Acid modulates the squamous epithelial barrier function by modulating the localization of claudins in the superficial layers

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Acid is a major cause of gastro-esophageal reflux disease. However, the influence of acid on the esophageal stratified epithelial barrier function and tight junction (TJ) proteins is not fully understood. Here, we explore the influence of acid on barrier function and TJ proteins using a newly developed model of the esophageal-like squamous epithelial cell layers that employs an air–liquid interface (ALI) system. Barrier function was determined by measuring trans-epithelial electrical resistance (TEER) and diffusion of paracellular tracers. TJ-related protein (claudin-1, claudin-4, occludin and ZO-1) expression and localization was examined by immunofluorescent staining, and by western blotting of 1% NP-40 soluble and insoluble fractions. We also examined the influence of acid (pH 2–4) on the barrier created by these cells. The *in vitro* ALI culture system showed a tight barrier (1500–2500 $\Omega \cdot \text{cm}^2$) with the expression of claudin-1, claudin-4, occludin and ZO-1 in the superficial layers. Claudin-1, claudin-4, occludin and ZO-1 were detected as dots and whisker-like lines in the superficial layers, and as a broad line in the suprabasal layers. These localization patterns are similar to those in the human esophagus. On day 7 under ALI culture, TJ proteins were detected in the superficial layers with functional properties, including decreased permeability and increased TEER. Dilated intercellular spaces were detected at the suprabasal cell layers even under the control conditions of ALI cells. pH 2 acid on the apical side significantly reduced the TEER in ALI-cultured cells. This decrease in TEER by the acid was in parallel with the decreased amount of detergent-insoluble claudin-4. Claudin-4 delocalization was confirmed by immunofluorescent staining. In conclusion, TJs are located in the superficial layers of the esophagus, and acid stimulation disrupts barrier function, at least in part by modulating the amount and localization of claudin-4 in the superficial layers.

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As one of the functions of the esophageal squamous stratified epithelium, the barrier function is very important to separate tissue from the outside world. The evidence for tight junctions (TJs) in the esophageal epithelium contributing to the barrier function of stratified epithelium has been controversial at times.^{1,2} Although occludin was reported to not be present in esophageal epithelium at first,¹ elucidation of the TJ components has made it possible to confirm the existence of occludin here, and to study the function of the TJs in the esophagus.²

Gastro-esophageal reflux disease (GERD) is caused by reflux of the stomach contents, which causes troublesome symptoms and/or complications. As gastric acid has a major

role in the pathogenesis of reflux esophagitis, it is believed that luminal pH control is important in the management of reflux esophagitis. Elucidation of the process by which esophageal epithelial cells are damaged and of the regulating factors of esophageal epithelial barrier function is important to more fully understand reflux esophagitis and non-erosive reflux disease (NERD). In NERD patients, who lack esophageal mucosal damage, the mechanism leading to heartburn can be represented by the presence of histological abnormalities favoring the diffusion of hydrogen ions through the esophageal epithelium and the consequent stimulation of sensory neurons. In both animal models and humans, dilatation of intercellular spaces (DIS) has been

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noted in acid-exposed tissues by means of transmission electron microscopy (TEM).^{3–6} However, the mechanism responsible for the selective localization of DIS in the basal and suprabasal cell layers is still unknown.⁷ Furthermore, whether DIS is related to the diffusion of hydrogen ions through esophageal epithelium is uncertain.

It has not been easy to examine the direct effects of mediators or to determine the mechanism of the regulation of the esophageal barrier *in vivo*. In addition, there has been no feasible model exploring the esophageal mucosal barrier function *in vitro*. Although cell lines from cancer tissues are easily maintained in culture, they often do not have the morphological or biochemical characteristics of the original tissue. Esophageal cancer cell lines do not consist of stratified epithelial layers and are not sufficient to study the barrier function of esophageal epithelial cells. Furthermore, esophageal stratified epithelial layers may have different functions, and the differences may be related to the different localization of TJ proteins from the basal to superficial layers. Therefore, an *in vitro* system consisting of these stratified squamous epithelial cells is required for analysis of the formation of epithelial layers, the barrier functions of the cell layers and the TJ protein expression in layers, and for determining functional differences between the layers.

