

Repeated exposure to acid and bile selectively induces colonic phenotype expression in a heterogeneous Barrett's epithelial cell line

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Barrett's epithelium is a precancerous, specialized columnar metaplasia in the distal esophagus. We demonstrate the changes in cellular phenotype in a non-neoplastic Barrett's cell line (BAR-T), following exposure to acid and bile salt, the two important components of gastroesophageal refluxate. Cell phenotypes in BAR-T cell line were quantified by fluorescence-activated cell sorting (FACS) using monoclonal antibodies against markers: cytokeratin 8/18 (CK8/18) for columnar, CK4 for squamous, mAbDas-1 for colonic epithelial cell phenotype and p75NTR for esophageal progenitors. Cells were exposed for 5 min each day to 200 μ M glycochenodeoxycholic acid at pH 4, pH 6 and pH 7.4 or only to acid (pH 4) for up to 6 weeks. The BAR-T cell line comprised 35 \pm 5.2% CK8/18, 32 \pm 3.5% mAbDas-1, 9.5 \pm 3% CK4 and 4 \pm 2.5% p75NTR-positive cells. Single exposure to acid and or bile did not change cell phenotypes. However, chronic treatment for at least 2 weeks significantly enhanced ($P < 0.05$) the expression of colonic phenotype and CK8/18-positive cells, as evidenced by FACS analysis. Bile salt at pH 4 and bile salt followed by acid (pH 4) in succession were the strongest stimulators ($P < 0.01$) for induction of colonic phenotype cells. Squamous (CK4⁺) phenotype did not change by the treatments. Cox-2 expression was induced after acute treatment and increased to twofold during chronic treatment, particularly in response to acidic pH. We conclude that BAR-T cells can be utilized as an 'in vitro' model to study the effect of environmental factors and their influence on the cellular phenotype and molecular changes in the pathogenesis of esophageal cancer.

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Barrett's epithelium (BE) is an acquired metaplastic change at the squamocolumnar junction of the distal esophagus secondary to chronic gastroesophageal reflux disease (GERD).^{1–3} Epidemiological studies indicate a strong relationship between GERD and esophageal adenocarcinoma,^{4–7} with the risk of malignant transformation being 30- to 125-fold higher in GERD patients complicated with BE.^{8,9} The origin and pathological progression of BE and the contribution of gastroesophageal reflux in the disease process have been extensively studied in animal models.^{1,10–13} The metaplastic process of BE appears to be a protective adaptation¹⁴ or a regenerative healing mechanism.¹⁵ It is hypothesized that 'pleuripotent cells' from the native esophageal stratified squamous epi-

thelium or ductal epithelium of the esophageal submucosal glands may give rise to the specialized columnar epithelium.^{16,17} However, this can be further established by delineating the cellular phenotype and molecular events involved in the metaplasia \rightarrow dysplasia \rightarrow carcinoma sequence.¹⁸

Acid and bile, the two primary components of gastroesophageal refluxate, act synergistically in inducing mucosal injury.¹⁹ Molecular events resulting from GERD in humans have been studied in esophageal biopsies and adenocarcinoma cell lines. A single pulse of either bile or acid independently increases cell survival and proliferation, as well as decreases apoptosis, perhaps by inducing Cox-2 expression, via the MAPKinase^{20–23} and ERK pathway.²⁴ Both acid

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and bile have been proposed to promote intestinal-type differentiation in esophageal keratinocytes by inducing the transcription factors NF- κ B and Cdx-2.^{25,26} Acid has been shown to induce villin expression in normal esophageal biopsy tissues grown in organ culture²⁷ and bile, at neutral pH, to cause DNA damage in esophageal cell lines.²⁸ In all of these experiments, acid and/or bile exposure have been only short-term for few minutes or hours primarily using cancer cell lines and organ cultures. There are, however, no reports of cellular phenotype or molecular changes resulting from long-term (weeks) exposure to acid and bile as in the *in vivo* situation in patients with GERD.

We developed, in our laboratory, a colonic epithelial-specific antibody, mAbDas-1 (also called 7E12H12, IgM isotype), that reacts only with the colon epithelium and not with any other part of the gastrointestinal tract, including columnar epithelium of small intestine, stomach and the squamous epithelium of the esophagus.^{29,30} The reactivity is more intense near the cell membrane. mAbDas-1 reacts with a glycoprotein, termed colon epithelial protein (CEP), that is selectively expressed in the colon epithelium.³¹ Although it does not react with normal esophagus, including gastroesophageal junction mucosa, mAbDas-1 can identify BE as well as adenocarcinoma arising from BE with 97% sensitivity and 100% specificity, suggesting that BE is indeed a metaplasia of colonic phenotype.³² This has been confirmed by several independent studies.^{33–39} Furthermore, mAbDas-1 can detect colonic metaplasia before the histological appearance of BE, suggesting the existence of a 'Pre-Barrett's' stage.^{34,35} The data suggest that expression of CEP preceded histological BE and persisted during development of metaplasia and progression to carcinoma.

