Activation of the Interleukin-6/STAT3 Antiapoptotic Pathway in Esophageal Cells by Bile Acids and Low pH: Relevance to Barrett’s Esophagus

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Abstract

Objectives: The molecular factors contributing to the development of Barrett’s esophagus (BE) are unclear. Our previous studies showed that BE tissues secrete interleukin-6 (IL-6) and express proteins associated with IL-6 signaling, including IL-6 receptor, activated signal transducer and activators of transcription 3 (STAT3), and antiapoptotic proteins Bcl-xL and Mcl-1. Here, we test the hypothesis that bile acids and gastric acids, two components of refluxate associated with gastosophageal reflux disease, activate the IL-6/STAT3 pathway.

Materials and Methods: Immunohistochemistry was used to assess levels of phosphorylated STAT3 in esophageal tissue samples from BE patients with different grades of dysplasia. Seg-1 esophageal adenocarcinoma cells were evaluated for STAT3 activation and IL-6 and Bcl-xL expression by molecular biology techniques, including Western blot, reverse transcription–PCR, and ELISA after exposure to control media (pH 7.4), media supplemented with a 0.1 mmol/L bile acid cocktail with media at pH 4 or media at pH 4 with bile acid cocktail.

Results: Immunohistochemical analysis showed that activated, phosphorylated STAT3 is expressed in nuclei of dysplastic BE and cancer tissues. Treatment of Seg-1 cells with media containing bile acid cocktail and acidified to pH 4 resulted in increased activation of STAT3, IL-6 secretion, and increased expression of Bcl-xL. Inhibition of the STAT3 pathway using STAT3 small interfering RNA or Janus-activated kinase inhibitor resulted in increased apoptosis.

Conclusions: The IL-6/STAT3 antiapoptotic pathway is induced by short exposure to bile acid cocktail and low pH. This alteration, if persistent in vivo, may underlie the development of dysplastic BE and tumor progression.

Barrett’s esophagus (BE) is a premalignant condition closely associated with the development of esophageal adenocarcinoma (1–3). Patients with this cancer have a poor prognosis with a median survival of <1 year (4). However, the precise mechanism of development of BE and esophageal adenocarcinoma is unclear.

Two major components of refluxate associated with gastroesophageal reflux disease, gastric acid, and bile acids are implicated in the development of BE (5–9). Bile acids induce oxidative stress, DNA damage, and mitochondrial damage (10–15). Furthermore, gastric acid and/or bile acids are known to alter signaling pathways, resulting in increased expression of cyclooxygenase 2, prostaglandin E2, activation of mitogen-activated protein kinase, and nuclear factor-κB pathways (16–20). These signaling pathways are associated with increased proliferation and decreased apoptosis of esophageal cells.

Our previous studies indicated that BE cells are resistant to apoptosis induced by bile acids (21). In addition, we showed that BE biopsies secrete interleukin 6 (IL-6; ref. 22). This prosurvival cytokine activates the antiapoptotic signal transducers and activators of transcription 3 (STAT3) pathway, which results in increased expression of antiapoptotic proteins (e.g., Mcl-1 and Bcl-xL; ref. 22). Because reflux disease is a major risk factor for the development of BE and BE tissue has reduced apoptosis competence, we tested whether exposure to low pH in combination with bile acids activates the STAT3 signaling pathway.

We hypothesize that exposure to bile acids and low pH results in activation of the IL-6/STAT3 pathway. We tested this hypothesis in vitro using Seg-1 cells. In addition, we evaluated the expression of activated STAT3 in human esophageal biopsies obtained from BE patients with different grades of dysplasia and adenocarcinoma. We show that BE tissue,
esophagus (Table 1). The Seg-1 cell line was kindly provided by David Beer (University of Michigan). The Seg-1 cells were cultured at 37°C in 5% CO2 in DMEM media (Life Technologies) supplemented with 10% (v/v) heat-inactivated bovine calf serum (Hyclone Laboratories), 2 mmol/L L-glutamine, penicillin (100 units/mL), and streptomycin (100 μg/mL). The 0.1 mmol/L bile acid cocktail consisting of an equimolar mixture of taurocholate, glycocholate, glycochenodeoxycholate, and deoxycholate. This cocktail reflects the mixture of bile acids to which the distal esophagus is ordinarily exposed during gastroesophageal reflux based on the studies of Kauer et al., Nehra et al., and Thiesen et al. (8, 23, 24). This cocktail was completely soluble in medium at pH 4 or pH 7.4. No precipitate was formed after centrifugation at 14,000×g for 30 min. For all experiments, we used 10-min exposure to pH 4 and 0.1 mmol/L bile acid cocktail. The recovery time was 15 min for RNA experiments and 170 min for all other experiments.

