Bile acid induces expression of COX-2 through the homeodomain transcription factor CDX1 and or orphan nuclear receptor SHP in human gastric cancer cells

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The carcino-related homebox gene, CDX1, encodes for an intestinal-specific transcription factor and is involved in the induction of intestinal metaplasia (IM) of the stomach in gastric cancer. Gastric IM induced by bile reflux is a precancerous gastric adenocarcinomai lesion and has been associated with the induction of cyclooxygenase-2 (COX-2). In this study, we demonstrate the molecular mechanisms underlying the transcriptional regulation of COX-2 by bile acid in gastric cells. We noted that the ectopic expression of CDX1 enhanced COX-2 gene expression and that bile acid was associated with the induction of CDX1 expression. Furthermore, the induction of CDX1 by bile acid was mediated by the orphan nuclear receptor, small heterodimer partner (SHP). Finally, it was verified that the expression of COX-2, CDX1, SHP and CCAAT element-binding protein beta messenger RNA in human IM lesions were significantly higher than in lesions associated with gastritis. Collectively, these results reveal that bile acid induces an increase in the gene expression of COX-2 via the sequential transcriptional induction of SHP and CDX1 in precancerous lesions of human gastric cancer.

Introduction

Gastric cancer is one of the most frequently experienced malignancies and causes of cancer-related mortality in the world (1). The pathogenesis of gastric cancer is complex, and unlike some other solid tumors, the molecular mechanisms inherent to the development of stomach cancer have yet to be clearly elucidated. Gastric cancer has been classified previously into two histological groups—diffuse and intestinal type, respectively (2,3). Intestinal metaplasia (IM) is a pre-malignant condition of the stomach and is associated with chronic atrophic gastritis and gastric carcinoma (3,4). Gastric pathogenesis is believed to result from a combination of environmental factors, including Helicobacter pylori infection, excessive salt intake, low vegetable and fruit intake and the accumulation of specific genetic alterations (3,5). Furthermore, associations between bile reflux and IM have been reported, and bile reflux has been generally considered to be a risk factor in the production of IM in the stomach and also is believed to function as an initiator of gastric carcinogenesis (6,7). However, the precise mechanism by which bile reflux promotes gastric tumor formation remains to be characterized.

CDX1 and CDX2, members of the carcino-related homebox family, are known to be crucial to the control of normal intestinal differentiation and the maintenance of the intestinal phenotype (8–10). CDX1 and CDX2 are not expressed in the normal stomach, but it has been demonstrated that aberrant expression of these factors induces IM of the stomach, as well as gastric carcinoma (3,11–14). Hence, CDX1 and CDX2 are most probably involved in the induction of IM of the stomach. Little is currently known regarding the molecular mechanisms of action of CDX1 and CDX2 in the progression from normal gastric epithelium to IM.

Cyclooxygenase-2 (COX-2) catalyzes a pivotal step in the formation of arachidonic acid to prostaglandins and is a typical molecular target of the non-steroidal anti-inflammatory drugs (15,16). COX-2 is not normally expressed in the majority of normal tissues and can be induced readily by a variety of agents, including growth factors, tumor promoters and inflammatory stimuli, but is upregulated in several cancers, most notably gastric cancer (1,15–18). Furthermore, increased COX-2 expression has been detected in intestinal-type gastric carcinoma and dysplasia precursor lesions, thereby suggesting that COX-2 may perform a function in the early events associated with gastric carcinogenesis (17).

The CCAAT element-binding protein beta (C/EBPβ) is a member of the C/EBP family of transcriptional factors and is a crucial regulator of biological processes, including proliferation, differentiation and tumor invasiveness (19,20). Recently, the consensus sequence of C/EBPβ has been described in the COX-2 promoter and C/EBPβ has been shown to regulate COX-2 expression in gastric cancer cells (21). In accordance with these findings, C/EBPβ overexpression is recognized as a marker in IM and performs an active role in gastric carcinogenesis. However, the molecular relationship of C/EBPβ in relation to CDX1-mediated gene regulation during IM remains to be elucidated. In this study, we demonstrated that bile acid-mediated increases in CDX1 expression are required for the induction of COX-2 expression. Moreover, our results demonstrated that the molecular mechanism by which CDX1 expression is mediated involves transcriptional regulation by the orphan nuclear receptor (NR), small heterodimer partner (SHP).