Cox-2 has been implicated in the neoplastic progression in Barrett's esophagus.^{22,24,40} Increased levels of Cox-2 have been reported in Barrett's tissues⁴¹ and in several cases of esophageal adenocarcinoma,^{22,40,42–44} although no causal link has yet been established. Cox-2 expression is generally associated with inflammatory or stress responses.⁴⁵ Cox-2 overexpression is linked with inhibition of apoptosis, increased invasiveness of malignant tumors and enhanced synthesis of tissue prostaglandins^{46–49} in gastrointestinal cancers.

Cytokeratin 8/18 (CK8/18), a marker of columnar epithelium, is not expressed by the squamous epithelium of the esophagus. Overexpression of CK8/18 protein has been reported in some adenocarcinomas,⁵⁰ but its expression in esophageal adenocarcinoma is unknown. CK8/18 has been observed in multi-layered epithelium in BE by immunocytochemistry.⁵¹ This focal multi-layered epithelium within BE comprises cells that concurrently express both squamous and columnar cytokeratin markers, much like the esophageal mucosal gland duct epithelium³⁴ and the non-neoplastic telomerase-immortalized Barrett's cell line, (BAR-T).⁵²

We utilized the BAR-T cell line as a model to identify the specific cell phenotype in a heterogeneous⁵² cell population

that could demonstrate molecular and/or cell phenotypic changes following chronic exposure to acid and bile. We utilized specific cell-phenotype markers: CK8/18 for columnar, CK4 for squamous, mAbDas-1 for colonic phenotype and p75NTR suggested as marker for esophageal progenitors^{53,54} and quantified the cells displaying each phenotype. We compared the expression of all of these markers sequentially in the BAR-T cells, following a single 5-min exposure (acute exposure) to acid (pH 4) or bile at pH 7.4, or bile at pH 4, and then continued similar exposure, every day, for up to 6 weeks (chronic exposure). Cox-2 protein expression was monitored in parallel as an indicator for cellular response to external stress induced by acid and bile salt treatment.

MATERIALS AND METHODS

Cell Line, Media and Cell Culture

BAR-T cells were grown in special supplemented keratinocyte medium (KBM2) from Cambrex Bioscience (East Rutherford, NJ, USA), as per the protocol described by Jaiswal *et al.*²⁰ Hydrochloric acid was used to adjust the pH of the culture medium to experimental conditions. The bile acid, glycochenodeoxycholic acid, GCDA (Sigma, St Louis, MI, USA), was diluted to optimum working concentration of 200 μ M²⁰ with the culture medium adjusted to either pH 4 (bile pH 4), pH 6 (bile pH 6) or pH 7.4 (bile) immediately before being added to the cell culture. For acute exposure, 0.1×10^6 cells growing on six-well plates were incubated in acid and/or bile for 5 min in 24 h. For chronic exposure, cells were exposed for 5 min everyday, for up to 6 weeks. No treatment was done on the day the cells were passed. The time was optimized from similar studies, showing that 5 min was sufficient for induction of signal transduction pathways regulating cellular machinery without cell damage.^{22–24} The cells were rinsed with phosphate buffered saline (PBS) before and after incubation with desired treatment medium. The control untreated cells were grown in parallel in the special medium as mentioned above at pH 7.4. To examine the possible priming effect of acid or bile in possible combinations, we investigated acid treatment followed by bile (A \rightarrow B), bile followed by acid (B \rightarrow A) or acid followed by acid (A \rightarrow A), with a PBS wash between treatments. The cells were replenished with culture medium and allowed to grow in the incubator at 37°C and 5% CO₂ for the duration of the experiment.

Antibodies

The primary antibodies used were mouse monoclonal antibody, mAbDas-1 (IgM isotype), developed in our laboratory against a human colonic epithelial protein, CEP.^{29,31} The Cox-2 monoclonal antibody (IgG isotype) was obtained from Cayman Chemicals (Ann Arbor, MI, USA). Monoclonal antibodies against CK8/18 (IgG clone M20) and CK4 (IgG) were obtained from Abcam (Cambridge, MA, USA), and p75NTR antibody (IgG) was obtained from Novus Biologicals

(Littleton, CO, USA). Anti-actin IgG (clone AC-40) was obtained from Sigma. Isotype-specific murine IgM, IgG (MOPC-IgM and IgG), biotinylated rabbit anti-mouse IgM and streptavidin peroxidase kit were obtained from Dako Corp. (Carpinteria, CA, USA). Carbocyanine fluorophore 2-conjugated goat anti-mouse IgG and phycoerythrin (PE)-conjugated goat anti-mouse IgM (secondary antibodies) were obtained from Jackson Immunologicals (Westgrove, PA, USA). PE-IgM for mAbDas-1 and CY2-IgG for CK8/18 were used for colocalization experiments. Immunogold (10 nm)-labeled secondary IgM was obtained from Electronmicroscopy Sciences (Fort Washington, PA, USA).

