

MULTIMODAL EVALUATION OF ESOPHAGEAL EPITHELIAL RESPONSE IN GASTROESOPHAGEAL REFLUX DISEASE

Ph.D. Thesis

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1. LIST OF ABBREVIATIONS

2D: 2-dimensional

3D: 3-dimensional

7AAD: 7-amino actinomycin D

AV: autophagic vesicle

BAC: bile acid cocktail

BE: Barrett's esophagus

BCECF-AM: 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein, acetoxymethyl ester

$[Ca^{2+}]_i$: intracellular Ca^{2+} concentration

CBE: Cl^-/HCO_3^- exchanger

CQ: chloroquine

DCF: 2',7'-dichlorodihydrofluorescein diacetate

EAC: esophageal adenocarcinoma

EECs: esophageal epithelial cells

GERD: gastroesophageal reflux disease

IFN- γ : interferon- γ

IHC: immunohistochemistry

IRF-1: interferon regulatory factor-1

IL: interleukin

pH_i: intracellular pH

NHE: Na^+/H^+ exchanger

NBC: Na^+/HCO_3^- cotransporter

OTC: organotypic culture

PBMC: peripheral blood mononuclear cell

ROS: reactive oxygen species

SE: squamous epithelium

2. LIST OF FULL PAPERS CITED IN THE THESIS

- I.** **Dorottya Laczkó**, András Rosztóczy, Klaudia Birkás, Máté Katona, Zoltán Rakonczay Jr., László Tiszlavicz, Richárd Róka, Tibor Wittmann, Péter Hegyi, Viktória Venglovecz. Role of ion transporters in the bile acid-induced esophageal injury. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 2016 Jul 1;311(1):G16-31. IF: **3.798**
- II.** Jianping Kong*, Kelly A. Whelan*, **Dorottya Laczkó**, Brendan Dang, Angeliz Caro Monroig, Ali Soroush, John Falcone, Ravi K. Amaravadi, Anil K. Rustgi, Gregory G. Ginsberg, Gary W. Falk, Hiroshi Nakagawa, John P. Lynch. Autophagy levels are elevated in Barrett's esophagus and promote cell survival from acid and oxidative stress. *Molecular Carcinogenesis* 2016 Nov;55(11):1526-1541. IF: **4.808** (*these authors contributed equally).
- III.** **Dorottya Laczkó**, Fang Wang, F. Bradley Johnson, Nirag Jhala, András Rosztóczy, Gregory G. Ginsberg, Gary W. Falk, Anil K. Rustgi, John P. Lynch: Modeling esophagitis using human 3D organotypic culture system. *American Journal of Pathology (Under revision)*.

3. LIST OF FULL PAPERS NOT RELATED TO THE THESIS

- IV.** Andrea Szentesi, Emese Tóth, Emese Bálint, Júlia Fanczal, Tamara Madácsy, **Dorottya Laczkó**, Imre Ignáth, Anita Balázs, Petra Pallagi, József Maléth, Zoltán Rakonczay Jr, Balázs Kui, Dóra Illés, Katalin Márta, Alexandra Demcsák, Andrea Párniczky, Gabriella Pár, Szilárd Gódi, Dóra Mosztbacher, Ákos Szücs, Adrienn Halász, Ferenc Izbéki, Nelli Farkas, Péter Hegyi. Analysis of Research Activity in Gastroenterology: Pancreatitis is in Real Danger Plos One 2016 (Accepted for publication PONE-D-16-26557R1) IF: **3.057**

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4. INTRODUCTION

Gastroesophageal reflux disease (GERD) is one of the most common disorder of the gastrointestinal system. It refers to a condition where the retrograde flow of gastric and duodenal content provokes symptoms and/or complications. According to population-based studies, GERD-related symptoms are extremely common in adults and they are sufficient to impair significantly the health-related quality of life. Weekly occurring reflux-related symptoms are reported by nearly 20% of the population in developed Western countries. Moreover their incidence and prevalence increases in such parts of the world where they were previously uncommon, particularly in South-East Asia and the Far East.

