarkers would therefore be helpful in identifying patients who are at high risk for progression.

BILE ACIDS:

Bile acids are natural detergents essential for the digestion of lipids; however, epidemiological, clinical and animal studies have indicated that chronic reflux of bile, such as in GERD, is important in the development of BE and EAC (8, 9, 10, 11). Our lab has previously reported that bile acids induce oxidative stress, (12) activate STAT3 signaling (13), alter cytokine levels (13, 14) and induce DNA damage (15) in BE.

The esophageal epithelium is exposed to hydrophobic bile acids and acid during esophageal reflux. Evidence suggests that bile acid concentrations are increased in the refluxate of patients with Barrett's esophagus and are even higher in those with EAC (11). Hydrophobic bile acids, such as deoxycholic acid (DCA), induce autophagy in the normal cell (16, 17). DCA is considered to be a major factor involved in GI carcinogenesis. However, chronic exposure of the cells to bile acids leads to the selection of clones with an apoptosis-resistant phenotype, as is shown by the studies outlined in this paper (18).

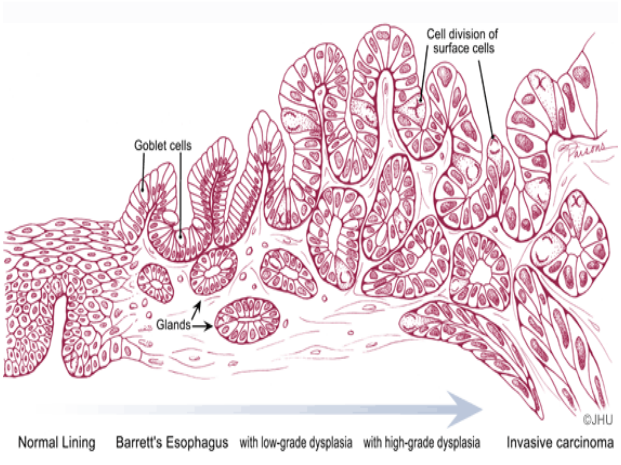


Figure 1: The progression of esophageal squamous epithelium to BE and EAC

AUTOPHAGY:

Autophagy is a highly regulated process that involves the degradation and recycling of cellular proteins and organelles as a means of survival. A major function of autophagy is to eliminate damaged proteins and organelles during periods of cellular stress as well (19), mostly by limiting DNA damage and chromosomal instability, as well as preventing necrosis and inflammatory responses (20, 21). Experimental and animal studies indicate that autophagy has an adaptive role in protecting organisms against diverse pathologies, including cancer, neurodegeneration and aging (22); in fact, basal levels of autophagy actually contribute to the maintenance of intracellular homeostasis, and is required for cell cleansing and remodeling (23).

AUTOPHAGY AND CANCER:

The role of autophagy in cancer is not yet clear due to its controversial involvement in both cell survival and cell death. Under conditions of starvation, autophagy seems to represent the pro-survival mechanism described above, operating to supply cells with metabolic substrates. However, excessive autophagy will eventually lead to cell death as characterized by the massive accumulation of autophagosomes (24). This autophagic cell death is another mechanism responsible for tumor suppression (21).

Some recent studies have shown that autophagy induction and autophagosome formation are greatly altered in cancer. Genetic links between autophagy defects and cancer suggest that autophagy is an important process involved in tumor suppression, as autophagy defects have been found to correlate with increased tumorigenesis (20, 22). The deletion of Beclin-1, UVRAG or Bif-1 (the major regulators of autophagy) has been observed in many cancers (25). The expression of other proteins which function as tumor suppressors, namely PTEN, TSC1-TSC2, p53 and death associated protein kinase (DAPK), are also altered (26).

Autophagy has been closely linked with apoptosis. Anti-apoptotic Bcl-2 family members are able to negatively regulate Beclin-1 dependent autophagy by binding the BH3 domain in Beclin-1 to the BH3 binding groove of Bcl-x_L (27). We have previously shown that Bcl-x_L is overexpressed in EAC (28).

