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Changes in cell characteristics due to retinoic acid; specifically, a decrease in the expression of claudin-1 and increase in claudin-4 within tight junctions in stratified oral keratinocytes

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Background and Objective: It has been reported that retinoic acid disintegrates the desmosome formation of squamous epithelium, resulting in inhibition of stratification. In contrast, it is not known whether retinoic acid influences the integration of tight junctions. Therefore, our objective of this study is to disclose effects of retinoic acid on the formation and maintenance of tight junction.

Material and Methods: In the present study, the alteration of expression of tight junction constituent proteins and keratin peptides in immortalized oral mucosal epithelial cells (GE1) induced by 1 μM retinoic acid was analyzed by immunofluorescence, electron microscopy and reverse transcription-polymerase chain reaction (RT-PCR).

Results: The stratifying GE1 cells expressed claudin-1, claudin-4, claudin-5, occludin and zonula occludens 1 in the control culture. The RT-PCR showed that retinoic acid significantly reduced the expression of claudin-1 mRNA, whereas it dramatically enhanced expression of claudin-4 mRNA. Immunofluorescence showed that claudin-1 was present at cell-to-cell contact sites in the flattened uppermost layers of the control culture. In the culture with retinoic acid, the flattened uppermost cells were absent and there claudin-1 was less present, but claudin-4 was prominently present in all layers. Claudin-5 was present in a variety of patterns, regardless of the presence of retinoic acid. Along with the change of claudin species, the expressions of keratin 7, keratin 8 and keratin 18, as markers for the simple epithelium, were clearly stimulated by retinoic acid.

Conclusion: Retinoic acid changed the expression of tight junction constituent molecules, such as claudin-1 and claudin-4, in oral keratinocytes. These findings suggest that long-term application of retinoids in clinical therapy should be carefully performed.

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The oral mucosa protects deeper tissues and organs in the oral region from the environment of the oral cavity. The epithelium of the oral mucosa acts as the major barrier against a resident population of microorganisms within the oral cavity that would cause infection, and protects against toxic substances produced by these microorganisms.

Cell junctions in vertebrates can be classified into four functional groups: tight junctions, adherens junctions, desmosomes and gap junctions (1). Of these, the function of tight junctions is to seal the intercellular space in epithelial and endothelial cellular sheets. They are therefore essential apparatus to function as selective barriers to establish compositionally distinct fluid compartments in a wide variety of tissues and organs. In vertebrate simple epithelium, tight junctions are the most apical structure of the lateral membrane, and the intercellular membrane space of these structures was thought to be almost completely closed and was thought to create a primary barrier to the diffusion of solutes through the paracellular route (2–4). However, it has been shown that tight junctions provide a semipermeable size- and ion-specific barrier, which varies depending on their claudin molecular composition (5,6).

In recent years, tight junction-associated proteins, such as claudin-1, claudin-4, occludin and zonula occludens 1 (ZO)-1 (7,8), have been identified in stratified epithelia. Quite recently, claudin-5 has also been demonstrated in squamous epithelia (9). The functional importance of typical constitutive tight junction proteins and hence of tight junctions in the epidermis has been confirmed by observations on mice deficient in claudin-1 (10). In these mice, the epidermal barrier was severely affected, which resulted in the death of animals owing to loss of water within 1 day of birth.

Vitamin A deficiency caused squamous metaplasia with an increased cell proliferation and hyperkeratosis in a variety of epithelia (11). In contrast, in a past study, therapeutic retinoids caused skin fragility in the epidermis of patients with disorders of keratinization (12). Thereafter, it was reported that retinoic acid reduced the number of tonofilaments and desmosomes in a human epidermal keratinocyte cell line (HaCat cells; 13), and that the disintegration of desmosomes was resulted from the reduction of both keratin peptides and desmosomal cadherins, such as desmoglein and desmocollin, in oral mucosal keratinocytes (14). These findings indicated that the cell differentiation of oral keratinocytes to squamous epithelial cells was inhibited by the introduction of retinoic acid. Therefore, we questioned what happened in oral keratinocytes according to inhibition of cell differentiation to squamous epithelia. To assess whether retinoic acid influences the tight junctions of another cell-to-cell adhesion structure, which acts as the selective barrier between the internal tissues of oral mucosa and oral cavity, we examined the regulation by retinoic acid of the expression of the tight junction-associated proteins, claudins, occludin and ZO-1. Simultaneously, the expression of keratin 7, keratin 8 and keratin 18 peptides, which are markers of simple epithelial cells (15), was examined to reveal any change in cell characteristics in oral mucosal keratinocytes cultured with retinoic acid.