It has been reported that primary rabbit tracheal epithelial cells can be differentiated to squamous cells *in vitro* and express several markers for squamous differentiation.⁸ Based on this background, we recently established a stratified squamous epithelial cell-like culture system *in vitro* using the air–liquid interface (ALI) culture method with primary human bronchial cells, and characterized the similarity to human esophageal epithelial cell layers. Optimizing culture conditions, such as selecting appropriate media, is known to be critical for stratified epithelial cell culture to regulate differentiation and to form suitable epithelial barriers with TJs.⁹ After characterizing the localization of TJ proteins in stratified epithelial cell layers, we examined the influence of acid on the barrier function of the cell layers, and on the expression and distribution of TJ proteins.

MATERIALS AND METHODS

Cell Culture

Normal human bronchial epithelial (NHBE) cells were purchased from Lonza Walkersville (Walkersville, MD, USA). To obtain a large amount of cells, the cells were seeded at a density of ~ 3000 cells/cm² in bronchial epithelial growth media (BEGM) (Lonza Walkersville) without antibiotics or all-trans-retinoic acid (ATRA) at 37 °C in a balanced air humidified incubator with an atmosphere of 5% CO₂, expanded to passage 3, and then frozen for later use.

Traswell™-Clear (1.12 cm², 0.4- μ m pore size) (Costar, Cambridge, MA, USA) inserts were coated with collagen, human fibronectin and BSA, and cells then seeded at a density of 4×10^4 cells/insert in BEGM. The medium was changed every 2 days until the cells reached confluence. ALI

culture, which has previously been shown to induce multi-layering and differentiation to squamous epithelial cells,⁹ was introduced by removing the apical medium and decreasing the outer medium to the level of the epithelial cells. The medium was switched to ALI medium, which consists of BEGM and DMEM (1:1) with ATRA (0.3 nM). The ALI medium was changed every day, and any medium that leaked through to the apical side was removed.

Measurement of Trans-Epithelial Electrical Resistance (TEER)

The resistance across the stratified epithelium was measured using MILLICELL-ERS (Millipore Corporation, Bedford, MA, USA) with ‘chopstick’ electrodes according to a previously described method.¹⁰ The value obtained from a blank insert was subtracted to give the net resistance, which was multiplied by the membrane area to give the resistance in area-corrected units ($\Omega \cdot \text{cm}^2$).

When resistance was stable (at $> 1500 \Omega \cdot \text{cm}^2$), the culture medium from the upper (apical) compartment of the monolayer was removed and replaced with a medium containing acid (pH 2–4) or control medium. Cell viability was evaluated using the CellTiter-Glo luminescent cell viability assay (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Each experiment was performed in quadruplicate.

Epithelial Solute Permeability

Epithelial permeability was measured in response to acid exposure using fluorescein-5-(and-6-)-sulfonic acid (FSA) (Mr 478 Da, Invitrogen, Carlsbad, CA, USA) as a permeable tracer that passes across the stratified epithelial layers. FSA permeability was measured as previously described.¹¹ In brief, the insert was placed on a 12-well plate with 0.8 ml of ALI medium in the lower chamber. A 500- μ l aliquot of medium containing 1 mg/ml FSA was added to the apical compartment, and then the apparatus was placed in a CO₂ incubator at 37 °C. After incubation for 60 min, a 100- μ l 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, USA). Permeability measurements were made under control conditions and following exposure to acid (pH 2–4) for 4 h.

Immunofluorescent Staining of Junctional Proteins

Human normal esophageal tissue was resected during surgery from patients with esophageal cancer. All subjects gave written informed consent as required by our ethics committee. The NHBE cells were cultured under ALI conditions and were exposed to acid or appropriate buffer controls. Samples were fixed with 10% formalin solution and embedded in paraffin. Sections 3- μ m thick were cut and stained for TJ-related proteins. After antigen retrieval by autoclave in HistoVT One (Nacalai Tesque, Kyoto, Japan), primary rabbit

anti-claudin-1, -occludin and -ZO-1 polyclonal antibodies (Invitrogen), and mouse anti-claudin-4 and -ZO-1 monoclonal antibodies (Invitrogen) were used at a concentration of 4–16 $\mu\text{g}/\text{ml}$. Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA), Alexa 488-conjugated goat anti-mouse IgG, Alexa 594-conjugated goat anti-mouse IgG and Alexa 488-conjugated goat anti-

rabbit IgG (Invitrogen) secondary antibodies were used at a 1:2000 dilution.

