Bile salts disrupt human esophageal squamous epithelial barrier function by modulating tight junction proteins

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Chen X, Oshima T, Shan J, Fukui H, Watari J, Miwa H. Bile salts disrupt human esophageal squamous epithelial barrier function by modulating tight junction proteins. Am J Physiol Gastrointest Liver Physiol 303: G199–G208, 2012. First published May 10, 2012; doi:10.1152/ajpgi.00454.2011.—Reflux of acid and bile acids contributes to epithelial tissue injury in gastro-esophageal reflux disease. However, the influence of refluxed material on human esophageal stratified epithelial barrier function and tight junction (TJ) proteins has not been fully elucidated. Here, we investigated the influence of acid and bile acids on barrier function and TJ protein distribution using a newly developed air-liquid interface (ALI) in vitro culture model of stratified squamous epithelium based on primary human esophageal epithelial cells (HEECs). Under ALI conditions, HEECs formed distinct epithelial layers on Transwell inserts after 7 days of culture. The epithelial layers formed TJ, and the presence of claudin-1, claudin-4, and occludin were detected by immunofluorescent staining. The NP-40-insoluble fraction of these TJ proteins was significantly higher by day 7 of ALI culture. Exposure of HEECs to pH 2, and taurocholic acid (TCA) and glycocholic acid (GCA) at pH 3, but not pH 4, for 1 h decreased transepithelial electrical resistance (TEER) and increased paracellular permeability. Exposure of cell layers to GCA (pH 3) and TCA (pH 3) for 1 h also markedly reduced the insoluble fractions of claudin-1 and -4. We found that deoxycholic acid (pH 7.4 or 6, 1 h) and pepsin (pH 3, 24 h) significantly decreased TEER and increased permeability. Based on these findings, ALI-cultured HEECs represent a new in vitro model of human esophageal stratified epithelium and are suitable for studying esophageal epithelial barrier functions. Using this model, we demonstrated that acid, bile acids, and pepsin disrupt squamous epithelial barrier function partly by modulating TJ proteins. These results provide new insights into understanding the role of TJ proteins in esophagitis.

esophageal epithelium; claudin; air-liquid interface

THE HUMAN ESOPHAGUS IS LINED with a layer of nonkeratinizing stratified squamous epithelium, which serves as an effective barrier against the influx of luminal contents. The esophageal epithelium consists of the basal, suprabasal, and superficial layers (14). In the pathogenesis of reflux esophagitis, acids, bile acids, and pepsin play a major role in damaging the esophageal epithelium (34). However, the mechanisms by which the luminal contents affect the barrier function of the stratified epithelial cell layers remain unclear.

Tight junctions (TJs) separate the apical and basolateral cell surface domains to establish cell polarity and establish barrier function. TJs are composed of several proteins, including occludin, claudins, junctional adhesion molecule (22), and the scaffold protein zonula occludens (ZO). Of these proteins, claudins, which form specialized cellular structures that regulate the permeability of cellular layers, are the most important structural and functional components of TJ strands (1, 6, 10, 30). Claudins are an integral transmembrane protein family consisting of at least 27 subtypes in mammals (22, 40) and are essential for establishing and maintaining epithelial cell polarity by controlling the movement of proteins and lipids within the plasma membrane (40).

To evaluate esophageal epithelial barrier function, monolayers of esophageal cancer cells or immortalized cells are typically exposed to chemical injury factors from the apical surface (40). However, these cell types do not represent the true esophageal mucosa and are not representative for the study of barrier function. Thus an in vitro model that consists of stratified epithelial cell layers is necessary to study esophageal barrier function. We recently established esophageal-like nonkeratinized stratified epithelial multilayers with a squamous epithelial phenotype in vitro using normal human bronchial epithelial (NHBE) cells cultured under air-liquid interface (ALI) conditions (3, 28, 29). It was demonstrated that the ALI-cultured NHBE cells formed tight barriers such as in esophageal biopsy specimens and were damaged by treatment with acid (28). However, as NHBE cells are of columnar epithelial cell origin, studies using these cells may have limited relevance to squamous cells.

Here, we attempted to establish an in vitro multilayer model of human esophageal stratified epithelium using normal human esophageal epithelial cells (HEECs). Using this model, we examined the influence of acid, bile acids, and pepsin on the barrier function of esophageal stratified epithelial cells and evaluated the influence of acidic bile salts on the expression of TJ proteins.