AUTOPHAGY AND ESOPHAGEAL CANCER:

There are very few studies on esophageal squamous cell carcinoma (ESSC) and autophagy; however, it has been shown that increased expression of Beclin-1 is an important determinant of survival in patients with ESSC (19). No other studies have yet reported on autophagy and EAC. We posed that acute exposure to an environment that induced cellular stress would lead to autophagy activation, while long-term exposure to the same environment would lead to up-regulation of pathways responsible for inhibiting autophagy. A study involving pancreatic cancer in rats furthered this suspicion, as the study showed that cells from pancreatic adenocarcinoma have decreased autophagic activity compared to cells taken from premalignant nodules, which had expressed increased autophagic capacity (29). These findings correlated with our hypothesis that autophagy levels would first increase during the premalignant stages of carcinogenesis, and then decrease during the adenocarcinoma transition.

AIMS OF CURRENT STUDY:

We have previously shown that acute exposure to bile acids induces autophagy (17). We therefore speculate that by removing dangerous cytosolic components or whole damaged cells, autophagy protects cells from oxidative and genotoxic stress. Deregulation of such a process would then be an apparent factor contributing to cancer development.

In this study, we explored the possibility of a link between chronic exposure to bile acids and a cell's loss of the capability to undergo autophagy, allowing for the proliferation of cells that have accumulated mutations and the eventual development of cancer. We sought to confirm or disprove this link by identifying alterations in signaling pathways known to inhibit a cell's ability to undergo autophagy. The overall goal of this research is to explore the role of autophagy in EAC development and the effect of hydrophobic bile acids in autophagy signaling.

Furthermore, we have observed that exposure to hydrophobic bile acids leads to the induction of apoptosis and autophagy in colon cancer cells as well (22, 30). We also observed that the long-term effect of repeated exposure to these acids lead to the selection of cells with an apoptosis-resistant phenotype (31). These observations also gave weight to our expectations that short-term effects of bile acids would lead to the induction of autophagy, while long-term, repeated exposure would result in the selection of cells which are incapable of initiating the autophagic and apoptotic processes required for cell death.

To study the chronic effects of DCA *in vitro*, we developed CP-A cells resistant to DCA. This new CP-AR cell line is capable of surviving and proliferating in medium containing 0.2mM DCA, a concentration that would kill the parental CP-A cell line within the course of a few hours.

The experiments that dealt with these cell lines were complimented by the analysis of tissue biopsies

taken from patients with different grades of BE dysplasia and EAC, as well as patients with normal esophageal epithelium. In this manner, the manifestations of chronic exposure to bile acids could be observed both *ex vivo* and *in vitro*.

METHODS:

Cell lines and chemicals. CP-A cells were kindly provided by Dr. Rabinovitch and are derived from BE cells. The cells were cultured in a special medium consisting of the following in one liter of purified water: 1 package (1M) MCDB-153 (Sigma), 15.7mL sodium bicarbonate (7.5% solution, Invitrogen/Gibco), .4 μg/mL hydrocortisone, 20 ng/mL EGF, 10M cholera toxin, 20 μg/L adenine, 10% fetal bovine serum, 100 unit/mL penicillin-streptomycin, 25 μg/mL fungizone, and 10mg/mL insulin0transferrin-selenium. This medium was then acidified to a final pH of 7.2 by adding the appropriate amounts of 4N HCl. The medium was sterilized with a bottle-top filter before being used for cell culture.

Cells were split once 80-90% confluent using 1mM trypsin. The CP-AR cell line was developed by growing the cells in the regular CP-A medium supplemented with various concentrations of DCA. The cells were subject to growing in this supplemented medium at all times. The cells first became accustomed to concentrations of .01mM DCA before being exposed to ever increasing concentrations, eventually gaining the ability to proliferate and survive normally in 0.2mM DCA.

(a) *Evaluation of tissue biopsies by immunohistochemistry.* Tissue biopsies of sixty patients with different grades of BE and EAC were evaluated for the expression of Beclin-1 (1:100; ProScience, Woburn, MA) and phosphorylated-mTOR^{Ser2448} (1:100; Cell Signaling, Boston, MA). Beclin-1 is a pro-autophagy protein that is required for the formation of the autophagosome in autophagy. mTOR, when phosphorylated, inhibits apoptotic processes by activating general cell proliferation, survival, protein synthesis, and transcription.