Fluorescence-Activated Cell Sorting

Fluorescence-activated cell sorting (FACS) was used to quantify the number of cells expressing various marker proteins. Cells were fixed and permeated with Cytotfix/Cytoperm reagent (BD Biosciences, Pharmingen, San Diego, CA, USA) for 20 min at 4°C. After fixation, cells were washed twice with PBS. Approximately 0.5×10^6 cells in reaction buffer (1% goat serum, 2 mM EDTA in PBS) were incubated for 1 h, or overnight, at 4°C with primary antibodies. The cells were rinsed twice with 30 volumes of FACS wash buffer (0.5% BSA, 2 mM EDTA and 0.005% NaN₃ in PBS) and incubated with corresponding fluorescence-labeled secondary antibodies for 45 min at room temperature. After the final rinse, cells were examined in the Cytomics FC500 cytometer. As the cells were stained with varying intensity and there was no distinct sharp peak, the number of positive cells was calculated using the Overton method of cumulative histogram subtraction.⁵⁵

Immunocytochemical Localization of CEP in BAR-T Cells

- Immunoperoxidase staining: For immunostaining, cytospin preparation of BAR-T cells was fixed and stained using the method previously described.^{29,32} Briefly, the cells were incubated with 1:20 dilution of mAbDas-1 overnight at 4°C followed by biotin-conjugated rabbit anti-mouse secondary IgM (1:50) for 1 h, with avidin-biotin enzyme reagent for 30 min and finally with peroxidase substrate for color development.
- Immunofluorescence staining: Indirect immunostaining of BAR-T cell cytospins was performed using the same reagents and method as mentioned under FACS assay. Stained slides were viewed under a Zeiss fluorescence microscope at $\times 20$ with appropriate filters.
- Electron microscopy: More precise localization of the CEP was performed by electron microscopy (EM) using the immunogold labeling technique. Biopsy tissue from a patient with BE was fixed and embedded in histogel (for preparing 5- to 6- μ m sections). Tissue sections were incubated with mAbDas-1 (1:20) overnight and subsequently with immunogold-labeled secondary antibody (1:5) overnight. After this, tissue was prepared following standard procedure for EM.

Western Blot

Total protein from BAR-T and colon cancer cells LS180 was obtained as follows. Cells were homogenized in lysis buffer (200 mM HEPES, 5 mM KCl, 0.5 mM MgCl₂, EDTA 0.1 mM, SDS 0.1% and protease inhibitor cocktail (Sigma)), centrifuged and the supernatant was collected. The total amount of protein was quantitated and 10 μ g of total protein was resolved in 10% SDS-PAGE gel electrophoresis.^{31,56} After transferring the proteins to the nitrocellulose membrane overnight, the blots were blocked in 1% low-fat milk and then incubated with primary antibody mAbDas-1 overnight at 4°C. The membrane was incubated with biotinylated secondary antibody for 1 h and the chemiluminescence reaction (Perkin Elmer Chemiluminescence kit) was performed following the manufacturer's protocol. Autoradiographs were developed after 5–15 min. The same blots were also incubated with anti-actin antibody as protein loading control.

Proliferation Assay

The proliferation of the cells was measured using 5-bromo 2'-deoxyuridine (BrdU) incorporation-based colorimetric assay (Roche Diagnostics, Indianapolis, IN, USA). Approximately 0.1×10^4 cells from each group were plated in a 96-well plate in triplicate; after acid and/or bile salt treatment for 24 h, BrdU was added for 2 h. Thereafter, the cells were fixed for 30 min at 15–25°C, 100 μ l anti-BrdU POD-conjugated antibody (diluted 1:100) was added per well and incubated for 90 min in the dark, at room temperature. After rinsing the wells three times with wash buffer, 100 μ l substrate solution was added per well and incubated at 15–25°C for 5–30 min. Absorbance was read on a Molecular Devices kinetic microplate reader at 450 nm.

Statistical Analysis

GraphPad Instat3 software (San Diego, CA, USA) was used for all statistical analyses. Analysis of variance was followed by Tukey's multiple comparisons test to determine the significance of the data obtained, represented by * ($P < 0.05$) or ** ($P < 0.01$) for comparisons between untreated control and experimental or treated groups and § ($P < 0.05$) for comparisons between only acid- or only bile-treated and other combination treatment groups. The bars indicate \pm s.e.m.

RESULTS

Telomerase-Immortalized Benign Barrett's Cell Line is a Heterogeneous Mix of Cells Expressing Columnar, Colonic and Squamous Epithelial Cell Phenotypes

CK8/18 and mAbDas-1 staining was observed at low intensity in the majority of the cells. Following quantification from at least six experiments,⁵⁵ it was observed that approximately $32 \pm 3.5\%$ of the BAR-T cells were of colonic phenotype (mAbDas-1 positive) and $35 \pm 5.2\%$ expressed columnar marker CK8/18. Only $9.5 \pm 3\%$ of the cells were positive for squamous cell marker CK4, and a very small population of cells ($4 \pm 2.5\%$), stained for p75NTR, which has been reported