Material and methods

Cell culture

The GE1 cell line, originating from gingival epithelial tissue of temperature-sensitive SV40 large T-antigen gene transgenic C57BL/6 mice (16), was cultured in a chemically defined medium, SFM101 (Nissui, Tokyo, Japan) with 1% fetal bovine serum and 10 ng/mL epidermal growth factor at the permitted temperature of 33°C. The calcium concentration of the SFM101 medium was 1.1 mM. Cells grew exponentially, appeared in the confluent state at about day 10 of culture, and then formed a three-dimensional architecture of stratified epithelia. The cells were plated and cultured for 4 days and further cultured with 1 nM, 0.01, 0.1 and 1 μM retinoic acid (all-*trans*-retinoic acid; Sigma Aldrich, Tokyo, Japan) for 5 days in the medium without fetal bovine serum. Retinoic acid was dissolved with ethanol. The concentration of ethanol added to the medium was $< 0.1\%$.

Immunofluorescence

Cells were cultured for 5 days with and without retinoic acid on eight-well chamber slides (BD, Tokyo, Japan) and were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 30 min. The cells were pretreated by microwave treatment (500 W for 3 min) and permeated with 0.1% Triton X-100 in PBS for 5 min. After treatment with 10% normal serum from animals of the same species as the secondary antibody for 20 min to block non-specific staining, cells were incubated for 2 h at 37°C with a mixture of primary antibodies against claudin-1 (1:100 dilution; Zymed Laboratory, South San Francisco, CA, USA) and claudin-5 (1:100 dilution; Zymed Laboratory) or with a mixture primary antibodies against claudin-1 and ZO-1 (1:100 dilution; Santa Cruz Biotechnology, San Francisco, USA) for double immunofluorescence, or they were incubated with a primary antibody against claudin-4 (1:100 dilution; Zymed Laboratory). Another cells were incubated with a primary antibody against keratin 7 (1:50 dilution; PROGEN Biotechnik GmbH, Hiderberg, Germany) or keratin 8 (1:50 dilution; PROGEN Biotechnik GmbH) or keratin 18 (1:50 dilution; PROGEN Biotechnik GmbH) for 2 h at 37°C, which are markers for the simple epithelium. Next, these cells were incubated with Alexa Fluor® 488- or Alexa Fluor® 594-conjugated secondary antibodies (1:100, Molecular Probes Inc., Eugene, OR, USA) for 1.5 h at 37°C, and were counterstained with 0.1 μg/mL 4′-diamidino-2-phenylindole (DAPI) for 20 min at 25°C. Finally, slides were mounted in a reagent (Prolong Gold Antifade reagent; Molecular Probes, Inc.), and sections were inspected with a laser-scanning confocal microscope (LSM510; Zeiss, Göttinggen, Germany). Negative control for immunostaining was incubated

with non-immune serum instead of the primary antibody.

Immunoelectron microscopy

Cells cultured in the medium with or without 1 μM retinoic acid for 12 days were fixed in dishes with 4% paraformaldehyde at 4°C for 1 h. Then, they were scraped and harvested by centrifugation (300 *g* for 2 min at 4°C). After washing three times with PBS, pellets were incubated in 30% sucrose in 0.1 M phosphate buffer. Then, immunoelectron microscopy was processed by the methods described previously (17,18). Briefly, specimens were incubated in 20% polyvinylpyrrolidone at room temperature for 2 h. The specimens were then rapidly frozen in a rapid-freezing apparatus (Reifert KF-80; Leica, Wien, Austria), using liquid propane (−190°C) cooled with liquid nitrogen. After trimming of the specimen at −70°C, ultrathin cryosections were cut with an ultramicrotome (Ultracut EM-UC; Leica) equipped with a cryo-attachment (EM-FC6; Leica) at −120°C. Sections were then negatively stained with 2% polyvinylalcohol and 0.2% uranyl acetate. Ultrathin sections mounted on grids were washed with PBS and incubated with primary anti-claudin-1 antibody (1:100 dilution) for 24–48 h at 4°C after blocking with 10% normal goat serum. Then, they were washed with PBS following the incubation in a 5 nm colloidal gold-conjugated secondary antibody (Amarsham Biosciences, Little Chalfont, UK) for 2 h at room temperature. After washing with PBS, sections were observed in an electron microscope (H-7100; Hitachi Ltd, Tokyo, Japan).

Isolation of RNA and reverse transcription-polymerase chain reaction (RT-PCR)

The GE1 cells were treated with retinoic acid for 5 days. Total RNA was purified from the cells using an RNeasy minikit (Qiagen, Tokyo, Japan). RT-PCR was performed as follows. Using Superscript II reverse transcriptase (GIBCO-BRL, New York, NY, USA), complementary DNA (cDNA) was synthesized from 1 μg of total RNA in the presence of oligo$(dT)_{8-12}$ hexamer (Amersham Pharmacia Biotech, Piscataway, NJ, USA) in a 20 μL reaction volume at 37°C for 60 min. One microliter of cDNA solution was amplified with *Taq* polymerase (Takara, Tokyo, Japan) in a volume of 12.5 μL using each set of primers (Table 1). Primers for mouse claudin-1, claudin-4, claudin-5, occludin (19), ZO-1 (20), mouse keratin 7, mouse keratin 8, mouse keratin 18, and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (21) were used. After degeneration at 94°C for 2 min, amplification of cDNA was performed with 20∼33 sequential cycles at 95°C for 30 s, 55°C for 30 s and 72°C for 30 s, followed by an additional extension step at 72°C for 5 min. The PCR products were then analyzed by electrophoresis on 1.8% agarose gels.

