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Bile salts sensitize esophageal squamous cells to CD95 (Fas/Apo-1 receptor) mediated apoptosis via a protein trafficking mechanism

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Abstract

Introduction—The role of non-acidic reflux contents on the pathophysiology of Barrett’s Esophagus remains poorly understood. We hypothesized that esophageal squamous epithelium differs from Barrett’s columnar epithelium in response to bile salts with respect to subsequent changes in the cell surface expression of CD95 (Fas/Apo-1) and sensitivity to CD95-mediated apoptosis.

Methods—Immortalized esophageal squamous cells (HET-1A) and Barrett’s Esophagus cells (BAR-T), and esophageal adenocarcinoma cells (Flo-1) were treated with toxic and non-toxic bile salts at concentrations observed in gastroesophageal refluxate. CD95 cell-surface expression and apoptotic response to activating anti-CD95 antibody treatment was determined by FACSscan analysis.

Results—Bile salt exposure resulted in a dose-dependent increase in CD95 cell-surface expression in HET-1A cells, but not BAR-T or Flo-1 cells. This response occurred rapidly, within a time-frame inconsistent with *de novo* protein synthesis and was blocked by protein kinase C (PKC) inhibition. Surprisingly, PKC inhibition in Flo-1 cells resulted in an increase in CD95 cell surface expression. Following bile salt exposure, a corresponding increase in the induction of CD95-mediated apoptosis was observed in HET-1A cells; PKC inhibition sensitized Flo-1 cells to apoptosis.

Conclusions—Our findings suggest that esophageal squamous cells are sensitized to CD95-mediated apoptosis following bile salt exposure. This response differs from that in columnar epithelial cells, and may offer a potential mechanism of selection pressure that contributes to the pathophysiology of Barrett’s Esophagus.

Keywords

Barrett’s Esophagus; Gastroesophageal Reflux Disease; Esophageal Adenocarcinoma

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INTRODUCTION

Barrett's esophagus (BE) is a metaplastic, columnar epithelium that replaces the normal stratified squamous epithelium of the lower esophagus (32), and is generally accepted to be a pre-malignant epithelium from which esophageal adenocarcinoma (EA) may arise. Significant effort to improve our understanding of the biology behind this metaplastic transformation is warranted, as an alarming increase in the incidence of EA is occurring in the developed world, and EA has now replaced squamous cancer as the most common histological type of esophageal cancer in the United States (1). Gastroesophageal reflux disease (GERD) and obesity have been identified as the major risks factors for the development of EA (32).

Extensive evidence has established that BE develops as a complication of GERD. The role of acidic reflux into the esophagus in the pathogenesis of BE is widely accepted (31), however, more recent investigation has suggested bile salts may play an important role in the pathophysiology of this metaplasia (29). While numerous studies have improved our knowledge in this regard, the mechanism(s) and relative contribution of bile salts in the pathophysiology of BE remains poorly understood.

We have previously reported that CD95 (Fas/Apo-1) expression is altered in virtually all cases of EA, and that these changes occur early in the progression of BE to EA (13). CD95 (Fas/Apo-1) is a transmembranous cell-surface receptor and member of the "death receptor" family that includes tumor necrosis factor receptors (TNFR1 and TNFR2), and DR4 and DR5 (24). CD95 transmits an apoptotic signal following binding by Fas ligand (FasL) (24). Consistent with this function, near universal resistance to Fas-mediated apoptosis has been observed in malignancy (25). Our previous work demonstrated that decreased cell-surface expression of CD95 in EA is associated with resistance to CD95-mediated apoptosis. This decreased cell-surface expression is not due to reduced total protein levels, rearrangements in the gene or altered transcription, but, rather appears to be due to alterations in post-translational trafficking resulting in accumulation of CD95 within the cytoplasm (13).

Two reports in the literature, in combination with our previous work, led us to design the present study. First, in experiments investigating the pathophysiology of cholestatic inhibition of liver regeneration, hepatocytes were shown to traffic CD95 from cytoplasmic stores to the cell surface in response to exposure to bile salts through a protein kinase C (PKC) dependent mechanism (16). This response to bile salt exposure sensitized hepatocytes to CD95 mediated apoptosis, and established post-translational regulation of CD95 via PKC regulated trafficking of storage pools of CD95 to the cell surface. Second, CD95 and FasL interactions were shown to be essential to the pathophysiology of pancreatic acinar to ductal metaplasia that progresses to pancreas cancer following pancreatic duct ligation in a murine model (3). Thus, taken together with our previous work, these findings led us to speculate that CD95 may play a role in the pathophysiology of BE. We hypothesized that exposure to bile salts would result in increased CD95 cell-surface expression in squamous esophageal epithelial cells, but not in BE or EA cells.

