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Title

Enolase-1 expression in the stratum corneum is elevated with parakeratosis of atopic dermatitis and disrupts the cellular tight junction barrier in keratinocytes

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Title

Enolase-1 expression in the stratum corneum is elevated with parakeratosis of atopic dermatitis and disrupts the cellular tight junction barrier in keratinocytes

Abstract

Objective: Previous studies have shown that enolase-1 (ENO1) in the stratum corneum (SC) is more highly expressed in patients with atopic dermatitis (AD) than in healthy individuals, suggesting that it is a novel biomarker for evaluating skin condition in patients with AD. However, the mechanism underlying high ENO1 expression in the SC and its pathological relevance in AD are unclear. In this study, the relationship between ENO1 expression and keratinization of epidermis was investigated and the role of high ENO1 expression in keratinocytes was characterized.

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Methods: ENO1 expression and morphological characteristics were examined in SC from the cheeks of 24 patients with AD. Additionally, the localization of ENO1 in the excised human epidermis was observed. Moreover, to analyze the role of ENO1 in cellular barrier function, tight junction proteins (TJs) and trans-epithelial electrical resistance (TEER) in keratinocytes with ENO1 overexpression were evaluated. Furthermore, the localization of ENO1 and plasminogen in keratinocytes was evaluated by immunostaining, and the cellular barrier function in keratinocytes was examined after treatment with tranexamic acid (TXA).

Results: ENO1 expression was substantially correlated with the rate of nucleated corneocytes in AD. In addition, ENO1 localized in the basal to spinous layers, but was its expression dramatically decreased in healthy human SC. ENO1 overexpression in human epidermal keratinocytes reduced the expression of TJs (claudin-4, E-cadherin, tricellulin, and occludin) and TEER, and treatment with anti-ENO1 IgG reversed these effects. ENO1 co-localized with plasminogen in keratinocytes. Treatment with TXA rescued the ENO1-induced reductions in TJ and TEER expression.

Conclusion: We found a substantial correlation between ENO1 expression and the rate of nucleated corneocytes in AD and decreased ENO1 expression with nuclear disappearance. These results suggest that high ENO1 expression in the SC of AD is

caused by deficient keratinization, which is an AD characteristic. Moreover, ENO1 overexpression in keratinocytes promoted dysfunction of TJ dynamics, leading to reduced integrity of the cellular barrier, and these effects might be mediated by plasmin activity. We propose that ENO1 is a useful indicator of parakeratosis and might have a potential role in cellular TJ barrier function in the epidermis.

Keywords

Enolase-1; biomarker; skin barrier; cell culture; skin physiology/structure

Introduction

Atopic dermatitis (AD) is a chronic, inflammatory cutaneous disease caused by complex interactions among genetic, immunologic, and environmental factors [1,2]. For optimal AD treatment, clinical characteristics and skin conditions must be correctly evaluated.

AD severity can be assessed using several biomarkers, including total and allergen-specific serum IgE [3], serum thymus and activation-regulated chemokines [4], lactate dehydrogenase [5], serum CD30 [6,7], macrophage-derived chemoattractants [8], and interleukin-12,-16,-18-, and -31 [3,9-11]. In addition, several stratum corneum (SC)

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biomarkers have been used to evaluate local skin condition [12-14]. SC samples can be collected non-invasively by applying and removing tape from the skin surface. Enolase-1 (ENO1) levels in the SC (scENO1) are higher in patients with AD than in healthy controls, and scENO1 expression is a potential marker for AD [15,16].

ENO1 is a key glycolytic enzyme in the cytoplasm of cells; it is a multi-functional protein [17] expressed on the cell surface of many tumors, pathogens, and activated immune cells [18-22]. ENO1 is a plasminogen (PLG) receptor and concentrates PLG at the cell surface [23-27]. However, it is unclear whether ENO1 functions as a PLG receptor in the epidermis, and whether its function is related to the pathology of AD. Plasmin (PL) is a serine protease that degrades proteins of the extracellular matrix [28-31] and down-regulates cell-cell adhesion by cleaving E-cadherin [32]. The ENO1-PLG interaction is mediated by the binding of PLG kringle domains to the C-terminal lysine residues of ENO-1, and lysine analogs, such as TXA, inhibit PLG binding to ENO-1 [33, 34]. TXA was shown to block plasmin formation by human leukemia U937 cells and human blood monocytes [35]. Tight junction (TJ) proteins connect keratinocytes in the stratum granulosum and control the paracellular passage of ions and solutes, which indirectly affect water transport [36-38]. Furthermore, it has been proposed that TJs could play an important role in the formation of desmosomes by maintaining protease access in

the granular layer, which is important for compartmentalization of the SC [39-41]. The disruption of TJ in epidermal cells causes skin barrier dysfunction and various cutaneous diseases, including AD [42].