Statistical methods

For semi-quantitative analysis, the amount of amplified DNA of bands in the gel was measured by a densitometer (AE-6920M-06S, ATTO, Tokyo, Japan). The ratios of the DNA amount amplified with each set of primers to the amount of DNA amplified with a set of primers for GAPDH were calculated. The value of the ratio in the control group was set at 1, and the values in dose groups were calculated relative to the control group value. The significance of alterations was tested using one-way analysis of variance.

Results

Immunofluorescence for claudin proteins

Phase contrast microscopy showed that GE1 cells were stratified, involving differentiated flattened upper cells in pavement pattern, at about 10 days of culture after plating (Fig. 1A). In contrast, in the culture with retinoic acid, cells were inhibited from stratifying and no uppermost pavement pattern

Table 1. Primers used for polymerase chain reaction

Gene	Primer sequnce		Size (bp)	Accession no.	Reference
Claudin-1	Forward	5′-TCCTGTCCCCGGAAACAA-3′	105	AF072127	—
	Reverse	5′-AGATCTCTTCCTTTGCCTCTGTCA-3′			
Claudin-4	Forward	5′-CAGTGCAAGTAGTACGACTCGAT-3′	114	BC132378	—
	Reverse	5′-TACCACTGAGAGAAGCATCCCC-3′			
Claudin-5	Forward	5′-GTCTGCAGCGTTGGAAATTCT-3′	196	BC002015	—
	Reverse	5′-ATACACCTTGCACTGCATGTGC-3′			
Occludin	Forward	5′-CAGGTTCGCTTATCTTGGGGA-3′	406	—	19
	Reverse	5′-TCCGTAGCCAAAGCCACTAT-3′			
ZO-1	Forward	5′-GAATGATGGTTGGTATGGTGCG-3′	192	—	20
	Reverse	5′-TCAGAAGTGTGTCTACTGTCCG-3′)			
Keratin 7	Forward	5′-TTCGCCTCCTTCATCGACA -3′	101	AF509888	—
	Reverse	5′-TGCTCTTGGCTGACTTCTGT-3′			
Keratin 8	Forward	5-TCATACGAAGACCACCAGCG-3′	167	BC106154	—
	Reverse	5′-ATCTTCTTCACAACCACGGC-3′			
Keratin 18	Forward	5′-CTGGAGGATGGAGAAGATTT-3′	189	BC089022	—
	Reverse	5′-CTTTTATTGGTCCCTCAGTT-3′			
GAPDH	Forward	5′-ACCACAGTCCATGCCATCAC-3′	452	—	21
	Reverse	5′-TCCACCACCCTGTTGCTGTA-3′			

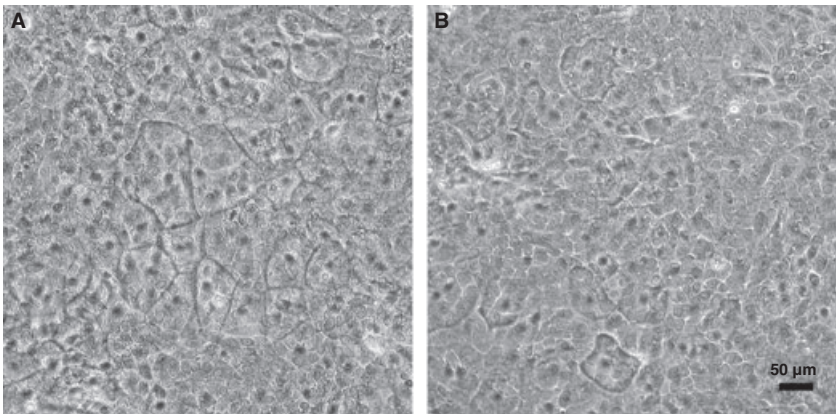

Fig. 1. Morphology of stratified oral keratinocytes, GE1. Scale bar represents 50 μm. (A) Pavement pattern appears in uppermost layers in the control culture. (B) Flattened uppermost cells are absent and no pavement pattern is seen in the culture with 1 μM retinoic acid for 5 days.

