Pathogenesis of Barrett Esophagus

Deoxycholic Acid Up-Regulates Goblet-Specific Gene MUC2 in Concert With CDX2 in Human Esophageal Cells

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Hypothesis: Bile acid exposure can induce caudal-related homeobox 2 (CDX2) messenger RNA (mRNA) expression, a transcription factor that plays a crucial role in the development of Barrett esophagus. We investigated mucin 2 (MUC2) and CDX2 mRNA expression before and after treatment with deoxycholic acid in 4 human esophageal cell lines.

Design, Setting, and Participants: Four human esophageal cell lines—(1) normal squamous cells immortalized by SV40 (Het-1A), (2) adenocarcinoma (SEG-1), and (3 and 4) squamous cell carcinoma (HKESC-1 and HKESC-2)—were exposed in culture for 1 to 24 hours to 100µM to 1000µM deoxycholic acid. Total RNA was extracted before and after bile acid treatment and reverse transcribed to complementary DNA.

Main Outcome Measure: MUC2 and CDX2 mRNA expression as determined by semiquantitative reverse transcription–polymerase chain reaction.

Results: MUC2 mRNA expression was absent before deoxycholic acid exposure in all 4 cell lines. MUC2 expression increased in a dose- and time-dependent manner with deoxycholic acid in all cell lines. Deoxycholic acid activated MUC2 up-regulation, which correlated directly with CDX2 up-regulation in all 4 cell lines.

Conclusions: Bile acids up-regulate both intestinal differentiation factor CDX2 and goblet cell–specific gene MUC2 in normal esophageal and cancer cell lines. Further, bile acid–stimulated MUC2 up-regulation correlates directly with CDX2 up-regulation. The simultaneous up-regulation of both CDX2 and MUC2 after bile acid exposure provides molecular evidence of the role of bile acid in the pathogenesis of Barrett esophagus.

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Barrett esophagus (BE) is characterized by the metaplastic replacement of squamous epithelial cells by intestinal epithelium–containing goblet cells. The existence of goblet cells in this premalignant lesion is the morphological hallmark of BE. The gene mucin 2 (MUC2) is thought to be expressed nearly exclusively in goblet cells and thus is considered a goblet cell–specific marker gene. Clinically, MUC2 can be used as a molecular marker to label goblet cells in histologic sections of Barrett epithelium.

Although the molecular pathogenesis of BE is still largely unknown, recent studies suggested that caudal-related homeobox 2 (CDX2) may play a crucial role in its development. CDX2 is the nuclear transcription factor thought to play a key role in the development, proliferation, and differentiation of intestinal epithelium. Several lines of evidence come together to suggest that CDX2 and bile acids may be involved in the pathogenesis of BE. First, it has been previously shown that both CDX2 messenger RNA (mRNA) and protein is up-regulated in Barrett mucosa when compared with normal squamous or non–goblet cell–containing cardiac epithelium. Second, decades of clinical evidence strongly suggest that bile acids present in the fluid refluxed into the esophagus of patients with gastroesophageal reflux disease are an etiological factor in the development of BE. Finally, we have also recently shown that deoxycholic acid (DCA) and other bile acids can induce intestinal differentiation factor CDX2 mRNA expression in human esophageal cell culture.

Given that goblet cells are the histological hallmark of BE and that the MUC2 gene is goblet cell–specific, we hypothesize that bile acid (DCA) exposure would lead to both CDX2 and MUC2 gene up-regulation in esophageal cells. To test this
hypothesis, we investigated \textit{MUC2} and \textit{CDX2} mRNA expression before and after DCA treatment in 4 human esophageal cell lines.

\section*{METHODS}

\subsection*{CELL LINES AND CULTURE}

Four human esophageal cell lines (Het-1A, SEG-1, HKESC-1, and HKESC-2) were used in this study. The characterization of the 4 esophageal cell lines has been described previously. \textsuperscript{12-15} Briefly, Het-1A, purchased from the American Type Culture Collection (Manassas, Va), is a human esophageal squamous epithelial cell line immortalized by viral SV40 transfection. \textsuperscript{12} SEG-1 is a BE adenocarcinoma cell line (a kind gift from Dr David Beer, University of Michigan, Ann Arbor). \textsuperscript{13} HKESC-1 and HKESC-2 are esophageal squamous cell carcinoma cell lines established by one of the authors (Y.H.). \textsuperscript{14,15} All 4 cell lines were cultured in low-glucose Dulbecco’s Modified Eagle Medium for 24 hours before DCA exposure. All 4 cell lines were harvested at the end of each time point with 0.05% trypsin solution (Invitrogen).