Based on these previous results, we speculated that ENO1 enhances TJ barrier dysfunction by inducing PL activity in the epidermis. Accordingly, the aims of this study were to clarify the mechanisms underlying the high ENO1 expression in the SC of AD patients and the effect of the elevated ENO1 expression on TJ barrier function.

Materials and methods

Clinical study

Subjects

The study subjects included 24 patients with AD. The patients were Japanese females, 32.6 ± 10.01 years old (mean $\pm$ S.D.). AD was evaluated comprehensively by degree of dryness, desquamation, erythema, pimples and itching of the face, and diagnosed at five levels, i.e., asymptomatic, minor, mild, moderate, and severe, by a dermatologist. The score of 9 AD patients were minor, whereas 11 patients were mild, 4 were moderate. The study protocol conformed to the ethical guidelines of Toho University's ethics committee

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and was conducted according to the principles of the Declaration of Helsinki. Informed consent was obtained from each patient prior to enrollment in the study. Exclusion criteria included scarring or inflammation at the test site.

SC sample preparation

The SC samples were obtained from participants by consecutively applying two adhesive tapes (Horny Layer Checker; Asahi Biomed, Tokyo, Japan) to a $2.5 \times 2.5 \text{ cm}^2$ area of the upper cheek and stripping it off at room temperature after removal of make-up.

Determination of scENO1 levels by ELISA

To examine scENO1 expression, proteins were extracted from SC samples by vortexing the tape strips with glass beads in 500 μL of radioimmunoprecipitation (RIPA) buffer (Thermo Fisher Scientific, Waltham, MA, USA). Total protein was quantified by the bicinchoninic acid assay (Thermo Fisher Scientific). In most cases, approximately 50–300 μg (average 125 μg) of total protein was recovered in 500 μL of SC extract from each skin site. These samples were directly assayed by ELISA for ENO1. The 96-well EIA plates (Corning, New York, NY, USA) were coated with 100 μL of ENO1

monoclonal antibody (mAb) (Abnova, Taipei, Taiwan) at 25°C overnight. On the next day, the plates were washed with phosphate-buffered saline (PBS)/0.1% Tween 20 and blocked with 1% bovine serum albumin (BSA) for 1 h at 25°C. The plates were washed, and each SC extract and ENO1 recombinant protein (Abnova) as a standard were applied to the plates and incubated at 25°C for 2 h. After washing, 100 µL of biotinylated ENO1 polyclonal antibody (pAb) (Abnova) was applied to the plates and incubated for 2 h. After incubation, the plates were washed and 100 µL of streptavidin-HRP (R&D Systems, Minneapolis, MN, USA) was applied and incubated for 20 min at 25°C. After the plate was washed, 100 µL of TMB One Solution (Promega, Madison, WI, USA) was applied to the plates and incubated for 20 min at 25°C. Then, 50 µL of 2 N H₂SO₄ was applied to the plates and the absorbance was measured at 450 nm (SpectraMax; Molecular Devices, Sunnyvale, CA, USA).

Morphological analyses of corneocytes

To analyze the morphological characteristics of the SC, corneocytes in SC samples were stained with brilliant green and gentian violet (BG) staining following the methods of Kikuchi et al. [43]. SC samples were stained with 0.5% brilliant green (Merck KGaA, Darmstadt, Germany) and 1% gentian violet (Kanto Chemical Co., Inc., Tokyo, Japan)

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and observed using a digital microscope at 500× (Keyence, Tokyo, Japan). Three morphological factors were measured in the stained samples following the methods of Masayuki et al. [44], i.e., rate of nucleated corneocytes, desquamative state, and corneocyte area. To evaluate rate of nucleated corneocytes, each image was manually counted to determine the number of all corneocytes and the number of nucleated corneocytes (Fig. 1a), and then the nucleated corneocytes rate was calculated. For the analysis, the average value of the rate from three images of each sample was used. To evaluate the desquamative state, each image with BG-stained corneocytes (Fig. 1b left) was binarized with two threshold values, i.e., recognizing the area of total corneocytes (Fig. 1b center) and the area of stratified corneocytes (Fig. 1b right), and the areas were quantified using ImageJ (NIH, Bethesda, MD, USA). For analysis, the ratio obtained by dividing the area of stratified corneocyte area by the area of total corneocytes was used. To evaluate the corneocyte area, the edges of each corneocyte were traced manually and the area was measured by using ImageJ (Fig 1c). For the analysis, the average value of the cell area contained in three images was used.