MATERIALS AND METHODS

Cell Lines and Culture

The Het-1A cell line is a SV-40-immortalized, non-neoplastic, normal squamous epithelial cell line that has been previously described (34). Het-1A cells were obtained from the American Type Culture Collection (Manassas, VA) and maintained per the recommended growing conditions in LHC-9 growth medium (BioSource; Camarillo, CA). The non-neoplastic Barrett's esophagus cell line (BAR-T), created via hTERT-immortalization of a

human sample of Barrett's esophagus, was provided by Drs. RF Souza and CP Morales and is also described elsewhere (15). BAR-T cells were maintained in co-culture with a mitomycin C-treated Swiss 3T3 feeder layer. The feeder cells were removed with 0.02% EDTA (titrated with NaOH to pH 7.4) before sub-culturing pure BAR-T cells 48 hours prior to experiments. Cells were maintained in keratinocyte basal media (KBM-2) (Cambrex; Walkersville, MD) supplemented with 5% FBS (Atlanta Biologicals; Norcross, GA), 0.1 nM cholera toxin (Calbiochem; San Diego, CA), 100 U/ml penicillin-streptomycin (GIBCO-BRL; Gaithersburgh, MD), 70 µg/ml bovine pituitary extract (Hammond Cell Technologies; Windsor, CA), 400 ng/ml hydrocortisone, 20 µg/ml EGF, 10 mg/ml adenine, 5 µg/ml insulin, and 5 µg/ml transferrin (all from Sigma-Aldrich Corp.; St Louis, MO). An established human EA cell line (Flo-1) was also studied and maintained in culture as previously described (13). BJAB cells, a B-cell lymphoma cell line that is sensitive to CD95-mediated apoptosis was also maintained in RPMI with 5% FBS and utilized as a positive control.

Treatments

Stock solutions of bile salts (1.0 mM) were freshly prepared immediately prior to each treatment by dissolving guanochenodeoxycholate (GDC) or taurodeoxycholate (TDC) obtained from Sigma-Aldrich Corp. (St. Louis, MO) in standard growth media. The solutions were titrated with 1.0 N NaOH to a 6.5 pH, and the solutions were protected from sources of light to prevent degradation. Stock solutions were then added directly to 1 cc of fresh, standard culture media in the culture well to produce final bile salt concentrations ranging from 50 to 250 µM. Treatment duration ranged from 5 to 60 minutes. Cells maintained in complete media served as controls.

For PKC inhibition experiments, cells were pretreated for 10 minutes by exposure to 1 µM of the PKC-inhibitor bisindolymaleimide (BIM) (BIM dissolved in DMSO and diluted 1:10 in PBS to create a 1 mM stock solution) prior to exposure to 250 µM of bile salts as described above for total treatment durations ranging from 15 to 120 minutes. Cells treated with vehicle alone served as additional controls. All presented data represent results from a minimum of 3 individual experiments performed on separate days.

Quantification of Cell Surface Expression of CD95

CD95 cell-surface expression was analyzed using flow cytometry as previously described (13). In brief, adherent cells were gently freed from culture plates using 10 mM EDTA (titrated with NaOH to pH 7.4) in phosphate buffered saline (PBS) to preserve the extracellular epitopes of membranous proteins, and then washed in a buffer consisting of PBS containing 10^{-3} mM NaN_3 and 0.5% normal rabbit serum. The cells were then incubated on ice with a 1:1000 dilution of anti-Fas antibody (Apo-1, Kamiya Biomedical Co.; Seattle, WA) for 30 minutes, washed in buffer (5 minutes $\times$ 3), and then incubated with an appropriate FITC-conjugated anti-mouse secondary antibody (BioSource; Camarillo, CA) at a 1:2500 dilution for 30 minutes at 4°C while being protected from exposure to light. Controls consisted of unstained cells, and cells treated with secondary antibody alone. After three final washings in PBS containing 10^{-3} mM NaN_3 , CD95 expression was analyzed using a Becton Dickinson FACSort™ flow cytometer (Becton Dickinson; San Jose, CA). Data is reported as mean fluorescence, and was analyzed using the WinMDI (The Scripps Research Institute; San Diego, CA) software program.