was observed (Fig. 1B). In serial observations from the basal to the uppermost layer by the confocal microscope, claudin-1 was clearly present at the flattened cell-to-cell contact sites in the uppermost layers in the control culture (Fig. 2Ab,Ad). In cells cultured with retinoic acid, claudin-1 was absent at cell-to-cell contact sites in the upper layers, though it was present in the cytoplasm (Fig. 2Bb,Bd). Claudin-4 was not present in the uppermost layers, but slightly present at the cell-to-cell contact sites in the medial layers in the control culture (Fig. 3Ab,Ac). In contrast, in the culture with retinoic acid, claudin-4 appeared to be present at cell-to-cell contact sites of all layers (Fig. 3Bb,Bc). Claudin-5 was not found in the control culture (Fig. 2Ac), but was present in medial layers in the culture with retinoic acid (Fig. 2Bc). Sometimes, claudin-5 appeared as dotted lines (Fig. 2Bc). When double immunofluorescence was used, only claudin-1 was present at the intercellular borders in the uppermost layers of the control culture (Fig. 2Ad), and co-localization of claudin-1 and claudin-5 was not observed. The tight junction protein ZO-1 was scarcely present in the control culture (Fig. 4-Ac), whereas it was clearly present at cell-to-cell borders in the upper layers of the retinoic acid-treated culture (Fig. 4Bc,Bd). The sites of the intercellular immunofluorescence of ZO-1 seemed to overlap with that of claudin-1 (Fig. 4Ad).

Immunoelectron microscopy

Immunogold labeling for claudin-1 in the stratified epithelium of GE1 cells cultured with or without 1 μM retinoic acid for 12 days was performed. More

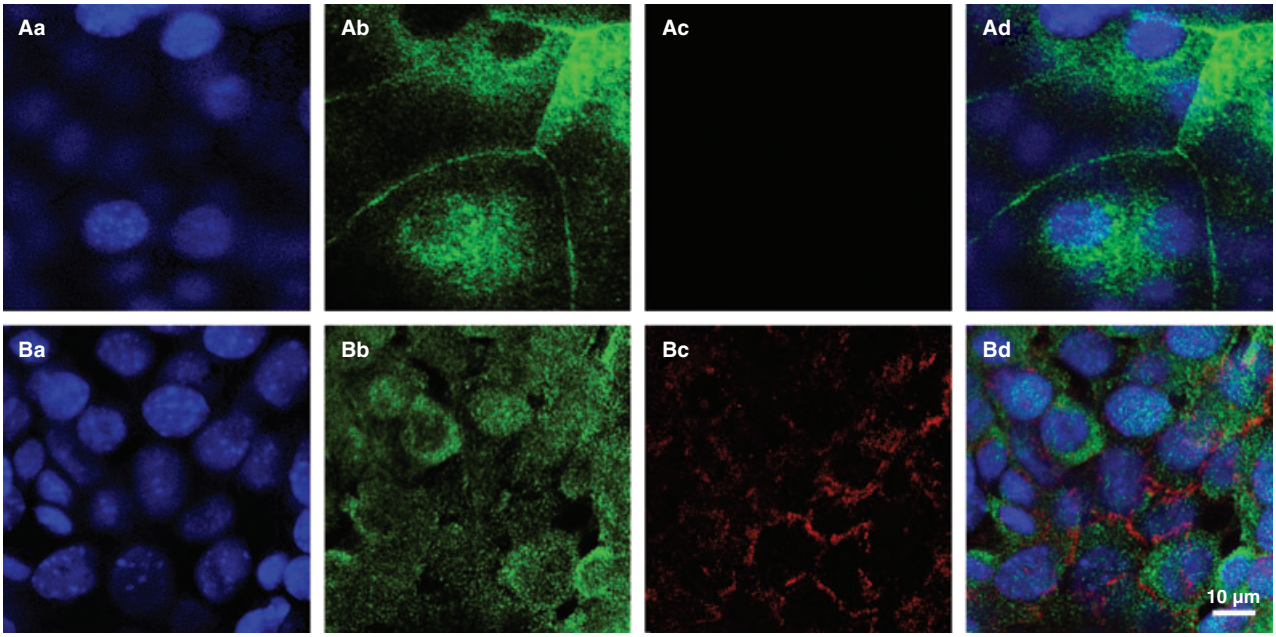

Fig. 2. Double immunofluorescence of claudin-1 and claudin-5 in GE1 cells in the control culture (A) and in the culture with retinoic acid (B), in which the cells were stimulated with retinoic acid for 5 days before double immunofluorescence staining was performed. (Aa,Ba) Staining of nuclei with 4′-diamidino-2-phenylindole (DAPI) (blue). (Ab,Bb) Staining with anti-claudin-1 (green). (Ac,Bc) Staining with anti-claudin-5 (red). (Ad,Bd) Staining with anti-claudin-1 (green) and anti-claudin-5 (red) and counterstain with DAPI (blue). A positive immunoreaction to claudin-1 is observed at the intercellular border in the flattened uppermost cell layers only in the control culture. Immunofluorescence for claudin-5 is observed at intercellular border in the medial layers in the culture with retinoic acid. DAP1 is used for the counterstain. Scale bar represents 10 μm.