In some experiments, fresh samples were also embedded in OCT compound (Sakura Finetek USA, Torrance, CA, USA) and immediately frozen with dry ice-acetone. Sections 6- μm thick were cut with a cryostat, dried on the slide, rehydrated in PBS for 30 min and permeabilized in PBT (PBS + 0.2% Triton X-100) for 10 min. After incubation with 1% bovine serum albumin to block nonspecific binding, the sections were incubated with Alexa Fluor 546 phalloidin (Invitrogen). 4',6-diamidino-2-phenylindole (DAPI), dilactate (Invitrogen) was used for nuclear staining. 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 (Olympus, Tokyo, Japan) or a confocal laser scanning microscope (FV1000; Olympus). Pictures were recorded on a Windows computer.

Transmission Electron Microscopy

Samples were collected from the ALI-cultured NHBE cells. These samples were cut into 3-mm-sized pieces and immersed in 2.5% phosphate-buffered glutaraldehyde solution for 24 h at 4 °C. Cell layers were then washed in phosphate buffer, fixed in 1% glutaraldehyde and 2% paraformaldehyde for 30 min at 4 °C, washed three times with 0.1 M phosphate buffer for 10 min at 4 °C, fixed in 1% osmium tetroxide for 30 min at 4 °C, washed three times with 0.1 M phosphate buffer for 10 min at 4 °C, dehydrated in ethanol and propylene oxide, and embedded in Epon 812 for 72 h. Sections 1- μm thick were cut and stained with toluidine blue. Ultra-thin sections 70-nm thick were then cut with a diamond knife, stained with uranyl acetate and lead citrate (Reynold's stain), and observed by TEM (H7100; Hitachi, Tokyo, Japan).

Western Blot Analysis

Total protein was obtained under reducing conditions. The cells were divided into soluble and insoluble fractions by the method of Sakakibara *et al.*¹² Protein concentrations were measured with a protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA).

The NP-40 soluble and NP-40 insoluble fractions were then loaded onto 12.5% SDS-PAGE gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes (Amersham Life Science, Arlington Heights, IL, USA). Next, blocking was performed by placing each membrane in TBS-T buffer containing 5% skim milk at room temperature

Figure 1 Influence of acid on TEER and FSA permeability. **(a)** TEER tended to be higher after exposing the cells to pH 3 acid. TEER was significantly decreased at 2 and 4 h after pH 2 acid stimulation compared with the basal level, and pH 2 acid stimulation significantly decreased TEER compared with pH 7.4. Data were obtained from four separate experiments, and expressed as mean $\pm$ s.e. ^{**} $P < 0.01$ vs pH 7.4 (4 h). ^{††} $P < 0.01$ vs pH 2 (0 h). **(b)** FSA permeability was not affected by pH 3 acid (4 h) stimulation compared with the control (pH 7.4). pH 2 acid stimulation for 4 h significantly increased FSA permeability. Data were obtained from four separate experiments, and expressed as mean $\pm$ s.e. ^{**} $P < 0.01$ vs control (pH 7.4, 4 h). NS: not significant.