GERD is divided into three subcategories: (1) non-erosive GERD, which refers to a condition where pathologic reflux is present in the absence of endoscopically visible mucosal injury. Nearly 60% of GERD patients fall into this category. (2) Erosive GERD accounts for 35% of GERD. These patients have macroscopically visible, mucosal injury during upper endoscopy. Although, most patients have non progressive mild to moderate forms, a minority may progress and end up in severe complications such as esophageal perforation, bleeding or esophageal strictures. (3) Barrett's esophagus (BE) which is the replacement of the multilayered squamous epithelium (SE) with specialized intestinal type of columnar epithelium at the distal part of the esophagus. BE is associated with a 30- to 40-fold increased risk for the development of esophageal adenocarcinoma (EAC), therefore, BE is considered to be the most severe complication of GERD. Although, the exact pathomechanism of BE is still unknown the major pathogenetic factors considered to be the excessive acid and bile exposure and the subsequent chronic inflammation triggered by the chemical injury.

4.1. The role of ion transporters in the pathogenesis of GERD

Under physiological conditions highly efficient barriers exist in the esophagus which can protect esophageal epithelium from harmful compounds. Epithelial resistance, which was first described by Orlando *et al.* is one of the key element of the defense. Esophageal resistance can be divided into three functional categories: the pre-epithelial, epithelial and post-epithelial defense. Pre-epithelial protection is consist of unstirred water layer and mucous layer with HCO_3^- in it. The main role of this defense mechanism is to sustain a substantial pH gradient from lumen to surface

cells. Post-epithelial defense refers to the adequate blood supply of the tissue which plays an essential role in the regulation of interstitial pH through removal of acidic byproducts.

Epithelial defense is the most important component of esophageal resistance. It has both structural and functional components and the transport proteins on the apical and basolateral membranes of esophageal epithelial cells (EECs) play a crucial role in it. Studies on iontransporters have been performed on normal esophageal epithelium; however, the activity or expression of ion transporters in the columnar epithelia under pathophysiological conditions is less characterized.

4.2. The role of autophagy in the pathogenesis of GERD and BE

Autophagy is a lysosome dependent cellular mechanism which degrades the damaged or obsolete organelles and proteins in the cells. Basal level of autophagy has a fundamental physiological role in cellular homeostasis like development, immune defense, programmed cell death and prevention of neuron degradation. Furthermore, autophagy is induced as an adaptive response to cellular stress, either from nutrient/growth factor deprivation, hypoxia, oxidative stress, accumulation of protein aggregates, and endoplasmic reticulum stress. During autophagy the potentially toxic cytoplasmic constituents are encapsulated by pre-autophagosomal structures called “phagophores” that mature into double-membrane vesicles called “autophagosomes” and fuse with lysosomes where the content is degraded.

In normal tissues, autophagy is responsible for maintaining cell homeostasis and prevents carcinogenesis by inducing apoptosis. Therefore, the dysregulation of autophagy has been linked to a growing list of diseases such as eosinophilic esophagitis, inflammatory bowel disease or Parkinson’s disease. Autophagy can also act as a double edged sword in the cells: once cancer develops, many cancer cells upregulate autophagy to survive hypoxia and nutrient limitation. Interestingly, despite the growing evidence between cancer and autophagy, the importance of autophagy in pre-cancerous lesions like BE is less characterized. Furthermore, in GERD, the gastric acid and bile contribute to the inflammation and cellular stress of esophageal epithelium which are important activators of autophagy. However, to date there is only a single publication on the role of autophagy in BE mainly focusing on the role of Beclin1 autophagy gene only.