Immunohistochemical staining. For the evaluation of phosphorylated STAT3 (p-STAT3) and STAT3 in tissues, standard immunostaining assays involving biotin-avidin–linked peroxidase detection were used as described previously (25). Briefly, paraffin-embedded sections were deparaffinized, rehydrated, and placed in 3% hydrogen peroxide for 30 min to block endogenous peroxidase. Antigen retrieval was done using a microwave protocol. Slides were then incubated for 30 min with 5% goat serum, followed by overnight incubation at 4°C until immunostaining. A similar method was used for the evaluation of p-STAT3 and STAT3 expression in Seg-1 cells. For this experiment, the Seg-1 cells were trypsinized, cytospun onto slides using a Cytospin 2 (Shandon), and fixed with 4% formaldehyde in PBS for 20 min. Next, the cells were permeabilized in 100% methanol at -20°C, air-dried, and stored at -20°C until immunostaining.

For fluorescent microscopic studies, the cells were grown on coverslips. At treatment with control medium or medium acidified to pH 4 and/or bile acid cocktail, the cells were fixed with 100% methanol and incubated overnight with antibodies against STAT3 (1:100, mouse monoclonal antibody; Cell Signaling Technology, Inc.). Slides were rinsed with PBS, and the Alexa Fluor 594 secondary antiamoine (1:100, Molecular Probes, Inc.) was applied for 60 min. After three final PBS rinses, the coverslips were placed on slides using Vectorshield HardSet mounting medium containing 4',6-diamidino-2-phenylindole (Vector Laboratories, Inc.) to counterstain nuclei. The cells were then evaluated by fluorescent microscopy using appropriate filters.

Materials and Methods

Patient tissue biopsies and endoscopy. Fifty-six BE patients with various grades of dysplasia and adenocarcinoma were included in the present study. Endoscopic biopsies of BE and control tissues (duodenum, squamous mucosa) were taken from patients with BE and esophageal adenocarcinoma who were undergoing surveillance procedures or surgery. Patients were males, 41 to 83 years old. These patients had given written informed consent using a form approved by the local institutional Human Subjects Committee. Endoscopic biopsies of BE were taken from BE patients using a therapeutic endoscope and a large capacity biopsy forceps. The biopsies were immediately fixed in 10% buffered formalin. BE was defined histologically as the presence of intestinal-like metaplastic epithelium containing goblet cell biopsies. Biopsies were stained with H&E and Alcian blue (pH 2.5) for goblet cells, and the degree of dysplasia was evaluated by two pathologists.

Cell line and chemicals. The human esophageal adenocarcinoma Seg-1 cell line was kindly provided by David Beer (University of Michigan). The Seg-1 cells were cultured at 37°C in 5% CO2 in DMEM media (Life Technologies) supplemented with 10% (v/v) heat-inactivated bovine calf serum (Hyclone Laboratories), 2 mmol/L L-glutamine, penicillin (100 units/mL), and streptomycin (100 μg/mL). The 0.1 mmol/L bile acid cocktail consisting of an equimolar mixture of taurocholate, glycocholate, glycochenodeoxycholate, and deoxycholate. This cocktail reflects the mixture of bile acids to which the distal esophagus is ordinarily exposed during gastroesophageal reflux based on the studies of Kauer et al., Nehra et al., and Thiesen et al. (8, 23, 24). This cocktail was completely soluble in medium at pH 4 or pH 7.4. No precipitate was formed after centrifugation at 14,000×g for 30 min. For all experiments, we used 10-min exposure to pH 4 and 0.1 mmol/L bile acid cocktail. The recovery time was 15 min for RNA experiments and 170 min for all other experiments.

