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Autophagy Regulates Transglutaminase-1 Expression and Vitamin C Enhances Autophagic Activity in Keratinocytes

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Abstract

Vitamin C, known for its various effects on the skin, promotes epidermal differentiation and enhances skin barrier function. However, the underlying mechanism remains unclear. Autophagy is an intracellular degradation system that maintains cellular homeostasis. Although autophagy decrement has been associated with barrier defects in skin diseases, the mechanism by which autophagy regulates skin barrier function remains insufficiently understood. Therefore, this study aimed to investigate the relationship between autophagy and transglutaminase-1 (TGase-1), a molecule required to form the cornified envelope that contributes to skin barrier function. We also examined the effect of vitamin C on autophagy in epidermal keratinocytes. Autophagy modulation through the knockdown of autophagy-related molecules (ATG5, ATG7, and ATG13) significantly decreased TGase-1 expression in human epidermal keratinocytes. Furthermore, vitamin C treatment enhanced the autophagic activity of epidermal keratinocytes and suppressed TGase-1 expression decrease in ATG13 knockdown cells. In conclusion, TGase-1 expression can be regulated by autophagy, and vitamin C may be involved in skin barrier function through autophagy activation.

Abbreviations: TGase-1: transglutaminase-1; CE: cornified envelope; ATG5: autophagy related 5; ATG7: autophagy related 7, ATG13: autophagy related 13, LOR: loricrin; FLG: filaggrin; TBP: TATA-box binding protein; LC3: microtubule-associated protein 1 light chain 3; CQ: chloroquine; VC: vitamin C; CTL: control.

Introduction

The epidermis, which is primarily composed of keratinocytes, is essential for maintaining the skin's overall barrier function. Keratinocytes undergo differentiation as they move away from the basal layer toward the upper epidermal layers. During the process of differentiation, keratinocytes change structure and produce various factors that are crucial for maintaining the skin barrier's integrity and functionality[1]. The cornified envelope (CE) is a critical structure for barrier function in the outermost layer of epidermis[2,3]. CE is composed of various structural proteins, including involucrin and loricrin. These proteins are covalently cross-linked to form insoluble and aggregated structures beneath the cell membrane. Transglutaminase (TGase), including TGase-1, -3, and -5, catalyzes this reaction, thereby forming highly resilient CE, which contributes to the overall strength of the stratum corneum[3]. Several studies have demonstrated the importance of TGase-1 for CE formation. For instance, TGase-1 knockout mice show impaired barrier function and neonatal mortality caused by the absence of CE formation[4]. In addition, TGase gene mutations have been associated with a skin disorder called lamellar ichthyosis, which is characterized by

impaired CE formation and severe barrier dysfunction[5]. These findings highlight the indispensable role of TGase-1 and CE formation in maintaining the protective barrier function of the skin.

Vitamin C, also known as ascorbic acid, is a water-soluble vitamin that is essential for various physiological functions in the body. In healthy skin, relatively high concentrations of vitamin C are present, contributing to the maintenance of skin health and functionality[6]. Vitamin C has numerous beneficial effects on the skin, including antioxidant effects and the inhibition of excessive melanogenesis[6-8]. Moreover, it enhances skin barrier function by promoting epidermal differentiation[9, 10]. However, the detailed mechanisms underlying the role of vitamin C in promoting epidermal differentiation and skin barrier function remain unclear.

Autophagy is a cellular degradation mechanism that targets various cellular components, including proteins, lipids, damaged organelles, and pathogens, and maintains cellular homeostasis[11-13]. In the context of epidermal keratinocytes, autophagy has been implicated in the regulation of responses to UV radiation and cellular senescence[14-16]. It is also involved in the degradation of internalized melanosomes, which regulate pigmentation[17-19]. Recent reports indicate the involvement of autophagy in epidermal differentiation[20-22]. However, the precise role of autophagy in the regulation of skin barrier function remains largely unknown.

In this study, we aimed to investigate the contribution of autophagy to skin barrier function by examining the impact of autophagy reduction on TGase-1 expression. In epidermal keratinocytes with autophagy impairment through autophagy-related molecule (ATG5, ATG7, or ATG13) knockdown using siRNA, we observed decreased TGase-1 mRNA and protein expression. Furthermore, treatment of autophagy-reduced cells with vitamin C prevented TGase-1 expression decrease. Thus, the expression may be regulated by autophagy, and vitamin C may play a vital role in skin barrier function through autophagy activation.