Assessment of CD95-Mediated Apoptosis

Cells were treated with 250 µM of bile salts with or without pretreatment with 1 µM BIM (PKC-inhibitor). Control cells were treated with vehicle alone, and BJAB cells served as a positive control in each experiment to confirm the efficient induction of CD95-mediated

apoptosis. Additional culture wells served to confirm by FACScan analysis the impact of bile salt and BIM treatment on cell-surface expression of CD95. Thirty minutes after treatments, cells were exposed to apoptosis-inducing, activating anti-CD95 antibody (Apo-1, Kamiya Biomedical Co.; Seattle, WA) and recombinant protein G (rPG), or rPG alone as a control. Twenty-four hours after this exposure, the cells sensitivity to FasL induced apoptosis was determined by FACScan analysis. Adherent cells were freed from culture using 0.1% trypsin, washed in PBS (pH 7.4) containing 10^{-3} mM NaN_3 and pelleted by centrifugation. Analysis of apoptosis was performed, detected and quantified using an annexin V-FITC and propidium iodide (PI) staining apoptosis detection kit (BD Biosciences; San Diego, CA) according to the manufacturer's instructions. Analysis was again performed using a Becton Dickinson FACSort™ flow cytometer (Becton Dickinson; San Jose, CA). Quadrant analysis was performed using the WinMDI software program.

Statistical Analysis

Comparisons between treatment groups were made using a two-tailed Student's *t* test. $p < 0.05$ was used to determine statistical significance.

RESULTS

Treatment with Bile Salts Increases Surface Expression of CD95 in HET-1A Cells, but Not BAR-T or EA Cells

We hypothesized that bile salt treatment would result in increased cell-surface expression of CD95 in esophageal squamous epithelial cells (Het-1A), but fail to increase cell-surface expression of CD95 in the Barrett's Esophagus cell line (BAR-T). Immortalized Esophageal squamous cells (Het-1A) and Barrett's Esophagus cells (BAR-T), and esophageal adenocarcinoma cells (Flo-1) were treated with Guanochenodeoxycholate (GCDC) or Taurodeoxycholate (TDC) at concentrations of 50, 100, and 250 μM and harvested at 5, 30 or 60 minutes. Control cells in each group were treated with equivalent volume of vehicle alone (pH 6.5). Cell-surface expression of CD95 was then determined via flow cytometry FACScan, using a phycoerythrin-conjugated anti-CD95 antibody. An increase in the surface expression of CD95 in Het-1A cells was observed following exposure to each of the two bile salts (Figure 1). However, neither the EA cell line nor the BAR-T cell line demonstrated a change (increase or decrease) in the surface expression of CD95 following bile salt exposure. Thus, bile salts were shown to increase the surface expression of CD95 in normal squamous epithelial cells, but not in esophageal adenocarcinoma or Barrett's esophagus cells.

Increases in Surface Expression of CD95 in Normal Squamous Epithelial Cells Following Bile Salt Treatment Occurs Rapidly in a Dose Dependent Manner

We next aimed to determine if CD95 cell-surface expression following exposure to bile salts would respond in dose dependent fashion. Het-1A cell-surface CD95 expression was observed to be dose-dependent for GCDC, with a peak effect detected at a concentration of 250 μM . For TDC, maximum effect was observed at a concentration of 50 μM , the lowest dose tested (Figure 2a). Surface expression of CD95 was not affected regardless of dose in either Flo-1 or BAR-T cells (data not shown). Harvesting Het-1A cells at various time points revealed this increase in the surface expression of CD95 began within 5 minutes following treatment and reached peak levels within 30 minutes (Figure 2b).