AG-490 was obtained from Calbiochem. A stock solution (1 mg/mL) was prepared in DMSO, filter sterilized, and stored at -80°C. Hoechst 3322 was from Molecular Probe, Inc. All other chemicals were of the highest purity available and were obtained from Sigma Chemical, unless otherwise noted.

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A simple grading system (0 to 3), routinely used in our laboratory based on percentage of positively stained cells, was used to grade the level of expression of p-STAT3 in BE glands (0, 0 to 5% cells stained; 1, 5% to 30% cells stained; 2, 30% to 60% cells stained; 3, >60% cells stained). Staining was evaluated independently by three experienced investigators.

Analysis of conditioned media for IL-6 secretion. ELISAs were used to determine the concentrations of IL-6 in conditioned media after incubation of the cells or biopsies in media. A Quantikine HS ELISA kit for the detection of IL-6 was obtained from R&D Systems, Inc., and the assay was done according to the manufacturer’s instructions. Seg-1 cells (1 × 105) were seeded in six-well plates and, the next day, were exposed to either control medium or medium at pH 4 and/or supplemented with 0.1 mmol/L bile acid cocktail. After 10 min, cells were washed twice with normal medium and incubated for an additional 170 min in normal medium to mimic pulse exposure. The supernatant was repeated four times, the measurements were done in duplicate, and the results were expressed as picogram per 10⁶ cells after counting total cells in the sample using a NucleoCounter (New Brunswick Scientific) according to the manufacturer's instructions.

Western blot analysis. Western blot analysis was done as previously described (26). Briefly, Seg-1 cells treated with control medium (pH 7.4), medium at pH 4, control medium with bile acid cocktail, or medium at pH 4 with bile acid cocktail were lysed using lysis buffer [50 mmol/L Tris (pH 8), 5 mmol/L EDTA, 150 mmol/L NaCl, 0.5% NP40] supplemented with phenylmethylsulfonyl fluoride (1 mmol/L), leupeptin (1 μg/mL), and aprotinin (0.01 units/mL). Protein aliquots (30 μg/lane) were loaded on 10% to 15% SDS-polyacrylamide gels for size fractionation by electrophoresis. The proteins were blotted onto Immobilon-P PVDF transfer membrane (Millipore). The membranes were immunostained with anti-Bcl-xL monoclonal antibody (1:100; Santa Cruz Biotechnology) and then incubated with goat anti-mouse conjugated to horseradish peroxidase (Pierce). Antibody complexes were detected using the Renaissance chemiluminescence detection system (Amersham, Pharmacia Biotech). After this, the membranes were stripped using Re-blot Western blot recycling kit (Chemicon International) and immunostained with β-actin antibody (1:10,000; Sigma). Finally, the membranes were stained for 20 min with Brilliant Blue G dye to confirm equal protein loading.

Real-time reverse transcription–PCR. Total RNA was isolated from cells treated with low pH and bile acids using a Qiagen RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. To eliminate DNA contamination, all samples were treated using RNase-free DNase (Qiagen). RNA concentration was determined using UV spectrophotometry at 260 nm, and the A260/A280 ratio was used for the determination of sample purity (SPECTRAmax PLUS, Molecular Devices). To verify the quality of the RNA, it was subjected to electrophoresis on a 1.2% agarose gel containing ethidium bromide and formaldehyde (2.2 mol/L) in 1× MOPS buffer [40 mmol/L MOPS (pH 7.0), 10 mmol/L sodium acetate, and 1 mmol/L EDTA (pH 8.0)].

Real-time reverse transcription–PCR was used to quantify relative levels of IL-6 and Bcl-xL mRNAs as described previously (27). Briefly, after reverse transcription of 1 μg of total RNA, real-time PCR was performed using primers and probes specific for IL-6 and Bcl-xL (Table 1). The relative levels of IL-6 and Bcl-xL mRNAs were calculated by the ΔΔCT method and expressed as fold changes compared with control samples.
amplification was done using human IL-6 and Bcl-xl. TaqMan predeveloped assay reagents (Applied Biosystems) according to the manufacturer’s instructions. Samples were subjected to 45 cycles at 95°C for 15 s, followed by 1 min at 60°C using an ABI Prism 7000 Sequence Detection System (Applied Biosystems). PCR reactions for each sample were done in duplicate. The experiment was repeated thrice. All IL-6 and Bcl-xl mRNA levels were normalized according to the level of 18s RNA within each sample used as an endogenous control. Cleavage of the sequence-specific probe by Taq polymerase created an increase in fluorescent signal, which was observed during the exponential phase of amplification and allowed determination of a threshold value for all samples. This threshold value, once normalized, was expressed as a fold change of gene expression relative to control samples.