MATERIALS AND METHODS

Cell culture. HEECs and Epithelial Cell Medium-2 (EpiCM-2) were purchased from ScienCell Research Laboratories (Carlsbad, CA). HEECs were routinely cultured in EpiCM-2 without antibiotics at 37°C in a balanced air humidified incubator under an atmosphere of 5% CO₂. Cells that had reached ~80% confluence were harvested using a 0.05% trypsin-EDTA solution and stored frozen in cell bank-2 solution (serum-free type; Nippon Zenyaku Kogyo, Fukushima, Japan). HEECs at passages 4 to 7 were used for experiments.

Transwell-Clear inserts (1.12 cm², 0.4-μm pore size; Costar, Cambridge, MA) were coated with collagen (0.033 mg/ml), human fibronectin (0.01 mg/ml), and BSA (0.03 mg/ml) in PBS for 1 h at room temperature (RT). The coating mixture was removed, and inserts were then equilibrated with EpiCM-2 (0.2 ml apically and 0.3 ml basolaterally) for 1 h at RT. Following removal of EpiCM-2, HEECs were diluted to 80,000 cells/ml in EpiCM-2, and 0.5 ml of the cell suspension was then seeded onto each insert. EpiCM-2 (1.5 ml) was added into the basal chamber. Medium was changed every 2 days until the cells reached confluence (~4 days). ALI culture was initiated by removing the apical medium and decreasing the basal chamber me-
dium volume to 600 μL. ALI medium was composed of EpiCM-2 and DMEM (1:1) and was changed daily. Any medium that leaked through to the apical side was carefully removed.

Reagents and antibodies. Rabbit anti-claudin-1 (catalog no. 519000), mouse anti-claudin-4 (catalog no. 329400), mouse anti-occludin, and Alexa 488-conjugated anti-mouse IgG antibodies were purchased from Invitrogen (Carlsbad, CA). Cy3-conjugated mouse anti-rabbit IgG antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Taurocholic acid (TCA) and glycocholic acid (GCA) were purchased from Calbiochem (Milan, Italy), and deoxycholic acid (DCA) was purchased from Wako Pure Chemical Industries (Tokyo, Japan). All other reagents were purchased from Sigma (St. Louis, MO), unless stated otherwise.

Measurement of transepithelial electrical resistance. Resistance across the stratified epithelium was measured using a Millicell-ERS (Millipore, Bedford, MA) equipped with "chopstick" electrodes in accordance with a previously reported method (31). The value obtained from a blank insert was subtracted to give the net resistance, which was then multiplied by the total membrane area to give the resistance in area-corrected units (Ω·cm²). The change in electrical resistance is reported relative to the measured baseline resistance.

To equilibrate ALI-cultured cells on the membrane for transepithelial electrical resistance (TEER) measurements, ALI medium (pH 7.4) was added into the apical chambers followed by recording of TEER values, and cells were incubated in a 5% CO₂ incubator at 37°C for 1 h. Apical medium was then changed to ALI medium containing acid, acidic bile salts, or pepsin. TEER values were recorded for each well before (time 0 h; control value) and after the treatment for 1 to 24 h, as described previously (3).

Immunofluorescent staining of junctional proteins. HEECs were cultured under ALI conditions and were exposed to stimulants or appropriate buffer controls. Samples were fixed with 10% formalin solution and embedded in paraffin. Sections (3 μm thick) were cut and stained with hematoxylin and eosin and for TJ-related proteins. After antigen retrieval by autoclave in HistoVT One (Nacalai Tesque, Kyoto, Japan), primary rabbit anti-claudin-1 and -occludin polyclonal antibodies and mouse anti-claudin-4 monoclonal antibodies were used at a concentration of 4–16 μg/ml. Cy3-conjugated goat anti-rabbit IgG and Alexa 488-conjugated goat anti-mouse IgG secondary antibodies were used at a 1:2,000 dilution. The specificity of the reaction was tested by incubation with HBSS, nonimmune rabbit serum, or mouse IgG1 (Dako, Glostrup, Denmark). Slides were viewed using a fluorescence microscope (OPTIPHOT-2; Nikon, Tokyo, Japan). Pictures were recorded on a Windows computer.