4.3. Modeling the pathogenesis of GERD

Research into GERD and its complications has been restricted by the availability of suitable laboratory approaches to model these conditions. Much of the past work has relied upon the availability of human patient biopsies, which are difficult to obtain and primarily suitable for descriptive studies. Immortalized cell lines, representing normal squamous, Barrett's, and EAC are available, however 2D cultured cells can not effectively model complex interactions between epithelial cells and their microenvironment. Animal models are another important tool of biomedical research. However, animal models for the diseases of the esophagus are limited as well, in part due to anatomic differences between mice and humans at the squamo-columnar junction. Organotypic culture (OTC) system is an innovative multicellular system which attempts to better model human tissues. Under 3D OTC conditions, human esophageal keratinocytes undergo a complete differentiation and stratification producing a fully mature epithelium. Its advantage relates to the normal polarization and differentiation of cells and gene expression patterns are similar to the *in vivo* conditions. OTC is an *in vitro* tool which is still physiologically relevant and helps to better recapitulate the complex interactions between epithelial cells and their microenvironment.

5. AIMS

The specific **aims** of our studies:

- I. In the first part of the thesis we attempted to identify the ion transport mechanisms in columnar epithelial cells derived from Barrett's metaplasia and to characterize the effect of main internal risk factors (such as HCl, bile acids) on these transporters. Furthermore, we aimed to compare their mRNA and protein expression profile of ion transporters with human squamous and columnar epithelial cells obtained from endoscopic biopsies of normal esophageal mucosa and BE.
- II. In the second part of the thesis, we planned to explore the functional role of the autophagic response in cellular oxidative stress and cell survival in esophageal cells representing different severity stages of adenocarcinoma in an *in vitro* model of acid reflux.
- III. Finally, our last objective was to establish whether OTC could be applied as a novel platform for the study of inflammatory environment in GERD.

6. MATERIALS AND METHODS

6.1. Cell lines

Immortalized human squamous esophageal epithelial cells (STR), human, non-dysplastic BE cell line (CP-A), human, dysplastic BE cell line (CP-D) were adapted to serum-free conditions in keratinocyte serum-free medium (KSFM). OE19 human adenocarcinoma cells were maintained in RPMI 1640 with 2 mM glutamine and 10% fetal calf serum. Medium was replaced in every 2 days on all cell lines. Cultures were continually incubated at 37 °C and gassed with the mixture of 5% CO₂ and 95% air.

6.2. 3-dimensional organotypic culture (OTCs)

The fibroblast feeder layer and peripheral blood mononuclear cells (PBMCs) were embedded within a collagen/Matrigel matrix and was allowed to mature for 7 days, after which epithelial cells were seeded on top and allowed to grow confluence for another 4 days. On day 11, the culture media level is reduced bringing the keratinocytes to air-liquid interface which stimulates epithelial differentiation into a multilayer epithelium typical for the esophagus. On day 15, OTCs were harvested for histology, RNA and/or protein isolation.

6.3. Patients

Fourteen patients with endoscopic evidence of esophageal metaplasia were enrolled in the First Department of Medicine, University of Szeged, Hungary. Four biopsy samples were obtained from the macroscopically visible metaplastic columnar epithelium of the esophagus and another four from the normal squamous lining. Two of each sample were formalin-fixed and submitted for histological evaluation including IHC. The remaining two samples were immediately placed and stored in RNA-later solution for real-time PCR analysis at -20°C. All procedures were performed with informed patient consent and under approved human subject's protocols from University of Szeged (No.: 2348).

6.4. Measurement of pH_i and $[Ca^{2+}]_i$ with microfluorimetry

In order to estimate changes of pH_i and $[Ca^{2+}]_i$, cells were loaded with BCECF-AM and FURA2-AM at room temperature and the fluorescence emission ratios (490/440 and 340/380) were measured at 535 and 515 nm, respectively. In order to estimate the activity of Na^+/H^+ exchangers (NHEs), the Na^+/HCO_3^- cotransporter (NBC) and Cl^-/HCO_3^- exchanger (CBE) the NH_4Cl prepulse technique was used.

