Bile salts induce or blunt cell proliferation in Barrett’s esophagus in an acid-dependent fashion

Kaur, Baljeet S., Rodica Ouatu-Lascar, M. Bishr Omary, and George Triadafilopoulos. Bile salts induce or blunt cell proliferation in Barrett’s esophagus in an acid-dependent fashion. Am J Physiol Gastrointest Liver Physiol 278: G1000–G1009, 2000.—Barrett’s esophagus (BE) results from acid and bile reflux and predisposes to cancer. We investigated the effect of bile salts, with or without acid, on cell proliferation in BE and assessed mechanism(s) involved. To mimic physiological conditions, biopsies of esophagus, BE, and duodenum were exposed to a bile salt mixture, either continuously or as a 1-h pulse, and were compared with control media without bile salts (pH 7.4) for 24 h. Similar experiments were also performed with acidified media (pH 3.5) combined with the bile salt mixture as a 1-h pulse. Cell proliferation was assessed by a [3H]thymidine incorporation assay with or without bisindolylmaleimide (BIM), a selective protein kinase C inhibitor. Bile salt pulses enhanced cell proliferation in BE without affecting cell proliferation in esophageal or duodenal epithelia. In the presence of BIM, there was complete obliteration of the bile salt-induced BE hyperproliferation. In contrast, 1-h pulses of bile salts in combination with acid significantly inhibited proliferation in BE but had no effect on esophagus or duodenum. We conclude that in BE explants, brief exposure to bile salts, in the absence of acid, increases proliferation, whereas exposure to a combination of bile salts and acid together inhibits proliferation.

Gastroesophageal reflux disease; duodenogastric reflux

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BARRETT’S ESOPHAGUS (BE), or specialized intestinal metaplasia, is a heterogeneous premalignant epithelium associated with gastroesophageal reflux and esophageal adenocarcinoma (36). Adenocarcinoma in BE is thought not to arise de novo but rather to follow a multistep process in which the metaplastic epithelium sequentially develops low-grade dysplasia, high-grade dysplasia, early adenocarcinoma, and eventually invasive cancer (29). Currently, with our limited understanding of the pathophysiology of this disease, it is not possible to predict which patients are at risk for cancer and over what time scale. Patients are therefore regularly surveyed with endoscopy and multiple biopsies to assess for the presence and grade of dysplasia/adenocarcinoma (22).

Several studies (16, 41) addressing the natural history and development of BE have examined the role of duodenogastroesophageal refluxate in its pathophysiology. Although its exact pathogenesis is unclear, BE appears to be associated with severe, long-standing reflux of acid, bile, and pancreatic secretions compared with those of patients with esophagitis or normal controls. If acid reflux alone does indeed induce BE, one potential prediction is that acid-suppressive therapy may reverse the metaplastic phenotype of BE to a squamous cell phenotype. However, although acid suppression sometimes leads to the generation of squamous islands, there seems to be no significant reduction in the overall length of Barrett’s epithelium (26). Similarly, antireflux surgery neither induces regression of BE nor eliminates the risk of adenocarcinoma (27, 44). Furthermore, there is no indication that treatment for acid reflux reduces the risk of esophageal cancer. In fact, in a recent study (21), patients who received medical treatment had a higher risk than those who did not receive treatment.

Acid directly affects cell proliferation and differentiation of BE in a dynamic fashion that depends on the pattern of acid exposure (14). For example, whereas long-term acid exposure results in a relatively differentiated phenotype and decreased cell proliferation, short pulses of acid exposure induce cell hyperproliferation. On the basis of these findings, a model was proposed whereby the variable patterns of acid exposure that mimic physiological conditions could at least in part account for the heterogeneous nature of the Barrett’s epithelium and the unpredictable progression to dysplasia. A potential therapeutic implication of this model is that acid-suppressive therapy must be powerful and continuous enough to completely abolish any acid pulses to effectively diminish cell proliferation and promote cellular differentiation in this disease. One important potential consequence of this model is that complete acid suppression may also decrease the risk for dysplasia and adenocarcinoma.