The tissues included those people with conditions fitting into the following categories: no dysplasia (BE), low-grade dysplasia (LGD), high-grade dysplasia (HGD) and EAC. The endoscopic biopsies of the duodenum and esophageal squamous mucosa were also provided. The biopsies were taken from patients who were undergoing surveillance procedures or esophageal resections. To quantify positively and negatively stained cells, we used a scoring system of 0-3.

We made use of the Mann-Whitney test statistic to test pair-wise differences in the protein expression between the groups of cells, with 20 samples/group and 99% power to detect 1 unit of difference in any pairs. For these test statistics we assumed a standard deviation of .5 staining units.

(b) *Evaluation of tissue biopsies using transmission electron microscopy (TEM).* Fresh biopsies from BE/EAC patients were evaluated for sensitivity to autophagy. We preformed an *ex vivo* stress bioassay test in which freshly obtained biopsies were incubated for four hours in control media or in media supplemented with HBSS (Hank's Balanced Cell Solution; inducer of autophagy) or 0.5mM DCA. Autophagy was then evaluated in normal duodenum and BE tissue by identification of autophagosome formation via transmission electron microscopy.

(c) *Evaluation of the expression of autophagic and proliferative markers in CP-A and CP-AR cells using Western blot analysis*. Protein was extracted from both CP-A and CP-AR cell lines to determine the basal expression of autophagic and proliferative markers in the cells, namely Beclin-1, p-AKT, and LC3II/LC3I. Protein was also extracted from CP-A cells treated with 0.2mM DCA for four hours immediately following treatment in order to observe the effects of acute exposure on normal cells in relation to chronic exposure as modeled by the CP-AR cell line.

To separate the protein from the cells, media was removed from each respective plate into a centrifuge tube and cold PBS was used to scrape and rinse remaining cells from the plate into the same tube that was then centrifuged at 1000 rpm for 15 min. The media was then aspirated off and the pellet resuspended in 5mL cold PBS before centrifuging again at the same speed for 5 minutes. The PBS was aspirated and the pellet then resuspended in 100μL lysis buffer with 0.01M protease inhibitor. The lysis buffer consists of 50mM Tris (pH 8.0), 5mM ECTA, 150 mM NaCl, and .5% NP-40 (IGEPAL). The cells were then passed through a 21G syringe 5 times and let to sit on ice for 1 hour. The mixture was then centrifuged for 15 minutes at 4°C maximum speed and the supernatant removed prior to storage at -80°C until required.

Western blot analysis was performed as previously described (32). The membranes were immunostained with antibodies against phospho-AKT (1:500; Cell Signaling, Boston, MA), beclin-1 (1:500; Cell Signaling, Boston, MA), and β-actin (1:1000; Calbiochem, Gibbstown, NJ), and then incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (Pierce Biotechnology). The membranes stripped using the RE-blot Western blot recycling kit (Chemicon International, Temecula, CA) and re-probed with β-actin antibody or stained with Brilliant Blue G dye to confirm equal protein loading. The densities of individual bands were determined using QuantiScan software (Biosoft, Cambridge, UK).

(d) *Ratio of autophagic/normal cells of CP-A and CP-AR cells assessed by quantification of cells with the punctuate pattern of GFP-LC3*. GFP-LC3 expression vector (Cell Biolabs, Inc.) containing a kanamycin resistant gene as a selection marker was transformed into a strain of E.coli and plated on an agarose plate containing kanamycin. Those that had successfully taken up the vector were able to successfully form colonies on the plate. A single one of these colonies was picked and a starter culture of 2-5mL LB medium containing the appropriate selective kanamycin was inoculated. This

culture was incubated for approximately 8 hours at 37°C and shaking at 300rpm. This starter culture was diluted 1/500 into selective LB medium and grown for 12-16 hours in the same conditions as the previous step. The bacterial cells were harvested via centrifugation at 6000x *g* for 15 min at 4°C and the cell pellets frozen at -20°C. The GFP-LC3 was later extracted from the cells using the Qiagen EndoFree Plasmid Maxi Kit. Determination of DNA yield was determined by spectrophotometry at 260nm and 280nm. Separation of the plasmid was confirmed through analysis on an agarose gel.

