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Acid and Bile Salt Induced CDX2 Expression Differs in Squamous Cells from Patients with and without Barrett's Esophagus

Xiaofang Huo, Ph.D.¹, Hui Ying Zhang, Ph.D.¹, Xi Zhang, M.D.¹, John P. Lynch, M.D.⁵, Eric D. Strauch, M.D.⁶, Jian-Ying Wang, M.D., Ph.D.^{6,7,8}, Shelby D. Melton, M.D.^{2,3}, Robert M. Genta, M.D.^{2,3}, David H. Wang, M.D.^{1,4}, Stuart J. Spechler, M.D.¹, and Rhonda F. Souza, M.D.^{1,4}

¹Department of Medicine, VA North Texas Health Care System and the University of Texas Southwestern Medical School, Dallas, TX

²Department of Pathology, VA North Texas Health Care System and the University of Texas Southwestern Medical School, Dallas, TX

³Caris Life Sciences, Inc., Irving, TX

⁴Harold C. Simmons Comprehensive Cancer Center, University of Texas Southwestern Medical Center, Dallas, TX

⁵Department of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania

⁶Cell Biology Group, Department of Surgery, University of Maryland School of Medicine, Baltimore, MD

⁷Department of Pathology, University of Maryland School of Medicine, Baltimore, MD

⁸Baltimore Veterans Affairs Medical Center, Baltimore, MD

Abstract

Introduction—It is not clear why only a minority of patients with gastroesophageal reflux disease (GERD) develop Barrett's esophagus. We hypothesized that differences among individuals in molecular pathways activated when esophageal squamous epithelium is exposed to reflux underlie the development of Barrett's metaplasia.

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Correspondence to: Rhonda F. Souza, M.D. Dept. Of GI, MC# 111B1, Dallas VA Medical Center, 4500 South Lancaster Road, Dallas, TX 75216, Phone 214-857-0301, FAX 214-857-0328, rhonda.souza@utsouthwestern.edu.

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Author Involvement:

Huo: study design; technical and material support; analysis and interpretation of data; critical revision of manuscript; important intellectual content; study supervision

Zhang: technical and material support; analysis and interpretation of data; important intellectual content; study supervision

Zhang: technical and material support; important intellectual content

Lynch: technical and material support; critical revision of manuscript

Melton: technical and material support; critical revision of manuscript

Genta: technical and material support; critical revision of manuscript

Wang: technical and material support; critical revision of manuscript

Spechler: study concept; analysis and interpretation of data; critical revision of manuscript; important intellectual content

Souza: study concept/design; analysis and interpretation of data; drafting of manuscript; important intellectual content

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Methods—We used esophageal squamous cell lines from patients who had GERD with Barrett's esophagus (NES-B3T & NES-B10T) and without Barrett's esophagus (NES-G2T & NES-G4T) to study effects of acid and bile salts on expression of the CDX2 gene. Bay 11-705, Ad5IkB α -SR, and site-directed mutagenesis were used to explore effects of NF- κ B inhibition on CDX2 promoter activity; DNA binding of the NF- κ B subunits p50 and p65 was assessed by ChIP.

Results—Acid and bile salts increased CDX2 mRNA, protein, and promoter activity in NES-B3T and NES-B10T cells, but not in NES-G2T or NES-G4T cells. Inhibition of NF- κ B abolished the increase in CDX2 promoter activity. Increased CDX2 promoter activity was associated with nuclear translocation of p50, which bound to the promoter. We also found CDX2 mRNA in 7 of 10 esophageal squamous biopsy specimens from patients with Barrett's esophagus, but in only 1 of 10 such specimens from patients who had GERD without Barrett's esophagus.

Conclusions—Acid and bile salts induce CDX2 mRNA and protein expression in esophageal squamous cells from patients with Barrett's esophagus, but not from GERD patients without Barrett's esophagus. We speculate that these differences in acid- and bile salt-induced activation of molecular pathways may underlie the development of Barrett's metaplasia.