Given the acid pulse-induced effect on cell proliferation and the potential presence of bile in duodenogastroesophageal refluxate, we investigated the effect of bile salts on proliferation of BE epithelia in an ex vivo organ culture compared with normal esophageal (squamous) and duodenal (columnar) controls. The effect of bile
salts on cell proliferation was also contrasted with the effect of acid alone or with acid plus bile salts. Simultaneous 24-h pH and bile monitoring of distal esophagus has shown that, although acid is the primary factor in the development of BE, bile reflux parallels acid reflux and may have a synergistic role (9). Because of their known role in epithelial injury and carcinogenesis, bile acids may act as tumor promoters by increasing cell proliferation via protein kinase C (PKC) activation (10). Similarly, dihydroxy-bile acids activate cyclooxygenase-2 transcription in a human esophageal adenocarcinoma cell line, in association with PKC activation (46).

Our results show that bile salts, administered as a 1-h pulse, induce cell proliferation in BE, possibly through a PKC-dependent mechanism, in a fashion that is similar to the effects noted with an acid pulse-induced proliferation. In contrast, bile salts do not affect cell proliferation of normal esophagus or duodenum. Paradoxically, the bile salt-induced proliferation burst in BE is completely blunted if bile salts are mixed with acid. Thus bile salts, independent of acid, contribute to the proliferative alterations in BE in a dynamic fashion.

MATERIALS AND METHODS

Patients and tissue collection. Endoscopic mucosal samples from normal esophagus (squamous controls), BE, and duodenum (columnar epithelial controls) were obtained from individuals with BE undergoing endoscopic surveillance at the Veterans Affairs Palo Alto Health Care System and Stanford University Hospital as part of an endoscopic surveillance program. Biopsies were collected under a protocol approved by the Administrative Panel on Human Subjects in Medical Research at Stanford University.

Endoscopy and biopsy. Endoscopy was performed using a video-image endoscope, with patients receiving intravenous sedation and continuous cardiorespiratory monitoring. BE was endoscopically defined as the presence of red columnar islands or tongues with squamous esophageal mucosa or by the presence of lighter squamous islands within a circumferential red columnar mucosa present above the endoscopically identified gastroesophageal junction, as previously described (39). For the diagnosis of BE, endoscopic and histological evidence for such epithelium was required. Endoscopic biopsies were taken from each quadrant (4 biopsies) every few centimeters, covering the length of the Barrett's epithelium (22). In addition, mucosal biopsies were obtained from the duodenum and the proximal (squamous) esophagus and used as controls. Endoscopic mucosal samples were immediately divided into two parts. One part was formalin fixed for histopathological assessment; the other part was maintained in tissue culture medium (see Organ culture) for subsequent experimental use.

Histopathology. All specimens were analyzed independently by a staff pathologist to categorize normal esophageal mucosa, esophageal inflammation, metaplasia, dysplasia, and esophageal adenocarcinoma. To confirm the presence of specialized intestinal metaplasia, Alcian blue staining was performed on all BE samples (30). Only BE biopsy samples with incomplete intestinal metaplasia, as defined by a specialized (intestinalized) surface and pit epithelium with goblet cells on hematoxylin and eosin (HE) and Alcian blue stains, were included in the organ culture studies. Morphological assessment by HE stain was performed to ensure histological integrity of organ culture tissues for each time point up to 24 h.

Reagents. All bile acid and salts (sodium glycocholate and taurocholate, glycocholic acid, and taurochenodeoxycholate) and anti-proliferating cell nuclear antigen (PCNA) monoclonal antibodies were obtained from Sigma Chemical (St. Louis, MO). Anti-PKC polyclonal antibodies that recognize all PKC isoforms were obtained from Oncogene Research Products (Cambridge, MA). The specific PKC inhibitor bisindolylmaleimide (BIM) was purchased from Boehringer Mannheim (Indianapolis, IN), and the PKC activator phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma Chemical.