\subsection*{RNA EXTRACTION AND COMPLEMENTARY DNA SYNTHESIS}

Total RNA was extracted using the Micro-to-Midi Total RNA Purification System (Invitrogen) from the 4 cell lines immediately prior to bile acid exposure (t₀) and at time points 1 (t₁), 2 (t₂), 4 (t₄), 8 (t₈), and 24 (t₂₄) hours after the end of the DCA exposure period. Then, 0.25 µg of total RNA was reverse transcribed to complementary DNA (cDNA) using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, Calif) according to the manufacturer’s protocol.

\subsection*{REVERSE TRANSCRIPTION–POLYMERASE CHAIN REACTION}

Two microliters of cDNA were amplified in a 20-µL polymerase chain reaction mixture containing 1 × iQ SYBR Green Supermix (Bio-Rad Laboratories) and 0.3 µM primers. The cDNA was amplified as follows: 95°C for 10 minutes, followed by 30 cycles of 1-minute denaturation at 94°C; 1-minute annealing at 48°C (for primers of \textit{MUC2}), 55°C (for primers of \textit{CDX2}), or 58°C (for primers of \textit{β-actin}); and 1-minute extension at 72°C. The final step of extension was for 10 minutes at 72°C. The primers were \textit{MUC2}-F, 5’-ACC ACC CTG TTC AAC GTC ATC-3’, and \textit{MUC2}-R, 5’-CAA ATG CTG GTA CCA AAG TGT G-3; \textit{CDX2}-F, 5’-ACC AGG ACG AAA GAC AAA TAT CTA-3’, and \textit{CDX2}-R, 5’-TGT AGC GAC TGT AGT GAA ACT CCT TCT-3’; \textit{β-actin}-F, 5’-CAA ATA TGA GGC ATT GTT ACA GGA-3’, and \textit{β-actin}-R, 5’-TGG TCT CAA GTC AGT GTA CAG GTA

\section*{RESULTS}

Representative reverse transcription–polymerase chain reaction results following exposure to DCA are shown in Figure 1. \textit{β-actin} was used as a positive control and the gene sonic hedgehog was also tested, proving to serve as a negative control. \textit{MUC2} mRNA expression was absent prior to bile acid exposure in all 4 cell types. In normal esophageal squamous cells (Het-1A), \textit{MUC2} expression was highly up-regulated by 1000 µM of DCA exposure for 1 hour, as well as at lower doses, 300 µM for 8 hours, and in a dose- and time-dependent fashion. In esophageal squamous carcinoma cells (HKESC-1 and HKESC-2), \textit{MUC2} expression was up-regulated at 1000 µM of DCA exposure for 1 hour, and the up-regulation maintained for the 2- to 24-hour time points (Figure 2).

\textit{CDX2} up-regulation was similar following exposure to DCA in each of the 4 esophageal cell lines in similar
dose and time patterns to MUC2. Sonic hedgehog expression remained unchanged at all doses, all times, and all cell lines, reflecting the fact that MUC2 and CDX2 up-regulation is not a nonspecific effect (Figure 2).

MUC2 expression was semi quantitated by scanning the gel bands into computer software for quantitation as relative expression levels (Figure 3 and Figure 4). Time- and dose-dependent up-regulation of MUC2 mRNA expression following DCA exposure is shown in Figure 1 for normal squamous Het-1A cells and Figure 2 for esophageal adenocarcinoma SEG-1 cells, respectively. As shown in Figure 3, Het-1A cell MUC2 expression increased predominantly at 1000µM of DCA treatment but did so slightly at 300µM for 8 and 24 hours. SEG-1 cell MUC2 up-regulation can be detected after 8-hour exposure to DCA at the 300µM and 1000µM concentrations (Figure 4).

**COMMENT**

We have shown that exposure to DCA results in up-regulation of MUC2 mRNA expression in 4 human esophageal cell lines, including those derived from normal squamous, adenocarcinoma, and squamous carcinoma tissues. Further, the DCA-induced increased expression of MUC2 mRNA correlates very well with up-regulation of the transcription factor CDX2. These findings provide evidence...
to link bile acids, goblet-specific MUC2 gene expression, and CDX2 expression in human esophageal cells.