Materials and methods

Construction of reporter plasmids and expression vectors

pGL2/COX-2 promoter (−327 to +59) construct was generously provided by Dr. D.S. Min (Pusan National University, Korea). Two nested 5′-end deletion constructs of the COX-2 promoter were generated by polymerase chain reaction (PCR) using the plasmid pGL2/COX-2 (−327 to +59) as the template. Two nested 5′-end deletion constructs were generated using the restriction enzyme BglII. A BgIII site was added to the 5′ end of COX-2 antisense. Two constructs generated by PCR were amplified with the same 3′ end and COX-2 antisense (5′-CTCCGCAAGCTAGCATAAAAC-3′) and COX-2 antisense (5′-GAAAGTCTCGCTGCCAGAGTTT-3′). The PCR product was double digested with Mull and BglII and cloned into pGL2 luciferase reporter (Promega, Madison, WI).

pDNA3/CDX1 and pGL3B/hCDX1 promoter (−1630 to +12) construct were a kind gift from M.A. Yoo (Pusan National University, Korea). pcDNA3/HA/CDX1 was constructed by inserting PCR fragments of open reading frame into EcoRI- and XhoI-digested pcDNA3/Ha expression vector. All plasmids were confirmed by automatic sequencing analysis.

Cell culture and bile acid treatment

Human gastric carcinoma cell lines MKN45 (a gift from B.J. Park, Pusan National University, Korea) and AGS (a gift from Y.H. Choi, Dongeui University, Korea) were maintained in RMPI 1640 supplemented with 10% heat-inactivated fetal bovine serum (Gibco BRL, Grand Island, NY) and 1% (vol/vol) penicillin–streptomycin (Gibco BRL) at 37°C in a humidified atmosphere containing 5% CO2 and fed every 2 days. Deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) were from Sigma (St Louis, MO). Treatments containing 5% CO2 were performed with 0.2% lactoalbumin hydrolysate for 24 h to avoid the confounding variable of serum-induced signaling.

RNA preparation and reverse transcription–PCR

Total RNA from MKN45 and AGS cells was prepared using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s recommendation.
The complementary DNA was synthesized from 2 μg of total RNA with avian myeloblastosis virus reverse transcriptase (Promega) using a random hexamer (Bioneer, Daejeon, Korea) at 42°C for 1 h. The PCR primer for CDX1 gene amplification were 5′-AGGACAATGGCCTGCTA-3′ (sense) and 5′-CATAGGCCAACATCTCTCTCTC-3′ (antisense); for SHP gene amplification, 5′-ACCTACGTGACACTCAGCCTAGC-3′ (sense) and 5′-CAACAGCAGCGTGGCGAATGACTG-3′ (antisense); for COX-2 gene amplification, 5′-CCCTTCCTGCTGCTAGT-3′ (sense) and 5′-CTGGCCTGCTAGTATTGCTC-3′ (antisense); for MUC2 gene amplification, 5′-CATTCTTCAAGGAAACCTTACCCAC-3′ (sense) and 5′-TCAAAGGAACTACATGAGATACTGGTG-3′ (antisense) and GAPDH gene amplification, 5′-GTCACCTGACTTAC-3′ (sense) and 5′-TCTCTCCTGTGGCTCTTG-3′ (antisense). The complementary DNAs were amplified by PCR under the following conditions: 32 cycles of denaturation at 95°C for 40 s, annealing at 57°C for 40 s and extension at 72°C for 50 s in a thermal cycle. The PCR products were examined by electrophoresis on a 1.2% agarose gel.