Organ culture. Organ culture was performed essentially as previously described (14, 40). Briefly, multiple mucosal biopsies were cut into two fragments using aseptic technique and randomly assigned to acid, bile acid, or control groups to avoid sampling bias. Four to six biopsy fragments were placed on a sterilized stainless steel grid within 12-well plates so that culture medium (1 ml) covered the surface of the biopsy. Plates were then placed on racks in a sterile sealed jar (Torsion Balance, Clifton, NJ) and perfused with 95% O2-5% CO2 and then cultured at 37°C. For all experiments, we used medium 199 supplemented with 10% heat-inactivated FCS, insulin (1 µg/ml), streptomycin (500 U/ml), and penicillin (250 U/ml). Tissues were exposed to a bile salt mixture (sodium glycocholate and taurocholate, glycocholic acid, and taurochenodeoxycholate; total final concentration, 1 mM; pH 7.4) as a 1-h pulse, followed by incubation in normal medium (pH 7.4). This bile acid mixture concentration was selected because it represents near-physiological constituents and concentrations in patients with BE (16, 38, 25). In all experiments, the bile salt mixture remained in solution, and there was no influence of pH on its solubility. For acidic culture conditions, the medium was acidified for 1-h pulse with 0.1 M HCl (~20% vol/vol, pH 3.5). In all experiments, a volume of distilled water (~20% vol/vol) was added to the control nonacidified medium to achieve an osmolality equal to that of the acid-treated medium. Tissues were exposed to acid alone (in 1-h pulse, pH 3.5), bile salts alone (in 1-h pulse, pH 7.4), acid plus bile salts (in 1-h pulse, pH 3.5), or control medium (pH 7.4). Experiments were conducted for 24 h with collection time points of 1 and 24 h. For the PKC experiments, a 1-h pulse of bile salts was administered either in the absence or presence of the PKC inhibitor (BIM) or activator (PMA). To confirm tissue viability after organ culture, lactate dehydrogenase (LDH) assays were performed using an aliquot of tissue culture medium taken at the end of the culture period and an LDH assay kit (Sigma Chemical) (11). Explants homogenized in medium with Triton X-100 and sonicated before measuring the LDH of the medium were used as positive controls. Control tissues were processed at the same pH as the experimental samples to adjust for pH sensitivity of the LDH assay. The absorbance values (at 490 nm) were expressed as units per milliliter.

Protein extraction and immunoblot analysis. This was performed to assess PCNA or PKC expression in BE epithelia treated with 1-h pulse of bile salts with or without acid. Samples from 1- and 24-h time points of the organ culture were assessed. For each time point, tissues were homogenized (4°C) in 1% deoxycholate, 1% Nonidet P-40, and 0.1% SDS in pH 7.4 PBS containing 5 mM EDTA, 15 µg/ml aprotonin, 10 µM leupeptin, 10 µM pepstatin, and 0.1 mM phenylmethylsulfonyl fluoride (collectively termed DNS buffer). The protease inhibitors were added just before solubilization, and homogenization was done using Kontes glass tissue grinders. Lysates were centrifuged at 16,000 g (20 min at 4°C), and
protein concentrations were measured by the bicinchoninic acid protein assay as recommended by the manufacturer (Pierce, Rockford, IL). Proteins were separated using 12% SDS-PAGE and then transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) in transfer buffer containing 12.5 mM Tris·HCl (pH 8.3), 100 mM glycine, and 20% methanol (40 V for 12 h at 4°C). After transfer, membranes were blocked for 3 h in MT buffer (5% dry nonfat milk in PBS containing 0.1% Tween 20) and then incubated for 2 h (22°C) in 1:1,000 dilution of the anti-PCNA antibody or 1:2,000 dilution of the anti-PKC antibody. Membranes were washed in PBS buffer (containing 0.1% Tween 20) and then incubated with peroxidase-conjugated secondary antibody (1:3,000 dilution) for h at 22°C. After being washed once in PBS buffer (containing 0.1% Tween 20) for 1 h, bands were visualized using an enhanced chemiluminescence system (Dupont-New England Nuclear, Wilmington, DE).

Cell proliferation assays. The proliferation rates (1, 6, 18, and 24 h time points) of normal esophagus, BE, and duodenum organ cultures were assessed by [3H]thymidine incorporation. For this, all endoscopic biopsies were placed in organ culture dishes and then labeled with 1 µCi/ml [3H]thymidine (Dupont-New England Nuclear) followed by incubation for up to 24 h (6). For the bile salt-pulse experiments, [3H]thymidine was added to the culture medium with or without the bile acid mixture only during the 1-h pulse. Fresh medium (pH 7.4) and [3H]thymidine were then added for the additional culture period up to 24 h for either bile acid-pulse or the control groups. The same process was followed for the bile salt plus acid-treated groups. At the end of the incubation, explants were homogenized, passed through a 19-gauge needle, and processed using a cell harvester (Skatron, Tranby, NJ). The incorporated radioactivity was measured and expressed as counts per minute (cpm) per milligram of protein, as determined by protein assay. For the PKC-related studies, 1 µM of BIM or PMA was used.

Statistical analysis. Statistical evaluation was performed using Student's t-test and Wilcoxon's rank-sum test where appropriate, with P < 0.05 being considered significantly different (Sigmastat Scientific Software). Graphically, all results are expressed as means ± SE and are representative of pooled data from at least three experimental groups (Sigmaplot Scientific Software).