PKC Inhibition Blocks Bile-Salt Induced Increases in Fas Surface Expression in Normal Squamous Epithelial Cells, but Not in Adenocarcinoma Cells

Bile salts have been shown to induce trafficking of cytosolic pools of CD95 to the cell surface via a PKC modulated pathway. As such, we hypothesized that by inhibiting PKC activity, a bile salt-induced increase in the surface expression of CD95 would be blocked. Esophageal squamous cells (Het-1A) and adenocarcinoma cells (Flo-1) were each treated with 250 μ M of either GCDC, or TDC for a duration of 30, 60 or 120 minutes, with concurrent exposure to the PKC-inhibitor Bisindolymaleimide (BIM). Control groups were exposed to bile salts without concurrent exposure to BIM. Following treatment, cell surface expression of CD95 was again determined via FACScan analysis, using phycoerythrin-conjugated anti-CD95 antibody. Within Het-1A cells, inhibition of PKC was shown to block bile salt-induced increases in surface expression of CD95 (Figure 3a). Surprisingly, BIM treatment appeared to have the opposite effect in Flo-1 cells following bile-salt exposure (Figure 3b); increasing the surface expression of CD95. Harvesting of Flo-1 cells at multiple time points following concurrent BIM and bile-salt treatment revealed this increase in the surface expression of CD95 plateaued within 60 min (Figure 3b). Thus, PKC inhibition was shown to block bile-salt induced increases in the cell surface expression of CD95 within normal squamous epithelial cells, but PKC inhibition increased cell-surface expression of CD95 in an adenocarcinoma cell line.

Increased Surface Expression of CD95 Correlates with Increased Sensitivity to FasL-Induced Apoptosis

In order to confirm that an increase in CD95 expression correlates with an increase in sensitivity to FasL-induced apoptosis, an Annexin V-Propidium Iodide apoptosis detection kit was used to quantify cell viability in Het-1A and Flo-1 cells (Figure 4). We observed that bile-salt treatment resulted in increased mean CD95 expression and to FasL-induced apoptosis. PKC inhibition using pretreatment with BIM attenuated this response. In Flo-1 cells, PKC inhibition via treatment with BIM was shown to exacerbate sensitivity to FasL-induced apoptosis.

DISCUSSION

We have shown that esophageal squamous cells differ from metaplastic, esophageal columnar cells and adenocarcinoma cells in the response to exposure to bile salts with respect to cell-surface expression of the apoptosis-inducing receptor CD95. These differences occur with treatment doses consistent with those present in gastro- and enteroduodenal-refluxate of patients with GERD (8,9). We have further demonstrated that this response is dose dependent, and occurs in a time-frame consistent with a post-translational trafficking event that is dependent upon PKC activity. Finally, we demonstrated that increased cell-surface expression of CD95 sensitized the cells to CD95-mediated apoptosis.

Our findings support the possibility that an intrinsic difference in sensitivity to CD95-mediated apoptotic signals between squamous and enteric columnar epithelia in response to bile salt reflux may contribute to the pathophysiology of BE. This is consistent with the findings of Leach, *et al.* that FasL/CD95 interactions are essential to pancreatic ductal metaplasia that precedes invasive malignancy in a murine model of spontaneous pancreatic cancer following pancreatic duct ligation (3). However, this conclusion must be tempered given our dependence on immortalized cell lines grown in culture, and lack of *in vivo* data. Further study is certainly necessary before we can confidently conclude that this difference between esophageal squamous and BE epithelia clearly exists and contributes to the

development of columnar metaplasia of the esophagus. Current animal models of BE preclude confirmation of our findings *in vivo*.

Our data further suggests that cell-surface expression of CD95 is regulated by post-translational trafficking of the protein modulated by PKC activity. Thus, our findings are consistent with those observed in hepatocytes by Gores *et al.* (16). The role of PKC in post-translational trafficking of proteins, including delivery and retrieval of proteins to and from the cell surface is well established (19). We suspect that this response to bile salts is cell-type specific, and given normal physiology, that all columnar enterocytes lack this response to bile salt exposure. Our observations in the EA cell line Flo-1 may suggest that PKC-dependent endocytosis of CD95 contributes to the low level of CD95 surface expression observed in esophageal adenocarcinoma. As CD95 has been shown to contribute to chemotherapy-induced cell death (17,27) and PKC inhibition has been explored as therapy for cancer (2,6,22), this observation suggests further investigation exploring the potential of synergistic effects between these therapies may be warranted.

