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Original Article

Claudin-7 in keratinocytes is downregulated by the inhibition of HMG-CoA reductase and is highly expressed in the stratum granulosum of the psoriatic epidermis

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ABSTRACT

Background: Cholesterol is *de novo* synthesized in the upper epidermis and plays an important role in maintaining the normality of skin. Studying the impact of the inhibition of cholesterol *de novo* synthesis in the epidermis may help understand how skin homeostasis is regulated.
Objective: In this study, we created a gene expression profile to investigate the effect of hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors on epidermal homeostasis.
Methods: A microarray analysis was performed using normal keratinocytes with or without HMG-CoA reductase inhibitor (pitavastatin) treatment. Real-time PCR confirmed the reproducibility of genes with altered expression in keratinocytes treated with HMG-CoA reductase inhibitors. Among these genes, we focused on reduced expression of claudin 7 histologically confirmed by immunohistochemical staining, in situ hybridization, and immunoelectron microscopy.
Results: Claudin-7 was highly expressed in the stratum granulosum of psoriatic lesions but was not expressed in the normal epidermis. Immunoelectron microscopy revealed that claudin-7 was localized in the keratohyalin granules of psoriatic lesions.
Conclusion: These results indicate that claudin-7 expression was regulated by HMG-CoA reductase in the epidermis and might play a pathogenic role in the keratohyalin granules found in the epidermal granular layer of psoriasis.

1. Introduction

Cholesterol plays an important role in maintaining epidermal homeostasis and is involved in the stability of skin cell membranes [1]. Cholesterol is supplied in epidermal keratinocytes via *de novo* synthesis or incorporation pathway [2]. For example, keratinocytes in the upper dermis supply cholesterol via the *de novo* synthetic pathway using hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase and contribute to the formation of mature intercorneocyte lipids. Conversely, the basal cell layer, which is the lower layer of the epidermis, is located near the blood vessels of the dermis. The keratinocytes in the basal cell layer are supplied with blood cholesterol mainly (as the incorporation pathway) via low-density lipoprotein receptors (LDL-R) [3,4]. Therefore, the basal cell layer highly expresses LDL-R but only expresses HMG-CoA reductase at low levels. Thus, it can be speculated that the supply of cholesterol in the suprabasal layer of the epidermis largely depends on the *de novo* synthetic pathway via HMG-CoA reductase activity [4].

To date, the unique localization of LDL-R on the epidermis in some dermatoses (e.g., atopic dermatitis, psoriasis) has been revealed [4]. LDL-R staining is positive in the basal layer of healthy controls and samples of atopic dermatitis [4]. In psoriasis, LDL-R is localized in all layers of the epidermis [4]. Considering this unique observation in psoriasis, it was our understanding that the possible relationship between the requisition of cholesterol and pathogenesis of psoriasis.

To investigate this hypothesis, we first examined the impact of an HMG-CoA reductase inhibitor, pitavastatin, on gene expression in human keratinocytes by using microarray analysis. Among the genes showing expression fluctuations in microarray analysis, we selected

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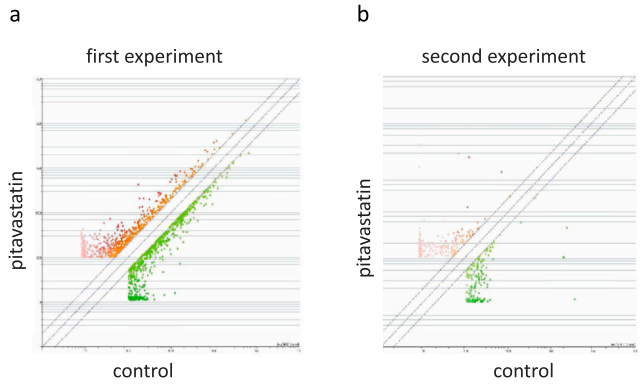

Fig. 1. RNA microarray analysis in cultured normal human epidermal keratinocytes after pitavastatin treatment. Two independent experiments were performed (a: first experiment, b: second experiment). Compared with the control, the red dots indicate the genes whose expression had more than doubled. The green dots indicate the genes whose expression had been reduced to less than half. (For interpretation of the references to colour in this figure, the reader is referred to the web version of this article.)

five genes that appeared to show similar change in expression for all examinations. We then focused on claudin-7 owing to the fact that only claudin-7, among the five genes, showed expression variation and reproducibility in validation experiments using real-time PCR. Histological analysis of claudin-7 revealed that it is highly expressed in the psoriatic stratum granulosum.