6.5. Bile acid treatments

In order to mimic the chronic bile acid exposure in GERD *in vitro*, cells were treated with bile acid cocktail (BAC) at pH 7.5 and 5.5. Cells were treated with bile acids for 10 min pulses, 3 times a day up to 7 days. The composition of BAC was: 170 μM glycocholic acid (GC), 125 μM glycochenodeoxycholic acid (GCDC), 100 μM deoxycholic acid (DC), 50 μM glycodeoxycholic acid (GDC), 25 μM taurocholic acid (TC), 25 μM taurochenodeoxycholic acid (TCDC) and 8 μM taurodeoxycholic acid (TDC). The composition and concentration of BAC mimics the bile acid profile of GERD.

6.6. RNA isolation, reverse transcription and quantitative real-time PCR

Total RNA was purified from individual cell cultures, biopsy samples and OTC epithelium (after manually peeling off from the collagen base) using the RNA isolation kit of Macherey-Nagel (Nucleospin RNA II kit, Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. High-Capacity cDNA Archive Kit (Applied Biosystems Foster City, CA, USA) was used for reverse transcription according to the manufacturer's instructions. For NHE-1, 2, NBC, Slc26a6 and IRF-1 genes quantitative real-time PCR (qPCR) reaction was performed using Taqman probe sets for the ion transporters and SYBR Green Master Mix for IRF-1 gene.

6.7. Western Blot analysis

20 μg of denatured protein was fractionated on a NuPAGE Bis-Tris 4–12% gel. Following electrotransfer, Immobilon-P membranes were blocked with PBST containing 5% milk, followed by overnight incubation with the following primary antibodies: rabbit anti-NHE1 and IRF-1 at 4°C. Mouse anti-GAPDH mouse alpha tubulin were used as an internal control. Targeted proteins were visualized using a chemiluminescence detection system.

6.8. Immunohistochemistry

5 µm paraffin-embedded sections of human, esophageal biopsy samples and OTCs were deparaffinised. After heat mediated antigen retrieval sections were incubated with primary mouse monoclonal anti-NHE1, chicken anti-NHE2, rabbit anti-CD45, rabbit anti-Ki-67 antibodies and biotinylated secondary antibodies and an avidin-horseradish peroxidase conjugate following the manufacturer's protocol. The signal was developed using the 3, 3'-diaminobenzidine substrate kit. Sections were counterstained with hematoxylin. The specificity of the primary antibodies was assessed by using mouse IgG1 or chicken IgY isotype controls.

6.9. Flow cytometry analysis

To detect autophagy flux Cyto-ID[®] green autophagy dye was used according to the manufacturer's instruction. Intracellular levels of ROS in epithelial cells were determined by flow cytometry with 2', 7'-dichlorodihydrofluorescein diacetate (DCF) dye. Cell death measures at 24 h were carried out using staining with 7-amino-actinomycin D (7AAD). 7AAD fluoresces upon binding to DNA and is normally excluded by living, intact cells. Following stainings, cells were washed then analyzed in DPBS containing 1% BSA using a FACS Calibur

6.10. Statistical analysis

Unpaired student's t-test was used for comparisons between two groups. Data from multiple groups were analyzed using one-way ANOVA with Tukey's post-hoc test. All data were represented as mean ± standard error of the mean (SEM). p values ≤ 0.05 were accepted as significant.