Small interfering RNA transfection. To test whether bile acid–induced apoptosis is regulated by STAT3 signaling, we used small interfering RNA to reduce expression of STAT3 in Seg-1 cells. A STAT3 small interfering RNA duplex was chosen against the DNA target sequence 3′-AACTTGGAGCCCTGGTGTGA-5′, which does not code for any known gene. A small interfering RNA duplex generated with 3′-dTdT overhangs was prepared by Qiagen, Inc. The sequence and identity of the small interfering RNA was confirmed using MALDI-TOF spectrometric analysis.

For nonsilencing control purposes, we used a 21-bp small interfering RNA that contained no homology to any known sequence. The nonsilencing inactive control ordered from Qiagen was targeted to the DNA sequence 3′-AATTCATCGGAACGTGTCACGT-5′, which does not code for any known gene.

Seg-1 cells were transfected with the above-described small interfering RNA duplexes, following the protocol in Qiagen’s TransMessenger Transfection Reagent Handbook. Briefly, the day before transfection, cells were seeded in 1-mL medium at 200,000 cells per well of a 24-well plate. After 24 h incubation at 37°C and 5% CO2, cells were gently washed with 2 mL of PBS, and then small interfering RNA transfection solutions, prepared according to the manufacturer’s instructions, were immediately added to the cells. The cells were incubated for 3 h under normal growth conditions. Untreated control wells were incubated with aliquots of 300 μL serum-free/antibiotic-free medium for the same period. At the end of the incubation period, the medium was removed from the cells, the cells were washed once with PBS, and 1-mL fresh medium containing serum and antibiotics was added to the cells. The cells were incubated under their normal growth conditions for 24 additional hours, and then apoptosis experiments were done.

Quantification of apoptosis using brightfield microscopy. For induction of apoptosis, Seg-1 cells were treated for 3 h with 0.5 mmol/L deoxycholic acid (DCA). Cells were then trypsinized, cytospun onto slides using a Cytospin 2 (Shandon), fixed with 100% methanol for 2 min, air-dried, and stained with Giemsa stain. Two hundred cells were evaluated for apoptosis using brightfield microscopy (100× oil immersion), as described previously (28). The criteria used to identify apoptotic cells included chromatin condensation, formation of apoptotic bodies, and cellular shrinkage, as previously described by our group (29). All apoptosis experiments were repeated at least thrice.

Statistical analysis. Numerical data are expressed as the mean ± SE. The statistical significance of the difference between groups was determined by the two-tailed Student’s t test at the 95% confidence level. The relationship of p-STAT3 staining to grade of dysplasia was analyzed by contingency table methods. Significance testing was based on Cochran-Mantel-Haenszel statistics for ordered contingency tables. These statistics account for the ordered levels of staining (0 to 3) and can also account for ordering of categories based on grade of dysplasia or cancer.

Results

Expression of p-STAT3 in BE and esophageal adenocarcinoma. Using immunohistochemical staining with a monoclonal antibody against p-STAT3, we evaluated biopsies from BE patients with different grades of dysplasia or with esophageal adenocarcinoma for activated STAT3 (nondysplastic BE, n = 15; low-grade dysplasia, n = 14; high-grade dysplasia, n = 12;...
esophageal adenocarcinoma, $n = 15$). We also evaluated, as control tissues, esophageal squamous epithelium ($n = 8$) and duodenal mucosa ($n = 8$) for expression of this transcription factor. The antibody against p-STAT3 detects this transcription factor only when STAT3 is phosphorylated at Tyr 705. Increased nuclear staining of p-STAT3 was shown in high-grade dysplasia BE glands and adenocarcinoma compared with squamous and duodenal epithelia (Fig. 1). The strongest nuclear staining of p-STAT3 was observed in patients with adenocarcinoma (median = 3; Table 1). Significance testing using Cochran-Mantel-Haenszel statistics showed that there was a significant trend (increased staining with increasing dysplasia or cancer) across the four categories (nondysplastic BE, low-grade dysplasia, high-grade dysplasia, ADCA; $P = 0.008$).