Keywords

Barrett's esophagus; gastroesophageal reflux; CDX2; NF- κ B

Introduction

Up to 40% of adult Americans experience symptoms of gastroesophageal reflux disease (GERD), a well established risk factor for adenocarcinoma of the esophagus (1). When GERD induces inflammation in esophageal squamous epithelium (reflux esophagitis), the damaged squamous cells usually are replaced by the regeneration of more squamous cells. In some individuals, however, the reflux-damaged squamous epithelium heals through a metaplastic process in which intestinal-type columnar cells replace reflux-damaged squamous cells. This results in the condition called Barrett's esophagus, which predisposes to esophageal adenocarcinoma (2).

Metaplasia is the process in which one adult cell type replaces another, usually in the setting of chronic inflammation (2). The repeated cycles of injury and repair that accompany chronic inflammation are thought to induce alterations in cellular gene expression patterns, and metaplasias occur when those alterations affect the homeotic genes that control cellular phenotype (3).

Cdx genes, homeotic genes of the para-homeobox family, appear to direct the formation of the simple columnar epithelium that characterizes the adult intestine (4;5). In humans, strong expression of CDX1 and CDX2 has been found in both the small and large intestine, whereas the normal esophagus and stomach do not express those proteins (6). The metaplastic epithelium of Barrett's esophagus can be intestinal in type, so it is not surprising that CDX mRNA and protein expression are observed in biopsy specimens of Barrett's metaplasia (7-11).

Recent studies suggest that GERD can induce CDX expression in esophageal squamous epithelial cells. Acid and bile salts, the major noxious agents in refluxed gastric juice, have been shown to increase Cdx2 promoter activity in rat and mouse esophageal keratinocytes (12;13), and to increase CDX2 mRNA expression in the HET-1A human esophageal squamous cell line (14-16). HET-1A cells also exhibit increased expression of CDX2 mRNA when exposed to bile acids and nitric oxide, a toxic molecule produced in the esophageal lumen during reflux episodes (16). In animal models of reflux esophagitis and Barrett's esophagus,

the reflux-injured esophageal squamous epithelium demonstrates increased Cdx2 expression prior to the appearance of intestinal metaplasia in the esophagus (17-19). In humans, CDX2 expression has been demonstrated in biopsy specimens of inflamed esophageal squamous epithelium, and CDX2 expression in the esophagus has been shown to precede the expression of other intestinal markers like MUC2, sucrase-isomaltase, defensin-5, and alkaline phosphatase (11;16). These studies suggest that GERD can induce the expression of CDX2 in esophageal squamous epithelial cells, and that such expression may be an early event in the development of Barrett's esophagus.

It remains unclear why only a minority of patients with GERD develop Barrett's esophagus. Patients with Barrett's esophagus often have severe GERD, with long durations of esophageal exposure to acid and bile, and some studies suggest that the esophageal squamous epithelium of Barrett's patients may be genetically predisposed to develop metaplasia after injury by GERD (20). We have hypothesized that there are differences (heritable or acquired) among individuals in the molecular pathways activated when esophageal squamous epithelium is exposed to gastroesophageal reflux, and that those differences determine whether reflux esophagitis heals through the process of regeneration or metaplasia. In support of our hypothesis, we have reported significant differences between GERD patients with and without Barrett's esophagus in how acid exposure activates the MEK-ERK mitogen activated protein kinase (MAPK) pathway in their esophageal squamous epithelium (21;22). In the present study, we have used telomerase-immortalized esophageal squamous cell lines established from GERD patients with and without Barrett's esophagus to explore differences in how acid and bile salt exposure induces CDX2 expression. We have found that acid and bile salts induce CDX2 mRNA and protein expression in esophageal squamous cells from GERD patients with Barrett's esophagus, but not from GERD patients without Barrett's esophagus. We also have found baseline differences in CDX2 expression between GERD patients with and without Barrett's esophagus in biopsy specimens of their esophageal squamous epithelium.

Materials and Methods

Patients

This study was approved by the institutional review board of the Dallas VA Medical Center. Patients scheduled for elective endoscopy who had GERD with and without Barrett's esophagus were invited to participate. During endoscopy, biopsy specimens of the squamous mucosa in the distal esophagus were taken with jumbo biopsy forceps (Olympus FB-50K-1) and snap frozen in liquid nitrogen (21). None of the patients had erosive esophagitis when the biopsy specimens were taken.