RESULTS

Effect of bile salts on cell proliferation. We initially examined the effect of increasing concentrations of bile salts on cell proliferation in explants of normal esophagus, BE, and duodenum from three patients. In these experiments, [3H]thymidine incorporation was assessed as cpm per milligram of tissue protein in mucosal biopsies that were continuously exposed to a bile acid mixture containing 12.5 mM Tris·HCl (pH 7.4) and 0, 1, and 5 mM of a bile acid mixture only during the 1-h pulse. Fresh medium (pH 7.4) and [3H]thymidine were then added for the additional culture period up to 24 h for either bile acid-pulse or the control groups. The same process was followed for the bile salt plus acid-treated groups. At the end of the incubation, explants were homogenized, passed through a 19-gauge needle, and processed using a cell harvester (Skatron, Tranby, NJ). The incorporated radioactivity was measured and expressed as counts per minute (cpm)/mg protein (see MATERIALS AND METHODS).

The concentration of 1 mM and short (1-h) exposure for all subsequent experiments described herein. This bile acid mixture concentration was selected because it represents near-physiological concentrations (4, 16). Figure 2 shows cell proliferation assessed by [3H]thymidine incorporation (in cpm/mg tissue protein) in normal esophagus (Fig. 2A), BE (Fig. 2B), and duodenum (Fig. 2C) after 1-h pulse exposure to bile salts, followed by continuous culture in standard medium for various time points. The BE explants exhibited significantly higher [3H]thymidine incorporation in the presence compared with the absence of bile salts (4,292 vs. 2,269 cpm/mg protein, respectively, at 24 h) than in either normal esophagus (2,421 vs. 2,209 cpm/mg protein, respectively, at 24 h) or duodenum (2,487 vs. 2,441 cpm/mg protein, respectively, at 24 h; P < 0.001) (Fig. 2). Of note, the bile salt-induced effect on cell proliferation of BE (in contrast to normal esophagus and duodenum, Fig. 2) is highly reminiscent of the effect that is noted on exposure of the same epithelia to an acid pulse (14). Bile salt exposure did not cause any significant tissue damage based on the minimal LDH release (<1% of the total, Triton X-100-extracted, positive controls of normal esophagus, BE, and duodenum explants; not shown). Furthermore, light microscopy of HE-stained sections revealed that tissue architecture was well maintained for all tissues cultured in the presence of bile salts (Fig. 3, A and B).

Effect of acid combined with bile salts on cell proliferation. Bile salts in combination with acid in vitro may be more toxic to esophageal cells than acid alone, because they increase the ionic permeability of the esophageal mucosa and make it prone to damage by other compo-
Fig. 3. Tissue viability is preserved in mucosal biopsies cultured ex vivo for 24 h. Morphological changes of esophagus, BE, and duodenum biopsy specimens after 24 h of organ culture are shown. After organ culture, samples for histopathology were placed in formalin, then sectioned and stained with hematoxylin and eosin (see MATERIALS AND METHODS).

A: bile salt (1 mM, pH 7.4) pulse-treated normal esophagus.
B: bile salt (1 mM, pH 7.4) pulse-treated BE.
C: bile salt plus acid (1 mM, pH 3.5)-treated BE.
D: bile salt plus acid (1 mM, pH 3.5)-treated duodenum.
E: bisindolylmaleimide (BIM) (1 µM, pH 7.4)-treated BE. At 24 h, there is no evidence of morphological damage in any of the samples.

Fig. 2. Bile salt pulse enhances [³H]thymidine incorporation in BE. Mucosal biopsies from normal (squamous) esophagus (A), BE (B), and normal duodenum (C) were incubated for 24 h with either medium alone (pH 7.4) (−BS) or 1-h pulse of bile salts (final concn, 1 mM; pH 7.4) (+BS), followed by incubation in regular medium, as described in MATERIALS AND METHODS. [³H]thymidine was added to the culture medium with or without bile salt mixture for the 1-h pulse. Fresh medium (pH 7.4) and [³H]thymidine were then added for additional culture period up to 24 h for the bile salt-pulse and control groups. After 24 h, explants were rinsed thoroughly, homogenized, and processed. Incorporated radioactivity was measured and expressed as cpm/mg tissue protein (see MATERIALS AND METHODS). There is a significant enhancement of cell proliferation of BE, starting as early as 1 h after exposure when comparing [³H]thymidine incorporation in the presence or absence of bile salts (P < 0.001). In contrast, the bile salt pulse has no effect on cell proliferation of mucosal explants from normal esophagus or duodenum.
nents of the refluxate, such as acid (19). Figure 4 shows that a 1-h pulse of the combination of acid and bile salts (pH 3.5), followed by restitution to pH 7.4 and continuous culture in regular medium, dramatically decreased [\(^3\)H]thymidine incorporation only in BE explants (Fig. 4B, P < 0.001). In contrast, the combination of acid and bile salts had no effect on normal esophagus (Fig. 4A) or duodenum (Fig. 4C). Under these experimental conditions, there was no evidence of acid-induced bile salt precipitation and no effect on cell viability as determined by LDH release (not shown). Light microscopy of HE-stained sections revealed that tissue architecture was well preserved (Fig. 3, C and D).