2. Materials and methods

2.1. Microarray analysis

Normal human keratinocytes (NHKs; DS Pharma Biomedical Co., Osaka, Japan) were used. According to the preliminary studies, the administration concentration was set to 0.1 μM, at which level pitavastatin does not affect cell proliferation abilities. The cells (1.0×10^6) were seeded in a 10 cmφ dish and cultured in 10 ml of EpiLife medium (Thermo Fisher Scientific, USA) for 3 days. Pitavastatin solution was then added to the culture medium to reach a final concentration of 0.1 μM, and the solution was cultured for 6 h. The same amount of DMSO alone was added as a control and cultured (culture conditions: 5% CO_2, 37 °C). The cells treated with pitavastatin and the cells treated with the vehicle had the same passage number (each n = 2). After culturing, the cells were collected, and total RNA was extracted using the SV Total RNA Isolation System (Promega, Tokyo, JAPAN). The resulting RNA was labeled with Cyanine-3 and was used for the microarray analysis. Three normalization procedures were performed on the samples. First, genes with signal intensities of 0.01 or less were corrected to 0.01. The normalized value of the corresponding pitavastatin-treated sample was then divided by the normalized value of the measured vehicle-treated sample on each chip. Genes with low expression levels with a signal intensity of less than 100 were filtered out in both the vehicle-treated samples and the pitavastatin-treated samples. A comparative analysis of the expression variability in genes that passed filtering was performed.

2.2. Real-time PCR analysis

Gene variability verification experiments were performed using real-time PCR for genes extracted by biological evaluation of the microarray analysis. Real-time PCR quantification was performed to compare the expression levels of the claudin-7 gene between the pitavastatin-treated cells and the vehicle-treated cells. Using 800 ng of each RNA, first-strand cDNA was synthesized by reverse transcriptase. Using this as a template, real-time PCR was performed using a primer specific to human claudin-7, and 18 S rRNA was used as an internal standard.

2.3. Histopathological analysis

For the skin disease tissues, surplus tissues from skin biopsies or surgical resections for which comprehensive consent was obtained were used. Normal skin tissue was collected from volunteers after obtaining comprehensive consent. The skin tissue was fixed with 4% neutral formalin, embedded in paraffin, and sliced into 3 μm sections with a microtome. After deparaffinization on a slide glass with xylene and alcohol, the tissues were washed with phosphate buffered saline (PBS) 3 times for 5 min each time. Endogenous peroxidase activity was blocked with 3% H_2O_2, and the tissues were washed again with PBS 3 times for 5 min each time. Blocking was performed with protein block serum-free solution (DAKO, USA), and anti-TNFAIP3 antibodies or anti-claudin-7 antibodies (Abcam, USA) were added to react for 1 h. After washing with PBS three times, TNFAIP3 and claudin-7 were visualized by color development with diaminobenzidine (DAB) using a ChemMate EnVision Kit (DAKO, USA).