Cell Culture

We used 4 non-neoplastic, telomerase-immortalized normal esophageal squamous (NES) cell lines that were created from endoscopic biopsy specimens of squamous epithelium in the distal esophagus of GERD patients with long-segment Barrett's esophagus (defined as ≥ 3 cm of specialized intestinal metaplasia in the esophagus) (NES-B3T and NES-B10T) and from GERD patients without Barrett's esophagus (defined as having no endoscopic evidence of Barrett's esophagus, with heartburn occurring at least once a week for ≥ 3 months and /or erosive esophagitis on endoscopy) (NES-G2T and NES-G4T) (21;22). As previously reported for NES-B3T and NES-G2T cell lines (22), the NES-B10T and NES-G4T lines express squamous cell markers (cytokeratins 13 and 4), demonstrate cell to cell contact inhibition, and do not exhibit anchorage-independent growth in soft agar (data not shown). Cells were maintained in monolayer culture at 37° C in humidified air with 5% CO₂ in growth medium co-cultured with a fibroblast feeder layer as previously described (22). For individual

experiments, cells were equally seeded into collagen IV-coated wells (BD Biosciences, San Jose, CA) and maintained in growth medium.

Acid and Bile Salt Exposure of NES Cells

For individual experiments, cells were cultured in one of four different experimental media: 1) Acidic full growth medium (brought to a pH of 4.0 with 1M HCl), 2) Neutral bile salt medium (containing conjugated bile acids with a total concentration of 400 μ M at pH 7.2), 3) Acidic bile salt medium (the same bile acid solution at pH 4.0), or 4) Control medium which consisted of neutral full growth medium (pH 7.2). The media were added for 10 minutes to equally-seeded wells of NES cells, then removed and replaced with neutral pH medium until the next treatment (unless otherwise indicated). NES cells were treated with either experimental or control medium three times per day for 7 days (unless otherwise indicated). For the 24-hour exposures, the pH of the acidic full growth and acidic bile salt media was adjusted to 5.5. The bile salt media contained a mixture of conjugated bile salts including glycocholic acid, taurocholic acid, glycochenodeoxycholic acid, taurochenodeoxycholic acid, glycodeoxycholic acid, and taurodeoxycholic acid (Sigma, St. Louis, MO and Calbiochem, San Diego, CA) in a 20:3:15:3:6:1 molar concentration as previously described (14). The pH levels, bile salt mixture, and durations of exposure were chosen to simulate typical clinical episodes of gastroesophageal reflux (23-25).

RNA Extraction and Reverse Transcription PCR

Total RNAs were isolated from cell lines and biopsy specimens using Trizol (Invitrogen, Carlsbad, CA) and quantitated by spectrophotometry. Following amplification, CDX2, CK20, and MUC-2 PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide; β -actin or GAPDH transcripts served as internal controls for the cell lines, GAPDH for the biopsy specimens. (Supplemental materials)

Immunoprecipitation, Nuclear/Cytoplasmic Fractionation, and Western Blot

Cells were exposed to experimental or control medium for 24 hours. The medium was removed and the cells were washed in cold PBS. For CDX2, equal amounts of protein were immunoprecipitated overnight at 4° C with saturating amounts of anti-CDX2, followed by a 1 hour incubation with protein A-sepharose beads at 4° C. For p50 and p65, nuclear extracts were isolated from cell lines using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo Fisher Scientific, Rockford, IL) per manufacturer's instructions. Proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes and incubated with primary antibodies to CDX2, p50, p65, tubulin, and lamin. (Supplemental materials)

DAC Treatment, DNA Extraction, and Methylation Specific PCR

For demethylation experiments, 1mM 5-aza-2-deoxycytidine (DAC, Sigma, St. Louis, MO) was added to neutral pH full growth medium for a total of 5 days. Genomic DNAs were extracted with the DNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Genomic DNA (1 μ g) was bisulfite treated using the EZ DNA Methylation kit (Zymo Research, Orange, CA) according to manufacturer's instructions. Methylation-specific PCR was performed using methylation-specific PCR primers designed to flank CpG site 25 within the CpG island of the CDX-2 promoter, which has been shown to undergo demethylation by acid and bile salt exposure in esophageal squamous cells (Supplemental materials) (14). Following amplification, PCR products were electrophoresed on 2% agarose gels and stained with ethidium bromide.