7. RESULTS

7.1. Role of ion transporters in the bile acid-induced esophageal injury

In the first series of experiments, the resting pH_i of CP-A and CP-D cell lines was calibrated by the high K^+ /nigericin method. We found 3 functionally active ion transporters on CP-A and CP-D cells: NHE, NBC and CBE. The NHE inhibitor, HOE-642 (1 μM) strongly inhibited the recovery from acid load in both cell line, whereas in the presence of 50 μM , the recovery was completely abolished, indicating that both NHE-1 and NHE-2 are expressed on CP-A and CP-D cells. In order to estimate the activities of NHE and NBC, the effects of the NBC inhibitor, H_2DIDS (500 μM) and HOE-642 (50 μM) on the recovery from acid load was tested separately and together. Both H_2DIDS and HOE-642 equally reduced the recovery from acidosis, whereas combined administration of these two agents completely abolished it.

Bile acids are known activators of many intracellular processes, including Ca^{2+} signaling which is an important intracellular pathway. Therefore, in the next step, we have investigated the effect of BAC on $[\text{Ca}^{2+}]_i$. 500 μM BAC induced an increase in $[\text{Ca}^{2+}]_i$ that was more pronounced under acidic conditions. Using the IP_3 receptor blocker, caffeine and the plasma membrane Ca^{2+} channel inhibitor, Gd^{3+} we have shown that BAC releases Ca^{2+} from the endoplasmic reticulum and also induces the entry of extracellular Ca^{2+} .

We have also investigated the effect of BAC on the activity of ion transporters. The acute effect of BAC was examined using the NH_4Cl pre-pulse technique. In HEPES-buffered solution, BAC dose-dependently decreased NHE activity by the inhibition of both NHE1 and NHE2. In contrast, the activity of NHE increased in CP-D cells after the administration of BAC. In $\text{HCO}_3^-/\text{CO}_2$ -buffered external solution BAC stimulated NBC and CBE in both CP-A and CP-D cell lines. Pretreatment of the cells with the Ca^{2+} chelator BAPTA-AM, a significant decrease was obtained both in the inhibitory and stimulatory effects of 500 μM BAC on the ion transporters, indicating that the effects of bile acids on ion transporters are Ca^{2+} -dependent.

7-days treatment with BAC increased the expression of NHE1, NHE2, NBC and the CBE isoform, *Slc26a6* compared to non-treated control cells at pH 7.5 in both CP-A and CP-D cells. At acidic pH alone or in combination with bile acids the expression levels of ion transporters did not change significantly in CP-A cells. In contrast, CP-D cells displayed a significant increase in NHE1 levels after bile acid treatment at pH 5.5. We have also shown that the enhanced mRNA

levels of NHE1 were associated with significantly increased protein expression. Increased mRNA expressions of NHE1, NHE2, NBC and Slc26a6 were also found in BE metaplasia compared to normal epithelium. In addition, both intestinal and non-intestinal metaplastic columnar mucosa displayed strong staining against NHE1 and NHE2 antibodies in contrast to normal SE.

7.2. Role of autophagy in the pathogenesis of GERD and BE

STR, CP-A, CP-D and OE19 cell lines were exposed to acidic challenge at pH=3.5 followed by treatment with chloroquine (CQ) which is a specific inhibitor of autophagy, or vehicle control. Six hours post-exposure cells were stained with DCF or Cyto-ID to determine intracellular ROS levels and autophagic responses, respectively. In both STR and CP-A cells, acid treatment significantly increased ROS levels at 6 hours post exposure. CQ alone had a mixed impact on cellular ROS levels-increasing them in STR but not CP-A cells. However, CQ in combination with acid stress induced an additional significant increase in ROS levels in both STR and CP-A cells compared to cells which were exposed to acid only. Autophagy levels were similarly responsive to these treatments in STR and CP-A cells. Cyto-ID autophagy levels were increased by all three conditions: acid exposure alone, CQ, as well as the combined treatments. The increase observed with CQ is due to the accumulation of blocked autophagic vesicles (AVs).