![Fig. 2](image-url). Expression of STAT3 and p-STAT3 in BE biopsies. Immunohistochemical staining was used to detect STAT3 and p-STAT3 in serial sections from two different patients (BE patients). Figure 2 shows biopsies from two patients with similar levels of total STAT3 (left) but differing levels of p-STAT3 (right), suggesting that the increased levels of p-STAT3 are not due to up-regulation of total STAT3 in patients’ biopsies.

Increased IL-6 mRNA and IL-6 after exposure of Seg-1 cells to low pH and/or bile acid cocktail. Our previous study showed that BE biopsies secrete increased amounts of IL-6 compared with esophageal squamous epithelium or duodenum (22). In our current experiments, we tested whether exposure to low pH and/or bile acid cocktail leads to increased expression and secretion of IL-6. Using reverse transcription–PCR, we measured IL-6 mRNA levels in cells exposed to control medium or to medium with low pH and/or bile acid cocktail. A statistically significant increase in IL-6 mRNA level was detected in Seg-1 cells after exposure to pH 4 alone ($P = 0.02$). The IL-6 mRNA levels after treatment with media acidified to pH 4 and supplemented with bile acid cocktail were also elevated. Although this trend was very clear in all three experiments, the variability between experiments was high, so that the difference in IL-6 mRNA levels between cells exposed to both pH 4 and bile acid cocktail compared with control cells did not reach statistical significance ($P = 0.08$; Fig. 3A).

Our data from four experiments indicate that a 10-min exposure of Seg-1 cells to a combination of bile acid cocktail and pH 4 followed by 170 min of incubation in normal medium leads to a doubling of secretion of IL-6 ($P < 0.05$; Fig. 3B). In contrast, exposure to bile acid cocktail alone or to pH 4 alone under the same conditions did not result in a significant increase in IL-6 secretion compared with the control.

Increased Bcl-xL mRNA and Bcl-xL after exposure to low pH and/or bile acid cocktail. Next, we evaluated Bcl-xL mRNA and protein expression after incubation with control medium or medium acidified to pH 4 and/or supplemented with bile acid cocktail. We found that Bcl-xL mRNA was significantly increased in these cells after exposure to the combination of low pH and bile acid cocktail (Fig. 4A). The data show the mean and SE from three different experiments. Western blot analysis showed that after exposure of Seg-1 cells to the

<table>
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<tr>
<th>Tissue</th>
<th>Median staining score</th>
<th>Patients ($n$)</th>
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</thead>
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<tr>
<td>Esophageal adenocarcinoma</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>BE with high-grade dysplasia</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
<td>BE with low-grade dysplasia</td>
<td>1</td>
<td>14</td>
</tr>
<tr>
<td>Nondysplastic BE</td>
<td>1</td>
<td>15</td>
</tr>
<tr>
<td>Squamous epithelium</td>
<td>0.5</td>
<td>8</td>
</tr>
<tr>
<td>Duodenum</td>
<td>1</td>
<td>8</td>
</tr>
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NOTE: The scoring used a simple grading system (0-3). Median values are indicated.
combination of bile acid cocktail and pH 4, the level of Bcl-xL is substantially increased compared with control, untreated cells (Fig. 4B). In addition, pH 4 treatment alone resulted in an increased expression of Bcl-xL at the protein level.

**STAT3 activation after exposure to low pH and bile acids in Seg-1 cells.** Transcription factor STAT3 is translocated to the nucleus after its activation. Thus, we evaluated the expression of STAT3 and p-STAT3 in Seg-1 cells after treatment with control medium or pH 4 and/or bile acid cocktail using fluorescent microscopy and brightfield microscopy. We found increased STAT3 staining in the nucleus of Seg-1 cells after 10-min exposure to pH 4 and bile acid cocktail (Fig. 5A). In the control cells, most of the STAT3 staining seemed to be in the cytoplasm (Fig. 5A) and only low STAT3 signal was detected in the nuclei, whereas intense signal of STAT3 and p-STAT3 was detected in the nuclei of cells treated with bile acid cocktail and pH 4 (Fig. 5A and B).