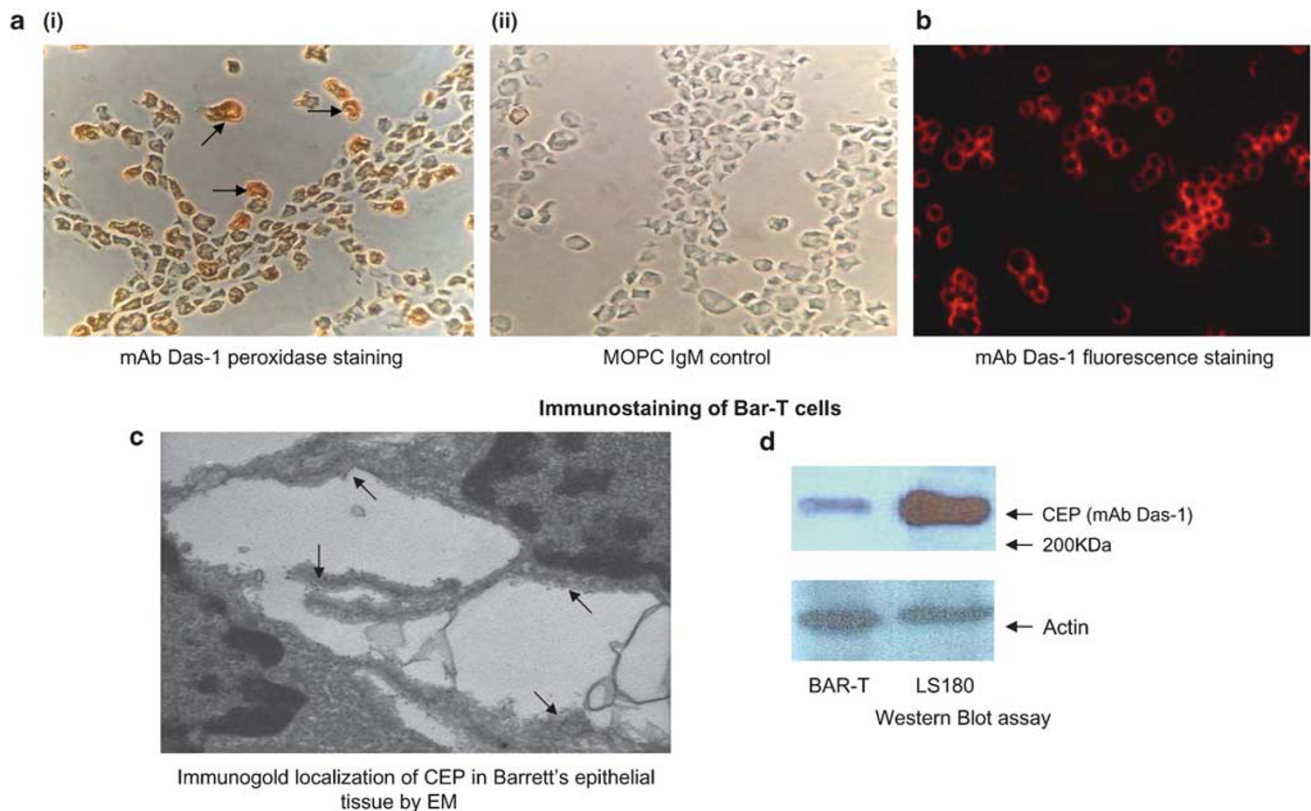


Figure 1 Immunostaining of Bar-T cells. (a) (i) mAbDas-1 peroxidase staining. (ii) MOPC IgM control. (b) mAbDas-1 fluorescence staining. (c) Immunogold localization of CEP in Barrett's epithelial tissue by EM. (d) Western blot analysis of BAR-T cells and LS180 colon cancer cells.

as an esophageal progenitor cell marker.^{53,54} Immunoperoxidase staining of mAbDas-1 (Figure 1ai) showed weak diffuse staining of cytoplasm with increased staining on the periphery of the cells, which is more evident by immunofluorescence assay (Figure 1b). Figure 1aii shows mouse IgM isotype control for immunoperoxidase assay. Immunogold localization of mAbDas-1 reactivity by electron microscope in BE biopsy tissue (Figure 1c) further confirmed enhanced expression of CEP toward the cell membrane. Western blot analysis of cell lysate from BAR-T cells and colon cancer cells LS180 (as a positive control) showed a distinct high molecular-weight protein (CEP) >200 kDa (Figure 1d). The colon cells where CEP was originally identified have much higher expression of the protein than the BAR-T cells.

Double-color FACS analysis showed that almost all of the mAbDas-1-positive cells were also stained for CK8/18 (Figure 2ai–iii). Figure 2aii shows fluorescence microscope picture of double staining for coexpression of CEP and CK8/18. The CK4-positive cells did not stain for CEP (data not shown).

Effect of Single and Repeated Exposure to Acid and Bile on the Expression of CEP and CK8/18

Multiple exposures on the same day resulted in considerable cell death (>80%) due to membrane disintegration, and further analysis was not performed with these cells. When the

BAR-T cells were treated daily with acid and/or bile for 5 min (only once a day), a gradual increase in the expression of CEP was observed after 1 week. The induction continued during the second week and reached a plateau by the third week, which persisted for up to 6 weeks (the maximum duration of the experiments). After 2 weeks of treatment, acid alone induced CEP expression to 1.55-fold ($P < 0.05$), and bile at pH 7.4 as well as at pH 6 to 1.4-fold ($P < 0.05$) when compared to untreated cells of the same duration. Treatment with bile at pH 4, however, caused a 2.1-fold ($P < 0.01$) increase in CEP-positive cells when compared with untreated cells growing for 2 weeks (Figure 3). The increased expression of CEP as observed by FACS (Overton method) is shown in Figure 2b (lower panel).