In both CP-D and OE19 cells, these cellular responses were different. As with the non-dysplastic cells, the acid treatment led to a significantly increased ROS levels at 6 hours post exposure in these cell lines. CQ alone had no significant effect on cellular ROS levels. However, when CQ was combined with acid stress, there was no additional increase in ROS levels experienced by either the CP-D or OE19 cells compared to cells which were exposed to acid alone. In CP-D cells, no change in Cyto-ID fluorescent signal was detected with any treatment, including after CQ treatment alone. In OE19 cells, not only there was no increase in relative Cyto-ID fluorescence after CQ treatment, but exposure to acid significantly reduced this relative fluorescence. Furthermore we examined the Cyto-ID fluorescent by confocal microscopy in OE19 cells after these same treatments and in parallel treated LC3-GFP labeled OE19 cells as a second measure of the autophagic response following the same experimental protocol. In contrast to Cyto-ID measurement LC3-GFP vesicles were increased weakly by acid treatment and strongly after CQ and combined treatment.

7AAD staining was used as a highly quantitative flow cytometry approach to assess cell death. 7AAD⁺ dead cells were quantified 24 hours after treatment with acid, CQ, or the combination of acid and CQ. For all cell lines examined, the combination of acidic stress and CQ treatment led to a very significant increase in 7AAD⁺ dead cells at 24 hours.

7.3. Modeling esophagitis using 3D organotypic culture system

In order to develop a human *in vitro* model of esophageal inflammatory conditions human PBMCs were included in the collagen/Matrigel extracellular matrix at the initiation of the culture, prior to the establishment of the epithelial cell layer. In order to induce a robust T_H1 acute inflammatory response, pro-inflammatory cytokines IL-2, IL-7 and IL-15 were included in the culture media. These three cytokines together supported the induction of an acute inflammatory response in the OTC-PBMC co-culture environment. The addition of the cytokines induced a significant increase in the number of CD45⁺ and Ki-67⁺ immune cells in the OTC-PBMC cultures. To show immune cell activation, the overlying epithelium in the OTC cultures were examined for expression of interferon regulatory factor 1 (IRF-1), as a surrogate marker for interferon- γ production. A highly significant increase was observed in the levels of IRF-1 mRNA and protein detected in the epithelium from the OTC+PBMC+IL cultures.

We next sought to identify the effects of activated immune cells on the overlying EECs. Cultures with cytokine-cocktail treated PBMCs displayed a significant increase in epithelial thickening, both in the basal and suprabasal compartments. Furthermore, a noticeable regenerative response marked by eosinophilic keratin pearl formation and excess keratin production was also observed.

OTC epithelium was examined for changes in proliferative rates by staining for the proliferation marker Ki-67 in the epithelial cell layer. There was a significant increase in Ki-67⁺ cells, as compared to controls, when quantified across multiple cultures. This increased level of cell death was confirmed and quantified by TUNEL staining in the suprabasal compartment in epithelium of the OTC cultures with the acute-inflammatory environment.

8. DISCUSSION

8.1. Role of ion transporters in the bile acid-induced esophageal injury

The preservation of physiological pH_i is critical for cell survival and it is mediated through intracellular buffers and ion transporters of the membrane. Ion transporters are part of the esophageal epithelial defense and play crucial role in the protection of gastric and bile acid induced esophageal injury.

Using functional and molecular biology methods, we determined that Barrett's derived EECs display three main functionally active ion transporters: the NHE, the NBC and the CBE isoform Slc26a6, similarly to other epithelial cells in the gastrointestinal tract. Using NHE isoform specific inhibitor HOE-642, we determined that NHE-1 and NHE-2 are participating in the restoration of pH_i which is rather attributable to NHE-1. This finding was in accordance with Goldman *et al.* who found that 60% of pH_i recovery is mediated through NHE-1. Furthermore, we determined that NHE and NBC contribute equally to the alkalization of CP-A and CP-D cells.