We also examined the effect of acid and/or bile salts on cell proliferation by assessing PCNA expression. For this, we performed organ culture experiments as described above using 1-h pulses of medium alone (pH 7.4), acid alone (pH 3.5), bile salts alone (pH 7.4), and acid plus bile salts (pH 3.5), followed by continuous culture in regular medium (pH 7.4). Figure 5 depicts representative immunoblots of mucosal lysates taken from normal esophagus, BE, and duodenum. An increase in PCNA expression is seen at both 1 and 24 h in response to a 1-h pulse of acid or bile salts compared with control medium. In contrast, an attenuation of PCNA expression at 1 and 24 h was noted in response to a 1-h pulse of acid in combination with bile salts.

Inhibition of PKC inhibits hyperproliferative response of BE to bile salts. Bile salts may act as tumor promoters by increasing cell proliferation via PKC activation as noted in colonic epithelial cell cultures (10). To study the potential role of PKC in the bile salt-induced hyperproliferative response of BE, we carried out the proliferation assays in the presence of a specific PKC inhibitor, BIM. As shown in Fig. 6B, BIM inhibited the hyperproliferative effect of bile salt pulse, whereas proliferation in the control groups (BIM treated in the absence of bile salts) was similar to what is seen in the absence of BIM (not shown but similar to data shown in Fig. 2). No effect on [\(^3\)H]thymidine incorporation was noted in BIM-treated normal esophagus (Fig. 6A) or duodenum (Fig. 6C). In addition, there was no evidence of morphological damage to any of the tissues exposed to BIM with (not shown) or without (Fig. 3E) the bile salt mixture. These experiments suggest that PKC activation may be a mechanism for the bile salt-induced proliferation in BE.

Fig. 4. Bile salt pulse in combination with acid inhibits [\(^3\)H]thymidine incorporation in BE ex vivo. Mucosal biopsies from esophagus (A), BE (B), and duodenum (C) were incubated with either 1-h pulse of bile salt mixture (1 mM, pH 7.4) or 1-h pulse of bile salts combined with acid (final bile salt concn, 1 mM; pH 3.5) (+BS/acid), followed by incubation in regular medium. [\(^3\)H]thymidine was also added to culture medium during 1-h pulse, and then fresh medium (pH 7.4) and [\(^3\)H]thymidine were added for additional culture period up to 24 h for experimental or control groups. At 24 h, explants were rinsed thoroughly, homogenized, and processed. Incorporated radioactivity was measured and expressed as cpm/mg tissue protein (see MATERIALS AND METHODS). Note significant inhibition of cell proliferation of BE. In contrast, bile acid in combination with acid pulse has no effect on cell proliferation of mucosal explants from normal esophagus or duodenum. –BS, medium alone.
PKC immunoreactivity is enhanced in response to either bile salts or acid pulses and attenuated in response to acid in combination with bile salts. Figure 7A depicts Western blots of PKC isoforms (molecular mass 93 and 80 kDa) in detergent lysates of mucosal biopsies taken from BE after 24 h of organ culture. Mucosal explants were retrieved after 1 and 24 h of organ culture, solubilized, and then analyzed by immunoblotting as described in MATERIALS AND METHODS. An increase in PKC expression was noted at 1 and 24 h in response to 1-h pulse of bile salts or acid compared with control medium. In contrast, an attenuation of PKC expression was noted at 1 and 24 h in response to 1-h pulse of acid in combination with bile salts (A + BS). Subscripted 1 and 24 indicate 1 and 24 h of culture, respectively.