***Ex vivo* study on excised skin**

Fluorescence immunostaining of human epidermal tissues

Human epidermal sections (from the abdomens of 23-, 24-, 28-, 61-, 64-, and 68-year-old healthy Caucasian women) fixed with 4% paraformaldehyde in PBS were purchased from KAC Co., Ltd. (Kyoto, Japan). The skin sections were washed with 0.05% Tween 20 in PBS and blocked with StartingBlock blocking buffer at room temperature. Then, they were stained with mouse anti-ENO1 mAb (Abnova) diluted in StartingBlock blocking buffer at a dilution of 1:500 for 1 h at 37°C. After washing, sections were labeled with secondary antibodies (Alexa Fluor 488 goat anti-mouse IgG) and DAPI (Thermo Fisher Scientific) at dilutions of 1:1000 and 1:5000, respectively, for 30 min at 37°C. After washing, the sections were observed using a confocal laser scanning microscope (Olympus FV-1000, Tokyo, Japan) with a 40× dry lens. The difference in ENO1 expression between the SC and the other layer was examined by evaluating fluorescence intensity using ImageJ.

Cell-based assay

Cell culture and transfection with *ENO1*

Normal human epidermal keratinocytes (NHEKs) of Caucasian origin (Lonza, Basel, Switzerland) were cultured in EpiLife medium (Life Technologies, Carlsbad, CA, USA) with Humedia-KG2 additives (Kurabo, Osaka, Japan) at 37°C under 5% CO₂. For a transient assay, ENO1 expression plasmids (3 µg/mL) or control plasmids (3 µg/mL) (Promega) were transfected using FuGENE HD transfection reagent (Promega) according to the manufacturer's instructions.

Immunoblotting for ENO1 and TJ detection

NHEKs were washed with PBS and resuspended in 200 µL of cell lysis buffer (120 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0.4% NP-40, and 1 mM Na₃VO₄) at 4°C for 30 min. The protein concentration was determined using a Bicinchoninic Acid Protein Assay Kit (Thermo Fisher Scientific). Samples were separated by 15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were blocked with StartingBlock blocking buffer (Thermo Fisher Scientific) and labeled with the primary antibodies diluted in StartingBlock blocking buffer at 1:5000 dilution overnight at 4°C.

The following primary antibodies were used: mouse anti-ENO1 mAb (Abnova), rabbit anti-tricellulin pAb (LifeSpan Biosciences, Seattle, WA, USA), rabbit anti-claudin-4 pAb (Abcam), rabbit anti-occludin pAb (Thermo Fisher Scientific), mouse anti-E-cadherin mAb (QED Bioscience, San Diego, CA, USA), and mouse anti- β -actin mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were washed in 0.1% Tween 20/PBS before labeling with the following secondary antibodies diluted in StartingBlock blocking buffer at a dilution of 1:10000 for 1 h at room temperature: horseradish peroxidase (HRP)-anti-rabbit IgG (Invitrogen) and HRP-anti-mouse IgG (Invitrogen). Reaction products were detected with Enhanced Chemiluminescence Prime Western Blotting Detection Reagent (GE Healthcare, Little Chalfont, UK) for 5 min. Band intensities were analyzed using an ImageQuant LAS-4000 mini-series (GE Healthcare).

Trans-epithelial electrical resistance (TEER) measurements

NHEKs were seeded on 12-well trans-well 0.4- μ m polyester membrane inserts (Corning) (2×10^5 cells/1.12 cm² well) 1 day after transfection. NHEKs were then cultured in medium containing 2.0 mM CaCl₂ for 7 days to induce differentiation. TEER was then measured using a Millicell-ERS voltmeter (Millipore, Billerica, MA, USA) in triplicate and calculated according to the formula: TEER = resistance $\times$ area ($\Omega \cdot \text{cm}^2$, with an area of 1.12 cm²).