Cells treated with bile at pH 4 also showed induction of CK8/18 protein expression, up to 1.62-fold ($P < 0.01$) at 2 weeks (Table 1). The acid only-treated group expressed a 1.37-fold ($P < 0.05$) higher CK8/18 protein in 2 weeks compared to the untreated cells, and bile pH 7.4 did not change the expression of CK8/18. Quantification of cells for single protein expression, either CEP or CK8/18, as well as coexpression of both CEP and CK8/18 in the same cells by dual-color protein localization FACS analysis after various treatments is shown in Table 1. Increased expression of both the proteins, CEP and CK8/18, was evident in acid pH 4- as well as bile pH 4-treated cells.

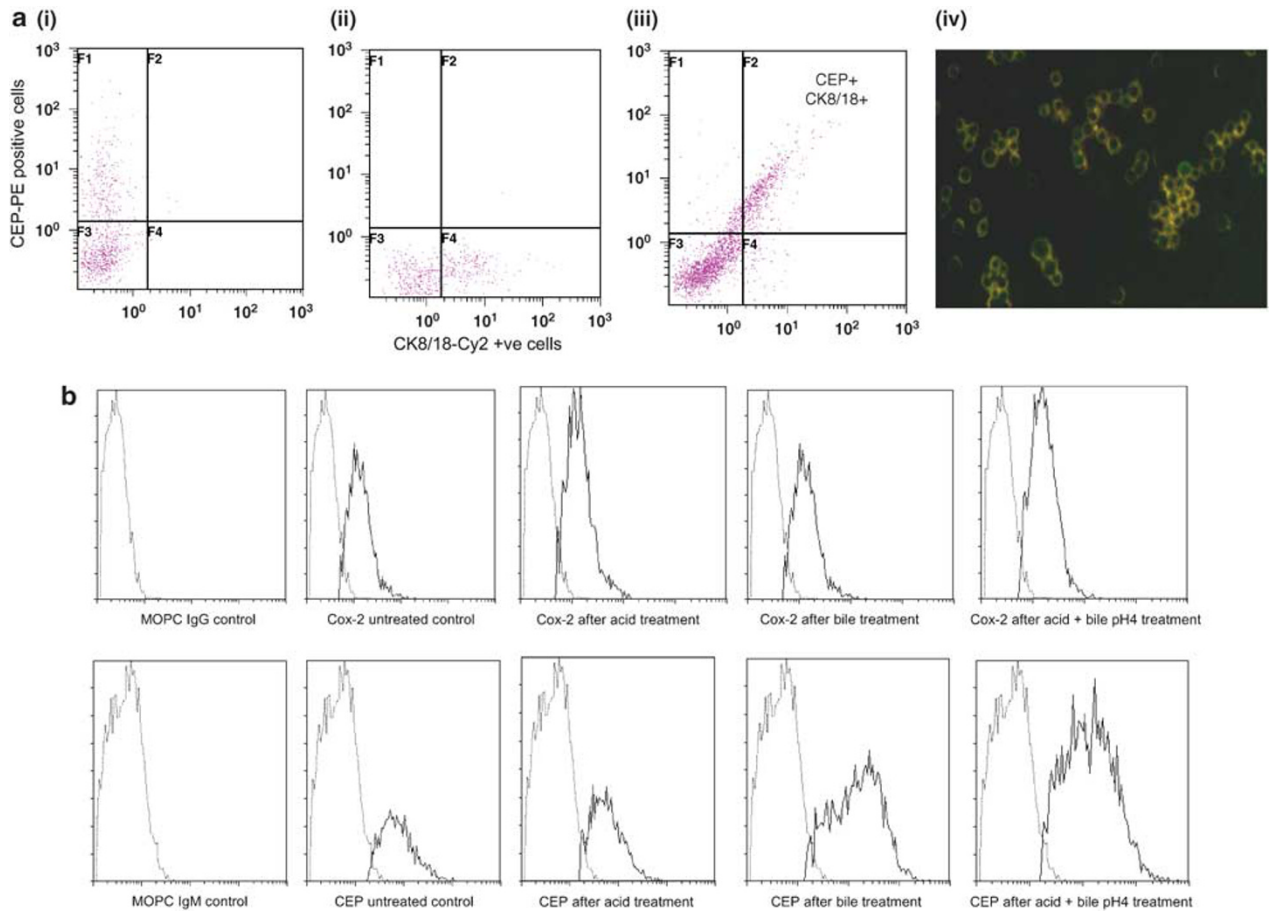


Figure 2 (a) Double-color immunofluorescence assay. (b) Effect of acid and bile on the expression of Cox-2 (upper panel) and CEP (lower panel) in BAR-T cells. (i) and (ii) show single color staining for CEP and CK8/18, respectively, (iii) and (iv) show double color-colocalization by FACS analysis and immunofluorescence microscopy, respectively.