We found that administration of bile acids induced dose-dependent Ca^{2+} -elevation in the cells which was more pronounced under acidic condition. Our findings were in agreement with observations of other laboratories that demonstrated that exposure CP-A cells or mouse EECs to DC or acidic media induced intracellular Ca^{2+} elevation. Furthermore, we investigated the potential source of intracellular Ca^{2+} -release. We showed that caffeine, an inhibitor of IP_3 -mediated Ca^{2+} responses, completely inhibited the bile acid-induced Ca^{2+} signaling in the absence of extracellular Ca^{2+} , suggesting the involvement of IP_3 receptors in the bile acid-induced Ca^{2+} release, similarly to colonic crypts, hepatocytes, or pancreatic ducts and acini. Gd^{3+} , a known inhibitor of plasma membrane Ca^{2+} entry channels, strongly blocked the bile acid-induced Ca^{2+} signaling indicating that bile acids also promote the influx of extracellular Ca^{2+} .

Administration of BAC dose dependently decreased the activity of NHEs (both NHE1 and NHE2), whereas stimulated the activities of NBC and Slc26a6 in CP-A cells. Inhibition of NHEs probably contributes to the acidification of the CP-A cells. In contrast, the acidification and the consequent cell damage is prevented by the increased activity of the HCO_3^- import system through the NBC and the elevated efflux of HCO_3^- through the $\text{Cl}^-/\text{HCO}_3^-$ exchanger, Slc26a6 which also plays an important role in the protection of the cells by the neutralization of the cell environment in the surface mucus layer. Interestingly, administration of BAC stimulated NHE activity in CP-

D cells. This difference to CP-A cells can be explained by the advanced stage of CP-D cells. However, the mechanism for this alteration and its potential physiological role cannot be explained by the present studies and are areas of focus for future work.

Furthermore, we have demonstrated that chelation of $[Ca^{2+}]_i$ by BAPTA-AM almost completely abolished both the inhibitory and stimulatory effect of BAC on ion transporters.

In order to mimic chronic bile acid challenge, we performed a 7-day treatment with BAC under neutral and acidic conditions to investigate the role of ion transporters in cellular adaptation. Following BAC treatment the mRNA expression of all of the investigated transporters in CP-A cells and the mRNA expression of NHE1 and NBC in CP-D cells were increased under neutral pH. In contrast, only the expression of NHE1 significantly increased in CP-A CP-D cells at pH 5.5. The increased expression of NHE1 was also confirmed at protein level. Furthermore, we investigated the expression profile of these transporters in human derived biopsy samples from the squamous and metaplastic mucosa of BE patients. We divided BE samples into intestinal and non-intestinal groups due to the different definitions of BE in Europe in contrast to the US. NHEs, NBC, and Slc26a6 displayed higher mRNA levels in both intestinal and non-intestinal metaplasia compared with normal tissue. Increased protein expression of NHE1 and NHE2 was also confirmed in BE. These results are consistent with the report by Goldman *et al.* that demonstrated upregulation of NHE-1 in BE compared with normal epithelium both at mRNA and protein levels in biopsy samples and cell lines. These findings support our hypothesis that the overexpression of ion transporters is probably a defensive or adaptive mechanism by which the cells try to compensate the toxic effects of bile acids.

8.2. Role of autophagy in the pathogenesis of GERD and BE

In the second part of the thesis we investigated the potential contribution of autophagy in the pathogenesis of BE. The date the role of autophagy in premalignant diseases like BE is less characterized. In present study we determined the functional role of autophagy in various esophageal cell lines. We provided evidence that autophagy inhibition with CQ further increased intracellular ROS level after acid pulse treatment in non-transformed cell lines (STR and CP-A). This significant increase is consistent with autophagy acting to reduce intracellular ROS stress after an injury. However, this significant increase was not observed in dysplastic cells (CP-D and OE-19), suggesting that these cells do not utilize autophagy to manage oxidative stress induced by