Fluorescence immunostaining of NHEKs

NHEKs were fixed with 4% paraformaldehyde in PBS for 1 h at 4°C, then washed with 0.05% Tween 20 in PBS and blocked with StartingBlock blocking buffer at 37°C. After blocking, cells were labeled with mouse anti-ENO1 mAb (Abnova), mouse anti-E-cadherin mAb (QED Bioscience), and rabbit anti-PLG pAb (Abcam) diluted in StartingBlock blocking buffer at a dilution of 1:500 for 1 h at 37°C. After they were washed with 0.05% Tween 20 in PBS, cells were incubated in secondary antibody solutions (Alexa Fluor 488 goat anti-mouse IgG, Alexa Fluor 568 goat anti-rabbit IgG) and DAPI at dilutions of 1:1000 and 1:5000, respectively, for 30 min at 37°C. After washing with 0.05% Tween 20 in PBS, NHEKs were observed using a confocal laser scanning microscope (FV-1000; Olympus) with a 40× dry lens.

Treatment of NHEKs with an ENO1 antibody and TXA

To assess the effect of an ENO1 inhibitor or ENO1-PLG binding inhibitor, NHEKs were incubated in the absence or presence of 10 µg/mL mouse monoclonal anti-ENO-1 (Abnova), 10 µg/mL IgG control (BD Biosciences, Franklin Lakes, NJ), or 10 mM TXA (Sigma-Aldrich, Taufkirchen, Germany) for 1 h at 37°C.

Statistical analysis

Data from experiments are expressed as the mean $\pm$ standard deviation (SD) for each test or control group. Statistical significance was evaluated using Student's *t*-test and the Wilcoxon signed-rank test. Correlation analysis was performed using the Pearson product-moment correlation test. All statistical analyses were implemented in BellCurve for Excel ver.7.0 (Social Survey Research Information Co., Ltd., Tokyo, Japan). Results were considered significantly significant when $P < 0.05$ (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Results

ENO1 expression decreased after keratinization in the human epidermis

To verify the relationship between ENO1 and the SC condition, SC samples were collected from 24 patients with AD. SC samples were examined with respect to the rate of nucleated corneocytes, desquamative state, and corneocyte area. scENO1 expression was correlated with the rate of nucleated corneocytes (Fig. 2a), but not with the desquamative state (Fig. 2b) or corneocyte area (Fig. 2c). In addition, to investigate the changes in ENO1 expression during keratinization, human epidermal sections were stained with

ENO1 and the difference in ENO1 expression between the basal to spinous layer and the SC were analyzed based on fluorescence intensity. ENO1 expression was detected in the basal to spinous layers, but was substantially lower in the SC in 6 healthy human skin tissue specimens. Fig. 3a shows ENO1 localization in the human epidermis from two representative cases, i.e., 24- and 61-year-old subjects, and Fig. 3b summarizes ENO1 expression in 6 human skin tissues.

ENO1 overexpression disrupts TJs and reduces TEER level in NHEKs

Based on previous reports, we speculated that ENO1 expression in keratinocytes is related to TJ barrier dysfunction. Therefore, in this study, to clarify this relationship, we performed transient overexpression assays. We confirmed that ENO1 expression in NHEKs was increased significantly by transfection. The expression levels of claudin-4, E-cadherin, tricellulin, and occludin in NHEKs with ENO1 overexpression and the absence or presence of anti-ENO1 IgG or control IgG were determined by immunoblotting. The expression levels of claudin-4, E-cadherin, tricellulin, and occludin in NHEKs were significantly reduced by ENO1 overexpression. The reduction in claudin-4, E-cadherin, tricellulin, and occludin levels by ENO1 overexpression was suppressed by addition of the ENO1 antibody, but not by addition of the IgG control (Fig.

4a). Moreover, the TEER levels in the NHEKs were also measured. NHEKs overexpressing ENO1 with or without the IgG control exhibited reduced TEER levels, but the addition of anti-ENO1 IgG abrogated the reduction of TEER levels in keratinocytes (Fig. 4b).