Barrett esophagus is an acquired condition in which the normal esophageal squamous epithelium is replaced by metaplastic intestinal columnar epithelial cells containing goblet cells. Goblet cells are characteristic of BE and serve as the histological hallmark identifying Barrett epithelium microscopically.1 Mucin production has long been recognized as a feature of intestinal epithelium and serves to both lubricate and protect the intestinal epithelium. Mucins are large molecules consisting of a protein backbone combined with O-linked oligosaccharides, which then polymerize to form the functional mucus layer.10 Ten to 12 mucin genes have been identified, each coding for the protein core of a specific mucin type, which can be broadly divided into those that are membrane bound and those that are secreted or gel forming. Genes coding for the secreted mucins lie in a cluster on chromosome 11.p15.5 and include MUC2, MUC5, and MUC6.11 MUC glycoproteins are variably expressed along the gastrointestinal tract. MUC1 has been shown to be expressed in intestinal goblet cells and enterocytes, MUC3 in intestinal enterocytes, MUC5 in gastric foveolar and mucous neck cells, and MUC6 in gastric antral and fundic gland epithelium. We chose to study the MUC2 protein because it is secreted from the goblet cells in BE, it has been shown to be present in and relatively specific for human Barrett epithelium, and it likely plays an important role in the cytoprotection against reflux of gastroesophageal contents, including bile acids, the latter being a candidate etiological factor for the development of BE.7-10

Immunohistochemical studies have shown that MUC2 is not expressed in normal esophageal squamous epithelium but is commonly found in goblet cells of colonic, intestinal, and Barrett epithelium.18,19 Little is known about the regulation of MUC2 transcript particularly during the development of BE. The MUC2 protein is a very large molecular weight glycoprotein with viscosity and gel-forming properties required for mucosal surface protection. The expression of MUC2 in BE is a feature of cellular differentiation as secretory mucins are normally produced by highly differentiated cells.16 Our data show that, even in immortalized normal esophageal squamous cells (Het-1A) and esophageal squamous carcinoma cells (HKESC-1 and HKESC-2), DCA stimulation can activate MUC2 transcription. Moreover, MUC2 mRNA upregulation correlates very well with CDX2 up-regulation. The simultaneous up-regulation of both CDX2 and MUC2 after DCA exposure in esophageal cells is consistent with previous findings of other groups showing CDX2 is directly involved in the transcriptional regulation of the MUC2 gene in gastric and colon cancer cells.20,21

The key steps in the molecular pathogenesis of BE are still largely unknown. Observations from numerous laboratories have shown that the intestinal differentiation factor CDX2 may play a key role in the early columnar differentiation of what is presumably an esophageal stem cell known to be present in the basal layer of esophageal epithelium. CDX2 is a nuclear transcription factor that has an important role in the early differentiation and maintenance of the intestinal epithelial phenotype.22 CDX2 is specifically expressed in the small and large intestines and has been shown to activate other intestinal differentiation genes, including MUC2.20,21 CDX2 is not expressed in normal esophageal mucosa but is abundantly re-expressed in intestinal metaplastic mucosa in the esophagus (ie, BE).16 Similarly, CDX2 is not expressed in normal gastric mucosa but re-expressed in abnormal intestinal metaplastic mucosa in the stomach.23,24 Further, gene transfection studies have shown that inducing CDX2 expression in mouse gastric mucosa can induce the transformation of gastric mucosa into an intestinal mucosa phenotype (ie, intestinal metaplasia).25,26 We and others have previously shown that both the CDX2 gene and protein expression is absent in normal esophageal squamous epithelium of tissue biopsies from patients but highly overexpressed in the intestinal metaplasia of BE.4,6,7,27,28 We have also recently shown that bile acids can induce CDX2 expression in human esophageal cell lines.11 These findings suggest that CDX2 may play a crucial role in the pathogenesis of intestinal metaplasia.