Figure 2 TJ protein expression pattern in human esophagus. Human esophageal tissue was double stained with ZO-1 (Cy3) / claudin-4 (Alexa 488) **(a, b, c)**, ZO-1 (red) / DAPI (blue) **(d)**, occluding (Cy3) / claudin-4 (Alexa 488) **(e, f, g)** and claudin-1 (Cy3) / claudin-4 (Alexa 488) **(h, i, j)**. Merged images were shown in **c, g** and **j**. All of claudin-1, claudin-4, occludin and ZO-1 were detected at junctions in human esophageal stratified squamous epithelial cells. Claudin-4, occludin and ZO-1 were detected as dots or whisker-like lines in superficial layers, and co-localized at the junctions of cell layers (arrowhead). Claudin-4 was also detected over a relatively broad cell surface area in the suprabasal and intermediate layers (arrow). Claudin-1 was predominantly detected at the cell-cell contact area in the suprabasal and intermediate layers in a continuous linear pattern (arrow) and not in the basal cell layer, and only faintly in the superficial layers. Yellow bar = 50 μm , white bar = 100 μm .

for 60 min. After blocking, the membranes were exposed to rabbit anti-claudin-1 antibody, or mouse anti-claudin-4 antibody, overnight in a refrigerator. The membranes were then washed with TBS-T buffer and exposed to horseradish peroxidase-labeled goat anti-rabbit IgG or anti-mouse IgG antibody (Cell Signaling Technology, Danvers, MA, USA). The membranes were again washed with TBS-T buffer and were then treated with western blotting detection reagent (ECL Plus; GE Healthcare UK, Amersham Place, Little Chalfont, UK). Next, the intensity of each band was scanned and quantified using image processing software (ImageJ, NIH). The quantity of each protein in each fraction was calculated relative to the average intensity of the control cells.

Statistical Analysis

Measured values are shown as mean $\pm$ s.e. Statistical analysis was performed using one-way ANOVA followed by Scheffé's *F* test for multiple comparisons. Significance was accepted at $P < 0.05$.

RESULTS

Influence of Acid on TEER and FSA Permeability in ALI Cells

The cells were in stratified multiple layers after 10 days of ALI culture. After the equilibration of cells by adding ALI medium (pH 7.4) to the insert for 1 h, the influence of acidic conditions was determined by measuring TEER and FSA permeability. TEER was not decreased by pH 3 acid, but TEER was significantly decreased at 2 and 4 h after pH 2 acid stimulation compared with the basal level, and pH 2 acid stimulation significantly decreased TEER compared with pH 7.4 (Figure 1a). FSA permeability was not affected by pH 3 acid (4 h) stimulation compared with the control (pH 7.4). pH 2 acid stimulation for 4 h significantly increased FSA permeability (Figure 1b). No cytotoxic effects with acidic solution at any time point were seen, as determined by a cell viability assay measuring ATP amount (pH 7.4: 149 569 counts/g protein vs pH 2: 179 553/g protein, NS).

TJ Protein Expression in Human Esophagus and ALI-Cultured NHBE Cells

To determine the expression pattern of TJ proteins in human esophageal stratified squamous epithelial cells, immunofluorescent double staining was performed. All of claudin-1, claudin-4, occludin and ZO-1 were detected at junctions in human esophageal squamous epithelial cells (Figure 2).

Claudin-4 was visualized as dots and whisker-like lines, and co-localized with ZO-1, occludin and claudin-1 at the junctions of superficial cell layers. Claudin-4 was also visualized as a relatively broad line on the cell surface in the suprabasal and intermediate cell layers, and was co-localized with occludin and claudin-1. ZO-1 was not detected in the basal to intermediate cell layers. Claudin-1 was predominantly visualized at the cell-cell contact area in the suprabasal and intermediate cell layers in a continuous linear pattern but not in the basal cell layer, and was only faintly stained in the superficial layers (Figure 2).

TJ proteins were also detected in the ALI-cultured NHBE cells (Figure 3a). It has been recently reported that TEER markedly increases between days 5 and 7 of ALI-cultured NHBE cells.⁹ In the present study, on day 5 of the ALI culture, claudin-1 and claudin-4 were detected at the cell-cell contact area in the suprabasal layers in a continuous linear pattern but occludin and ZO-1 were not. On day 7 of the ALI culture, occludin and ZO-1 were detected as dots and whisker-like lines, and were co-localized at the junctions of the superficial layers, while claudin-1 and claudin-4 were also detected as dots and whisker-like lines in the superficial layers, and as a continuous linear pattern in the suprabasal layers.