TXA suppresses the effect of ENO1 overexpression

We investigated whether ENO1 in NHEKs acted as a PLG receptor, and whether the effect of ENO1 overexpression on cellular barrier function is mediated by plasmin. The localization of ENO1 and PLG in NHEKs was observed by immunostaining. Fig. 5 shows that ENO1 and PLG were both expressed on the cell membrane, and the ENO1-rich regions corresponded to PLG-rich regions. Next, we investigated whether ENO1 overexpression-induced cellular barrier dysfunction was mediated by PL activity. TXA has been reported to inhibit the binding of ENO1-PLG; accordingly, TXA was applied to NHEKs overexpressing ENO1, and claudin-4, E-cadherin, tricellulin, occluding, and TEER levels were examined. The reduced expression levels of claudin-4, E-cadherin, and occludin in response to ENO1 overexpression were significantly reversed by TXA (Fig. 6a), and tricellulin was marginally suppressed. Moreover, the decline in TEER by ENO1 overexpression was also reversed by TXA (Fig. 6b).

Discussion

Previous reports suggest that ENO1 expression is higher in the SC of patients with AD than in the SC of healthy individuals; therefore, scENO1 expression is a potential marker for AD [15, 16]. Our preliminary study confirmed that scEno1 expression in the cheek of AD patients was high even when our ELISA method was used (data not shown). In this study, the mechanisms underlying the high scENO1 expression in AD and the role of ENO1 in cellular barrier function in keratinocytes were evaluated.

The relationship between scENO1 expression and morphological characteristics in the SC of AD patients was analyzed. scENO1 expression was substantially correlated with the rate of nucleated corneocytes. In addition, to verify that ENO1 expression decreases with normal keratinization in the epidermis, we observed ENO1 expression in excised healthy abdominal tissues by immunostaining. The immunostaining data indicated that ENO1 expression was high in the basal layer to the spinous layer, but low after DNA degradation. These results suggest that ENO1 expression in NHEKs decreases during keratinization. Therefore, it is possible that the high expression of scENO1 in patients with AD can be explained by incomplete keratinization in the SC of patients with AD. However, there are physiological and environmental differences between the epidermis

from the cheek and the abdomen. As we were unable to obtain cheek skin tissues from patients with AD in this study, it is necessary to verify the localization of ENO1 in cheek skin tissues with AD or parakeratosis in the future.

Next, we performed transient overexpression assays using NHEKs. ENO1 overexpression in NHEKs reduced TJ and TEER levels, and these effects were abrogated by addition of an anti-ENO1 IgG. These results suggest that ENO1 overexpression promotes dysfunction of TJ dynamics, leading to reduced integrity of the cellular barrier. Previous reports suggested that ENO1 acts as a PLG receptor and concentrates PLG in the pericellular region of tumors and activated immune cells [18-22]. PL is a serine protease that degrades extracellular matrix and TJ proteins [28-32]. Based on these observations, we speculated that ENO1 in keratinocytes may act as a PLG receptor and may cause TJ barrier dysfunction by promoting PL-mediated disruption of cell–cell junctions, as observed in tumor and activated immune cells. Thus, we observed the localization of ENO1 and PLG in NHEKs by immunostaining. ENO1 and PLG co-localized at the cell membranes of NHEKs, supporting the role of ENO1 in NHEKs as a PLG receptor. Moreover, we found that TXA, an ENO1-PLG binding inhibitor, reversed the disruption of TJ protein expression and TEER levels induced by ENO1 overexpression. Based on these results, we propose that the cellular barrier dysfunction in NHEKs associated with

ENO1 overexpression is caused by ENO1-induced PL activity. It has been proposed that TJs could play a role in the formation of desmosomes and compartmentalization of the SC [39-41]. Therefore, ENO1 might have a potential role in deficient keratinization by disruption of TJ barrier. However, some points require further clarification. For example, although TXA is an efficient ENO1-PLG binding inhibitor [33, 34], it has additional functions and therefore may influence other pathways. To further verify that ENO1 acts as a PLG receptor and promotes PL activity in keratinocytes, additional studies are needed, e.g., analyses with a highly specific ENO1-PLG binding inhibitor or gene disruption experiments. Moreover, it is necessary to verify that the results of the cell-based assay are consistent with processes on the human skin surface. Voegeli et al. reported that plasmin activity is correlated to trans-epidermal water loss [45]. Therefore, it is important to investigate the relationship between scENO1 and plasmin activity in future studies.

In summary, we found that scENO1 expression may be elevated by parakeratosis. We propose that scENO1 is a useful indicator of parakeratosis in AD. In addition, our results suggest that ENO1 overexpression promotes cellular barrier dysfunction by inducing plasmin activity in keratinocytes. We consider that further studies on ENO1 will enhance the understanding of the process of skin barrier formation.

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