**Transient transfection assay and luciferase assay**

MKN45 and AGS cells were seeded in a 24-well culture plate and transfected with reporter vector and β-galactosidase expression plasmid, along with each indicated expression plasmids using jetpe (Polyplus-transfection, Illkirch, France). The pcDNA3.1HisC plasmid DNA was added to achieve the same total amount of plasmid DNA per transfection. After 48 h of transfection, the cells were rinsed with ice-cold phosphate-buffered saline and lysed with 1× cell lysis buffer (Promega). Luciferase activity was determined using an analytical luminescence luminometer according to the manufacturer’s instructions. Luciferase activity was normalized for transfection efficiency using the corresponding β-galactosidase activity and all assays were performed at least in triplicate.

The construction of small-interference RNA (siRNA) targeted to CDX1 and SHP was carried out according to the instruction manual provided by the manufacturer (Bioneer). For the construction of CDX1 siRNA (the sense, 5′-CAACAGGAAACTGCGCAGACAGA-3′ and the antisense, 5′-UCACGGGCTGGCGCGAUCUCG-3′) and the antisense primer, 5′-UCACGGGAACACUCUUGUUCUC-3′), oligonucleotides were synthesized. The transfection of MKN45 cells was carried out by HiPerFect according to the instruction manual provided by the manufacturer (QIAGEN, Hilden, Germany).

**Chromatin immunoprecipitation assay**

MKN45 cells were collected and cross-linked with 1% formaldehyde at 37°C for 10 min and then rinsed with ice-cold phosphate-buffered saline and centrifuged for 5 min at 2000 g. Cells were then resuspended in 0.2 ml lysis buffer [1% sodium dodecyl sulfate (SDS), 10 mM ethylenediaminetetraacetic acid (EDTA), 50 mM Tris–Cl, pH 8.1, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Roche, Rotkreuz, Switzerland)] and sonicated with a microtip probe. After ultrasonic treatment, chromatin was collected by centrifugation for 10 min at 3000 g at 4°C and the supernatant was aspirated off. The chromatin pellet was resuspended in 0.5 ml lysis buffer and incubated for 5 min at room temperature. Nuclei were then incubated for 2 h at room temperature with anti-CDX1 (AB4122, Chemicon, Temecula, CA), anti-C/EBPβ (sc-150, Santa Cruz Biotechnology, Santa Cruz, CA), anti-COX-2 (sc-1745, Santa Cruz Biotechnology), anti-β-actin (Roche, Mannheim, Germany) and anti actin (A 2066, Sigma, Steinheim, Germany) in tris-buffered saline containing 0.05% tween 20 (TBST) supplemented with 1% non-fat dry milk. After washing three times with cold TBST, the blotted membranes were incubated with peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 30 min at room temperature. After washing three times with cold TBST, the bands were detected using an enhanced chemiluminescence system (Amershams, Piscataway, NJ).

**Results**

**DCA induces COX-2 expression**

Different bile acids exert different biological effects on cell growth, cell transformation and gene expression (22,23). In particular, DCA and CDCA perform a function in tumor promotion, and higher levels of bile acid have been reported in patients suffering from atrophic chronic gastritis and IM in gastric carcinoma (6,7). Although bile acids are the principal etiologic agents associated with IM and neoplasia in the gastrointestinal tract and alterations in COX-2 expression are observed during gastric carcinogenesis, the molecular mechanisms underlying the bile acid-mediated overexpression of COX-2 remain to be determined.