Western blot analysis of cell lysates. Cells were collected after 1 h of stimulation, and proteins were extracted with NP-40 lysis buffer (25 mM HEPES/NaOH pH 7.4, 150 mM NaCl, 4 mM EDTA, 25 mM NaF, 1% NP-40, 1 mM Na3VO4, protease inhibitor) or SDS lysis buffer (25 mM HEPES/NaOH pH 7.5, 4 mM EDTA, 25 mM NaF, 1% SDS, 1 mM Na3VO4) for the soluble and insoluble fractions, respectively. Protein concentrations were determined using a Bradford assay kit (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer’s instructions.

Equal quantities of protein were separated by electrophoresis on 12% SDS-PAGE gels. Gels were transferred to nitrocellulose membranes (Hybond ECL; GE Healthcare UK, Little Chalfont, UK). Ponceau S staining of the membranes was analyzed for the amount of loaded protein. Membranes were then blocked with 5% milk powder in PBS for 1 h at RT. Membranes were washed three times at 5 min/wash with wash buffer (0.1% milk powder in PBS) and were then incubated with the appropriate primary antibody overnight at 4°C. Membranes were washed again in 0.1% milk-PBS three times at 5 min/wash with wash buffer and were then incubated with the appropriate secondary horseradish peroxidase-conjugated anti-rabbit or anti-mouse IgG antibody for 1 h at RT. Finally, membranes were washed three times at 5 min/wash with wash buffer, and bands were detected with ECL-Plus Western Blotting Detection system (GE

Fig. 1. Morphology and barrier function of air-liquid interface (ALI)-cultured human esophageal epithelial cells (HEECs). A: images of HEECs cultured on membranes under ALI conditions for 3, 7, and 10 days. Bar = 50 μM. B: results of transepithelial electrical resistance (TEER) measurements on days 3, 7, and 10 at pH 7.4 for ALI-cultured HEECs (n = 4–12). **p < 0.01 vs. day 3.

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Healthcare UK) according to the manufacturer’s instructions. Blots were stripped (1% SDS, 0.5% 2-mercaptoethanol, PBS) for 2 h at 40°C and then rinsed three times in distilled water. Stripped membranes were reblocked for 1 h in 5% milk-PBS before subsequent reactions. Finally, chemiluminescence was detected with an ImageQuant Imager 350 system (GE Healthcare Life Sciences, Tokyo, Japan). All experiments were reproduced at least three times, and representative results are shown. Western blotting bands were analyzed by ImageJ software (Bio-Arts, Fukuoka, Japan).

Epithelial solute permeability. Epithelial paracellular permeability was measured in response to treatments using fluorescein-5-(and-6)-sulfonic acid (FSA) (Mr 478 Da; Invitrogen) as a permeable tracer that passes across the stratified epithelial layers. FSA permeability was measured as described previously (10). Briefly, after treatment exposure, media from both sides of the inserts were discarded, and inserts were placed on a 12-well plate with 0.7 ml of ALI medium in the lower chamber. A 300-µl aliquot of medium containing 0.2 mg/ml FSA was added to the apical compartment, and the apparatus was placed in a CO2 incubator at 37°C. After incubation for 60 min, a 100-µl sample was taken from the lower chamber, and the fluorescence intensity of FSA was determined at excitation 485 nm and emission 538 nm using a spectrophotometer (Fluoroskan Ascent; Thermo Fisher Scientific, Waltham, MA). Results are expressed as permeability ratio vs. controls (pH 7.4).

Statistical analysis. All values are presented as the means ± SE. Data were analyzed using the Student’s t-test for two independent groups or one-way ANOVA followed by Fisher’s paired least-significant difference or Scheffe’s F test for multiple comparisons. Significance was accepted at P < 0.05.

RESULTS

Stratified epithelial cell layer formation and appearance of barrier function. After 3 days of culture on Transwell inserts under ALI conditions, HEECs began to form stratified squamous epithelial cell layers, which were markedly more developed by day 7. Subsequently, the morphology on day 10 was the same as on day 7 (Fig. 1A). TEER of the cell layers increased in a time-dependent manner and was significantly elevated between days 3 and 7 (Fig. 1B). TEER reached a plateau after 7 days of ALI culture.