As mentioned earlier, previous studies have shown that CDX2 may transcriptionally regulate MUC2 gene expression in gastric and colon cancer cells.20,21 A more recent study has shown that CDX2 inactivation leads to down-regulation of MUC2 mRNA expression.29 Further studies have identified 2 CDX2 binding sites at −177/−171 and −191/−187 in the MUC2 gene promoter, both of which are required for the transcription of MUC2 mRNA.21 Collectively, these studies strongly suggest that CDX2 is an important transcriptional regulator of MUC2 gene expression. In fact, we were able to show that DCA up-regulated CDX2 correlated with MUC2 up-regulation in all 4 esophageal cell lines. Our results are consistent with the conception that CDX2 is the major regulator of MUC2 transcript.

Our data provide in-vitro evidence that there are interactions among bile acids strongly associated with the development of BE; CDX2, a key transcription factor for the intestinal epithelial phenotype; and MUC2, a specific molecular marker for goblet cells, the morphological hallmark of BE. While the exact mechanisms of the interactions among these 3 components (bile acid, CDX2, and MUC2) in esophageal cells remain to be elucidated, it is conceivable that bile acid stimulation of esophageal stem cells leads to intestinal transcription factor CDX2.
up-regulation, which promotes the cells to differentiate toward an intestinal epithelial lineage. Continued stimulation may up-regulate the MUC2 gene, promoting the cells to further differentiate into the BE phenotype and the emergence of goblet cells.

In summary, we have shown that DCA can up-regulate both intestinal differentiation factor CDX2 and goblet cell-specific gene MUC2 in normal esophageal and esophageal cancer cell lines. Moreover, DCA-activated CDX2 up-regulation correlates directly with MUC2 up-regulation. The concurrent up-regulation of both CDX2 and MUC2 after bile acid exposure provides molecular evidence of the role of bile acids in the pathogenesis of BE.

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REFERENCES


Discussion

David W. Easter, MD, San Diego, Calif: This work adds an important piece to the puzzle of a difficult clinical question—namely, what causes the neoplastic transformation of Barrett epithelium? This finding may be an edge piece, or even a corner piece of the puzzle; we just don’t know yet.

The authors have shown that cultured esophageal cells exposed to bile acids will express both MUC2 and CDX2 messenger RNA. This group and others have previously shown that CDX2 activation is relevant in the transformation of Barrett metaplasia and other mucosal tissue differentiation; and this current work ties CDX2 activation to the goblet cell–specific gene, MUC2. The tantalizing suggestion is that these genes are turned on by bile acids bathing normal esophageal mucosa, thereby starting the metaplasia-dysplasia-carcinoma sequence. The authors correctly don’t overstate their findings: the cause-and-effect relationship of this sequence is still missing.
Here are my allowed 3 questions:

1. The micromolar amounts in your study seem appropriate, but what about the multiple hour and continuous exposure times? Are these laboratory conditions relevant in human tissues? I want to believe the answer is "yes," but please reassure us. Maybe your next study will involve intermittent exposures and different acid reflux conditions.

2. What other physiologic or pathologic pathways are possibly described by the CDX2-MUC2 activation sequence? Maybe this up-regulation is protective, not harmful to the distal esophagus. Do you have any information about response at normal pH vs acidic or basic pH?

3. Can you suggest why low levels and even long exposure times of dilute deoxycholic acid seem to decrease MUC2 expression in some of your cell lines?

Dr. Carolyn Jones, Jeffrey Peters, and his group have shown that bile acids can turn on the genes of cultured human esophageal cells, genes that are known to cause proliferation and differentiation into intestinal goblet cells. Their manuscript correctly states that "the key steps in the molecular pathogenesis of Barrett esophagus are still largely unknown," but an intriguing new piece of the puzzle has been added by their fine work. Congratulations.

Dr. Peters: Thank you, David, for your insightful comments and questions. Did the concentrations and time exposure reflect what is going on inside the human esophagus? The concentrations certainly do. There is reasonable data from a number of sources around the world using continuous aspiration studies to show that concentrations as high as 1000, even 2000, micromolar do occur over a 24-hour period in the human esophagus. Deoxycholic acid, usually conjugated to an amino acid, which was not the case in our experiments, is the second most abundant bile acid found in the human esophagus, particularly in patients with Barrett esophagus.

The exposure times and the exposure patterns are indeed intermittent in patients but can be on the order of hours. Interestingly, bile exposure typically occurs at night, and it can be quite prolonged over the course of hours. Reproducing the exact exposure that happens inside a human esophagus in the laboratory is quite difficult, as it is with the pH as well.