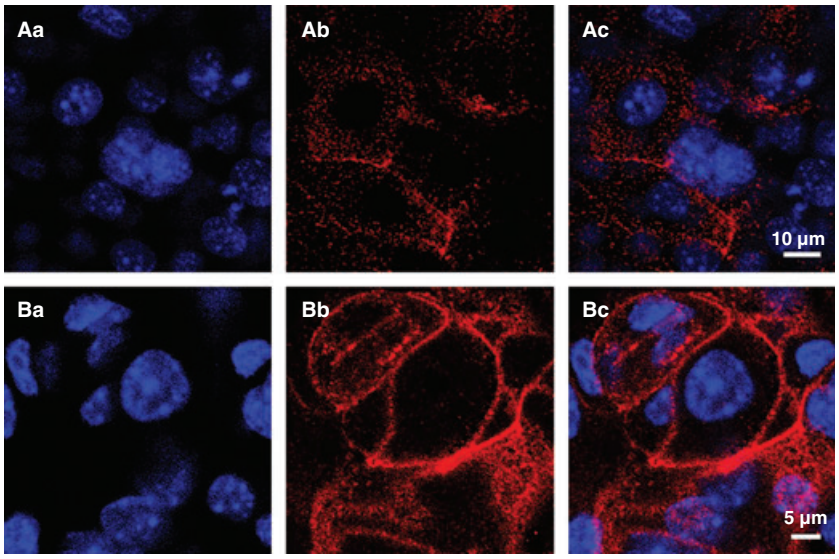

Fig. 3. Immunofluorescence for claudin-4 in GE1 cells in the control culture (A) and in the culture with retinoic acid (B), in which the cells were stimulated with retinoic acid for 5 days. (Aa,Ba) Staining of nuclei with DAPI (blue). (Ab,Bb) Staining with anti-claudin-4 (red). (Ac,Bc) Staining with anti-claudin-4 (red) and counterstain with DAPI (blue). Slight immunofluorescence is seen for claudin-4 in the medial layer in the control culture, but strong immunofluorescence at the cell-to-cell contact sites is seen in the culture with retinoic acid. DAP1 is used for the counterstrain. Scale bar represents 10 μm (A) and 5 μm (B).

5 nm gold particles (Fig. 5, arrows) were localized in the cytoplasm and on cell membranes in the control cells than in the retinoic acid-treated cells.

Expression of tight junction-associated proteins and keratin peptides assessed by RT-PCR

In the control culture, cells expressed claudin-1, claudin-4, claudin-5, occludin and ZO-1 mRNA (Fig. 6A,B). When cells were cultured with retinoic acid, expressions of claudin-1 and claudin-5 mRNA were significantly decreased in a dose-dependent manner. In contrast, the expressions of claudin-4 and occludin were significantly increased. Expression of ZO-1 mRNA was also increased, though with no significant difference. To analyze the alteration of cellular characteristics induced by retinoic acid treatment, the expression of keratin 7, keratin 8 and keratin 18 were examined in retinoic acid-treated cells by RT-PCR analysis.