PKC activation and BE proliferation. To further support the hypothesis that PKC may play a role in BE proliferation, we examined the effect of PKC activator PMA on [3H]thymidine incorporation in BE explants. As shown in Fig. 7B, a PMA pulse for 1 h (1 µM) significantly increased cell proliferation as judged by [3H]thymidine incorporation in BE explants starting at 18 h and increasing over time up to 24 h after exposure (P < 0.05). This effect was similar in magnitude to the effect noted with either continuous or pulse exposure to bile salts (see Figs. 1 and 2), suggesting that cell proliferation was maximally stimulated under these conditions. In contrast, no significant effect was noted with explants from normal esophagus or duodenum in response to 1-h PMA pulse.

DISCUSSION

Our ex vivo experiments demonstrate for the first time that bile salts affect cell proliferation in BE and that such effect correlates with increased PKC expression. While under neutral pH, 1-h pulses of bile salts result in enhanced cell proliferation in BE. In contrast, in the presence of acid (pH 3.5) bile salts decrease cell proliferation without causing morphological damage. These findings may be significant in terms of the heterogeneity and the variable risk of neoplastic progression of BE.

Despite ongoing efforts to characterize the molecular changes in BE, its pathogenesis and propensity to dysplasia and esophageal adenocarcinoma remain poorly understood (33). A growing number of abnormalities in cell proliferation, oncogene activation, tumor suppressor gene inactivation, and growth factor effects are gradually being described that suggest a multifactorial and multistage disease. To understand the malignant progression of BE to adenocarcinoma, an understanding of the spectrum and the relationship of local and genetic factors involved is critical. Understanding the role of local factors that may modulate Barrett's epithelium, such as acid and/or bile, will allow their modification by medical or surgical therapy and may in turn alter the natural history of this condition (12, 37, 42). In this regard, two main questions need to be addressed. 1) Are local factors important in the malignant progression of Barrett's metaplasia or do they only promote the metaplastic change from squamous to columnar epithelium? 2) Which local factors (dietary constituents, acid, bile, and/or other gastroduodenal refluxate constituents) are most important and how do they result in the genetic and molecular changes that lead to malignant transformation? Most studies (43) to date have concentrated on correlation of BE with local factors or molecular changes, whereas little information exists about their causal relationship. One could imagine a scenario wherein a local trigger (i.e., acid, bile salts) for an increase in cell proliferation predisposes cells to genetic mutations. As a result of these genetic alterations, cell proliferation is deregulated further and more genetic abnormalities accumulate. A vicious cycle is thus set up, and the accumulation of critical genetic errors (e.g., p53 mutations) may eventually lead to a clone of malignant cells.

In this study, we examined the relationship between BE and local factors to which this metaplastic tissue is exposed. Specifically, because BE usually develops in the context of chronic reflux of gastric acid and intestinal juices into the esophagus, we studied how bile salts (alone or in combination with acid) affect its proliferation. In the experiments described, we used tissue samples from patients with BE undergoing surveillance endoscopy and monitored their cellular behavior in the presence or absence of acid and bile in the laboratory setting of organ culture. We hypothesized that if these components of the duodenogastric reflux-
ate increase cell proliferation and in turn the risk of dysplasia in BE, effective inhibition of bile reflux with medical or surgical therapy would substantially decrease the risk for esophageal adenocarcinoma.

Hyperproliferative response of BE to bile salt pulse. With regard to cell proliferation markers, \( ^{3}H \) thymidine uptake and PCNA expression were evaluated. Although \( ^{3}H \) thymidine uptake during the S phase of the cell cycle is a sensitive method for evaluating cell proliferation, we also assessed PCNA levels, which accumulate progressively through the G1 to M phases of the cell cycle and disappear at the end of mitosis. Several studies previously demonstrated that PCNA expression is increased in BE, compared with other glandular epithelia, and can thus serve as a marker of cell proliferation in our ex vivo organ culture system. Because of the complex nature of the mucosal explants containing multiple cell types, caution is needed when interpreting the \( ^{3}H \) thymidine-uptake studies. However, previous reports using PCNA immunohistochemistry have confirmed that the glands are the main proliferative compartment in BE and that other cell types, such as lymphocytes, are not a significant proliferative population. It is not clear that the results of these reports can be extrapolated to the bile salt-induced effects, so PCNA immunolocalization studies are necessary. Although continuous exposure to bile salts increased proliferation in BE, we chose a short (1 h) bile salt pulse to mimic the physiological effect of short reflux episodes and in light of our previous data that, in response to similarly short acid pulses, BE epithelia proliferate. Studies with both pH and bile probes in BE have shown a variable pattern of bile exposure, sometimes for prolonged periods of time. Therefore, the physiological relevance of our ex vivo observations must be further substantiated.