Materials and Methods

Cell culture

Normal human epidermal keratinocytes (NHEKs), which were purchased from Kurabo, were cultured in HuMedia-KG2 (Kurabo) supplemented with human epidermal growth factor (0.1 ng/ml), insulin (10 mg/ml), gentamicin (50 mg/ml), amphotericin B (50 ng/ml), and bovine brain pituitary extract (0.4% [v/v]) at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

siRNA transfection

We transferred siRNA into NHEK cells by using lipofectamine RNAiMax (Thermo Fisher Scientific) according to the manufacturer's instructions. Three days after transfection, cells were treated with or without sodium ascorbate (FUJIFILM Wako Chemicals) and subjected to quantitative real-time reverse transcription PCR or immunoblotting. We used the following siRNAs: siATG5: s18159; siATG7: s20651, s20652; and siATG13: s18881. All siRNAs were purchased from Thermo Fisher Scientific.

Quantitative real-time reverse transcription–polymerase chain reaction (RT-PCR)

Total RNA from NHEK cells was extracted using the QuickGene-AutoS RNA Cultured Cell Kit (Kurabo, Osaka, Japan). Extracted total RNA samples were reverse-transcribed using Random Primer (#3801, Takara Bio) and ReverTra Ace (#TRT-101, Toyobo). Quantitative PCR was performed on QuantStudio 3 (Thermo Fisher Scientific) using PowerTrack SYBR Green Master Mix (A46109, Thermo Fisher Scientific). Relative mRNA was calculated after normalization to the human TBP gene as an internal control for quantification using the $2^{-\Delta\Delta}$CT method. Primers are detailed as follows: TGM1 Forward Primer: 5′-GCACCACACAGACGAGTATGA-3′, Reverse Primer: 5′-GGTGATGCGATCAGAGGATTC-3′; ATG5 Forward Primer: 5′-AAAGAT GTGCTTCGAGATGTGT-3′, Reverse Primer: 5′- CACTTTGTCAGTTACCAACGT CA-3′; ATG7 Forward Primer: 5′-CAGTTTGCCCCTTTTAGTAGTGC-3′, Reverse Primer: 5′-CCAGCCGATACTCGTTCAGC-3′; ATG13 Forward Primer: 5′-TTGCT ATAACTAGGGTGC AACCA-3′, Reverse Primer: 5′- CCCAACACGAACTGTCTG GA-3′; FLG Forward Primer: 5′-GGACAGGAACAATCATCGGGG-3′, Reverse Primer: 5′-CAACCTCTCGGAGTCGTCTG-3′; LOR Forward Primer: 5′-CGAAGG AGTTGGAGGTGTTT-3′, Reverse Primer: 5′-GGCTTCTTCCAGGTAGGTTAAG-3′; S100A Forward Primer: 5′-GACCCTCATCAACGTGTTCCA-3′, Reverse Primer: 5′-CCACAAGCACCACATAC TCCT-3′; and TBP Forward Primer: 5′-CCCGAAACGC CGAATATAATCC-3′, Reverse Primer: 5′-AATCAGTGCCGTGGTTCGTG-3′.

Immunoblotting

NHEKs were seeded in six-well plates at a density of 3.0×10^5 cells/well and cultured for 24 h. Thereafter, the cells were cultured with or without sodium ascorbate, or chloroquine diphosphate (Fujifilm Wako Chemicals) for the indicated times and then lysed on ice in RIPA buffer supplemented with protease inhibitor cocktail (P8340-1ML, Sigma). After centrifugation, the protein concentration of the supernatant was measured using a Takara BCA Protein Assay kit (T9300A, Takara Bio) and dissolved in sample buffer. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were blocked with 5% skim milk for 1 h and probed with the following primary antibodies: anti-LC3B

antibody (ab51520, Abcam), anti-β-actin antibody (#3700, Cell Signaling Technology), anti-TGase-1 (SC-25786, Santa Cruz Biotechnology), and anti-ATG13 antibody (#13273, Cell Signaling Technology). Subsequently, we incubated the membranes with secondary antibodies (Cell Signaling Technology), detected the signals by chemiluminescence using an ECL prime kit (RPN2232, Fisher Scientific), and captured images by using LAS500 (Cytiva).