ZO-1 was detected as dots and whisker-like lines on the cell surface as indicated by the localization of phalloidin and did not overlap with the nucleus, as indicated by DAPI staining (Figure 3b).

TEM showed wide intercellular spaces in the suprabasal cell layers with closer intercellular spaces in the superficial layers (Figure 3c).

Influence of Acid on TJ Proteins in ALI Cells

To determine the influence of acid on TJ protein expression and localization, cells were incubated under acidic conditions for 4 h, and the NP-40 solubility of TJ proteins then assessed (Figure 4). pH 2 acid stimulation did not change the NP-40 soluble fraction of claudin-1 or claudin-4, as determined by western blotting (Figure 4a). The NP-40 insoluble fraction of claudin-1 constantly decreased after stimulation with pH 2 acid for 1 to 4 h. The NP-40 insoluble fraction of claudin-4 gradually decreased after stimulation with pH 2 acid from 1 to 4 h (Figure 4b). pH 3 acid stimulation for 4 h did not change either the NP-40 soluble or insoluble fractions of claudin-4 (Figures 4c and d). The NP-40 insoluble fraction of

Figure 3 TJ expression pattern in ALI-cultured NHBE cells. (a) The ALI-cultured NHBE cells were stained for TJ protein expression. On day 5 (D5) of ALI culture, claudin-1 and claudin-4 were detected at the cell-cell contact area in the suprabasal layers in a continuous linear pattern but occludin and ZO-1 were not. On day 7 (D7) of ALI culture, occludin and ZO-1 were detected as dots and whisker-like lines, and co-localized at the junctions of the superficial layers. Claudin-1 and claudin-4 were also detected as dots and whisker-like lines in the superficial layers, and as a continuous linear pattern in the suprabasal layers. (b) Triple staining of ZO-1, phalloidin and DAPI. ZO-1 was detected on the cell surface (phalloidin) and did not overlap with the nucleus (DAPI). (c) TEM showed wide intercellular spaces in the suprabasal cell layers (arrows) and closer intercellular spaces in the superficial layers. Bar = 10 μ m.

Figure 4 Influence of acid on TJ proteins in ALI cells. **(a)** pH 2 acid stimulation did not change the NP-40 soluble fraction of claudin-1 or of claudin-4 proteins as determined by western blotting. **(b)** The NP-40 insoluble fraction of claudin-1 constantly decreased after stimulation with pH 2 acid for 1 to 4 h. The NP-40 insoluble fraction of claudin-4 gradually decreased after stimulation with pH 2 acid from 1 to 4 h. **(c)** The NP-40 soluble fraction (sol.) of claudin-4 was not changed after stimulation with pH 2 or pH 3 acid. **(d)** The NP-40 insoluble fraction (insol.) of claudin-4 was not changed after stimulation with pH 3 acid and significantly decreased after stimulation with pH 2 acid for 4 h. Data were obtained from three to four separate experiments, and expressed as mean $\pm$ s.e. * $P < 0.05$ vs pH 7.4. NS: not significant.

claudin-4 was significantly decreased after stimulation with pH 2 acid for 4 h (Figure 4d).

Influence of Acid on TJ Protein Localization in ALI Cells

To determine the influence of acid on TJ protein localization, immunofluorescent staining was performed. Claudin-4 and ZO-1 were expressed at junctions in the superficial layer under control (pH 7.4) conditions. Both claudin-4 and ZO-1 were co-localized at junctions (merged) (Figure 5). pH 2 acid stimulation changed the staining pattern of claudin-4 but not ZO-1, with claudin-4 being delocalized to the cytoplasm and the nucleus, as dots, by pH 2 acid stimulation (arrows). The ZO-1 staining pattern was not affected and did not overlap with claudin-4 (arrowhead) in some parts. The claudin-1 staining pattern was not visibly affected by acid stimulation (data not shown).