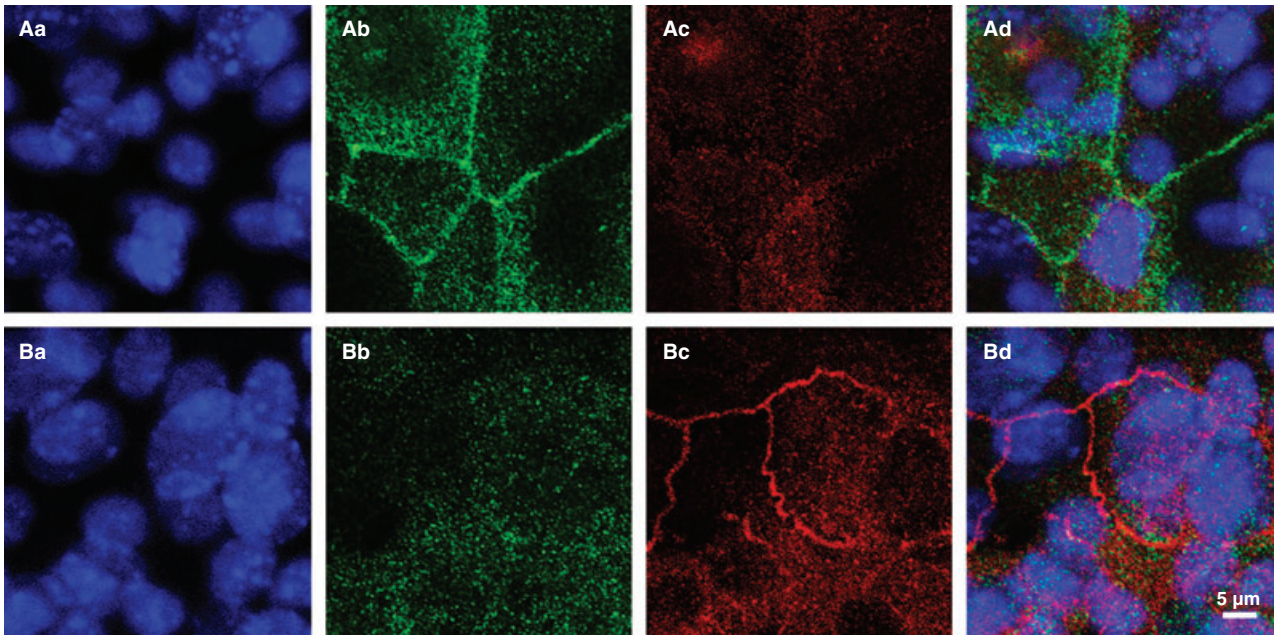

Fig. 4. Double immunofluorescence for claudin-1 and ZO-1 in GE1 cells in the control culture (A) and in the culture with retinoic acid (B), in which the cells were stimulated with retinoic acid for 5 days before double immunofluorescence staining was performed. (Aa,Ba) Staining of nuclei with DAPI (blue). (Ab,Bb) Staining with anti-claudin-1 (green). (Ac,Bc) Staining with anti-ZO-1 (red). (Ad,Bd) Staining with anti-claudin-1 (green) and anti-ZO-1 (red) and counterstain with DAPI (blue). Weak immunofluorescence for ZO-1 is present in the control culture, but is clearly seen at cell-to-cell contact sites in the culture with retinoic acid, where immunofluorescence for claudin-1 is weakly present. Scale bar represents 5 μm.

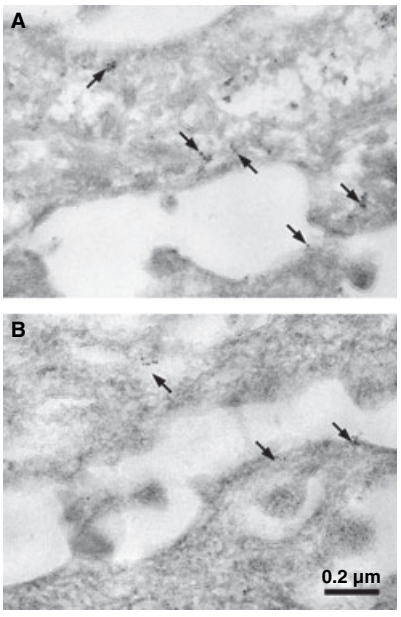

Fig. 5. Immunogold labeling for claudin-1 in GE1 cells. More 5 nm gold particles (arrows) are localized in the cytoplasm and on the cell membrane in control cells (A) than in cells treated with retinoic acid (B). Scale bar represents 0.2 μm.

In the control culture, GE1 cells expressed keratin 8 and keratin 18, and not keratin 7 mRNA, whereas retinoic acid apparently induced the expression of keratin 7 mRNA and enhanced those of keratin 8 and keratin 18 in dose-dependent manner (Fig. 7).

Immunofluorescence for keratins

In the control culture, keratin 7- and keratin 8-positive cells were absent (Fig. 8A,C), though a few cells weakly positive for keratin 18 were present (Fig. 8E). In contrast, in a retinoic acid-treated culture, a few cells positive for keratin 7 were induced (Fig. 8B), and keratin 8- and keratin 18-positive cells were apparently increased (Fig. 8D,F).

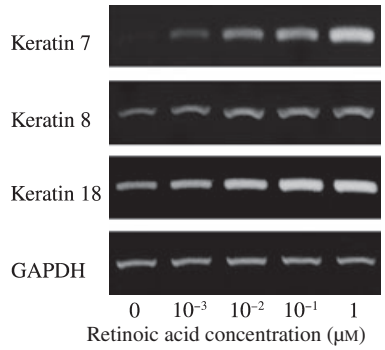

Fig. 7. Gel electrophoresis of the stimulating effect of retinoic acid on expression of keratin 7, keratin 8 and keratin 18. The cells were cultured with or without retinoic acid and were examined by RT-PCR analysis. Gel electrophoresis shows the results of a representative experiment. The expressions of keratin 8 and 18 are increased in a dose-dependent manner and that of keratin 7 is induced by the treatment with retinoic acid.

Discussion

Tight junctions are constituted of transmembrane components, such as claudins and occludin, and scaffolding proteins, such as ZO-1, ZO-2 and ZO-3 (6). Of these, claudins are critical components of tight junctions, and the claudin family consists of at least 24 members, with each showing a specific organ and tissue distribution (22). It has been presumed that every type of cells has its own peculiar species of claudin molecules, because the combination and mixing ratio of claudin species appear to be crucial in regulating the tightness of tight junctions (2,23,24). In this study, immunohistochemistry showed the presence of claudin-1, claudin-4, claudin-5 and ZO-1 at the cell-to-cell border in stratified GE1 cells and, furthermore, claudin-1, claudin-4, claudin-5, occludin and ZO-1 mRNAs were expressed as well. Findings indicated that these molecules were components of tight junctions in the cells. Treatment with retinoic acid reduced claudin-1 and, conversely, induced higher levels of expression of claudin-4. Furthermore, immunohistochemistry clearly showed claudin-4 at the intercellular border in retinoic acid-treated cells. Therefore, in oral keratinocytes treated with retinoic acid, it was recognized that claudin-4, not claudin-1, composed tight junctions, with increased occludin and ZO-1. The compositional change predicted the change of the tightness