For transfection studies, both CP-A and CP-AR cell lines were plated on 4-chamber slides in medium (+serum/-antibiotics). Once 70-80% confluent the slides were transfected with GFP-LC3 DNA for 8 hrs. The transfection mixture consisted of 2µL lipofectamine and 0.8µg DNA plasmid per 50µL of serum free, antibiotic free transfection medium (OPTI-MEM). 100µL of this solution was added to each chamber already containing 500µL transfection medium.

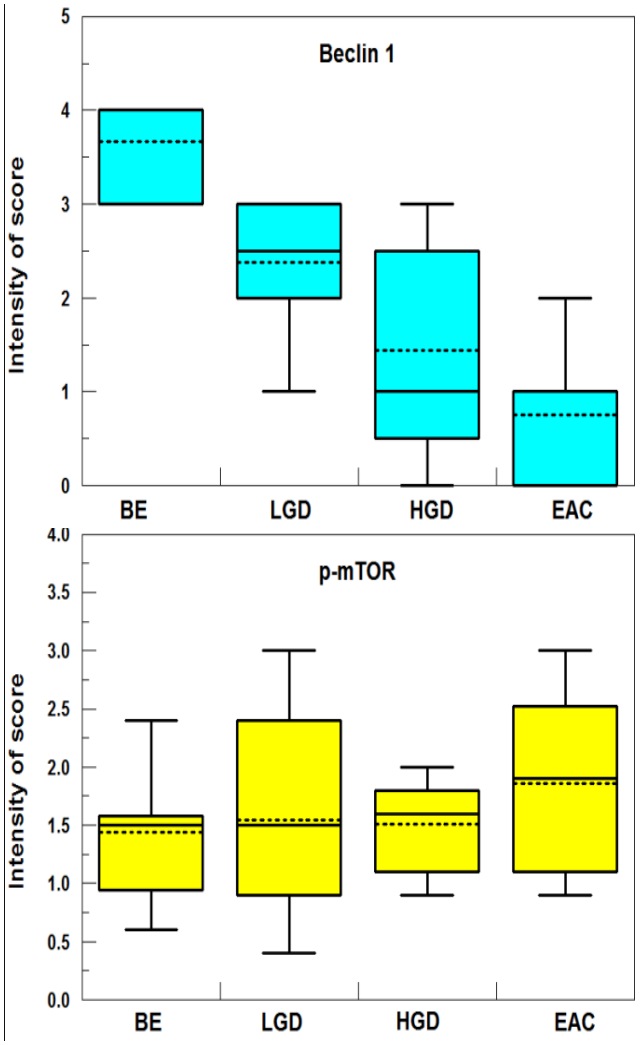
After 48 hours recovery in medium (+serum, -antibiotics), the cells were treated under various conditions including HBSS, 50nM rapamycin (inhibitor of mTOR), and 0.2mM DCA for 4 hours. HBSS and rapamycin were used as positive controls in these experiments,

Slides were washed 3x with cold PBS immediately following removal of the treatments. They were then fixed with -20°C methanol and placed in the -20°C freezer for 6 minutes. The slides were then let to completely dry for 15 minutes before being cover-slipped with DAPI. The cells were assessed under fluorescence and the ratio of autophagic/normal cells was determined through quantification of cells with/without the punctuate pattern. Autophagic cells show the punctuate pattern, which is an indication of autophagosome formation, while in normal cells the GFP-LC3 is dispersed in the cytoplasm.

Five different sets of slides were prepared and analyzed at different times. A random sample of 50 cells was counted four different times per chamber. Averaging of the four ratios was used to determine the percentage of autophagic cells in each treatment.

RESULTS:

(a) Evaluation of tissue biopsies by immunohistochemistry. Staining of Beclin-1 was found to be decreased in the progression from BE to EAC. Strong staining of Beclin-1 was found in the duodenum and normal squamous epithelium (not shown). The expression of p-mTOR was low in non-dysplastic BE, while elevated in EAC (Figure 2).