In an effort to assess the effects of different bile acids on the modulation of COX-2 expression, two gastric cancer cell lines—MKN45 and AGS cells—were transiently transfected with human COX-2 promoter (−327 to +59), as is shown in Figure 1A, DCA treatment induced an increase in the activity of the COX-2 promoter in a dose-dependent manner, with the maximum effect occurring at 200 μM in the MKN45 and AGS cells. However, CDCA did not induce COX-2 expression at the transcriptional level. As compared with the untreated cells, DCA also induced an increase in the levels of steady-state COX-2 messenger RNA (mRNA) and protein in a dose-dependent fashion in MKN45 and AGS cells (Figure 1B), and this induction began within the first 6 h, reaching a maximum intensity 24 h after stimulation (Figure 1C). These results demonstrated that COX-2 expression is stimulated by DCA treatment in a dose- and time-dependent manner in gastric cancer cell lines.

**CDX1 increases COX-2 expression via transcriptional regulation**

In an effort to understand the mechanism underlying the regulation of the COX-2 gene, the promoter region of the COX-2 gene was identified. From database analysis of the promoter region for known consensus sequences in the COX-2 promoter, several putative CDX and C/EBPβ response elements were detected in the COX-2 promoter. Other previous reports have indicated that C/EBPβ regulates the activity of the COX-2 promoter (21). In order to determine whether CDX1, CDX2 and C/EBPβ regulate the expression of the COX-2 gene, MKN45 cells were transfected with the COX-2 promoter–luciferase reporter plasmid, along with expression vectors encoding for CDX1, CDX2 and C/EBPβ, respectively. CDX1 and C/EBPβ expression resulted in a gradual and dose-dependent increase in the promoter activity of COX-2 (Figure 2). Consistent with the results of the reporter assay, the levels of COX-2 mRNA and protein were significantly increased in the presence of CDX1 overexpression (Figure 2B). These results indicate that CDX1 and C/EBPβ expression, but not CDX2 expression, upregulates COX-2 expression.

Direct analysis of the COX-2 promoter sequence (−327 to +59) revealed two highly conserved potential CDX1-binding sites. In order to search the CDX1-responsive site of the COX-2 promoter, a series of sequentially deleted constructs of the COX-2 promoter were prepared and subjected to reporter assays involving the cotransfection of the CDX1 expression plasmid into MKN45 cells (Figure 2C). Based on the cotransfection of the CDX1 expression plasmid and a series of COX-2-deleted promoter constructs into MKN45 cells, the COX-2 promoter region (−80 to +59), including just one CDX1 site, was considered a CDX1-responsive site on the transactivation assay (Figure 2D). In order to determine whether an increase in the transactivation of the COX-2 promoter as the result of CDX1 expression is
the result of direct DNA-binding activity, a chromatin immunoprecipitation assay was conducted. Isolated DNA was subjected to PCR both prior to (input) and after chromatin immunoprecipitation using primer sets designed to amplify the region with the COX-2 promoter harboring the CDX1-binding site. The results of the chromatin immunoprecipitation assay showed that CDX1 interacted directly with the COX-2 promoter (C0 to +59) (Figure 2E). However, no amplified PCR products were detected in the lane of pcDNA/HA-transfected cells. This result indicates that CDX1 induces an increase in COX-2 expression at the transcriptional level via direct promoter binding.

Next, in order to determine whether DCA increases COX-2 expression via the transcriptional regulation of CDX1, MKN45 and AGS cells were transfected with the human CDX1 promoter and then maintained either under control conditions or in the presence of different concentrations of DCA for 24 h. CDX1 promoter activity was increased in a DCA dose-dependent manner in two gastric cell lines (Figure 3A, upper panel). Furthermore, DCA (100 μM) induced an increase in the level of CDX1 mRNA in both cells as compared with the untreated cells (Figure 3A, lower panel).

In Figure 3B, MKN45 and AGS cells were transfected with the indicated amounts of the expression vectors encoding for CDX1 along with the human COX-2 promoter in the absence or presence of different concentrations of DCA for 24 h. CDX1 promoter activity was increased in a DCA dose-dependent manner in two gastric cell lines (Figure 3A, upper panel). Furthermore, DCA (100 μM) induced an increase in the level of CDX1 mRNA in both cells as compared with the untreated cells (Figure 3A, lower panel).