To determine the expression pattern of TJ proteins in ALI-cultured HEECs, immunofluorescent staining was performed (Fig. 2). Claudin-1, claudin-4, and occludin were detected on the surfaces of the stratified epithelial cells in a continuous lined pattern on day 3 of ALI culture (Fig. 2; top). On day 7, claudin-1, claudin-4, and occludin were detected as dots and whisker-like lines in the superficial layers and were detected in a continuous lined pattern in the suprabasal layers (Fig. 2; bottom). Increased TEER and the formation of the superficial layers with the relevant TJ proteins were detected on day 7 of ALI culture.

The change in TJ protein expression was also examined by Western blotting using NP-40-soluble and -insoluble protein fractions collected from the HEECs on days 3 and 7 of ALI culture (Fig. 3). Levels of claudin-1 and -4 in the NP-40-soluble fraction did not markedly differ between days 3 and 7, and occludin levels were significantly lower on day 7. In the NP-40-insoluble fraction, however, the levels of claudin-1 and occludin increased significantly on day 7 of ALI culture, compared with those on day 3 (Fig. 3).

Influence of acid and acidic bile salts on ALI-cultured HEECs. The influence of acid and acidic bile salts on TEER and permeability were examined in HEECs that had been cultured for 10 days under ALI conditions by exposing the apical surface to the test agents (Figs. 4 and 5). Treatment of cells with acid at pH 2, but not pH 3 or 4, significantly decreased TEER within 15 min and increased FSA permeability in 1 h (Fig. 4, A and B, respectively). Levels of claudin-1 in the NP-40-soluble fraction increased significantly with acid at pH 2, and levels of claudin-1 and -4 in the NP-40-insoluble fraction decreased significantly (Fig. 4, C and D). When the stratified epithelial cell layers were exposed to 4 mM GCA or

Fig. 2. Expression pattern of tight junction (TJ) proteins under ALI-cultured conditions. Immunofluorescent staining for claudin-1, claudin-4, and occludin was performed on days 3 (top) and 7 (bottom) for ALI-cultured HEECs. All proteins were detected in a lined pattern in the suprabasal layers on day 3. Claudin-1 was predominantly detected in the suprabasal layers on day 7, whereas claudin-4 and occludin were detected as dots or whisker-like lines in the superficial layers and colocalized at the junctions of cell layers. Bar = 50 µM.
4 mM TCA at pH 4, neither TEER nor permeability was affected (Fig. 5, A and B, respectively). However, 4 mM GCA and TCA at pH 3 significantly decreased TEER and increased permeability (Fig. 5, A and B, respectively).

To evaluate the expression pattern of TJ proteins under these conditions, Western blotting was performed with NP-40-soluble and -insoluble protein fractions collected from the HEECs on days 3 and 7 of ALI culture (Fig. 6). Compared with controls (pH 7.4), no differences in the levels of claudin-1 and -4 were detected in the soluble fractions between the three treatments (Fig. 6A). However, the levels of claudin-1 and -4 were significantly lower in the insoluble fraction by exposure to GCA (4 mM, pH 3) and TCA (4 mM, pH 3) (Fig. 6B).

The morphology of stratified epithelial cell layers was not affected by TCA (4 mM, pH 3) or by GCA (4 mM, pH 3) (Fig. 7, A–D). Claudin-1 and -4 were detected at junctions even after 1 h of stimulation with TCA (4 mM, pH 3) or GCA (4 mM, pH 3) (Fig. 7). TCA or GCA stimulation did not change the staining patterns of claudin-1 and -4, compared with control conditions.
Influence of combined TCA and GCA treatment on ALI-cultured HEECs. Combined treatment of HEECs with GCA (2 mM) and TCA (2 mM) at pH 3 resulted in a significant decrease and increase in TEER and permeability, respectively, when compared with exposure to pH 3 (Fig. 8). The combined influence of TCA (2 mM) and GCA (2 mM) at pH 3 on TEER and permeability was similar to that of GCA (4 mM, pH 3) treatment. In addition, the combination of TCA (1 mM) and

Fig. 4. Influence of acid on barrier function of ALI-cultured HEECs. A: results of TEER measurements for HEECs exposed to pH 2, 3, and 4 acid from 0 to 60 min (n = 4). B: fluorescein-5-(and-6)-sulfonic acid (FSA) permeability of HEECs exposed to pH 2, 3, and 4 acid for 1 h (n = 4). Levels of claudin-1 (C) and claudin-4 (D) in NP-soluble and insoluble protein fractions collected from the HEECs after exposure to pH 2 for 60 min (n = 4). *P < 0.05, **P < 0.01 vs. control (pH 7.4) (Cont.). ##P < 0.01 vs. control (pH 7.4) (Cont.).