Immunofluorescence microscopy

NHEKs were grown in an eight-well chamber and treated with or without sodium ascorbate fixed in cold methanol for 15 min. Cells were washed using phosphate-buffered solution (PBS) to remove excess fixative. After washing, the cells were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) for 15 min and blocked with 2% skim milk (Becton Dickinson and Company) in PBS for 10 min at room temperature. After blocking, cells were incubated with primary antibody overnight at 4°C, washed thrice, and incubated for 1 h with appropriate secondary antibodies conjugated with fluorophore. For nuclear staining, we used 4′,6-diamidino-2-phenylindole. Images were acquired using the All-in-one Fluorescence Microscope (BZ-X800, Keyence). The area or number of positive signals in the images was calculated in automated manner using BZ-X800 analyzer software (Keyence).

Statistical analysis

Statistical data were analyzed using Prism10 (GraphPad Software). All quantitative data were expressed as means ± SD and significant differences were determined by the two-tailed unpaired student's t-test or ANOVA followed by Dunnett's test. *P* values <0.05 were considered significant.

Results

Decreased TGase-1 expression in cells with decreased autophagy

The relationship between autophagy and TGase-1 was investigated using knockdown experiments targeting key autophagy-related genes (ATG5, ATG7, and ATG13) in epidermal keratinocytes. In assessing the knockdown efficiency of each target gene using siRNAs (Figure 1a), we found a decrease in gene expression. Furthermore, immunoblotting analysis confirmed autophagy reduction in cells transfected with each siRNA (Figure 1b). Notably, RT-PCR revealed that cells with ATG5, ATG7, or ATG13 knockdown exhibited a significant decrease in TGase-1 mRNA expression (Figure 1c). Consistently, TGase-1 protein levels decreased in ATG13 knockdown cells (Figure 1d). Conversely, the expression of loricrin (LOR), filaggrin (FLG), and S100A genes, which are markers of epidermal differentiation, did not significantly change (Figure 1e).

Sodium ascorbate modulates the autophagy flux and rescues TGase-1 expression in autophagy-deficient cells

Considering the observed decrease in TGase-1 expression associated with autophagy reduction, we explored the effects of vitamin C on autophagy and TGase-1 expression in epidermal keratinocytes. In assessing autophagy flux, the LC3-II levels (Figure 2a) or the number of autophagosomes indicated by LC3 puncta (Figure 2b) after lysosome inhibitor treatment were compared with the levels observed in the absence of the inhibitor. Treatment with sodium ascorbate, a form of vitamin C, enhanced autophagy flux. Moreover, TGase-1 expression decrease observed in cells with ATG5, ATG7, or ATG13 knockdown was significantly mitigated by sodium ascorbate treatment (Figure 3a). Consistent with these effects, sodium ascorbate increased the autophagy flux level in ATG13 knockdown cells (Figure 3b). Taken together, vitamin C may counteract TGase-1 reduction caused by autophagy deficiency.

Discussion

Autophagy is critical for maintaining cellular homeostasis; however, its role in the skin remains largely unknown. In this study, we investigated its involvement in skin barrier function by examining changes in the expression of barrier-related genes in cells with impaired autophagy. We specifically focused on the changes in the expression of TGase-1, a key player in CE formation.

Our findings revealed that epidermal keratinocytes with decreased autophagy caused by the knockdown of autophagy-related genes (ATG5, ATG7, and ATG13) exhibited significantly decreased TGase-1 expression. Therefore, autophagy may play a crucial role in TGase-1 expression regulation. Conversely, decreased autophagy did not affect the expression levels of other barrier-related genes, such as loricrin, filaggrin, and S100A, which are involved in epidermal differentiation. Further investigation is needed to elucidate the mechanisms by which autophagy specifically regulates TGase-1 expression without affecting the expression of other epidermal differentiation markers.