Transfection Assays

A plasmid containing the CDX2 5'-UTR promoter construct (pGL-3-CDX2 promoter, -562 bp) attached to a luciferase reporter was used for the transfection studies; pGL-3 Basic Luciferase Vector (Promega, Madison, WI) lacking the CDX2 promoter insert served as a control. The renilla reporter pRL (Promega, Madison, WI) plasmid was used to equalize for transfection efficiency. Cells were co-transfected with 0.4 μ g of the CDX2 plasmid and 25 ng of pRL using Lipofectamin 2000 (Invitrogen, Carlsbad, CA) per manufacturer's instructions. After 24 hours, cells were treated with either experimental or control medium for another 24 hours. Cell extracts were assayed for luciferase activities using the Dual-Luciferase Reporter Assay system (Promega, Madison, WI) per manufacturer's instructions, and data were expressed as relative light units (RLU) for firefly luciferase normalized to renilla luciferase.

Inhibition of NF- κ B

Bay11-7085 (Sigma) is a pharmacologic inhibitor that blocks I κ B α phosphorylation. Cells transfected with the CDX2 promoter construct were pre-treated with Bay11-7085 at a concentration of 5 μ M for 2 hour prior to exposure to acid, bile salts, or the combination of both for 24 hours (26). We confirmed the role of NF- κ B in mediating effects on CDX2 promoter activity using an I κ B super-repressor construct (27). Cells were infected with viral medium containing either recombinant-deficient adenovirus Ad5I κ B α -SR or the corresponding empty vector 2 hours before treatment with either experimental or control medium. (27). We also introduced a mutation specifically in the NF- κ B binding site of the pGL-3-CDX2 promoter construct using *in vitro*-site-directed mutagenesis. The mutagenesis reaction was performed using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) per manufacturer's instructions. (Supplemental materials)

Immunofluorescence

Cells were exposed to experimental or control medium for 24 hours, and then fixed in 4% paraformaldehyde for 15 min. The sections were incubated for 10 min in PBS, 2% bovine serum albumin (BSA), and 0.2% Triton X-100, washed, and incubated for 1 hour at 4°C with 1:250 dilutions of polyclonal rabbit anti-human antibodies to p65 or p50 (Cell Signaling Technologies, Beverly, MA). The binding of primary antibodies was detected by a 1:250 dilution of goat anti-rabbit IgG fluorescein isothiocyanate (FITC) conjugated secondary antibody (Sigma).

Chromatin immune-precipitation (ChIP) Assay

Cells were treated with experimental or control media for 24 hours, and ChIP assay was performed according to the protocol published by Nelson et al. (Supplemental materials) (28). Following chromatin shearing in NES cells, the sheared chromatin was cleared by centrifugation and the supernatant was immunoprecipitated overnight at 4°C with 1 μ g of polyclonal rabbit anti-human p50 or anti-human p65 (Abcam, Cambridge, MA) or rabbit IgG as an isotype control. Following immunoprecipitation, the DNA was precipitated by centrifuging the sample at 12,000g at 4°C for 1 min, collecting the supernatant, washing the beads with 120 μ l of H₂O, followed by another centrifugation. The pooled supernatants served as the template for the PCR; reaction sheared chromatin that did not undergo immunoprecipitation was used as a control. Following amplification, PCR products (97 bp) were electrophoresed on 2% agarose gels and stained with ethidium bromide.

Statistical Analyses

Data were collected from at least three independent experiments. Quantitative data are expressed as the mean $\pm$ standard error of the mean (SEM). Statistical analysis was performed using ANOVA and the Student-Newman-Keuls multiple-comparison test with the Instat for

Windows statistical software package (GraphPad Software, San Diego, CA). P values <0.05 were considered significant for all analyses.