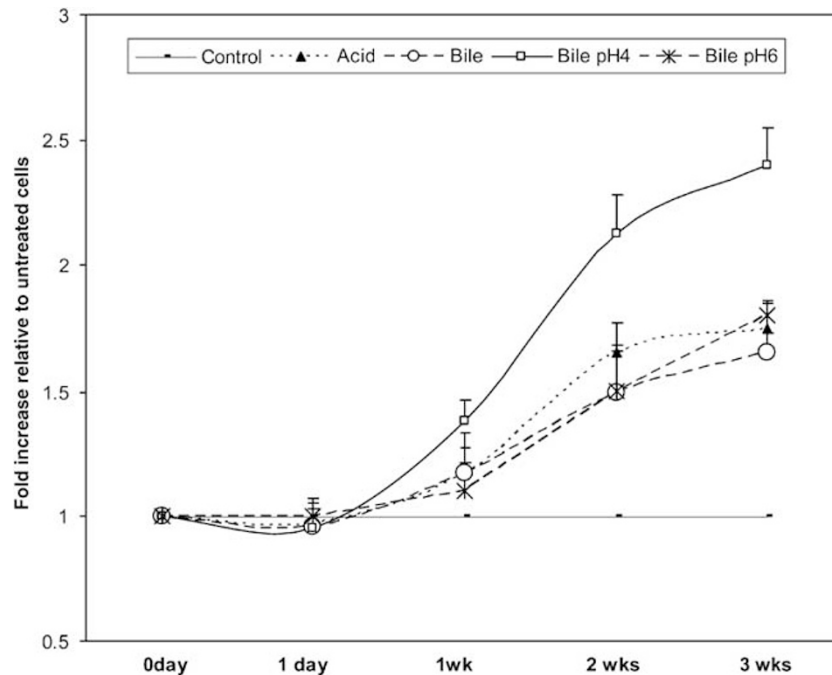


Figure 3 Effect of continued treatment of BAR-T cells with acid and bile on CEP expression.

Table 1 Effect of acid and bile salt treatment for 2 weeks on the expression of different cell phenotypes in the BAR-T cells

	CEP +ve	CK8/18 +ve	CEP +ve CK8/18 +ve	Cox-2 +ve	Cox2 +ve CEP +ve	Cox-2 +ve CEP -ve	CK4 +ve
Control	32 ± 3.5	35.5 ± 5.2	31 ± 1.9	23 ± 1.2	12 ± 2	11.7 ± 4.2	9.5 ± 2.96
Acid pH 4	49.6 ± 7.08*	48.2 ± 5.02*	40.8 ± 0.87*	39 ± 2.8*	19.3 ± 0.9	16 ± 2.3	9.3 ± 2.4
Bile pH 7.4	45.3 ± 1.94*	40.9 ± 4.5	38.3 ± 5.34*	33 ± 5*	14 ± 4	15 ± 3.2	10.3 ± 0.29
Bile pH 4	68 ± 5.4**§	56.7 ± 4.1**§	48 ± 0.49*	47 ± 1.8**§	22 ± 2.5*	21 ± 1.5*	11.3 ± 0.64

Table represents percent positive cells in each group ± s.e.m. obtained from $n = 6$ for single-staining and $n = 3$ for double-staining FACS experiments. The different treatment conditions are shown in horizontal rows and the cell phenotypes in each group recorded in vertical columns. For coexpression analysis, anti-mouse IgM labeled with PE (for anti-mAbDas-1 IgM) and anti-IgG labeled with Cy-2 were used.

* $P < 0.05$ or ** $P < 0.01$ for comparisons between untreated control and experimental or treated groups and § $P < 0.05$ for comparisons between the treated groups by Tukey's multiple comparisons test.

These observations indicate that the cells expressing columnar and colonic phenotype markers were mostly induced in response to acid or bile salt treatment, as there was no change in the number of cells expressing CK4 protein. Both acid and bile salt treatment could individually induce changes of similar magnitude in the expression of colonic and columnar phenotype markers in BAR-T cells. However, bile salt in acidic pH 4 was more effective when compared to either alone (§ $P < 0.05$) (Table 1).

Effect of Successive Exposure to Acid or Bile Salt Treatment

To examine the sequential effects of acid and bile salt treatment, BAR-T cells were exposed to acidic pH 4 for 5 min followed by repeat exposure with same (A → A), daily for 2 weeks, which induced CEP expression by 1.7-fold ($P < 0.05$) compared to untreated cells. Similar treatment for 2 weeks with bile at pH 7.4 following acid (pH 4) priming (A → B) increased CEP expression 1.96-fold ($P < 0.05$), and the third group with acid (pH 4) treatment following bile at neutral pH priming (B → A) induced CEP up to 2.4-fold ($P < 0.01$) when compared to untreated control cells. B → A was also more significant (§ $P < 0.05$) than either acid or bile alone in inducing CEP expression (Figure 4).