2.4. In situ hybridization

Paraffin-embedded blocks and sections of human skin for in situ hybridization (ISH) were obtained from Genostaff Co., Ltd. (Tokyo, Japan). The human skin was fixed with Tissue Fixative (Genostaff), embedded in paraffin by their proprietary procedures, and sectioned to 4 μm thick. For the ISH, the tissue sections were de-waxed with xylene and re-hydrated through an ethanol series and PBS. The sections were fixed in 4% paraformaldehyde in PBS for 15 min and then washed with PBS. The sections were treated with 6 μm/ml Proteinase K in PBS for 30 min at 37 °C, washed with PBS, re-fixed with 4% paraformaldehyde in PBS, washed again with PBS, and placed in 0.2 N HCl for 10 min. After washing with PBS, the sections were acetylated by incubation in 0.1 M tri-ethanolamin-HCl, pH 8.0, 0.25% acetic anhydride for 10 min. After another washing with PBS, the sections were dehydrated through a series of ethanol solutions. Hybridization was performed with probes at concentrations of 300 ng/ml in Probe Diluent-1 (Genostaff) at 60 °C for 16 h. After hybridization, the sections were washed in 5xHybriWash (Genostaff), equal to 5xSSD, at 60 °C for 20 min and then in 50% formamide, 2xHybriWash at 60 °C for 20 min, followed by RNase treatment in 50 μg/ml RNaseA in 10 mM Tris-HCl, pH 8.0, 1 M NaCl, and 1 mM EDTA for 30 min at 37 ℃. The sections were then washed twice with 2xHybriWash at 60 ℃ for 20 min, twice with 0.2xHybriWash at 60 ℃ for 20 min, and once with TBST (0.1% Tween20 in TBS). After treatment with 1xG-Block (Genostaff) for 15 min at (approximately) 26 °C, the sections were incubated with anti-DIG AP conjugate (Roche) diluted 1:2000 with x50G-Block (Genostaff) in TBST for 1 h at room temperature. The sections were then washed twice with TBST and incubated in 100 mM NaCl, 50 mM $MgCl_2$, 0.1% Tween20, and 100 mM Tris-HCl, pH 9.5. Coloring reactions were performed with NBT/BCIP solution (Sigma) overnight, and the sections were then washed with PBS. The sections were counterstained with Kernechtrot stain solution (Mutoh) and then mounted with CC/Mount (DBS).

2.5. Immuno-nanogold electron microscopy

The sections were fixed at 4 °C for 2 h or more with 4% paraformaldehyde (0.1 M PBS), washed with 0.1 M PBS, dehydrated in a series of ethanol solutions, and embedded with LR White resin. The cured block was sliced to a thickness of 80 nm with an ultramicrotome, recovered with a nickel grid, and then immunostained

Table 1
The genes common to the fluctuating gene groups are shown by microarray analysis. Thirteen genes who expression had more than doubled (upregulated) and nine genes whose expression had been reduced to less than half (downregulated) were extracted.

up-regulated	
Common name	Description
THC2317149	C40201 artifact-warning sequence (translated ALU class C) – human {Homo sapiens;}, partial (11%) [THC2317149]
BC071790	Homo sapiens cDNA clone IMAGE:4611512, partial cds. [BC071790]
GALNT4	Homo sapiens UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 4 (GalNAc-T4) (GALNT4), mRNA [NM_003774]
ENST00000315478	AY358560 GACL263 {Homo sapiens;}, partial (17%) [THC2263748]
ZNF664	Homo sapiens zinc finger protein 664 (ZNF664), mRNA [NM_152437]
MYR8	Homo sapiens myosin heavy chain Myr 8 (MYR8), mRNA [NM_015011]
MCF2L	Homo sapiens cDNA FLJ35420 fis, clone SMINT2001183. [AK092739]
VTN	Homo sapiens vitronectin (serum spreading factor, somatomedin B, complement S-protein) (VTN), mRNA [NM_000638]
WDR56	Homo sapiens WD repeat domain 56 (WDR56), mRNA [NM_020800]
C20orf103	Homo sapiens chromosome 20 open reading frame 103 (C20orf103), mRNA [NM_012261]
TAAR5	Homo sapiens trace amine associated receptor 5 (TAAR5), mRNA [NM_003967]
FATE1	Homo sapiens fetal and adult testis expressed 1 (FATE1), mRNA [NM_033085]
NYD-SP25	Homo sapiens protein kinase NYD-SP25 (NYD-SP25), transcript variant 1, mRNA [NM_033516]
down-regulated	
Common name	Description
BM981574	BM981574 UI-CF-EN1-adi-i-21–0-UI.s1 UI-CF-EN1 Homo sapiens cDNA clone UI-CF-EN1-adi-i-21–0-UI 3′, mRNA sequence [BM981574]
A_32_P114268	
A_24_P843921	
AF289591	Homo sapiens clone pp7651 unknown mRNA. [AF289591]
TNFAIP3	Homo sapiens tumor necrosis factor, alpha-induced protein 3 (TNFAIP3), mRNA [NM_006290]
LOC63928	Homo sapiens hepatocellular carcinoma antigen gene 520 (LOC63928), mRNA [NM_022097]
ENST00000310865	Homo sapiens mRNA full length insert cDNA clone EUROIMAGE 2004990. [AL389981]
CLDN7	Homo sapiens claudin 7 (CLDN7), mRNA [NM_001307]
CSF2	Homo sapiens colony stimulating factor 2 (granulocyte-macrophage) (CSF2), mRNA [NM_000758]