In Figure 3B, MKN45 and AGS cells were transfected with the indicated amounts of the expression vectors encoding for CDX1 along with the human COX-2 promoter in the absence or presence of different concentrations of DCA. The results demonstrated that CDX1 and DCA cooperatively increased COX-2 promoter activity in a dose-dependent fashion. In order to further confirm the induction of the COX-2 gene by CDX1 and DCA, MKN45 and AGS cells were transiently transfected with CDX1 expression vector or with empty vector as a control, then treated with 100 μM of DCA and subjected to reverse transcription (RT)-PCR and western blot assay. As shown in Figure 3C, the CDX1 expression and DCA treatment significantly stimulated COX-2 mRNA and protein levels. These results show that DCA stimulates COX-2 expression via the induction of CDX1 in gastric cells.

Next, in order to obtain additional insight into the function of CDX1 in the context of COX-2 expression, loss of CDX1 expression studies were conducted using a siRNA approach. The knockdown of CDX1 attenuated the transcriptional activity of the COX-2 promoter in the presence of DCA (Figure 3D). In order to verify the level of COX-2 expression along with siRNA directed against CDX1, RT–PCR and western blot assays were conducted. The knockdown of CDX1 resulted in a reduction in the levels of COX-2 mRNA and protein expression (Figure 3E). These results showed that CDX1 performs a critical function in DCA-induced COX-2 expression.

SHP is required for DCA-induced CDX1 expression

NRs are ligand-activated transcription factors, which are involved in the transcriptional regulation of several aspects of mammalian physiology, and have been identified to regulate a variety of genes by ligands (24,25). SHP is a member of the NR-related proteins and is an unique orphan NR that lacks a conserved DNA-binding domain and is expressed specifically in the liver and a limited number of other tissues (26,27). In the murine macrophage RAW 264.7 cell line, SHP interacts directly with the nuclear factor-kappa B (NF-κB) p65 subunit and functions as a positive transcriptional coregulator of NF-κB (28). In order to characterize the mechanism of CDX1 gene expression by bile acid, the promoter region of the CDX1 gene was...
identified. Via a search of the GeneBank database, the full-length CDX1 promoter harbored responsive sites for activator protein 1, acute myelogenous leukemia1/runt-related transcription factor1, NF-jB, sterol regulatory element binding protein and CDX (29,30). It was recently reported that bile acid increases the activation of NF-jB signaling and the active NF-jB subunit (p65) exerts a direct effect on CDX1 expression (30). On the basis of these findings, in order to assess the role of SHP in DCA-induced CDX1 expression, the effects of SHP on the regulation of human CDX1 promoter activity were assessed. MKN45 and AGS cells were transiently transfected with an expression vector encoding for SHP and the CDX1 promoter–reporter plasmid. The results provided in Figure 4A illustrate that SHP expression increased the luciferase activity derived from the CDX1 promoter. The protein and mRNA induction of COX-2 were also enhanced by SHP expression (Figure 4B). These results indicated that increased levels of CDX1 by SHP were associated with an increase in COX-2 expression.

Next, in order to determine whether DCA-induced CDX1 expression could be mediated by SHP, MKN45 and AGS cells were transfected with the indicated amounts of the expression vectors encoding for SHP, then cultured in the presence or absence of DCA. As is shown in Figure 4C, DCA alone induced the expression of SHP. Furthermore, the cooperation of SHP and DCA induced a substantially increased expression of CDX1 mRNA (Figure 4C). In order to further confirm the role of SHP in CDX1 expression in gastric cells, the siRNA of SHP was transfected into both MKN45 and AGS cells. The knockdown of SHP resulted in a reduction in the transcription of the CDX1 promoter in the presence of DCA (Figure 4D). The level of CDX1 expression along with siRNA directed against SHP was assessed via RT–PCR. Consistent with the results of the examination of CDX1 promoter activity, the loss of SHP expression caused a reduction in the levels of CDX1 mRNA (Figure 4E). These results showed that SHP is required for DCA-induced CDX1 expression. Thus, DCA induces COX-2 expression in gastric cells via the induction of CDX1 expression through the transcriptional regulation of SHP.