PKC overexpression as a potential mechanism for bile salt-induced enhanced proliferation in BE. Members of the PKC superfamily play key regulatory roles in a multitude of cellular processes and particularly in cell proliferation. In turn, bile salts are known to stimulate the proliferative activity of intestinal epithelia, an action likely related to the ability of these agents to act as tumor promoters in human carcinogenesis. In our experiments, pharmacological inhibition of PKC obliterated the bile salt-induced hyperproliferative response of BE to a level similar to that of the control group (Fig. 6), suggesting that PKC may be an

Fig. 6. Inhibition of protein kinase C (PKC) attenuates bile salt pulse-enhanced \( ^{3}H \) thymidine incorporation in BE. Mucosal biopsies from esophagus (A), BE (B), and duodenum (C) were incubated with either 1-h pulse of BIM (1 µM, pH 7.4) (+BIM) or bile salt pulse (final conc, 1 mM; pH 7.4) in combination with BIM (+BS/BIM), followed by incubation in regular medium. \( ^{3}H \) thymidine was added to culture medium for 1-h pulse. Fresh medium (pH 7.4) and \( ^{3}H \) thymidine were then added for additional culture period up to 24 h for either group. At 24 h, explants were rinsed thoroughly, homogenized, and processed. Incorporated radioactivity was measured and expressed as cpm/mg protein (Materials and Methods). In the presence of BIM, bile salt-induced enhancement of cell proliferation of BE is inhibited (compare B with Fig. 2B). BIM alone has no effect on cell proliferation of mucosal biopsies from esophagus, BE, or duodenum.
important mechanism for cell proliferation after bile salt exposure. In contrast, cell proliferation in BE was induced by PMA, a PKC activator, to a level similar to that reached by bile salt pulse exposure (Fig. 7B).

Together with the increased PKC expression in BE epithelia exposed to acid or bile salts but not acid and bile salts together (Fig. 7A), these data imply that PKC is exerting a mitogenic effect in BE and support previous observations (10) in intestinal epithelia exposed to bile acids. It remains to be determined, however, whether bile salts activate PKC directly or indirectly, possibly through an increase in diacylglycerol content of the BE epithelium.

Acid and bile salt combination decreases proliferation in BE. The attenuation of cell proliferation by the combined exposure to both acid and conjugated bile acids may be explained by the predominance of the nonionized form of conjugated bile acids in low pH values (5). Bile acids enter the mucosal cells in the nonionized form through the lipophilic lipid membrane and then accumulate intracellularly because of salt formation and subsequent entrapment (31). High concentrations of bile acids cause intracellular damage by the dissolution of cell membranes and mucosal tight junctions (4). Bile salts may also allow acid to gain access to the mucosal and submucosal regions, causing proteolytic damage and inhibition of cell proliferation (32). Furthermore, conjugated bile acids may precipitate out of solution at higher pH (42). In our experiments, however, we did not observe any precipitation of bile salts; nor did we observe any significant morphological

Fig. 7. Effect of bile salts and/or acid on PKC levels and activation on cell proliferation ex vivo. A: Western blots of PKC isoforms (molecular mass 93 and 80 kDa) in detergent lysates of mucosal biopsies from BE after 1 and 24 h of organ culture. Mucosal explants were retrieved after 1 and 24 h of organ culture, solubilized, and then analyzed by immunoblotting as described in MATERIALS AND METHODS. PKC expression is increased at 1 and 24 h in response to 1 h pulse of bile salts (BS) or acid (A), compared with control medium (Co). PKC expression is attenuated at 1 and 24 h in response to 1 h pulse of acid in combination with bile salts (A + BS). Subscripted 1 and 24 indicate 1 and 24 h of culture, respectively. B: mucosal biopsies from esophagus (E), BE, and duodenum (D) were incubated with either 1 h pulse of the PKC activator phorbol 12-myristate 13-acetate (PMA; 1 µM, pH 7.4) or control medium (Co) (pH 7.4), followed by incubation in regular medium; [3H]thymidine was added to culture medium for 1 h pulse. Fresh medium (pH 7.4) and [3H]thymidine were then added for additional culture period up to 24 h for either group. At 1, 6, 18, and 24 h, explants were rinsed thoroughly, homogenized, and processed. Incorporated radioactivity was measured and expressed as cpm/mg tissue protein (see MATERIALS AND METHODS). PMA induced enhancement of cell proliferation of BE (P < 0.05) but had no effect on cell proliferation of mucosal biopsies from esophagus or duodenum.