by a post-embedding method using a colloidal gold-labeled antibody. Approximately 30 μl of 1% normal goat serum (NGS) / 1% bovine serum albumin (BSA)-PBS was dropped onto the parafilm in the moist chamber, floated on water droplets with the sample surface of the grid facing down, and blocked at room temperature for 60 min. The primary antibody (claudin-7 monoclonal antibody, Thermo Fisher Scientific, USA) diluted with 1% BSA-PBS was similarly dropped onto parafilm and reacted at room temperature for 2 h. Five drops of 50 μl of 1% BSA-PBS water droplets were dropped on the parafilm, and the grid was washed 5 times for 1 min each time. Colloidal gold-labeled secondary antibodies (Gold colloid, BBI solutions, UK) diluted with 1% BSA-PBS were reacted at room temperature for 90 min, washed 3 times with 1% BSA-PBS, and then washed 3 times with 0.01 M PBS. To immobilize the colloidal gold on the slices, it was reacted with 2.5% glutaraldehyde for 15 min at room temperature and then washed 3 times with 0.01 M PBS. After staining with uranyl acetate and lead citrate to obtain tissue contrast, the tissues were observed via transmission electron microscopy.

3. Results

3.1. Microarray analysis and real-time PCR

Cultured normal human epidermal keratinocytes were co-cultured with pitavastatin or a vehicle, and each gene expression profile was created from the results of the gene array analysis (GEO ID: GSE180418). Genes whose signal intensity had more than doubled and those whose signal intensity had been reduced to less than half by pitavastatin treatment compared with vehicle treatment were extracted (Fig. 1). Two completely independent experiments were performed in this profiling. Of the 41,152 genes on the array, the signal intensity of 711 genes (red dots) had more than doubled and that of 706 genes (green dots) had been reduced to less than half;

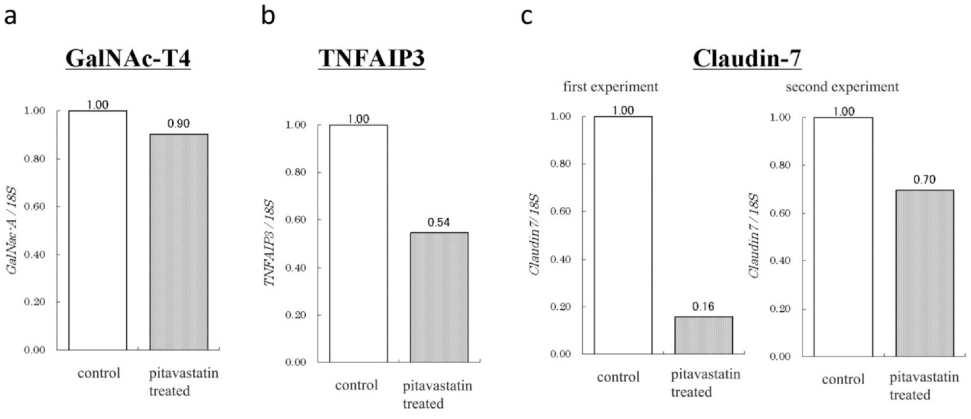

Fig. 2. Real-time PCR of claudin-7 in pitavastatin-treated and vehicle-treated (control) cells.