**Inhibition of the IL-6 signaling pathway sensitizes cells to apoptosis.** Next, we evaluated whether IL-6 signaling represents a survival pathway in Seg-1 cells. IL-6 signaling is primarily mediated by the Janus-activated kinase (JAK)/STAT3 pathway (30). Thus, we inhibited the JAK/STAT3 pathway using AG-490, a specific inhibitor of JAK, or by transfection with STAT3 small interfering RNA that specifically eliminates STAT3 mRNA.

Seg-1 cells were transfected with STAT3 small interfering RNA or control inactive small interfering RNA or pretreated with 5 μmol/L AG-490 for 18 h. Cells were then treated for 3 h with 0.5 mmol/L DCA to induce apoptosis. We used DCA as a model bile acid because DCA is a well-characterized hydrophobic bile acid, a multiple stress inducer, a known inducer of apoptosis at physiologic concentrations, and one of the components of duodenogastric refluxate (8, 29). Our major goal in this experiment was to evaluate the effect of STAT3 knockdown on the process of apoptosis.

Inhibition of IL-6 signaling, using AG-490 or STAT3 small interfering RNA, significantly increased DCA-induced apoptosis in Seg-1 cells (Fig. 6A). DCA treatment alone resulted in 25 ± 0.5% apoptotic cells, but DCA treatment after pretreatment with AG-490 or transfection with STAT3 small interfering RNA resulted in 61.3 ± 3.8% and 51.8 ± 5% apoptotic cells, respectively. Pretreatment with inactive small interfering RNA did not alter the extent of apoptosis of untreated control cells or cells treated with DCA (Fig. 6A). For the small interfering RNA experiments, we also used an immunocytochemical assay to evaluate expression of STAT3. As indicated in Fig. 6B, compared with control untreated cells, decreased STAT3 expression was found in cells transfected with STAT3 small interfering RNA but not with the inactive control, inactive small interfering RNA. Overall, the data indicate that inhibition of IL-6 signaling sensitizes cells to apoptosis.
Fig. 5. Translocation of STAT3 to the nucleus. Seg-1 cells were treated for 10 min with control medium, medium acidified to pH 4, and/or medium supplemented with bile acid cocktail. After treatment, cells were fixed and immunostained with STAT3 or p-STAT3 antibodies and evaluated by fluorescent microscopy (A) or brightfield microscopy (B'). For STAT3 (red signal), 4′,6-Diamidino-2-phenylindole (DAPI) was used to visualize the nuclei (blue signal). For p-STAT3 (brown signal), nuclei were stained using hematoxylin (blue signal). Note the intense staining of STAT3 and p-STAT3 in the nuclei of cells treated with pH 4 and bile acid cocktail.
The normal squamous epithelium of the distal esophagus is often exposed to gastric acids and bile acids during reflux episodes. Bile acids, primarily glycine conjugates, are present in the esophagus of 86% of patients with gastroesophageal reflux disease (23). These include glycocholic acid, glycochenodeoxycholic acid, and glycodeoxycholic acid (23). Furthermore, DCA and a mixture of taurine-conjugated bile acids are also present in the refluxate of BE patients (8). In our current study, we used a bile acid cocktail that included glycodeoxycholate, taurocholate, glycodeoxycholate, glycochenodeoxycholate, and DCA. This cocktail reflects the mixture of bile acids to which the distal esophagus is exposed during gastroduodenal reflux (8, 23, 24). Total bile acid concentrations in the refluxate of BE patients are in the range 0.03 to 0.82 mmol/L (8), although much higher concentrations have been reported in the refluxate of some BE patients (7). The bile acid cocktail used in our studies of altered expression and activation had a concentration in the lower physiologic range (0.1 mmol/L).

We and others have previously shown that expression of antiapoptotic proteins is increased in BE (19, 22). Van der Woude et al. suggested that alterations in apoptotic balance leads to the transformation from BE to adenocarcinoma, because increased expression of antiapoptotic Bcl-xL and decreased expression of proapoptotic Bax were hallmarks of progression to adenocarcinoma.