Results

Acid and Bile Salts Induce CDX2 mRNA and Protein Expression in Esophageal Squamous Cells from GERD Patients with Barrett's Esophagus, but Not from GERD Patients without Barrett's Esophagus

We determined the effects of acid, bile salts, or the combination of both on CDX2 mRNA and protein expression in esophageal squamous cells. We found no baseline CDX2 mRNA or protein expression in any of our NES cell lines (Figure 1A&B). We found that exposure to acidic medium, neutral bile salt medium, or acidic bile salt medium for 10 minutes, 3 times a day for 7 days increased CDX2 mRNA and protein expression in esophageal squamous cell lines derived from GERD patients with Barrett's esophagus (NES-B3T and NES-B10T), but not in squamous cell lines derived from GERD patients without Barrett's esophagus (NES-G2T and NES-G4T) (Figure 1A&B). To determine whether the induced CDX2 protein was functionally active, we determined mRNA expression levels of two CDX2 target genes - CK20 and MUC-2 (14). We found that exposure to acidic medium, neutral bile salt medium, and acidic bile salt medium increased levels of CK20 mRNA (Figure 1C) and MUC-2 mRNA (data not shown) in the NES-B3T and NES-B10T cells, but not in the NES-G2T and NES-G4T cells.

Acid and Bile Salts Do Not Alter the Methylation Status of the CDX2 Promoter in Esophageal Squamous Cells

CDX2 promoter demethylation has been described as a mechanism underlying acid- and bile salt-mediated increases in CDX2 mRNA expression in esophageal squamous cell lines (14). Therefore, we determined whether acid, bile salts, or both changed the methylation status of the CDX2 promoter in our squamous cell lines using methylation specific PCR. None of our experimental treatments changed the methylation status of the CDX2 promoter in any NES cell line, even though those treatments increased CDX2 mRNA in the NES-B3T and NES-B10T lines (Figure 1A and 2A). To confirm that those negative findings were not due to a technical problem, we treated the cell lines with 1 μ M DAC, a demethylating agent, for 5 days after which we performed methylation-specific PCR. Although the DAC treatment resulted in CDX2 promoter demethylation in all four lines, promoter demethylation alone did not induce CDX2 mRNA expression (Figure 2 B & C).

CDX2 mRNA Expression Occurs More Frequently in the Esophageal Squamous Epithelium of GERD Patients with Barrett's Esophagus than in GERD Patients without Barrett's Esophagus

We determined CDX2 mRNA expression levels in endoscopic biopsy specimens of esophageal squamous epithelium from GERD patients with and without Barrett's esophagus. Seven of the 10 patients with Barrett's esophagus demonstrated CDX2 mRNA expression, whereas such expression was found in only 1 of 10 GERD patients without Barrett's esophagus (Figure 3).

Acid and Bile Salts Induce CDX2 Promoter Activity in Esophageal Squamous Cells from GERD patients with Barrett's Esophagus, but Not from GERD Patients without Barrett's Esophagus

Transient transfections were performed using a pGL-3-CDX2 promoter -reporter construct attached to luciferase (-562 bp) to determine whether acid and bile salts induced activity of the CDX2 promoter. Transfected NES-B3T and NES-B10T cells exposed to acidic, neutral bile salt, and acidic bile salt media exhibited significantly increased CDX2 promoter activity compared to untreated control cells (Figure 4). In contrast, exposure to acidic, neutral bile salt,

and acidic bile salt media had no effect on CDX2 promoter activity in the NES-G2T and NES-G4T cells (Figure 4).

Inhibition of NF- κ B Prevents Acid and Bile Salt Induction of CDX2 Promoter Activity in Esophageal Squamous Cells from Patients with Barrett's Esophagus

In human gastrointestinal cancer cell lines, NF- κ B has been found to be a primary regulator of CDX2 transcription (29;30). Therefore, we explored whether NF- κ B regulated CDX2 promoter activity in response to acid and bile salt exposure in esophageal squamous cells. We inhibited NF- κ B in NES-B3T and NES-B10 T cells using: 1) Bay 11-7085 (a pharmacological inhibitor), 2) specific mutation of the NF- κ B binding site within the pGL-3-CDX2 promoter-reporter construct and 3) infection with the vector Ad5IkB α -SR, which contains an IkB super-repressor construct (27). Inhibition of NF- κ B by treatment with Bay 11-7085 and by mutation of the NF- κ B binding site prevented the increase in CDX2 promoter activity induced by any of the experimental media in both squamous cell lines (Figure 5A&B). Similarly, inhibition of NF- κ B by infection with Ad5IkB α -SR prevented the increase in CDX2 promoter activity induced by any of the experimental media (Figure 5 C&D). In contrast, all 3 experimental media significantly increased CDX-2 promoter activity in cells that were infected with the adenoviral empty vector, suggesting that inhibition of CDX2 promoter activity was indeed due to inhibition of NF- κ B and not due to a non-specific effect of the adenovirus vector (Figure 5C&D). These findings suggest that, in esophageal squamous epithelial cells from GERD patients with Barrett's esophagus, acid and bile salts activate NF- κ B, which then increases activity of the CDX2 promoter.