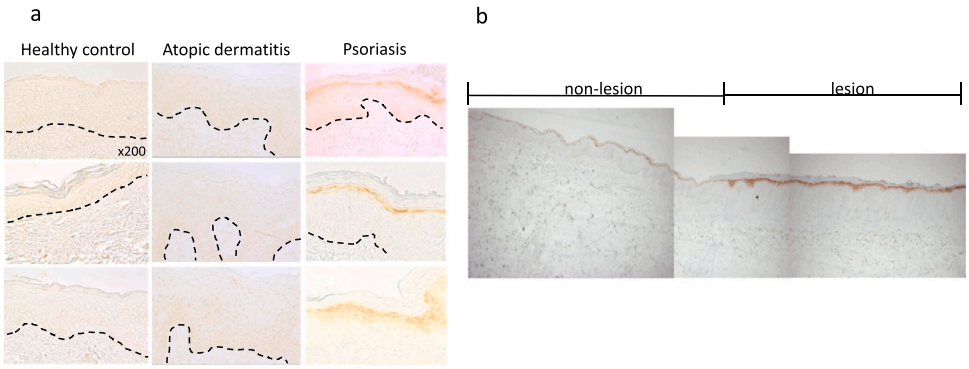

Fig. 3. (a) Immunohistochemical examination of claudin-7 (DAB stain; original magnification, ×200) in tissues from healthy controls, patients with atopic dermatitis, and patients with psoriasis. The dotted line shows the basal layer. (b) Immunohistochemical examination of claudin-7 (DAB stain; original magnification, ×40) in lesional and non-lesional psoriatic skin.

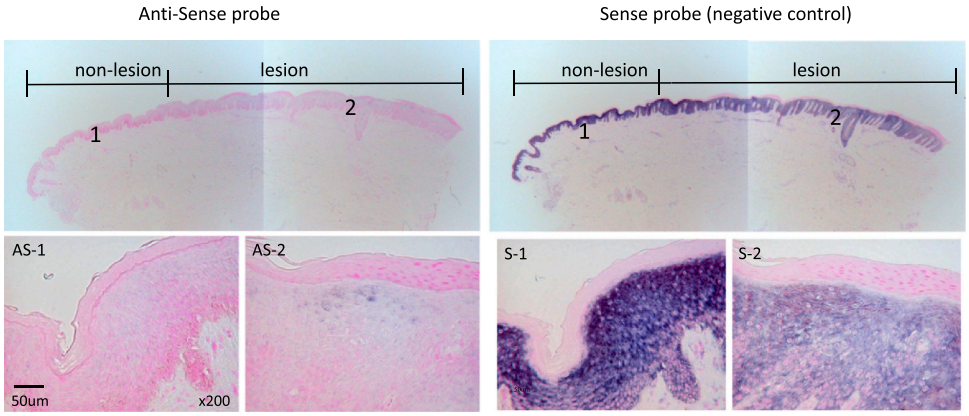

Fig. 4. In situ hybridization of claudin-7 in human psoriatic skin. The skin specimen derived from a psoriasis patient included both lesional and non-lesional skin. AS: antisense, S: sense.

these genes were extracted in the first experimental sample (Fig. 1a). In the second experimental sample, the signal intensity of 240 genes (red dots) had more than doubled and that of 201 genes (green dots) had been reduced to less than half; these genes were extracted (Fig. 1b). Genes common to the fluctuating gene group were further extracted. As a result, 13 genes were extracted whose signal intensity had more than doubled, and 9 genes were extracted whose signal intensity had been reduced to less than half (Table 1). After a biological evaluation of these 22 genes, TAAR5 (trace amine associated receptor 5), VTN (vitronectin), and GalNaAc-T4 (UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyl-transferase 4) were selected from among the genes whose signal intensity had more than doubled. Among the genes whose signal intensity had been reduced to less than half, TNFAIP3 (tumor necrosis factor, alpha-induced protein 3) and claudin-7 were selected. The genes selected by the above profiling were verified by real-time PCR (Fig. 2). There was no change in GalNaAc-T4 (Fig. 2a). TAAR5 and VTN could not be measured below the quantification limit. It was confirmed that TNFAIP3 suppressed gene expression by approximately 0.5 times (Fig. 2b); furthermore, claudin-7 suppressed gene expression by 0.16 times in experiment 1 and 0.7 times in experiment 2 (Fig. 2c).