Effect of Acid and Bile on Cox-2 Expression in BAR-T Cells

Twenty-three ± 1.2% of the BAR-T cells were stained for Cox-2 protein expression in control untreated condition. Unlike the other proteins, Cox-2 was induced early in the first week of treatment, 1.4-fold by acid (pH 4) alone and 1.5-fold by bile at pH 4, when compared to untreated cells of the same duration. It increased further to 1.75-fold ($P < 0.05$) by acid alone and twofold ($P < 0.01$) by bile at pH 4 at the second week, when compared to untreated control cells. The response to bile salt treatment at pH 7.4 as well as pH 6 was minimal until 2 weeks when it increased to 1.54-fold ($P < 0.05$) compared to the untreated cells (Figure 5). A representative FACS flow diagram of the increase is shown in Figure 2b (upper panel). These elevated levels of Cox-2 were

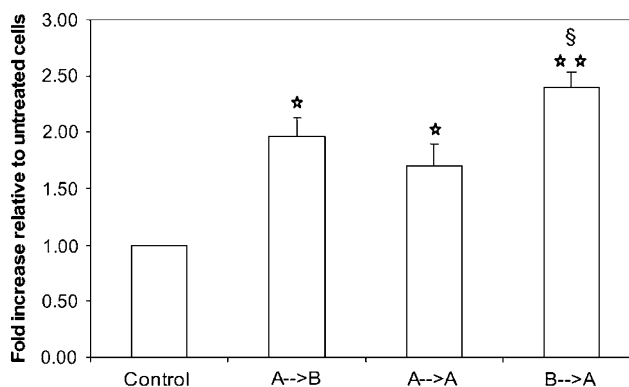


Figure 4 Effect of continued treatment of BAR-T cells for 2 weeks sequentially with acid or bile, on CEP expression. * $P < 0.05$ or ** $P < 0.01$ for comparisons between untreated control and experimental or treated groups and § $P < 0.05$ for comparisons between the treated groups.

maintained beyond 3 weeks with minor changes up to 6 weeks. Double-color immunofluorescence revealed that 12 ± 2% of the total (untreated) Cox-2-positive cells were CEP- and/or CK8/18-positive. After stimulation with acid and/or bile for 2 weeks, the expression of Cox-2 in CEP- and/or CK8/18-expressing cells (major population of cells) was doubled. However, Cox-2 expression was also increased about 1.5-fold in the CEP- and/or CK8/18-negative cells (Table 1).

Proliferation Assay

Considering BrDU incorporation in the control untreated group to be 100, the changes in the treated groups are acid pH 4, 97 ± 12; bile pH 7.4, 98 ± 10; A + B pH 4, 90 ± 10; and A + B pH 6, 105 ± 5. Therefore, acid and/or bile salt treatment for 2 weeks did not alter the proliferation of the BAR-T cells.

DISCUSSION

Intestinal metaplasia in the esophagus and stomach can be divided into complete or small intestinal and incomplete or colonic phenotype on the basis of enzyme assays and the

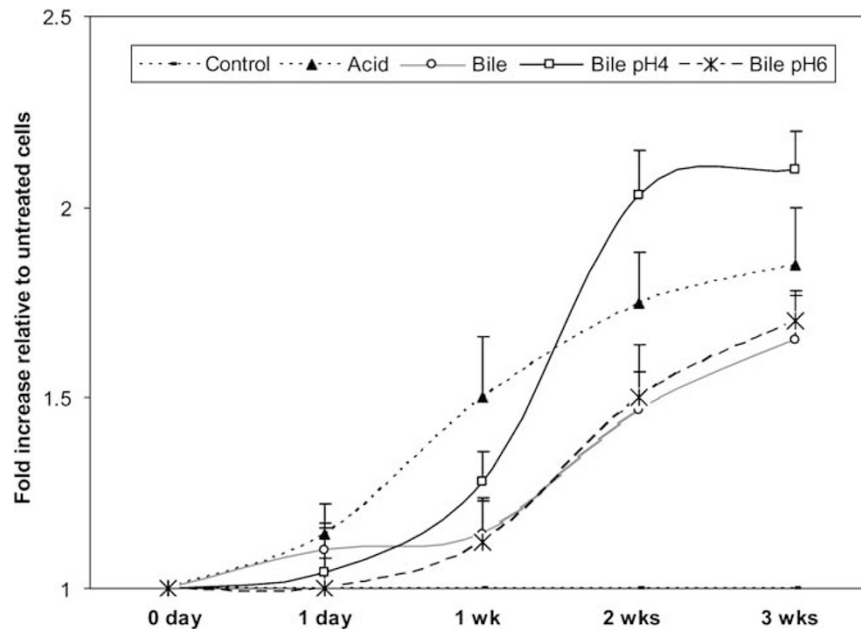


Figure 5 Effect of continued treatment of BAR-T cells with acid and bile on Cox2 expression.

difference in glycoproteins in the goblet cells.^{57–60} Incomplete or colonic phenotype appears to carry the highest pre-neoplastic potential.⁶¹ Colonic type of metaplasia as detected by mAbDas-1 has been reported to be associated with gastric,⁶² small intestinal⁶³ and also urinary bladder carcinoma.^{64,65} Ninety-three percentage of gastric intestinal metaplasia reactive to mAbDas-1 (colonic phenotype) was associated with gastric cancer.^{61,62} These findings support the potential of mAbDas-1 antibody as a preneoplastic marker for colonic phenotype of cells with malignant potential. This study demonstrates that acid and bile salt exposure, over an extended period of at least 2 weeks, can trigger progressive increase of colonic phenotype marker (mAbDas-1), in columnar type cells (CK8/18-positive) in a heterogeneous Barrett's cell line. This change in phenotype markers was not associated with any change in proliferation, although Cox-2 was significantly induced.