Acid and Bile Salts Induce Nuclear Translocation of the NF- κ B Subunits p50 and p65 in Esophageal Squamous Cells from Patients with Barrett's Esophagus

In certain cancer cell lines, the p65 and p50 subunits of NF- κ B exert regulatory effects on CDX-2 expression. Therefore, we explored whether acid and bile salts cause these subunits to undergo nuclear translocation in our esophageal squamous cells from patients with Barrett's esophagus. In untreated NES-B3T and NES-B10T cells, immunofluorescence and Western blotting revealed prominent cytoplasmic expression and slight nuclear expression of both the p50 and p65 subunits (Figure 6 A&B; immunofluorescence for NES-B10T data not shown). After exposure to acid and bile salts, in contrast, there was strong nuclear localization of p50 and p65 in both cell lines (Figure 6 A&B).

Acid and Bile Salts Induce Binding of the NF- κ B/p50 Subunit to the CDX2 Promoter in Esophageal Squamous Cells from Patients with Barrett's Esophagus

We used a ChIP assay to determine whether the p50 and p65 subunits bound to the CDX2 promoter. We observed some DNA binding of the p50 subunit to the CDX2 promoter at baseline. Following exposure to acid and bile salts, binding of the p50 subunit to the CDX2 promoter increased markedly (Figure 7). In contrast, there was minimal DNA binding by p65 to the CDX2 promoter at baseline, and that binding did not change in response to any of the experimental media (Figure 7).

Discussion

In earlier reports, we proposed that differences (heritable or acquired) among individuals in the signal transduction pathways activated in esophageal squamous epithelium by gastroesophageal reflux might determine the development of Barrett's esophagus (21;22). In those studies, we found differences between esophageal squamous cell lines from patients with and without Barrett's esophagus in how acid activates the MEK-ERK pathways that regulate cellular proliferation and differentiation (22). In the present study, we have shown that acid and bile salts induce CDX2 mRNA and protein expression in esophageal squamous cells from

GERD patients with Barrett's esophagus, but not from GERD patients without Barrett's esophagus. This demonstration that esophageal squamous cells from patients with and without Barrett's esophagus differ in acid- and bile salt-induced expression of CDX2, a key homeotic gene for intestinal development, suggests that such differences might underlie the development of Barrett's metaplasia.

For these studies, we used telomerase-immortalized, non-neoplastic esophageal squamous cell lines established from GERD patients with and without Barrett's esophagus. These cell lines maintain morphologic features and cytokeratin expression patterns typical of normal esophageal squamous cells and, unlike transformed cells, demonstrate cell contact inhibition and anchorage-dependent cell growth. For studies on how reflux contributes to the development of benign Barrett's metaplasia, we feel that using such cell lines is more appropriate than using transformed cells, which have numerous uncharacterized genetic abnormalities that might alter their molecular responses to stimuli. Those responses also might be affected by infection with simian virus 40 large T antigen (SV40Tag), which can cause DNA promoter methylation abnormalities. SV40Tag has been used to immortalize the HET-1A esophageal squamous cell line. Although it is conceivable that the forced expression of telomerase also might alter cellular responses, our group and others have reported that telomerase allows for the immortalization of normal, non-tumorigenic, epithelial cells without the introduction of major genetic alterations, and that these cells at least maintain normal cell cycle checkpoint responses (22;31-33).