Cooperation of CDX1 and C/EBPβ increases COX-2 promoter activity and protein expression

The transcription factor, C/EBPβ, has been associated previously with tumorigenesis and is also recognized to function as a transcription factor for COX-2 in cases of gastric cancer (19–21). In order to determine whether C/EBPβ performs a function in the CDX1-dependent transcription of the COX-2 promoter, we cotransfected cells with the COX-2 luciferase reporter plasmid, along with expression vectors encoding for CDX1 and C/EBPβ. The coexpression of CDX1 and C/EBPβ resulted in a cooperative transcription activity of the COX-2 promoter (Figure 5A). As had been predicted, the combined activity of CDX1 and C/EBPβ gave rise to increased COX-2 protein expression (Figure 5B). These results raise the possibility that CDX1 and C/EBPβ may be markers of an early event in IM and may also perform an active function in the regulation of COX-2 induction.

Fig. 2. CDX1 increases COX-2 expression. (A) MKN45 cells were transfected with the indicated amounts of the expression vectors encoding for CDX1, CDX2 and C/EBPβ along with the human COX-2 promoter–luciferase reporter vector. After 48 h of transfection, promoter activities were measured as luciferase activities normalized to β-galactosidase activities. Bars indicate the mean value of at least three independent experiments. The standard deviation is indicated by error bars. (B) MKN45 cells transiently transfected with the indicated amounts of expression vector encoding for CDX1. Total RNA was prepared from the cells, and then the CDX1 and COX-2 mRNA levels were detected by RT–PCR with GAPDH as a loading control (upper panel) and protein expression was tested with western blot analysis using specific antibodies for HA, COX-2 and actin (lower panel). (C) A schematic representation of the deletion constructs of the hCOX-2 promoter–reporter gene used in transient transfection assays. (D) Luciferase activity of the promoter-deletion constructs in MKN45 cells. The MKN45 cells were transfected with the indicated promoter-deletion constructs of the COX-2 gene along with the expression vector encoding for CDX1 or empty vector. (E) The chromatin immunoprecipitation assay. Empty vector pcDNA3/HA and pcDNA3/HA/CDX1 expression vector were transfected into MKN45 cells. Following formaldehyde cross-linking, soluble chromatin was prepared. After immunoprecipitation with HA antibody, precipitated DNAs were used in PCR analysis. The input lane shows the starting chromatin extracts.
Fig. 3. DCA increases COX-2 expression by inducing CDX1. (A) MKN45 and AGS cells were transfected with 0.1 μg of human CDX1 promoter–reporter vector. After 24 h of transfection, cells were treated for 24 h with DCA. Cell lysates were assayed for luciferase activity and the results were normalized to the β-galactosidase activity. Bars indicate the mean value of at least three independent experiments. The standard deviation is indicated by error bars (upper panel). The CDX1 mRNA levels were detected by RT–PCR with GAPDH as a loading control (lower panel). (B) MKN45 and AGS cells were transfected with the indicated amounts of the expression vectors encoding for CDX1 with the human COX-2 promoter–reporter vector in the presence or absence of different concentrations of DCA for 24 h. Cell lysates were assayed for luciferase activity. (C) MKN45 and AGS cells were transiently transfected with expression vector pcDNA3/HA alone or containing CDX1 expression vectors. The CDX1 and COX-2 mRNA levels were detected via RT–PCR (left panel) and protein expressions were tested with western blot analysis using antibodies specific for HA, COX-2 and actin (right panel). (D) MKN45 cells transiently transfected with siRNA oligonucleotide targeted to CDX1 (CDX1 siRNA) or a control siRNA with the human COX-2 luciferase reporter vector and treated for 24 h with or without 100 μM DCA. Promoter activities were measured as luciferase activities. (E) The COX-2 mRNA levels were detected by RT–PCR with GAPDH as a loading control (left panel). Western blot analysis using antibodies specific for CDX1, COX-2 and actin as indicated (right panel).
Relative expression levels of COX-2, CDX1, C/EBPβ, SHP and MUC2 mRNA in gastritis patients with and without IM