Vitamin C, known for its various roles in the skin, has been implicated in the regulation of epidermal differentiation. For instance, the addition of vitamin C to the culture medium of three-dimensional epidermal models increases the levels of glucosylceramides, ceramide 6, and ceramide 7[9]. Furthermore, vitamin C enhances TGase-1 expression in epidermal keratinocytes by activating protein kinase C[10]. However, the effect of vitamin C on autophagy remains unclear. In the present study, vitamin C enhanced autophagy flux in epidermal keratinocytes. Notably, this autophagy flux enhancement by vitamin C was also observed in cells with ATG13 knockdown using siRNA. Although the exact mechanism by which vitamin C enhances autophagy flux

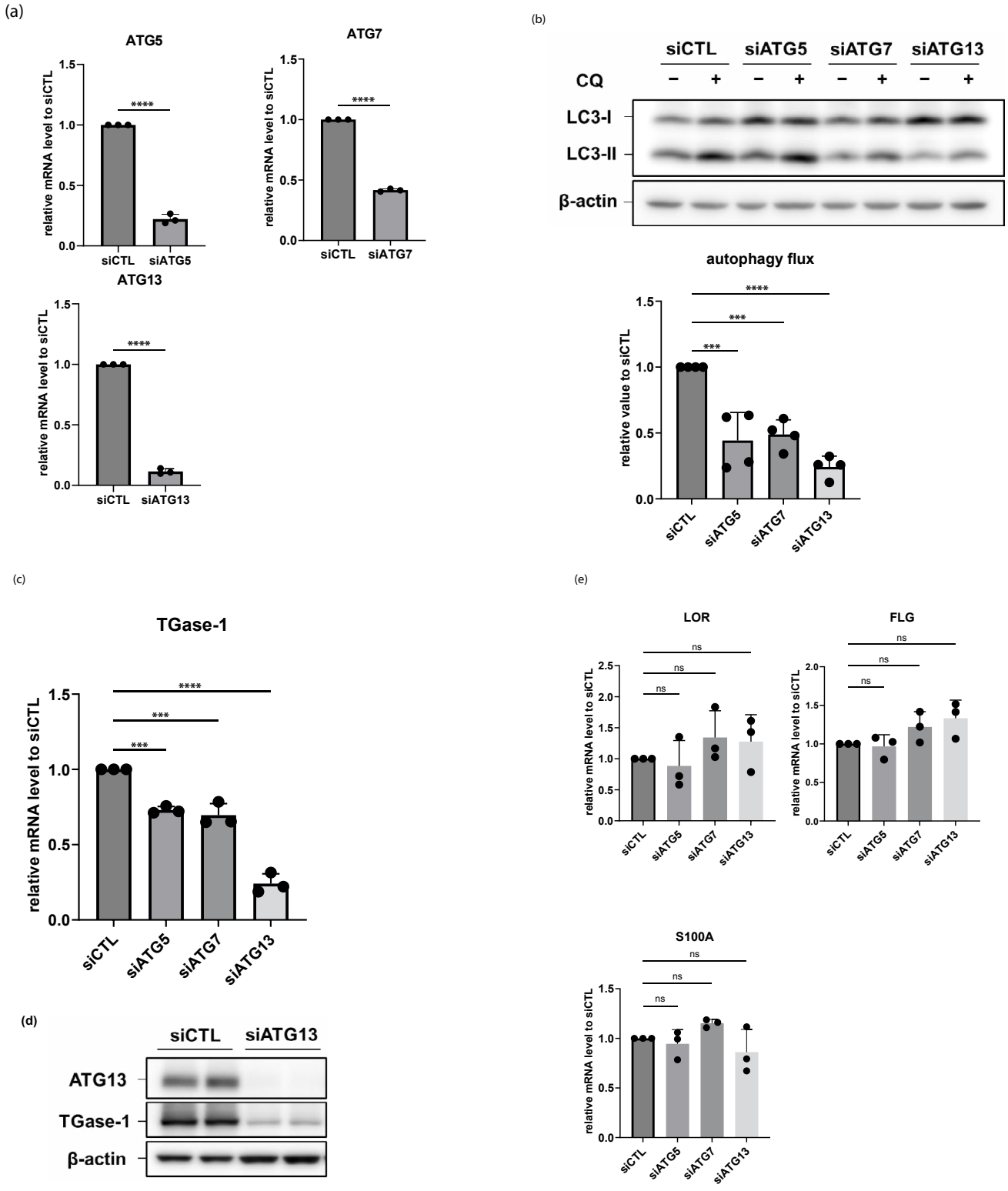

Figure 1. Impaired autophagy decreases TGase-1 expression. Human epidermal keratinocytes were incubated with siControl (CTL), siATG5, siATG7, or siATG13. After incubation, mRNA expression (a, c and