None of our telomerase-immortalized esophageal squamous cell lines expressed CDX2 mRNA or protein at baseline. After exposure to acid, bile salts or both for 7 days, CDX2 mRNA and protein were expressed in the lines derived from GERD patients with Barrett's esophagus (NES-B3T and NES-B10T), but not in those from GERD patients without Barrett's esophagus (NES-G2T and NES-G4T). Moreover, in the NES-B3T and NES-B10T lines, we found that exposure to acid, bile salts, or both increased mRNA expression of CDX2 target genes (CK20 and MUC-2), suggesting that the induced CDX2 is functional. We also studied CDX2 mRNA expression in esophageal squamous mucosal biopsy specimens taken during endoscopic examinations in GERD patients. In support of our *in vitro* findings, we found CDX2 mRNA expression in 7 of 10 squamous biopsy specimens from patients with Barrett's esophagus, whereas only 1 of 10 such specimens from patients who had GERD without Barrett's esophagus expressed CDX2 mRNA. Moons *et al.* also found expression of CDX2 mRNA in 6 of 19 esophageal squamous mucosal biopsy specimens taken from patients with Barrett's esophagus, but did not study CDX2 expression in GERD patients without Barrett's esophagus (34).

Others have reported that acid and bile salts increase CDX2 mRNA expression in HET-1A cells and in certain cancer cell lines by demethylation of the promoter (14;15). In contrast, we did not find that acid and bile salts changed the methylation status of the CDX2 promoter in any of our telomerase-immortalized cell lines, even though those agents clearly increased CDX2 mRNA levels in NES-B3T and NES-B10T cells. To confirm that those findings were not due to a technical problem, we treated the cells with the demethylating agent DAC. Although DAC demethylated the CDX2 promoter in all 4 cell lines, there was no associated increase in mRNA expression, suggesting that promoter demethylation alone is not sufficient to induce CDX2. The reasons underlying the disparity between our findings and those of earlier investigators are not clear, but may be related to the presence of the SV40Tag in HET-1A cells, and to the genetic abnormalities of cancer cells.

We transfected our cells with a human CDX2 promoter-reporter construct attached to luciferase to explore the effects of acid and bile salts on promoter activity. We found that those agents increased CDX2 promoter activity in NES-B3T and NES-B10 T cells, but not in NES-G2T or NES-G4T cells. Since this human CDX2 promoter construct contains an NF- κ B binding site,

we investigated whether acid and bile salts increase CDX2 transcriptional activity via NF- κ B activation. We inhibited NF- κ B activation using three different approaches, and found that all three abolished the transcriptional activation of CDX2 by acid and bile salts in NES-B3T and NES-B10T cells. These data suggest that reflux-induced transcriptional regulation of CDX2 is NF- κ B-dependent in esophageal squamous cells, as it is in human colorectal carcinoma cells and adenocarcinoma cells of the gastroesophageal junction (29;30).

The NF- κ B subunits p65 and p50 have been found to exert opposing effects on Cdx2 transcriptional activity, with p65 inhibiting and p50 stimulating that activity (29;30). In adenocarcinoma cells of the gastroesophageal junction, deoxycholic acid has been found to increase activity of the Cdx2 promoter by inducing nuclear translocation and promoter binding of only the p50 subunit (30). We found that acid and bile salts induced nuclear translocation of both the p65 and p50 subunits in NES-B3T and NES-B10T cells, but only the p50 subunit bound to the CDX2 promoter. These findings suggest that, in esophageal squamous cells from patients with Barrett's esophagus, acid and bile salts increase CDX2 mRNA expression through transcriptional activation via the nuclear translocation and binding of the p50 subunit of NF- κ B.