In order to compare the in vivo expression of IM markers and the transcription factors described in this study, human gastritis tissues were collected via endoscopic resection from each of 11 patients after they had provided their informed consent. The patients were defined by their possession of an aberrant high level of bile acid in the bottom of the stomach. The IM region and gastritis mucosa were extracted via endoscopy and the pathological identities were verified via tissue examination, and RT–PCR was conducted to assess the expression of COX-2, CDX1, SHP, C/EBPβ and MUC2 mRNA in mucosal tissues with and without IM. Figure 6A shows that the mRNA expression of COX-2, CDX1, SHP and C/EBPβ was significantly higher in the IM than in gastritis. As is shown in Figure 6B and C, COX-2 and SHP were expressed abundantly in the IM region. MUC2 expression, which is detected in goblet cells and considered to be an intestinal differentiation marker, was evaluated in cells possessing IM. The
The ratio of mRNA expression in IM is compared with its expression of gastritis as a control. However, the mechanism of IM and gastritis mucosa with hematoxylin and eosin staining. Total RNA was prepared from two gastric mucosa representing gastritis and IM, and then the COX-2, CDX1, SHP and MUC2 mRNA levels were detected by RT–PCR with GAPDH as a loading control. The ratio of mRNA expression in IM is compared with its expression of gastritis as a control.

results indicate that the concomitant expression of CDX1, SHP and C/EBPβ may be diagnostic markers of IM and may induce IM via a cascade of transcriptional regulation for precancerous target genes.

Discussion

Metaplasia is a potentially reversible change from one cell type to another, a process referred to as transdifferentiation. Metaplastic cells are more susceptible to neoplasia. Understanding the steps leading to metaplasia is crucial for the identification of early prognostic or diagnostic markers of tumor development (5,31). Chronic gastritis and associated IM are fairly common precancerous conditions for gastric carcinoma (3). IM of the stomach is a risk factor in the development of intestinal-type gastric cancer. The most commonly recognized metaplasia of the gut is characterized by the transdifferentiation of gastric epithelial cells to an intestinal phenotype. The phenotype of IM is characterized by mucin-secreting goblet cells and absorptive enterocytes with villin that express intestinal plasma membrane-specific enzymes and may also include the presence of peptide hormone-secreting enteroendocrine cells and antimicrobial-secreting Paneth cells (13,32). This phenotype is also verified by demonstrating COX-2 expression by bile acids in gastric cells.

It has been shown that the interaction of bile acid with the M3 muscarinic receptor subtype expressed in chief cells may participate in mucosal damage, manifesting as active inflammation, IMs, glandular atrophy and focal hyperplasias and other pathophysiological consequences of bile reflux. Refluxed bile may induce direct injuries of mucous epithelial cells and the tight junctions between these cells. It could also provoke inflammatory responses in the entirety of the mucous and submucous layers. Furthermore, by changing the chemical environment of the mucous surface, bile acid may influence the expression of SHP gene, thereby inducing the activation of a variety of signaling cascades and promoting cell proliferation. It would be valuable to conduct continued molecular analyses of the effects of bile on the gastrointestinal mucosa.

The pathogenesis of IM may be reversible via the treatment of *H. pylori* infection, as well as by dietary changes. Bile reflux into the stomach is associated significantly with the presence of IM. Furthermore, bile reflux directly induces IM and progression to neoplasia in the gastrointestinal tract in animal models (7,41,42). On the basis of these studies, bile reflux is considered to be the most important causative factor and an initiator of gastric carcinogenesis. In this study, we first demonstrate that the caudal-related homeobox gene, CDX1, an intestine-specific transcription factor, participates in the control of COX-2 expression by bile acids in gastric cells.