Fig. 5. Influence of acidic glycocholic acid (GCA) and taurocholic acid (TCA) on barrier function of ALI-cultured HEECs. A: results of TEER measurements. B: FSA permeability for HEECs exposed to pH 3, pH 4, GCA (4 mM, pH 3), GCA (4 mM, pH 4), TCA (4 mM, pH 3), and TCA (4 mM, pH 4) (n = 4). **P < 0.01 vs. control (pH 7.4) (Cont.); ##P < 0.01 vs. pH 3.
GCA (1 mM) did not result in marked changes in either TEER or permeability (Fig. 8).

Influence of DCA and pepsin (pH 3) on ALI-cultured HEECs. Treatment of HEECs with DCA (2 mM) at pH 6 and 7.4 significantly decreased TEER and increased permeability when compared with controls (pH 7.4) and weakly acidic conditions (pH 6) (Fig. 9, A and B). No differences in TEER or permeability were detected between DCA (2 mM) at pH 6 and 7.4. Levels of claudin-1 in the NP-40-soluble fraction were significantly increased by DCA (2 mM, pH 6), and levels of claudin-1 and -4 in the NP-40-insoluble fraction were significantly decreased (Fig. 9, C and D).

Finally, the influence of treatment with 0.5, 1.0, and 2.0 mg/ml pepsin (pH 3) on TEER and permeability of HEECs was examined over a 24-h period (Fig. 10). Exposure to pepsin resulted in a gradual decrease in TEER, and this decrease reached the level of significance after 24 h (Fig. 10A). Pepsin treatment also dose dependently and significantly increased permeability within 24 h (Fig. 10B).

DISCUSSION

Several esophageal cell lines, such as SV40 T-antigen immortalized normal human esophageal cell line and esophageal carcinoma cell lines, have been used to study esophageal epithelial barrier function (12, 32). However, as these cell lines typically grow in monolayers, the barrier function of multilayer stratified epithelial cells cannot be accurately assessed. This limitation can be overcome using a Transwell system with ALI conditions to generate a three-dimensional in vitro culture system (20), which we have previously used to form stratified epithelial cell layers with NHBE cells (28). Initial attempts to use a normal squamous epithelial cell line (Het-1A) in this system, however, were unsuccessful, as Het-1A did not form polarized stratified epithelial cell layers or express TJ proteins (29). Here, using HEECs, we successfully established stratified esophageal epithelial cell layers that exhibited a stable barrier function and may serve as an in vitro model of the human esophagus. The HEECs formed well-developed stratified squamous epithelial cell layers on the membranes after 7 days of culture and had a high TEER that was stable for up to 10 days of culture under ALI conditions.

Several distinct types of claudins have been reported to colocalize in various cell types (6, 38), and several reports, including ours, indicate that claudin-1, -4 and -7, occludin, and ZO-1 are expressed in the human esophagus (8, 28, 33). Although the expression profiles of TJ proteins are the same in each columnar epithelial cell (30, 31), cells in the different layers of stratified epithelium express only selected types of TJ proteins in distinct patterns (28). Here, there were no superficial layers on day 3 of HEECs with low TEER in ALI-cultured conditions. At the same time, claudin-1 and -4 were detected in the suprabasal layers in a lined pattern. In contrast, on day 7, the high TEER developed with superficial layer formation. The TJ proteins claudin-1, claudin-4, and occludin were detected as dots and whisker-like lines in the superficial layers. The TJ strand is reported to be restricted to locations where occludin is present although the function of occludin remains unclear (9, 35, 36, 39). Therefore, we focused on claudins colocalized with occludin at junctions to analyze the functions of TJ in epithelial layers. As functional TJ proteins are insoluble in nonionic detergents such as NP-40 and are involved in the regulation of paracellular permeability in epithelial cells (31, 42), proteins from ALI-cultured HEECs were divided into nonionic detergents.
gent (NP-40)-soluble and -insoluble fractions. Although the levels of claudin-1 and -4 in soluble fractions were similar between days 3 and 7, those of claudin-4 in the insoluble fractions had markedly increased by day 7. Several previous reports have indicated that the disruption of barrier function is related to delocalization and loss of TJ complexes between epithelial cells (1, 6, 10, 24, 30). These findings suggest that the insoluble fraction of TJ proteins may relate to the TJ proteins that appeared as dots and whisker-like lines in the superficial layers. Furthermore, we previously confirmed that a similar staining pattern for these TJ proteins was detected on the cell surfaces of human esophagus, and these TJ proteins in the superficial layers may play a key role in stratified epithelia barrier function (29). Here, we also found that levels of occludin in soluble fractions were similar between days 3 and 7, but in the insoluble fraction increased between days 3 and 7. However, the mechanisms underlying this increase and the function of occludin remain to be elucidated.