Both acid and bile salt were individually capable of inducing gradual increase in the number of colonic phenotype cells after continued treatment for 2 weeks. It is of interest that the most explicit induction of mAbDas-1-positive cells was seen with bile at pH 4, and also when acid exposure followed soon after bile in neutral pH (B→A). The latter findings provide experimental evidence for the clinical observation that GERD patients complicated with BE have a significant increase in bile salt regurgitation.² Taken together, the above findings suggest that *in vivo* bile salt exposure, accompanied by acid, has more injurious effect. In cell-culture experiments, discontinuation of the treatment with acid and bile salt after 2 weeks, at which time maximum mAbDas-1-positive cells were seen, caused regression of CEP. However, re-exposure to acid and bile salt promptly initiated induction of CEP expression within 5 days, much earlier than the cells

which are exposed to acid and bile for the first time (unpublished data). This may provide additional experimental evidence for the necessity of continued acid suppression. Patients with dysplastic BE are reported to show a reversal of the dysplastic process by proton pump inhibitor therapy;^{66–68} however, it is unknown if the lesions will reoccur once such a treatment is stopped.

The BAR-T cell line is reported to be a heterogeneous mix of cells of different phenotypes⁵² and/or cells in different stages of differentiation. We observed an increase in CK8/18 expression in the BAR-T cell line significantly in response to bile at pH 4, as compared to acid alone or bile salt at neutral pH. The cytokeratins comprise the intermediate filament network considered as a rather static structure responsible for mechanical stability of cells. However, the importance of CK8/18 in the maintenance of non-mechanical functions, such as functional integrity of cells and defense against toxic injury as in chronic cholestasis reflecting a cellular stress response, has been reported in a mouse model of liver injury.^{69,70} Upregulation of CK8/18 protein in our experimental model indicates the possibility of a similar protective phenomenon in the Barrett's esophageal cells in response to acid- and bile-induced toxicity. Further investigation of the correlation between CK8/18 upregulation and esophageal injury may explain the appearance of columnar metaplasia at the distal esophagus, as observed in BE.

Colocalization of CK8/18 and CEP indicated that the majority of the columnar cells were of colonic phenotype. However, after treatment, particularly with bile at pH 4, the number of cells expressing colonic phenotype was higher than that expressing CK8/18 phenotype, indicating the expression of CEP in the small percentage of cells that were negative for CK8/18 as well as CK4. It is unknown if these

CK8/18-negative but CEP-positive cells arose from progenitors as result of acid and bile salt treatment. Similar colocalization studies could not be performed with p75NTR-positive population due to very small number of such cells. Further enrichment of p75NTR progenitors is necessary to conclusively assess the contribution of these cells toward differentiation of any specific phenotype. The reactivity of mAbDas-1 against hepatic progenitor cells has been reported by us, earlier.⁷¹ In this study, we also demonstrated that fetal esophageal mucosa reacts with mAbDas-1 but not with adult esophagus. Thus, presence of mAbDas-1 reactivity in fetal esophageal mucosa, followed by its loss in the normal adult esophagus and reappearance of activity in BE as well as in esophageal adenocarcinoma may provide an important clue concerning the lineage analysis of BE.⁷¹ A future study, using P75NTR-enriched cell population, following their differentiation in response to acid and or bile, may provide important information regarding the origin of BE.

We observed induction of Cox-2 expression in our study both in the colonic/columnar and in the non-colonic/non-columnar cell populations of unknown phenotype in response to acid and/or bile treatment/s. There was, however, no significant induction of the CK4 cell phenotype with any of the treatments. Cox-2 upregulation, in response to acute acid and bile salt treatment, has been reported in normal esophagus,²⁴ BE,²⁰ as well as in esophageal adenocarcinoma cells.²¹ Cox-2 suppression therapy has been suggested for better prognostic outcomes in Barrett's carcinomas.⁴³ Upregulation of Cox-2 has been associated with increased proliferation mediated by activation of MAPK pathways, due to acid and bile salt exposure in SEG-1 cells.^{22,23} Such proliferation was not seen in the non-neoplastic BAR-T cells. These differences may be due to difference in cell lines used in the experiments (cancer cell line SEG-1 *vs* benign Barrett's cells) or due to the use of regular 5% serum-substituted media in our experiments to maintain long-term cultures *vs* serum-deprived cells used by others investigators for one-time treatment.

In summary, using a non-neoplastic Barrett's cell line (BAR-T), which is composed of both squamous (CK4) and metaplastic/columnar cells (villin- and alcian blue-positive),⁵² we demonstrate selective increase of CK8/18 type columnar cells, colonic phenotype (mAbDas-1-positive) and Cox-2 expression following chronic (up to 6 weeks) repeated exposure to acid and bile salt. Bile and acid together (A + B pH 4) were found to be more potent than either alone. However, acid exposure alone, immediately following bile priming, was also a potent stimulator of colonic phenotype. As mAbDas-1 reactivity has been shown to be strongly associated with precancerous conditions and adenocarcinomas of the esophagus, stomach and small intestine, induction of this phenotype in the Barrett's cell line may provide an experimental model for further understanding of the pathogenesis of Barrett's metaplasia → dysplasia → esophageal adenocarcinoma.

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