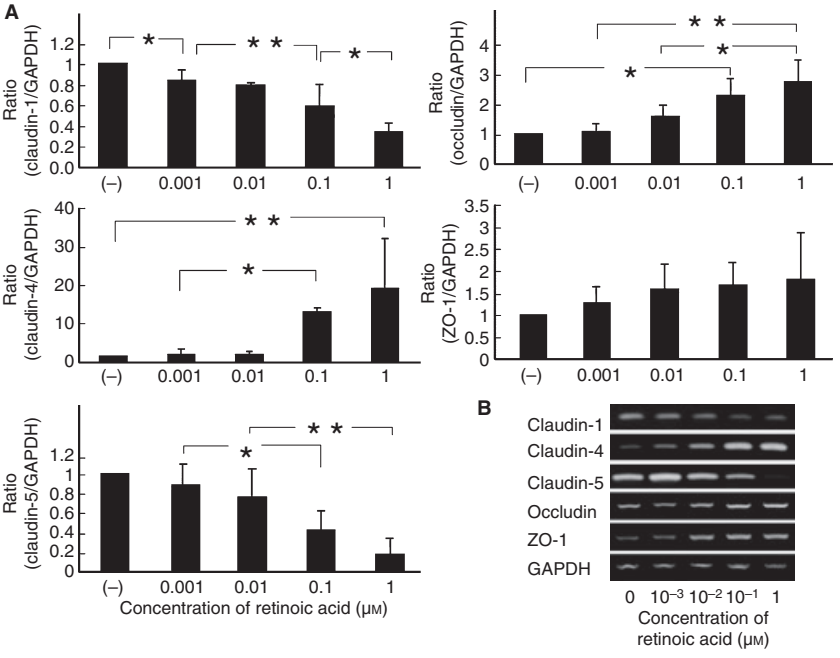

Fig. 6. Expression of tight junction-associated proteins in GE1 cells cultured with or without retinoic acid. The cells that were stimulated with retinoic acid for 5 days were harvested, and total RNA was purified and RT-PCR was performed. (A) Relative transcript level of claudin-1, claudin-4, claudin-5, occludin and ZO-1 are shown as ratios of each transcript level to that of GAPDH mRNA transcript. Each bar represents the mean + SD of three independent experiments. Significance of alterations was tested using one-way analysis of variance (*$p < 0.05$, **$p < 0.01$). (B) Graph of gel electrophoresis shows the results of one representative experiment out of three.