Acid is a major cause of esophagitis, and the results of animal studies suggest that only strong acid is capable of damaging the esophageal epithelium (41). In our previous study, acid at pH 1 significantly decreased TEER and increased the FSA permeability of ALI-cultured NHBE cells (28). In the present study, we also found that acid exposure adversely affected the NP-40 solubility of claudin-1 and -4 and the barrier function of stratified epithelial layers of HEECs. Our findings are consistent with those of Farre et al. (4), who reported that human esophageal mucosa is extremely sensitive to continuous exposure to acidic and even weakly acidic solutions. These data indicate that stratified epithelial layers of

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Fig. 7. Immunofluorescent staining of claudin-1 and -4 in ALI-cultured NHBE cells. Hematoxylin and eosin (HE) staining was performed with ALI-cultured HEECs. pH 3 TCA (4 mM, pH 3) or GCA (4 mM, pH 3) did not influence the morphology of stratified epithelial cell layers (A–D). Immunofluorescent staining of claudin-1 and -4 was performed (E–P). Claudin-1 was predominantly detected at the suprabasal and intermediate cell layers on the cell surface as a lined pattern and showed a faint dot pattern in the superficial layer (E–H). Claudin-4 was detected at the suprabasal layers as a lined pattern and at the superficial layers as a dot and whisker-like lined pattern (I–L). Merged views of claudin-1 and -4 are shown (M–P). No visible changes were detected among the groups. Bar = 50 μM.
depending on the pH of the solution in which they reside. In epithelium. TCA and GCA can damage the barrier function of esophageal sheets (5) and NHBE cells (29) and indicate that only acidic part with previous studies using rabbit esophageal mucosal 4, had significant adverse effects. These data are consistent in epithelial barrier function, TCA and GCA at pH 3, but not pH 3. Although pH 3 acid alone did not disrupt major bile acids in refluxed material are TCA and GCA (13, used in most studies of esophageal mucosa (5, 26). As the esophagitis, and higher concentrations of bile acids have been reported in patients with GERD (5, 11) and is often detected at high concentrations in the acid and acidic bile-induced disruption of esophageal TJ, further studies are necessary to elucidate the mechanisms of barrier disruption.

Refluxed fluids are often mixed in vivo; therefore, we combined TCA and GCA at pH 3 and demonstrated that barrier function was impaired at concentrations as low as 2 mM. Higher levels of secondary bile acids, including DCA, are often detected in patients receiving acid-suppression therapy (15). Here, although GCA did not affect the barrier function under weakly acidic and neutral conditions, treatment of HEECs with DCA under these mild conditions resulted in a decrease of the NP-40-insoluble fractions of claudin-1 and -4 and an impaired barrier function. These data indicate that the suppression of acid secretion in patients with GERD may be insufficient for the relief of symptoms and that therapy targeting bile salts may be useful in proton pump inhibitor-resistant patients with GERD.

Pepsin is commonly found in the esophagus of patients with GERD (5, 11) and is often detected at high concentrations in patients with stricture or Barrett’s esophagus (7). It has been reported that the maximum enzymatic activity of pepsin occurs at pH levels below 3 (41). Because exposure to pH 2 acid severely impaired the barrier function in our in vitro system, we exposed HEECs to pepsin at pH 3. Under this condition, the impairment of barrier function occurred only gradually, requiring 24 h to reach significant levels. Although these findings suggest that pepsin may only play a limited role in disrupting esophageal barrier function, further studies using in vivo models are necessary.