3.2. Immunohistological staining

Immunohistochemical staining of claudin-7 was performed on tissue samples from healthy controls, patients with atopic dermatitis, and patients with psoriasis. The healthy control and atopic dermatitis tissues did not express claudin-7, but claudin-7 was expressed in the epidermis of psoriatic lesions, particularly in the stratum granulosum (Fig. 3a). Positive staining for claudin-7 was not observed in the nonlesional (normal) skin of psoriatic tissue (Fig. 3b). Claudin-7 was not expressed in other hyperkeratotic skin diseases such as seborrheic keratosis, actinic keratosis, and squamous cell carcinoma (Supplemental file).

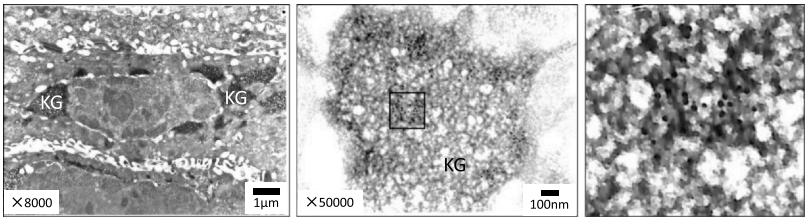

Fig. 5. Immuno-nanogold electron microscopy of claudin-7 in a human psoriasis lesion. KG: keratohyalin granule.

3.3. In situ hybridization (ISH)

ISH was performed to investigate the expression pattern of claudin-7 in the skin (Fig. 4). In this assessment, antisense (AS) and sense (S) probe were designed to judge the specificity of hybridization. The skin specimens derived from the psoriasis patients included both lesional and non-lesional skin. The AS probe displayed less hybridization to the normal epidermis (AS-1) but was intensely hybridized to the upper epidermis of psoriatic lesional skin (AS-2). The entire epidermis showed an intense positive reaction to the S probe (S-1), indicating the possible existence of an AS transcript for claudin-7. The positive signal intensity for the S probe was apparently reduced in lesional skin (S-2).

3.4. Immuno-gold electron microscopic analysis

Next, to confirm the precise localization of claudin-7 in the psoriatic epidermal stratum granulosum, an immuno-gold electron microscopic analysis was performed using skin tissue derived from a psoriatic patient. Claudin-7 positive signals (small black dots) were observed only inside the keratohyalin granules (Fig. 5).

4. Discussion

Cholesterol has been shown to play an important role in epidermal homeostasis. However, the role of HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, in the epidermis is unknown. To investigate this role, we co-cultured an HMG-CoA reductase inhibitor with epidermal keratinocytes to create a gene expression profile. A gene array analysis extracted genes whose expression had more than doubled compared with untreated keratinocytes. We focused on the claudin-7 gene.

Tight junctions of epithelial or endothelial cells contain tight junction constituent proteins that are characteristic of each organ. Two common functions of these proteins are the ability to adhere adjacent cells face-to-face and to control the intercellular transport of substances. Examples of tight junction constituent proteins include claudin-1, claudin-3, claudin-4, claudin-5, claudin-6, claudin-7, claudin-11, claudin-12, claudin-18, occludin, JAM-A, ZO-1, ZO-2, cingulin, MUPP-1, symplekin, aPKC, Par3, and Par6; many other tight junction constituent proteins have also been identified. Their expression sites differ greatly depending on the type of tight junction constituent protein. The expression level of tight junction constituent proteins changes in various skin diseases. For example, ichthyosis vulgaris and lichen planus have increased expressions of occludin, claudin-4, and Zo-1 [5,6] whereas decreased claudin-1 expression is observed in atopic dermatitis [7,8] and psoriasis vulgaris [5,6]. Claudin-7, a tight junction constituent protein, is knocked down in esophageal cancer-derived cells, leading to a decrease in E-cadherin and cell proliferation [9]. Claudin-7 can be a diagnostic marker for lung cancer because the expression of the claudin-7 gene is increased in lung cancer cells compared with normal lung epithelial cells [10]. Moreover, claudin-7 is highly expressed in the tumor cells of ovarian cancer patients, which promotes local infiltration [11]. Conversely, claudin-7 has been shown to inhibit tumor infiltration in the tumor cells of lung cancer patients [12]. Thus, there is no consensus on whether claudin-7 has a protective effect on tumor cells.