In summary, we have shown that acid and bile salts increase expression of the homeotic gene CDX2 in esophageal squamous cells from patients with Barrett's esophagus, but not in those from GERD patients without Barrett's esophagus. We have also documented *in vivo* that expression of CDX2 mRNA occurs more frequently in the esophageal squamous epithelium of patients with Barrett's esophagus. The acid- and bile- induced increase in CDX2 transcription in squamous cells from Barrett's patients is dependent on the activation of NF- κ B. Although both the p50 and p65 subunits of NF- κ B undergo nuclear translocation after treatment with acid and bile, only the stimulatory p50 subunit binds the CDX2 promoter. These findings support our hypothesis that differences among individuals in the molecular pathways activated when their esophageal squamous epithelium is exposed to gastroesophageal reflux determine whether reflux-induced damage heals through squamous regeneration or through the development of Barrett's esophagus. It remains unclear whether those differences are heritable or acquired.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Effects of acidic medium, neutral bile salt medium, or acidic bile salt medium on CDX2 mRNA and protein expression, and on CK20 mRNA expression in esophageal squamous cell lines from GERD patients with (NES-B3T & NES-B10T) and without (NES-G2T & NES-G4T) Barrett's esophagus. Acid, bile salts, and acidified bile salts increase: (1A) CDX2 mRNA, (1B) CDX2 protein, and (1C) CK20 mRNA expression. CaCo2 cells served as a positive control for CDX2 mRNA expression. C, untreated control cells; A, acidic medium; B, bile salt medium; A&B, acidified bile salt medium,

Figure 2. CDX2 promoter methylation status and mRNA expression in esophageal squamous cell lines. (2A) acid, bile salts, and acidified bile salts do not change CDX2 promoter methylation status in any of the cell lines. C, untreated control cells; A, acidic medium; B, bile salt medium; A&B, acidified bile salt medium, (2B) DAC demethylates the CDX2 promoter in all cell lines. (2C) Demethylation of the CDX2 promoter alone did not induce mRNA expression in any cell line. U, unmethylated allele; M, methylated allele.

Figure 3.

CDX2 mRNA expression in biopsy specimens of esophageal squamous mucosa from GERD patients with and without Barrett's esophagus. CaCo2 cells served as a positive control. M, marker; C, control; B, esophageal squamous biopsy from a GERD patient with Barrett's esophagus; G, esophageal squamous biopsy from a GERD patient without Barrett's esophagus

Figure 4.

Acid and bile salts increase activity of the human CDX2 promoter construct in NES-B3T and NES-B10T cells, but not in NES-G2T and NES-G4T cells. The bar graphs depict the mean + SEM of at least 3 individual experiments. (***, $p < 0.001$ compared with non-treated controls) pGL-3-CDX2, cells transfected with plasmid containing the CDX2 promoter construct attached to a luciferase reporter (-562 bp); pGL-3, cells transfected with pGL-3 plasmid vector lacking the CDX2 promoter; RLU, relative light units.

Figure 5.

Acid and bile salts increase CDX2 promoter activity by activating NF- κ B in NES-B3T and NES-B10T cells. (5A & 5B) NF- κ B inhibition by Bay 11-705 and site-directed mutagenesis of the NF- κ B binding site within the reporter construct pGL-3-CDX2 prevents activation of the CDX2 promoter in both cell lines. (5C & 5D) NF- κ B inhibition by infection with the adenoviral construct Ad5IkBa-SR prevents activation of the CDX2 promoter; the corresponding empty adenoviral vector served as a control. The bar graphs depict the mean + SEM of at least 3 individual experiments. (*, $p < 0.05$ compared with non-treated controls; **, $p < 0.01$ compared with non-treated controls; ***, $p < 0.001$ compared with non-treated controls) RLU, relative light units.

Figure 6.

Acid and bile salts increase nuclear translocation of the NF- κ B subunits p50 and p65 in NES-B3T cells. Representative experiments of (6A) immunofluorescence and (6B) Western blotting for p50 and p65. Note the prominent cytoplasmic and slight nuclear expression of both subunits in control cells. Following exposure to acid, bile salts, or acidified bile salts, there was strong nuclear localization of p50 and p65. DAPI staining demonstrates the total number of cell nuclei in the same field. Tubulin and lamin served as controls for the cytoplasmic and nuclear fractions, respectively. C, untreated control cells; A, acidic medium; B, bile salt medium; A&B, acidified bile salt medium.

Figure 7.

Acid and bile salts increase DNA binding of p50, but not p65 to the CDX2 promoter in NES-B3T and NES-B10T cell. Panels show representative experiments of ChIP assays for p50 and p65. Note that exposure to acid, bile salts, or acidified bile salts markedly increases binding by of p50, but not p65, to the CDX-2 promoter. C, untreated control cells; A, acid exposure; B, bile salt exposure; A&B, acidified bile salt exposure; M, marker.