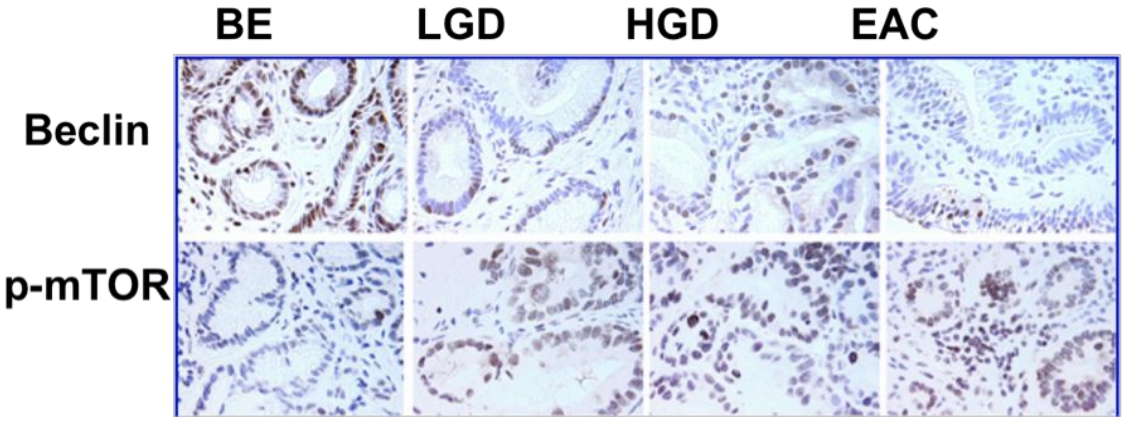


Figure 2: Tissues taken from patients with varying degrees of dysplasia were immunostained for expression of Beclin-1 and p-mTOR (left). The intensity scores for each are plotted above, showing a decrease in Beclin-1 and an increase in p-mTOR with progression of the disease.

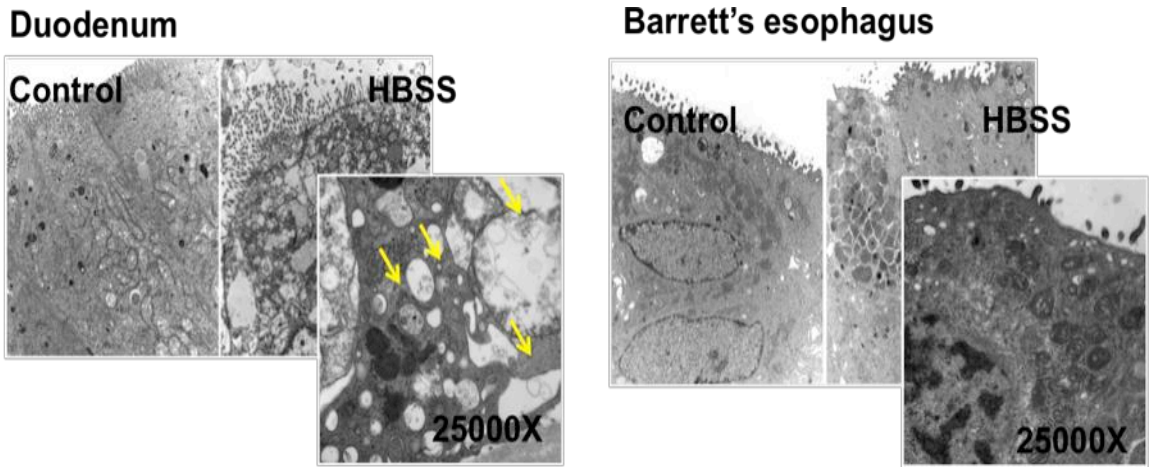


Figure 3: Tissues taken from the duodenum (control) and BE biopsies were starved with HBSS and observed under TEM for presence of autophagosome formation. Duodenum tissues responded by initiating autophagy, whereas BE tissues did not.