Claudin-7 is known to act as an ion channel [13], but its function has not yet been fully elucidated [14]. According to previous reports, claudin-7 is expressed in the intestine [15], lung [10,12], and kidney [16–18]. Verification with mice revealed that overexpression or knockdown of claudin-7 caused changes in the permeability of Na^+ and Cl^- ions [16–19]. However, there have been no reports on the expression status and role of claudin-7 in the skin or changes in its expression level in skin diseases.

This study found that claudin-7 was regulated by HMG-CoA reductase in the epidermis because the inhibition of the novel cholesterol synthesis pathway by pitavastatin resulted in decreased claudin-7 expression. Claudin-7 was not expressed in the normal epidermis or atopic dermatitis but was highly expressed in the stratum granulosum of the epidermis of psoriatic lesions. Moreover, claudin-7 was not expressed in non-lesion sections of psoriatic skin. Immunoelectron microscopy revealed that claudin-7 is localized in the keratohyalin granules of the stratum granulosum of the psoriatic epidermis.

The rapid turnover of the psoriatic epidermis promotes the degradation of intercorneal lipids. Additionally, the water content of the stratum granulosum decreases based on barrier dysfunction. This phenomenon promotes the HMG-CoA-mediated *de novo* synthesis of epidermal cholesterol in the psoriatic stratum granulosum. Our data suggest that claudin-7 may be involved in psoriasis epidermal lipid metabolism.

The present study had a few limitations. The non-specific action of pitavastatin may evidently be involved in the claudin-7expression. Aside from HMG-CoA reductase inhibition, pitavastatin reportedly suppressed the secretion of pro-inflammatory cytokines by inhibiting ERK/p38 MAPK pathway in T lymphocyte [20]. Therefore, pitavastatin could possibly decrease claudin-7 expression differently than that by HMG-CoA reductase. The biggest limitation of our study is that we were unable to clarify the function of claudin-7 in keratohyalin granules. We hypothesized that claudin-7 may play a supportive role in promoting HMG-CoA-mediated cholesterol synthesis in the psoriatic epidermis. Furthermore, it was noteworthy that the expression of claudin-7 was limited to keratohyalin granule in keratinocytes and was not observed in intercellular space. Consequently, this finding was the basis of the assumption that claudin-7 might be involve in intercellular ionic regulation. In support of this assumption, claudin-7 has been reported to be involved in the permeability of sodium ions, we predicted that it might be involved in regulating sodium concentrations in the stratum corneum. Contrary to our expectations, sodium ion levels in the stratum corneum, were nearly comparable between patients with claudin-7 expression in keratohyalin granules and those with normal skin (data not shown). Owing to the absence of evidence to prove the involvement of claudin-7 in intracellular ionic regulation of keratinocyte, increased expression of claudin-7 in psoriatic epidermis cannot be distinguished between cause and effect of psoriasis. Considering that the barrier function is impaired in psoriasis, claudin-7 might be involved in hydration or dehydration by regulating ion channels. Krasovec M [21] focused on skin rash as a side effect of HMG-CoA reductase inhibitors. They argued that HMG-CoA reductase inhibitors may have caused the rash by inhibiting epidermal cholesterol synthesis and affecting the barrier function of the skin, and their consequence supports our findings. There are still many unclear areas about the function of claudin-7. Further studies to clarify the remaining issues are warranted and will largely contribute to understanding the biological understanding of the psoriatic epidermis leading to the formulation of novel therapeutic interventions.

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Declaration of Competing Interest

HM is a patent holder of ID: 5678566. Other authors have no conflicts of interest to declare.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jdermsci.2021.10.002.

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