Could this be a protective response and what are the signaling mechanisms? The signaling mechanisms are something we are quite interested in. We may be moving toward an understanding of even what the receptor signaling may be. Whether it is a protective mechanism or not: who knows? It is almost certainly a response of a stem cell in the basal layer of the esophageal tissue wall. So it is a response. Whether you can call it protective or not is hard to know.

Dr. Easter also asked why deoxycholic acids sometimes decrease the MUC2 expression at low levels in the early time points. We don’t quite know, again, exactly how the gene is turned on, turned off, how the receptors are regulated. That is probably an artifact of the tissue culture system and perhaps even of the cells that we are using. I don’t think we can make too much about exactly what we see there.

Stephen G. Jolley, MD, Anchorage, Alaska: What happens if you do the same study but use cell lines from normal gastric mucosa, proximal small bowel mucosa, and squamous cells from the skin?

Dr. Peters: Don’t know. Good question. Our next set of experiments is probably going to be on intestinal stem cells, if we can get them, and not differentiated cells. Similar studies have been done studying bile-induced injury to both colon cancer and hepatocyte cell lines fairly extensively. To my knowledge, skin cells and gastric cells have not been used.

Robert V. Rege, MD, Dallas, Tex: You alluded to this in the answer to the first question, but pH is extremely important with bile cells. So you are using an unconjugated bile cell and its pKa is close to the range we are talking about so that a small change in pH will change the characteristics and toxicity of that bile cell. Do you have any information about response at normal pH vs acidic or basic pH?

Dr. Peters: Good question. Bile salt chemistry is extremely complex, especially if you throw it in with lipoproteins and all the other material that is in bile, not just the bile acids and bile salts. Again, reproducing this experimentally is extremely difficult. If you are using tissue culture, as you take the pH down to try to add pH or acid effects, the cells begin to die. Below pH 5, it can be quite hard to keep the cells viable for any appreciable amount of time, more than an hour or two. Thus the tissue culture system is slightly artificial, but I think we are beginning to understand the molecular events despite the fact that it doesn’t exactly reproduce what is going on in the human esophagus.

Steve DeMeester, MD, Los Angeles, Calif: I wonder if you explained or expounded a little bit on how this fits into our general 2-step theory of Barrett concept where you get cardiac mucosa and then cardiac mucosa becomes intestinalized. We have seen the CDX2 really only appreciably expressed once you have got the goblet cells developing in the cardiac mucosa. How do you fit this together with that 2-step theory?

Dr. Peters: The first step may be nongoblet cells containing cardiac epithelium. We don’t know yet whether CDX2 can precipitate a non-goblet cell epithelium. Presumably it may. But if it doesn’t, it leads my mind to believe that CDX2 is not the only gene. There are almost certainly other genes that are turned on that lead down the differentiation pathway, and I think we need to find them. Microarray and other pathway studies might lead us down to an understanding of what the other players are. Probably not just 1 gene.

C. Max Schmidt, MD, Indianapolis, Ind: This has already been hinted at, but from a mechanistic standpoint, have you looked at a CDX2 SiRNA or antisense to determine whether or not that reverses MUC2 expression?

Two, what happens with the protein? You talked about RNA in this study.

Three, speculate why the adenocarcinoma cell line is the easiest and the most sensitive to deoxycholic acid.

And finally, was there a change in the morphology of the cultured cells with MUC2 expression, and have you done any longer-term cultures to see if there is any change observed?

Dr. Peters: We anticipate using SiRNA and are anxious to do so. We haven’t yet. SiRNA technology, as you may know, has the ability to block the function of the various proteins and more downstream effects. We haven’t done that experiment yet, but it is planned.

We have measured protein. The protein expression was not part of this data but seems to mirror the messenger RNA expression.

Why is the SEG-1 cell line more sensitive? There is circumstantial evidence that this may be working through the EGFR receptor, and we are now working on experiments to see if that is indeed the case. If that is true, I would postulate that those adenocarcinoma cell lines express EGFR quite readily as we know most adenocarcinomas do.

Richard C. Thirlby, MD, Seattle, Wash: If you take 2 cohorts of patients with reflux, one with Barrett and one without Barrett, and they have some objective measure of equivalent amount of reflux, do the Barrett patients have more bile in their refluxate?

Dr. Peters: Twenty years of data, probably hundreds of studies—the unequivocal answer is yes.

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