DISCUSSION

We have determined the esophageal and stratified squamous epithelial TJ protein expression patterns *in vivo* and *in vitro*. Although normal human esophageal keratinocyte culture has been reported from surgical specimens,^{13,14} it is not easy to constantly obtain surgically acquired normal esophageal tissue. We recently established an esophageal-like stratified epithelial cell layer under ALI conditions using NHBE cells.^{9,15} Although the cells were not originally from the esophagus, these cells created stratified epithelial layers,

expressed cytokeratins like the esophagus, and the TJ expression pattern and gene expression pattern were similar to human esophagus.⁹ Here, the stratified epithelial cell layers acquired more than $1500 \Omega \cdot \text{cm}^2$ resistance, which is similar to the resistance obtained from esophageal biopsy specimens using an Ussing chamber.^{16,17} The trachea and esophagus originate from the foregut endoderm during early embryonic development. Tracheobronchial epithelium can be transformed into stratified squamous epithelium, like esophageal epithelial cells.¹⁸ Our data also indicate that the NHBE cells have the capacity to differentiate into stratified epithelium. This system can at least be used as a representative *in vitro* system of stratified squamous epithelial cells to assess the function and expression of TJ proteins.

Although the barrier to diffusion of tracers is present throughout the stratum corneum in rabbit esophageal epithelium,¹⁹ the TJ proteins, which contribute to the barrier function of stratified squamous esophageal epithelial cell layers, have not previously been investigated in detail. The distribution pattern of claudin-1, claudin-4 and ZO-1 in normal esophageal cell layers has been determined by immunohistochemistry.^{20,21} However, the results of examinations of the expression pattern of TJ proteins in esophageal epithelial cell layers have been inconsistent. Here, we performed immunofluorescent staining of TJ proteins in human esophageal epithelia and ALI-cultured stratified epithelial cell

Figure 5 Influence of acid on TJ protein localization in ALI cells. Claudin-4 and ZO-1 were detected at junctions on the superficial layers under control (pH 7.4) conditions in ALI cells. Both claudin-4 and ZO-1 were co-localized at junctions (merged) detected by confocal laser scanning microscopy. Claudin-4 was also detected in the suprabasal cell layers. After pH 2 acid stimulation (4 h), the localization of claudin-4 changed in surface area. Claudin-4 did not co-localize with ZO-1, and was stained in the cytoplasm and in the nucleus, as visualized with DAPI as dots (arrow). The ZO-1 staining pattern was not affected by pH 2 acid stimulation and did not overlap with claudin-4 (arrowhead) in some parts. The pictures of pH 7.4 and pH 2 were magnified in part as pH 7.4 (magnified) and pH 2 (magnified). Bar = 10 μ m.

layers. Both in human and ALI-cultured cells, claudin-4 was detected as dots and whisker-like lines in the superficial layers and was also detected in the suprabasal layers as a line, which differs from a previous report showing claudin-4 in the cytoplasm of cells in the basal cell layers and in the membrane in the superficial layers.²¹ Claudin-1 was visualized predominantly in the suprabasal cell layers as a line, in contrast to the intense dot localization of occludin, ZO-1 or claudin-4 detected in the superficial layers. Although claudin-3 has been detected in rat esophagus⁶ and claudin-18 has been detected in Barrett's esophageal epithelia, claudin-3 and

claudin-18 were not detected in our normal human esophagus or ALI-cultured NHBE cells (data not shown).

Columnar epithelial cells express the same TJ proteins in each cell.^{10,22} In contrast, the cells in the different layers of the stratified esophagus express only selected types of TJ proteins in different patterns.⁹ The TJ strand has been said to be restricted to wherever occludin exists, even if the function of occludin is still unclear.^{23–27} Even in the stomach and intestine, the expression pattern of the claudins is broad, running from the apical to basolateral membrane, while that of occludin is only dense at TJs in normal tissues.^{12,28}