It has been shown that the interaction of bile acid with the M3 muscarinic receptor subtype expressed in chief cells may participate in mucosal damage, manifesting as active inflammation, IMs, glandular atrophy and focal hyperplasias and other pathophysiological consequences of bile reflux. Refluxed bile may induce direct injuries of mucous epithelial cells and the tight junctions between these cells. It could also provoke inflammatory responses in the entirety of the mucous and submucous layers. Furthermore, by changing the chemical environment of the mucous surface, bile acid may influence the pathogenetic patterns of other damage-causing factors, including gastric acid and *H. pylori*, and may also potentiate the actions of these factors. Consequently, bile reflux exerts harmful effects on gastric mucosa throughout the entire stomach.

The transdifferentiation mechanism that characterizes IM might be explained by the switching of the expression of transcription factors such as homeobox genes. Several studies have reported previously that the expression of CDX1 and CDX2 plays a pivotal role in the development of IM and directs programs to induce IM with aberrant
differenciation, structures and proliferation activities (11–13). Villin, sucrase--isomaltase, alkaline phosphatase, MUC2 and trefoil family factor 3 have all been investigated in conjunction with IM, leading to gastric cancer (42–44). Recently, the intestine-specific homeobox genes, *CDX1* and *CDX2*, were shown to be associated closely with MUC2 and trefoil family factor 3 expression, and it was suggested that *CDX1* and/or *CDX2* perform crucial roles in the aberrant intestinal differentiation program and gastric carcinogenesis, due in part to the regulation of MUC2 and trefoil family factor 3 at the transcriptional level (44). These markers have been useful for the identification of metaplastic tissue. However, it remains unclear as to whether the expression of these factors is functionally significant. Interestingly, *CDX1*, but not *CDX2*, exerts a significant effect on the *COX-2* promoter in MKN45 cells, although the two genes harbor the same CDX-binding sites (TTTAT). These observations indicate that *CDX1* may cooperate with other transcription factors, including C/EBPβ, and that these factors are involved in the regulation of *COX-2* expression during gastric carcinogenesis.

Recently, the expression of the nuclear bile acid receptor, farnesoid X receptor, has been reported in association with gastric cancer. Bile acids activate this receptor and induce an increase in *SHP* gene expression, thereby causing the activation of a variety of signaling cascades and promoting cell proliferation (5,45). In this study, increased *SHP* expression has been demonstrated to potentiate the induction of *CDX1* protein in gastric cells. *CDX1* performs a critical role in increasing *COX-2* production with another transcription factor, C/EBPβ. The molecular mechanisms underlying the cooperation of *C/EBPβ* with *CDX1* for the enhancement of *COX-2* expression remain elusive. It is possible that (i) the functional direct interaction of *CDX1* and *C/EBPβ* on the *COX-2* promoter increases its gene transcription; (ii) since the *CDX1* promoter has a few binding sites for *C/EBPβ*, *CDX1* expression is augmented by *C/EBPβ* or (iii) *CDX1* and *C/EBPβ* may be able to form a transcriptional active complex on the *COX-2* promoter via a coactivators.

In conclusion, this study demonstrates the signaling mechanisms by which bile reflux induces *COX-2* expression to generate IM. Specifically, the results of this study demonstrate that bile acid activates *SHP* gene expression, which induces the expression of the intestinal-specific transcription factor, *CDX1*, in gastric carcinoma cells. In turn, *CDX1* is capable of inducing *COX-2*. Moreover, the present observations suggest that the identification of the factors required for IM and the clarification of the molecular mechanisms responsible for an early phenotypic change in gastric carcinogenesis may facilitate the development of therapeutic interventions.

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**References**


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