Bile salts have been shown to result in a number of other events within esophageal epithelial cells. Conjugated bile salts and the inflammatory cytokines TNF-alpha and IL-1beta increase CDX1 mRNA expression *in vitro* (26,30,33,35). CDX1, an important regulator of normal intestinal development (7,10) and deoxycholic acid up-regulates goblet-specific gene MUC2 expression in concert with CDX2 in human esophageal cells (12,18); thus bile salt exposure may also contribute to columnar differentiation (35). The bile acid receptor FXR is significantly overexpressed in Barrett's esophagus compared to normal mucosa, esophagitis and esophageal adenocarcinoma. In addition, the induction of apoptosis by the FXR inhibitor guggulsterone in a Barrett's esophagus-derived cell line suggests that FXR may contribute to the regulation of apoptosis in this epithelium (5). Finally, bile salt exposure increases proliferation through PI-3K (14), and p38 and ERK MAPK pathways in BAR-T cells (15). Thus, a number of mechanisms have been identified that could be driving a natural selection process that results in BE metaplasia following bile salt exposure.

Bile salts share significant molecular properties with hormones, and intracellular receptors that are activated by bile salt receptors have been identified (4,21). We intentionally limited these experiments to bile salt treatments at pH 6.5 to elucidate their effect in the context of acid suppression therapy. At this pH, the bile salts are likely of neutral charge with access to the cytoplasm (11,21,23). The ionic charge and subsequent cell permeability of these compounds is clearly dependent on pH, and further investigation into our observations at varying pH is necessary to better understand how the observed effects may differ in more acidic environments. Moreover, further elucidation is needed as to whether these effects are mediated by bile salt receptors or reflect changes in the lipid composition of the cell membrane or intracellular organelles such as lipid rafts, known to harbor cytoplasmic pools of CD95 (20,28).

In summary, TDC and GCDC exposure induces esophageal squamous cell sensitivity to CD95-mediated apoptosis via a post-translational mechanism dependent upon PKC. Barrett's columnar epithelial cells lack this response, offering a potential mechanism capable of contributing to the metaplastic transformation of the esophagus in response to GERD.

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Figure 1. FACSscan analysis of CD95 cell-surface expression in Het-1A, BAR-T and Flo-1 cells following guanochenodeoxycholate (GCDC) and taurodeoxycholate (TDC) bile salt exposure Both bile salts were observed to cause an increase in the cell-surface expression of CD95 in the squamous epithelial cell line Het-1A (GCDC $p = 0.01$, TDC $p = 0.004$). In contrast to Het-1A cell line, cell-surface expression of CD95 did not change following treatment in either the immortalized Barrett's esophagus cell line BAR-T ($p = 0.08$) or esophageal adenocarcinoma cell line Flo-1 ($p = 0.15$).

Figure 2a.

Figure 2b

Figure 2. FACScan analysis of temporal and dose response CD95 cell-surface expression in Het-1A cells following GCDC and TDC treatment

a) CD95 cell-surface expression in Het-1A cells was observed to be dose-dependent, with a peak in expression at 250 μ M and 50 μ M for GCDC and TDC, respectively ($p < 0.05$ at 50 μ M dose for both GCDC and TDC). *b)* Temporal analysis following treatment of Het-1A cells with TDC revealed the increase in cell-surface expression of CD95 appeared within 5 min ($p = 0.22$) and peaked within 30 min ($p = 0.02$).

Figure 3a

Figure 3b

Figure 3. FACScan analysis of CD95 cell-surface expression in Het-1A and Flo-1 cells following bile salt exposure with concurrent PKC inhibition

a) Inhibition of PKC activity with BIM blocked bile salt-induced increases in the surface expression of CD95 in Het-1A cells (control/BIM $p = 0.38$, control/GCDC $p = 0.01$, GCDC/BIM $p = 0.006$). *b)* Surprisingly, BIM treatment resulted in a significant increase in cell-surface expression of CD95 in Flo-1 cells ($p = 0.007$ at 30 min)

Figure 4. Annexin V/propidium iodide staining analysis of apoptosis/necrosis in Het-1A and Flo-1 cells following bile salt and BIM treatment

Flow cytometric analysis of apoptotic subpopulations with a combination of annexin V-FITC and propidium iodide staining revealed that bile salt treatment of Het-1A cells resulted in an increase in percent apoptosis ($p = 0.02$), whereas BIM treatment was shown to block any increase in FasL-induced apoptosis ($p = 0.28$). We also observed that both bile salts alone and concurrent BIM treatment increase apoptosis in Flo-1 cells ($p = 0.007$, $p = 0.03$ respectively).