Interestingly, in the present study, occludin was only localized at the superficial layers in ALI-cultured cells, suggesting that TJs may exist in the superficial layers. The barrier function of ALI-cultured NHBE cells has been reported to increase between day 5 and day 7 under ALI culture.⁹ Parallel to these data, in the present study, on day 5 during ALI culture, when the epithelial barrier is weak, occludin and ZO-1 were only slightly detected on the top of the layers, and claudin-1 and claudin-4 were localized in a lined pattern in the suprabasal layers. These data indirectly indicate that the claudin-1 and claudin-4 localized in the line pattern in the suprabasal layers might not be acting as TJs, as they do in the colon,^{10,22} and the functions of these claudins remain unclear. Occludin and ZO-1 were mainly localized in the superficial layers on day 7 under ALI culture, which is when the epithelial barrier is established. Claudin-4 and claudin-1 were also localized in the superficial layers in a dotted staining pattern, similar to occludin and ZO-1. The punctuated, dense and whisker-like line staining patterns of the TJ proteins in the superficial layers were similar to those seen in columnar epithelial TJs. These findings indirectly indicate that the dot and whisker-like line staining patterns of TJ proteins in the superficial layers of esophageal-like stratified epithelium may be related and important for barrier function. This is consistent with the data from skin.²⁴

As the functional TJ proteins are insoluble in nonionic detergent,^{10,12,29,30} in the present study, proteins from ALI-cultured cells were divided into nonionic detergent (NP-40)-soluble and -insoluble fractions, and the level of claudin-1 and claudin-4 in each fraction then measured. Claudin-4 levels were lower in pH 2-stimulated cells compared with control conditions, in a time-dependent manner, suggesting that acid stimulation delocalized the claudin-4 from the TJs in the superficial layers. Immunofluorescent staining of claudin-4 and ZO-1 indicated the delocalization of claudin-4 from TJs. These data suggest that claudin-4 is susceptible to acid stimulation and may be related to esophageal stratified epithelial cell barrier function.

The reason why pH 3 acid did not decrease, but did increase, the stratified epithelial barrier function is unclear at present. Interestingly, Farre *et al*³¹ have also reported that weakly acidic solutions (pH 5) decrease permeability, which may be consistent with our pH 3 data. We are currently investigating the mechanism of the increase in TEER by pH 3 acid stimulation, but have been unsuccessful to date.

DIS is only seen in the basal and suprabasal cell layers and not in the intermediate and superficial layers.⁴ In this ALI culture system, wide intercellular spaces in the basal and suprabasal cell layers were detected even under control conditions, in which the tight barrier function still existed. Furthermore, in human esophagus, claudin-4 and claudin-1 were detected in the suprabasal layer at the lateral membrane in a lined pattern, and they were even detected on day 5 of ALI culture in a lined pattern, where the barrier function was very weak. These findings indirectly indicate that the claudin-

4 and claudin-1 found in the suprabasal layer at the lateral membrane in a lined pattern may not be important to barrier function, nor are they related to the formation of DIS in the suprabasal layer. This is in contrast to the function of the claudins in the superficial layer, in which they have a role in maintaining barrier function.

In the present study, acid stimulation altered the barrier function and also altered claudin-4 in the superficial layers where DIS was not seen. Although the significance of relatively dilated intercellular spaces under ALI control conditions is unclear at present, and we cannot exclude the significance of DIS in the generation of heartburn symptoms in GERD and NERD patients, we can conclude that DIS is at least not related to the barrier function of stratified epithelial cells, claudins are not related to the formation of DIS and claudin delocalization but not DIS may directly affect the diffusion of hydrogen ions. Further studies are necessary to fully elucidate the role of claudin-4 in the superficial layers in GERD and NERD patients.

We tried to detect TJs in stratified epithelial cells by TEM. Dilated intercellular spaces were detected in the suprabasal cell layers where lined staining of claudins was seen, even under control conditions. Conversely, the intercellular spaces of esophageal superficial epithelial cell layers were very small and it was difficult to distinguish the TJs. These difficulties may explain why TJ structures have not been discovered in stratified squamous epithelia until recently.^{1,32} It might be useful to perform immunoelectron microscopy of claudins or occludin to determine the exact TJs in the esophageal epithelial cell layers.

In conclusion, using established stratified squamous epithelial cell layers *in vitro*, TJs may be located in the superficial layers of the esophagus, and acid stimulation disrupted the barrier function. Altered localization of claudin-4 in the superficial layers may be associated with the disruption of the barrier function.

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DISCLOSURE/CONFLICT OF INTEREST

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