(b) Evaluation of tissue biopsies using transmission electron microscopy (TEM). Typical changes associated with autophagy (presence of electron-dense lysosomes and large, autophagic vacuoles containing cellular debris) were observed in the duodenum after incubation for 4 hours with HBSS. The duodenum was used as a control tissue as it is a non-metaplasic form of columnar epithelium. No autophagic vacuoles were observed in the BE tissues which were incubated in HBSS. Similarly, autophagic vacuoles were identified in squamous epithelium incubated with DCA, while no autophagy was observed in the BE tissues. The control tissues and BE tissues incubated in normal medium showed no signs of autophagy (Figure 3).

(c) Evaluation of the expression of autophagic and proliferative markers in CP-A and CP-AR cells using Western blot analysis. The pro-autophagic protein, Beclin-1, was found to be expressed by nearly 5-fold in CP-A cells that were exposed to 0.2mM DCA for four hours, showing initiation of autophagy in acutely exposed cells (Figure 4). However, CP-AR cells which are constantly bathed in medium consisting of 0.2mM DCA showed no such increase in expression of Beclin-1. The density of band for these cells was nearly identical to that of the control cells.

Western blot analysis also showed about equal levels of expression for p-AKT between CP-A control cells and the CP-AR cell line. However, when exposed to 0.2mM DCA conditions for two hours, the CP-A cells showed a decrease in p-AKT by nearly half.

(d) Autophagic cells of CP-A and CP-AR cells were assessed by quantification of cells with the punctuate pattern of GFP-LC3. Although the CP-AR line has expresses higher basal levels of autophagy, they are indeed resistant to treatments with 0.2mM DCA and exhibit no increase in autophagy levels whatsoever after exposure to DCA or HBSS (Figure 5). On the other

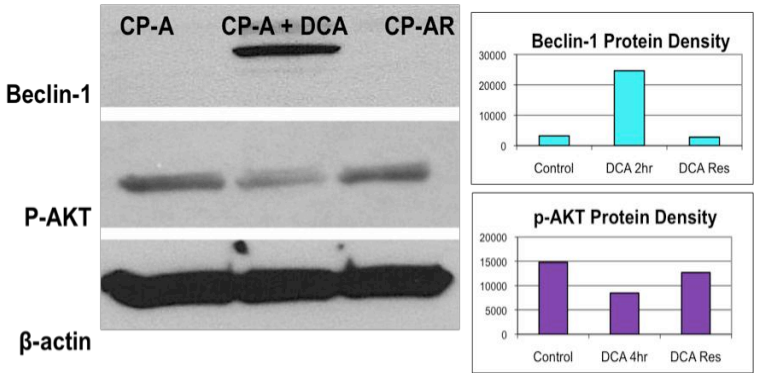


Figure 4: Expression of Beclin-1 and p-AKT were assessed through western blot (left) for CP-A cells, CP-A cells exposed to 0.2mM DCA for 4 hrs, and CP-AR cells. The band densities for each marker were recorded (right). CP-A cells express these markers in nearly equal amounts.

hand, when CP-A cells are exposed to 0.2mM DCA for four hours, autophagy levels were found to increase from a normal 15% to 40%.

CP-A cells were also found to initiate autophagy in response to the two positive controls, HBSS and 50nM rapamycin. It is interesting to note that the DC-AR cell line only initiated autophagy in response to the rapamycin treatment. Rapamycin is an inhibitor of the cell-survival protein m-TOR.

DISCUSSION:

BE has been characterized as a metaplastic lesion is which normal esophageal epithelium is replaced by intestinal epithelium. It appears to result from chronic and repeated exposure of the esophageal mucosa by a mixture of gastric acids, proteases, and bile acids over the course of several years (10, 33). A number of different theories have been proposed as to the origin of BE cells (34). It was originally proposed that cells from