Fig. 8. Hypothetical model for variable effect of bile salts with or without acid on BE in vivo. Effect of multiple pulses of bile salts in the absence of acid (●) and multiple pulses of bile salts in the presence of acid (▲) on cell proliferation are compared with baseline condition of no acid or bile salt exposure (○). This dynamic effect of bile salts with or without acid results in a heterogeneous cell population with potential implications on dysplasia risk (bottom).
damage by either histology (Fig. 3) or LDH assays (not shown) of the explants and surrounding medium.

An in vivo model for the dynamic effect of bile salts with or without acid on cell proliferation in BE. Extrapolating our data to the situation in vivo, one model that can be envisioned is that variable patterns of bile salts and acid exposure in BE may contribute to the complex, heterogeneous epithelium seen in BE with a consequent variable risk of neoplastic progression (Fig. 8). As such, BE epithelia pulsed with bile salts in the absence of acid would proliferate preferentially and might in turn have a higher risk of developing dysplasia (13, 45). In contrast, BE epithelia exposed to a mixture of acid and bile salts would proliferate slowly and hence would have lower likelihood of progressing toward dysplasia. BE cells that are relatively protected from either acid or bile salts would maintain their basal state of differentiation and proliferation. This model assumes that cells at the same level of the esophagus do indeed have variable degrees of exposure to gastroduodenal contents refluxing through a transiently incompetent lower esophageal sphincter. Primary or secondary esophageal peristalsis leading to variable esophageal clearance provides an ever changing, highly dynamic environment for the esophageal mucosa. Studies (24) using dual pH probes that are placed equidistant from the lower esophageal sphincter have yielded markedly different pH readings, implying that variations in the pattern of acid exposure do exist within the esophageal mucosa at the same level. This model expands our (14) previous observations that acid alone has a variable effect on differentiation and proliferation of BE epithelia and has wide implications for the development of dysplasia and esophageal adenocarcinoma.

In conclusion, we have demonstrated using an ex vivo BE culture model that bile salts, administered either continuously or as a 1-h pulse, induce cell proliferation in BE, possibly through a PKC-dependent mechanism. In contrast, bile salts have a minimal effect on cell proliferation of normal esophagus or duodenum. Paradoxically, bile acids mixed with acid block each other’s pulse-induced proliferative burst in BE explants. These results raise the possibility that variation in acid and bile exposure may contribute to the proliferative alterations and the molecular and structural heterogeneity seen in BE patients. There is laboratory evidence (20) that the presence of acid protects rodents from developing adenocarcinoma secondary to bile and carcinogen exposure. Although our data suggest a role for PKC activation as a mechanism for the bile salt-induced hyperproliferative response of BE, additional studies will be needed to assess whether PKC plays a direct role in this response and the nature of the PKC isoforms involved. One possible important therapeutic implication of our proposed model is that acid-suppressive therapy alone may not be adequate in blunting cell proliferation in BE unless it is associated with effective inhibition of bile reflux for those patients that do indeed have significant bile reflux into the metaplastic esophagus. Interestingly, all clinical studies (34, 35) to date have failed to show a reduction of dysplasia risk in patients with BE treated with acid-suppressive therapy. Furthermore, it has been suggested (1-3) that prolonged acid suppression in patients with BE may promote duodenogastroesophageal reflux, causing progression to Barrett’s adenocarcinoma. Further understanding of the influence and interaction of the various components of gastroduodenal reflux on the phenotype and behavior of BE epithelia will in turn allow for a more rational and evidence-based disease prevention and treatment.

We thank Kris Morrow for medical illustration and the faculty, fellows, and staff of the Endoscopy Units of the Veterans Affairs Palo Alto Health Care System and Stanford University Hospital for assistance in retrieval of mucosal specimens.

This study was supported in part by the Cancer Research Foundation of America (B. S. Kaur), the Estelle B. Simon Fund at Stanford University (G. Triadafilopoulos), and a Veterans Affairs Career Development and Merit Award (M. B. Omary).

Address for reprint requests and other correspondence: G. Triadafilopoulos, Gastroenterology Section (111-GI), Veterans Affairs Palo Alto Health Care System, 3801 Miranda Ave., Palo Alto, CA 94304 (E-mail: vagt@eland.stanford.edu).

Received 9 December 1999; accepted in final form 19 January 2000.

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