In conclusion, ALI-cultured HEECs represent a novel in vitro model of human esophageal epithelium and are suitable for studying stratified epithelial barrier function. Using this model, we demonstrated that acid and acidic bile salts disrupt squamous epithelial barrier function partly by modulating the levels of claudin-1 and -4. Our findings provide new insights into understanding the role of TJ proteins in esophagitis and

HEECs may be representative of human esophageal epithelial cell layers in vivo.

Gastro-esophageal refluxed material contains gastric acid, as well as bile acids and pepsin. In patients with severe gastro-esophageal reflux disease (GERD), mixed reflux of gastric and bile acids or pepsin is the predominant pattern and plays a key role in the pathogenesis of the disease (4, 5, 26, 37). Exposure to the combination of bile acids and gastric acid appears to be more harmful than gastric acid alone (3, 13, 16, 25, 26) and can damage the esophageal epithelial layers, leading to esophagitis, Barrett’s esophagus, and even esophageal cancer (19, 21). Different concentrations (ranging from 200 μM to 6 mM) of bile salts refluxed with acid have been reported in patients with esophagitis, and higher concentrations of bile acids have been used in most studies of esophageal mucosa (5, 26). As the major bile acids in refluxed material are TCA and GCA (13, 26), we employed TCA, GCA, and their combination in the present study. Although pH 3 acid alone did not disrupt epithelial barrier function, TCA and GCA at pH 3, but not pH 4, had significant adverse effects. These data are consistent in part with previous studies using rabbit esophageal mucosal sheets (5) and NHBE cells (29) and indicate that only acidic TCA and GCA can damage the barrier function of esophageal epithelium.

There are marked differences in behavior of bile acids depending on the pH of the solution in which they reside. In strongly acidic conditions, conjugated bile acids enter mucosal cells in a non-ionized form, which occurs at a pH close to or below their pKa (18). These refluxed bile acids can cause intracellular damage by the dissolution of cell membranes and TJ (26, 34). Asaoka et al. (2) reported that the total amount of claudin-1 and -4 proteins increased in a rat chronic acid reflux esophagitis model. This finding may be related to the functions of claudins in different fractions, as claudin-1 and -4 found in NP-40-insoluble fractions have been reported to be important for epithelial barrier function (28–30). Here, the total amount of claudin-1 and -4 was not affected by exposure to either 4 mM GCA or TCA at pH 3 (data not shown). In the present experiments involving acidic bile salts, the levels of claudin-1 and -4 in the insoluble fraction significantly decreased following treatment with acidic GCA or TCA. Although claudin-3 has been detected in rat esophagus and claudin-18 has been detected in Barrett’s esophageal epithelia (17, 23, 27), claudin-3 and claudin-18 were not detected in normal human esophagus or ALI-cultured cultured HEECs. As a limitation of the present study, although we previously detected the delocalization of claudin-4 in both colonic epithelial cell model and stratified epithelial cell model with NHBE cells after stimulation (29, 31), differences in the staining pattern of TJ proteins after acidic bile acid stimulation were not detected in our HEECs model. Because other proteins may also be involved in the acid and acidic bile-induced disruption of esophageal TJ, further studies are necessary to elucidate the mechanisms of barrier disruption.

Fig. 8. Influence of combined TCA and GCA on barrier function of ALI-cultured HEECs. A: results of TEER measurements. B: FSA permeability for HEECs exposed to a combination of 1 mM GCA and TCA at pH 3 (pH 3 + GCA 1 + TCA 1), 2 mM GCA and TCA at pH 3 (pH 3 + GCA 2 + TCA 2), and 4 mM GCA at pH 3 (pH 3 + GCA) (n = 3). **P < 0.01 vs. pH 3; ##P < 0.01 vs. pH 3.
may help to elucidate the mechanisms underlying chemical factor-induced injury in stratified epithelium.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: X.C., T.O., and J.S. performed experiments; X.C. and T.O., H.F., J.W., and J.S. analyzed data; X.C. prepared figures; X.C. drafted manuscript; X.C., T.O., H.F., J.W., and J.M. interpreted results of experiments; T.O., H.F., and J.W. conceived and designed research; T.O., H.F., J.W., and H.M. edited and revised manuscript; T.O. and H.M. approved final version of manuscript; T.O. and H.M.

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