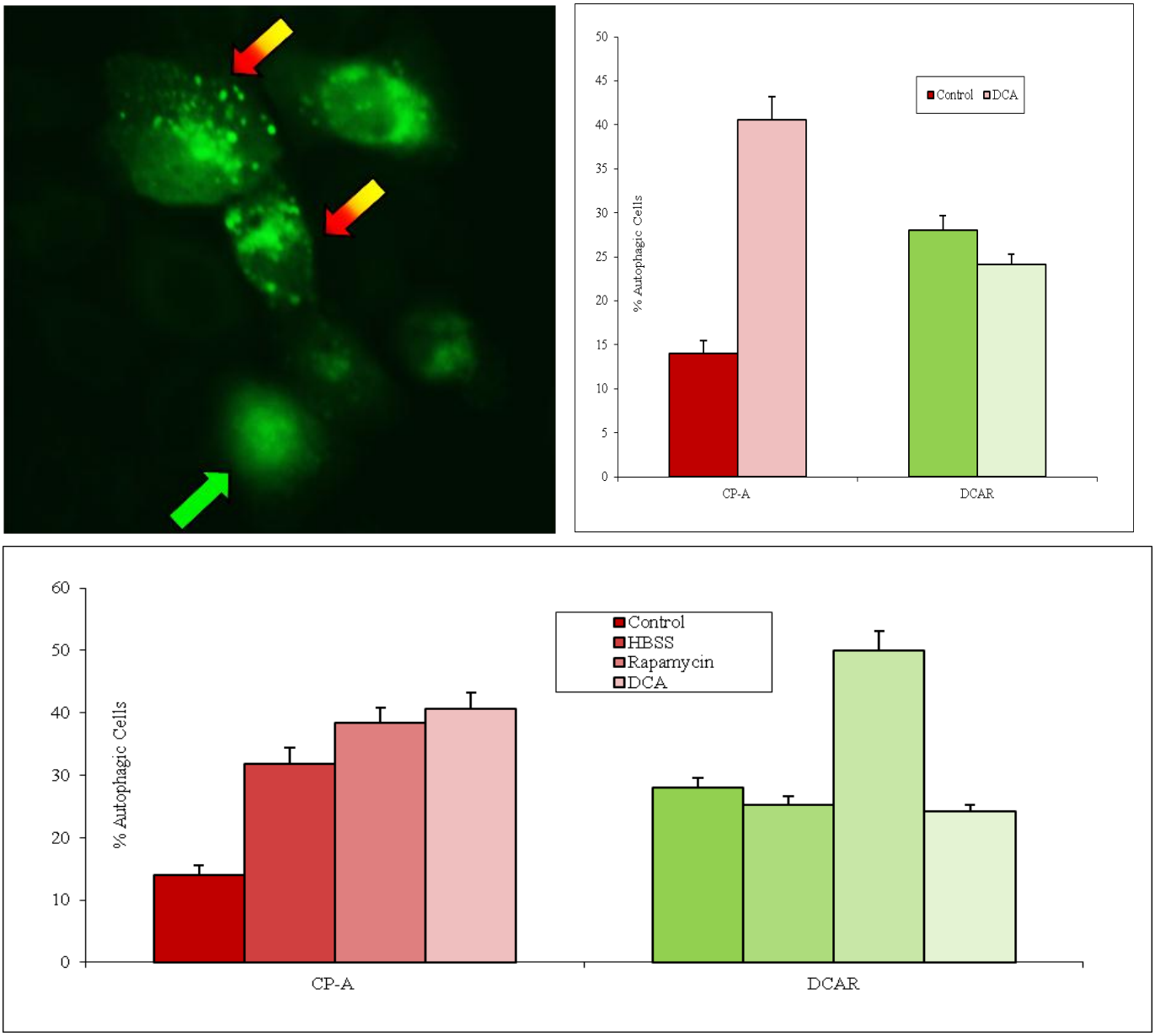


Figure 5: CP-A and CP-AR cell lines were transfected with GFP-LC3 and then treated under various conditions to see their response in terms of autophagy. Autophagic cells are characterized by the punctuate pattern indicated by the red arrows (upper left), while normal cells are green throughout. CP-A cells saw a drastic increase in autophagy levels when exposed to 0.2mM DCA for 4 hours (upper right), while CP-AR cells saw no change. CP-A cells also responded to positive controls of 50nM rapamycin and HBSS, while CP-AR cells only saw an increase in autophagy when exposed to rapamycin.

the gastroesophageal junction migrate to the distal portion of the esophagus as a result of the damage caused by the refluxate. Other suggestion included the abnormal differentiation of pluripotent stem cells in the squamous epithelium or submucosal glands. The most recent concensus is that BE evolves from native squamous cells that undergo a transdifferentiation as a result of chronic exposure to bile acids (35). Furthermore, it is hypothetical that bile acids contribute to progression of BE to EAC.