an acidic environment. Most significantly, we observed that the inhibition of autophagy after an acidic insult leads to significantly greater cell death at 24h in all cell lines tested. This suggests two important role for autophagy in esophageal cells in response to an acid insult: the modulation of the oxidative stress and enhancing cell survival. One unexpected finding from our study is that in dysplastic CP-D and OE19 cells, Cyto-ID, and LC3-GFP appear to mark different vesicle subsets. This is the only explanation available to explain the disconnect between these two well-established approaches to quantifying autophagy vesicle content of cells. We tend to favor the LC3-GFP results as being more representative of the autophagy response, given that control OE19 cells by observed by transmission electron microscopy had vesicles but these were not double-walled and did not contain cellular debris (data not shown). Thus the Cyto-ID dye identifies these vesicles based on a shared biochemistry with autophagosomes. However, quite unexpectedly only in OE19 cells, the Cyto-ID signal was lost after acid treatment. Together these findings do suggest autophagosome biochemistry may be different in the dysplastic cell line. The mechanism for this alteration, and the physiologic role it may play, cannot be determined by the present studies and needs to be further explored.

8.3. Modeling esophagitis using 3D organotypic culture system

Research into GERD has been limited by the availability of suitable laboratory approaches to model this conditions. The limitations of the available *in vitro* and *in vivo* approaches inspired the development of 3D OTC which is an *in vitro* system which is able to recapitulate *in vivo* growth and differentiation.

In the third part of the thesis we further expanded the original protocol of OTC in order to model esophageal inflammation and get a better insight into the interactions between the esophageal epithelium and the acute inflammatory environment. We found that OTC is an appropriate culture system to maintain immune cell viability and cytokine responsiveness, and we demonstrated that human PBMCs incorporated into the OTC culture respond appropriately to pro-inflammatory cytokines. Moreover, the epithelial responses to our inflammatory environment are in accordance with previous descriptions of *in vivo* responses to reflux esophagitis. We observed changes in epithelial morphology in our OTC cultures, including increased epithelial thickening, enhanced basal cell proliferation, elevated levels of epithelial cell apoptosis, and hyperkeratinization. Biopsy samples from reflux esophagitis patients have similarly displayed

basal cell hyperplasia, and increased epithelial cell apoptosis in the setting of an immune cell infiltration. Similar findings has been described in rat reflux models as well. Our observations here imply that a significant portion of these epithelial responses in GERD esophagitis are immune cell mediated, rather than directly caused by the actions of the gastric refluxate, and therefore support the alternative mechanism for the tissue injury recently proposed.

9. CONCLUSIONS AND NEW RESULTS

- In the first part of the thesis we characterized the role of ion transport mechanisms in metaplastic EECs. Our findings further confirm the notion that metaplastic change of the normal SE to BE may be an adaptive process to regulate pH_i after exposure to gastric and bile acid. Our study may help to better understand the metaplastic esophageal response to injury and the role of ion transporters in this process. We believe that pharmacological activation of ion transporters increases epithelial resistance in an acidic environment and therefore may protect the esophageal mucosa against the injurious bile acids.
- In the second part of the thesis we demonstrated that autophagy is activated in response to GERD-like acidic stress where it functions to reduce intracellular oxidative stress and improve cell survival. We hypothesize that autophagy may be a novel therapeutic target in BE that deserves to be explored. Our work exploring the activity of autophagy in BE and EAC is thus important both for mechanistic insights as well as the potential application of novel therapeutic agents to intervene in BE and EAC onset and progression.
- In the final part of the thesis we demonstrated that OTC is an ideal tool to model esophageal inflammation since it can recapitulate the characteristics of esophagitis in a well-controlled *in vitro* setting. In conclusion, our results further highlight the importance of immune-cell mediated esophageal injury and supports the further use of this exciting platform to characterize the underlying molecular events in inflammation-induced esophagitis, BE, and carcinogenesis .

In summary, our studies give a better insight into the pathogenesis of GERD and its complications and enhance our understanding of these conditions.

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