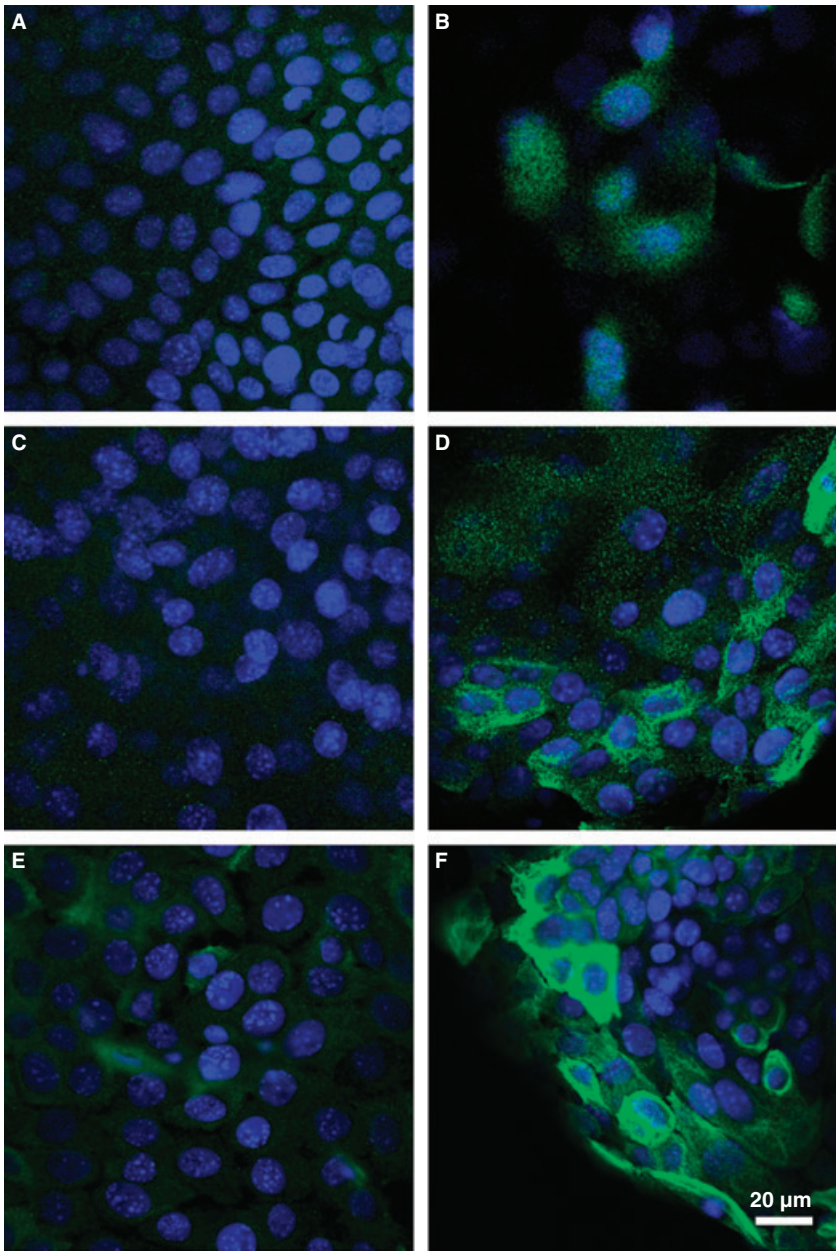

Fig. 8. Immunofluorescence of keratin 7, keratin 8 and keratin 18. The cells were cultured and incubated with retinoic acid for 5 days. Immunofluorescence staining of keratin 7 (A,B), keratin 8 (C,D) and keratin 18 (E,F) is shown in the control culture (A,C,E) and in the culture with retinoic acid (B,D,F). Scale bar represents 20 μm.

as selective barrier of the tight junction formed by retinoic acid-treated cells. However, the present study did not examine this particular aspect, and further experiments are necessary to determine what changes, if any, had occurred.

Claudin-1-based tight junctions are known to be important for the mammalian epidermal barrier, because in claudin-1-deficient mice, tracer (~600 Da) injected subcutaneously passed though these tight junctions and diffused to the skin surface (10). Therefore, it was thought that the decrease of claudin-1 was intimately associated with the decrease or loss of barrier function of stratified squamous epithelia, such as epidermis. In other instances, a significant amount of claudin-4 was displayed in normal mammary epithelial cells, whereas claudin-4 was reduced or absent in ductal adenocarcinoma (25), in the disrupted glandular structure in gastric adenocarcinoma (26) and in invasive and metastatic portions of pancreatic cancer (27). These studies indicated that claudin-4 was the important constituent of tight junctions in normal simple epithelia, such as glandular epithelium. From these studies, we speculated that oral keratinocytes were probably changed from squamous cells to simple epithelium-like cells by treatment with retinoic acid. Our previous study, in which retinoic acid inhibited the stratification of GE1 cells (14) and the expression of keratin 13 and keratin 4 observed in mucosal epithelia, supports this hypothesis. To confirm this hypothesis, we observed the pattern of keratin peptide in retinoic acid-treated GE1 cells. The present study showed that the expression of keratin peptides such as keratin 7, keratin 8 and keratin 18, which were known to be present in glandular epithelium, was stimulated. These data also support our speculation. So far, regarding the factors influencing the barrier function and integrity of structure of tight junctions, nothing was known except that basic fibroblast growth factor-2 preserved the expression of claudin-5 in blood vessels (28). In the present study, it was firstly and clearly demonstrated that retinoic acid regulated the expressions of tight junction constituent molecules. It also indicated that the replacement of claudin-1 by claudin-4 was associated with a morphological change from stratified squamous cells to simple epithelium-like cells.

Clostridium perfringens enterotoxin is the causative agent of symptoms associated with *C. perfringens* food poisoning in man (29). *C. perfringens* enterotoxin, produced in the intestinal tract, injures intestinal epithelial cells and causes fluid accumulation in the intestinal cavity, resulting in diarrhea (30). Claudin-4 was initially identified as a *C. perfringens* enterotoxin receptor, and it was demonstrated that the claudin-4/*C. perfringens* enterotoxin receptor specifically bound to the COOH-terminal half of *C. perfringens* enterotoxin (31). When the COOH-

terminal of *C. perfringens* enterotoxin peptide was applied to cultured MDCK cells expressing claudin-1 and claudin-4, the claudin-4 was specifically removed from tight junctions. When retinoids are selected for pharmaceutical therapy for some cornification disorders in the oral mucosa, claudin-1, composing the tight junctions, may be replaced with claudin-4. Then, in very rare cases, if at this time *C. perfringens* invades and its enterotoxin is produced, tight junctions will probably be disrupted, and the barrier function of oral mucosa will be injured. Although the present results of increased expression of claudin-4 and decreased claudin-1 induced by retinoic acid in keratinocytes were disclosed in mouse cells, it is important to remember this particular effect of retinoic acid when retinoid is used for clinical application in humans. In particular, the long-term application of medicine containing retinoic acid for oral mucosal disorders must be performed under careful observation of therapeutic progress.

In present study, the data concerning claudin-5 were changeable in the culture both with and without retinoic acid and also in both RT-PCR and immunofluorescence studies. The expression and functional roles of claudin-5 in squamous epithelial tissues may be regulated in a complex manner by unknown factors. Thus, we could not completely clarify the cause of the contradiction between the present results of RT-PCR analysis and immunofluorescent staining. Further study is necessary.

Acknowledgements

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