In order to test this notion, and in particular, to test the effect it has on suspending autophagy in cancer cells, we developed a resistant cell line able to survive and proliferate under constant exposure to high concentrations (0.2mM) of deoxycholic acid (DCA). These new, resistant cells were seen in our studies to activate the same signaling pathways and morphological changes similar to tissues taken from patients with pathologies ranging from BE to full-fledged EAC.

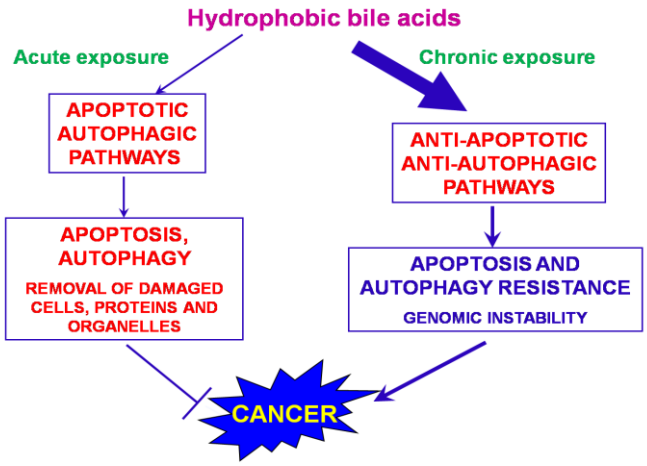


Figure 6: Proposed hypothesis for the role of acute and chronic bile acid exposure in the esophagus and its contribution to cancer progression.

Our immunohistochemical staining for p-mTOR and Beclin-1 showed increasing levels of p-mTOR in the progression from BE to EAC, while Beclin-1 expression decreased in the progression of BE to cancer. This is consistent with our hypothesis that the longer a cell is exposed to bile acids, the less responsive it will be to the environment and the more equipped it will be in surviving those conditions.

Our analysis of duodenum and BE tissues taken from patients and subjected to HBSS showed that while normal columnar epithelium responds to amino acid starvation through the initiation of autophagy, BE cells do not. This supports the hypothesis that BE cells are resistant to autophagy.

The expression of Beclin-1 in CP-A cells subjected to 0.2mM DCA for four hours, and the lack of Beclin-1 in the resistant cell line which is constantly exposed, supports the results seen in our immunostained tissues. Beclin-1 is activated by acutely exposed tissues, while those which have been chronically exposed lack the ability to do so. p-AKT levels were similar for CP-A and CP-AR cells, reinforcing the fact that chronically exposed cells gain the ability to endure the exposure. p-AKT levels were decreased by nearly half in the CP-A cells which were exposed for 4 hours.

To see if our novel CP-AR cells line lacked the ability to undergo autophagy, as seen in the BE biopsy tissues, both the CP-A and resistant lines were transfected with GFP-LC3 and observed under florescence after exposure to various treatments. We found that that the CP-AR cell line maintained a constant level of autophagy regardless of treatment, while CP-A cells increased their autophagy levels by nearly threefold. This is perhaps our most persuasive evidence showing that acute exposure induces autophagy in BE cells, while chronic exposure leads to the loss of the ability to do so (Figure 6)

In summary, exposure of the esophageal epithelium to bile acids is an important factor in BE pathogenesis, and the eventual formation of cells resistant to autophagy. The CP-AR cell line resistant to 0.2mM DCA can serve as an *in vitro* model to show that esophageal cells which are repeatedly exposed to bile acids express the anti-autophagic characteristics typical of BE. This model cell line can help contribute to better understanding of the molecular mechanisms underlying the development of resistance to autophagy and provide important insights into he development of therapies targeted at BE. On the basis of this study and also of those previously conducted by our lab, we propose that the reflux of gastric and duodenal reflux is a critical clinical step in patients with GERD.

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