In our current study, we observed that the antiapoptotic protein Bcl-xL is increased after pH 4 and bile acid treatment at the protein and mRNA levels. Because apoptosis resistance can lead to genomic instability, the loss of apoptosis competence in BE may be responsible, in part, for malignant transformation of this preneoplastic tissue. This antiapoptotic protein is regulated by multiple signaling pathways. The STAT, nuclear factor-κB, and Ets transcription factor families have been reported to directly regulate the bcl-xL gene (31). Activated Ras, integrin, vitronectin, and hepatocyte growth factor signaling cascades have also been linked to changes in Bcl-xL expression (31).

IL-6, an upstream cytokine in the JAK/STAT3 pathway, also seems to be involved in malignant transformation and tumor progression (30). Its overexpression has been implicated in the pathogenesis of various tumors, including multiple myeloma and cancers of the ovary, prostate, breast, colon, kidney, and lung (32–38). We hypothesize that exposure to bile acids and low pH results in induction of oxidative stress, which may lead to activation of the nuclear factor-κB and IL-6/STAT3 pathways.

Indeed, our recent study indicates that bile acids and gastric acid induce oxidative stress (39). This consequently results in an increased expression of antiapoptotic proteins, such as Bcl-xL, regulated by IL-6/STAT3 signaling. Expression of IL-6 by cancer cells suggests that IL-6 may act as an autocrine and paracrine growth factor to promote tumor progression by inducing the expression of antiapoptotic and angiogenic factors (40–44). Previously, we showed that both IL-6 and IL-6 receptor are expressed in BE epithelial cells (22). Furthermore, we found that IL-6 secretion and expression is increased in BE compared with squamous epithelial or duodenum (22). IL-6 signaling is primarily mediated by the

**Activation of the IL-6/STAT3 Antiapoptotic Pathway**

![Fig. 6.](image1.png)

**Fig. 6.** Increased apoptosis induced by DCA in Seg-1 cells transfected with STAT3 small interfering RNA or pretreated with an inhibitor of JAK, AG490. Seg-1 cells were pretreated with 5 μmol/L AG490 or pretreated with STAT3 small interfering RNA or control inactive small interfering RNA (siRNA) and then treated for 3 h with 0.5 mmol/L DCA to induce apoptosis. A percentage of apoptotic cells were determined by morphologic criteria after staining with Giemsastain (A). *, statistically significant differences between DCA-treated cells and control cells; #, statistically significant difference between DCA-treated cells and cells pretreated with AG490 and then treated with DCA (P < 0.05). B, immunohistochemical staining of representative Seg-1 cells stained for STAT3 after transfection with STAT3 small interfering RNA or inactive control small interfering RNA. Untreated cells are included as a control.

**Proposed model of development of apoptosis resistance by bile acids and low pH in BE.**

![Fig. 7.](image2.png)

**Fig. 7.** The major aim of this study was to determine whether short exposure to low pH and/or bile acids has significant biological effects on esophageal cells, particularly on IL-6/STAT3 signaling. Our studies using the Seg-1 esophageal cell line indicate that exposure to pH 4 in combination with bile acid cocktail increases IL-6 expression, STAT3 translocation to the nucleus, and expression of the antiapoptotic protein Bcl-xL. Furthermore, inhibition of the STAT3 pathway sensitzes esophageal cells to DCA-induced apoptosis. Immunohistochemical studies indicated that BE epithelial cells, especially dysplastic and cancer cells, frequently express increased levels of activated STAT3.

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Increased nuclear staining of activated STAT3 was detected in epithelial cells of BE biopsies, compared with that seen in the control tissues of squamous epithelium and duodenum. Importantly, intense nuclear staining was detected especially in dysplastic and cancer tissues.

In summary, our study is the first to show that bile acids, in combination with low pH, activate IL-6 signaling pathways associated with resistance to apoptosis in esophageal cells (Fig. 7). We speculate that chronic episodic exposure to bile acids, and low pH may result in abnormal cell signaling leading to increased STAT3 activation to apoptosis resistance. These alterations may enhance progression to cancer. However, we recognize that further studies may reveal other signaling pathways that result in apoptosis resistance as well as other signaling pathways activated in BE lines in response to gastric acid and bile acids.

References


Clinical Cancer Research

Activation of the Interleukin-6/STAT3 Antiapoptotic Pathway in Esophageal Cells by Bile Acids and Low pH: Relevance to Barrett's Esophagus

Katerina Dvorak, Melissa Chavarria, Claire M. Payne, et al.


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