e) and autophagy flux (b) were determined by qPCR and immunoblotting, respectively. Autophagy flux indicates the degradation of LC3-II, which is the substrate of autolysosomes, estimated by subtracting the LC3-II densitometric value (measured using ImageJ and normalized to β-actin value) without the lysosome inhibitor chloroquine (CQ) from the value with the inhibitor. Protein expression in siCTL cells and siATG13 cells was determined by immunoblotting (d). Data are presented as mean ± SD.***$P < 0.001$, ****$P < 0.0001$.

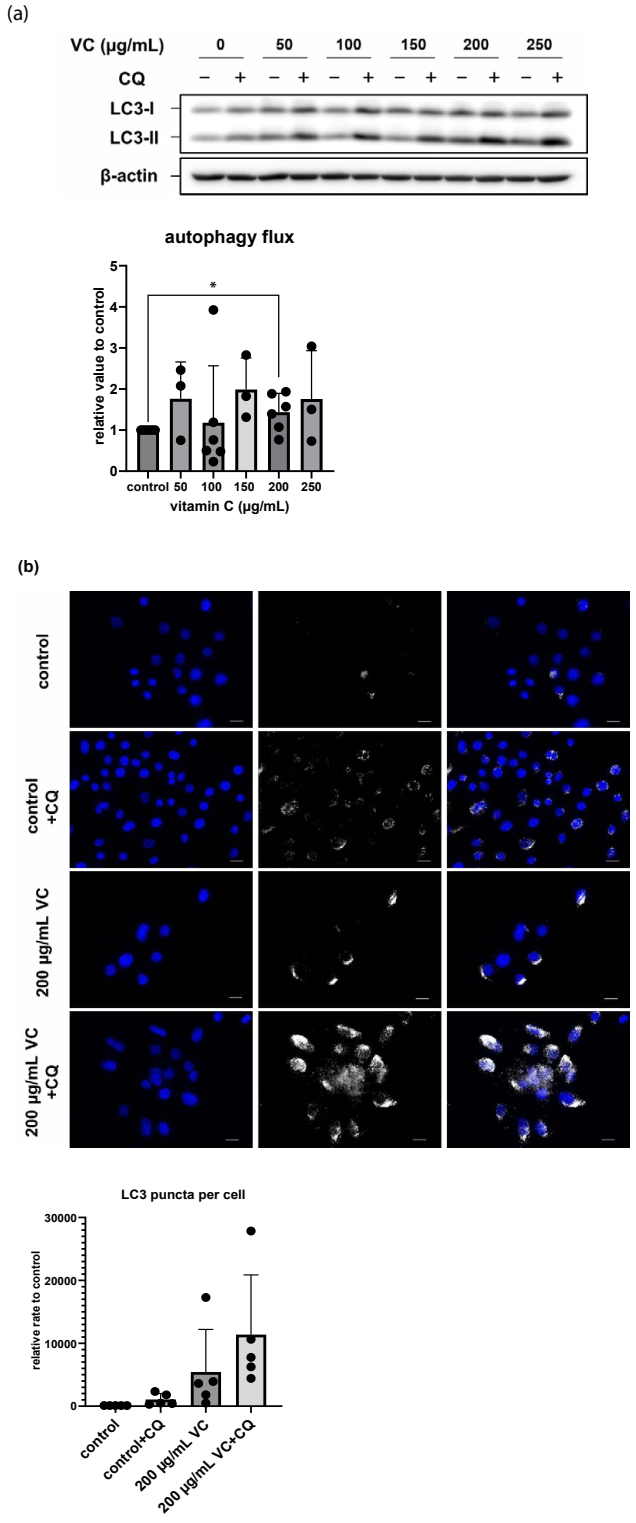

Figure 2. Vitamin C enhances autophagy in human epidermal keratinocytes. Keratinocytes were treated with or without sodium ascorbate (VC). After treatment, the cells were subjected to autophagy flux assay (a) and immunostaining with LC3 antibody and DAPI (b). LC3 puncta (gray) intensity per cell were calculated by BZ-X800 analyzer software (Keyence). Data are presented as mean ± SD. *$P < 0.05$. Scale bar = 20 μm.

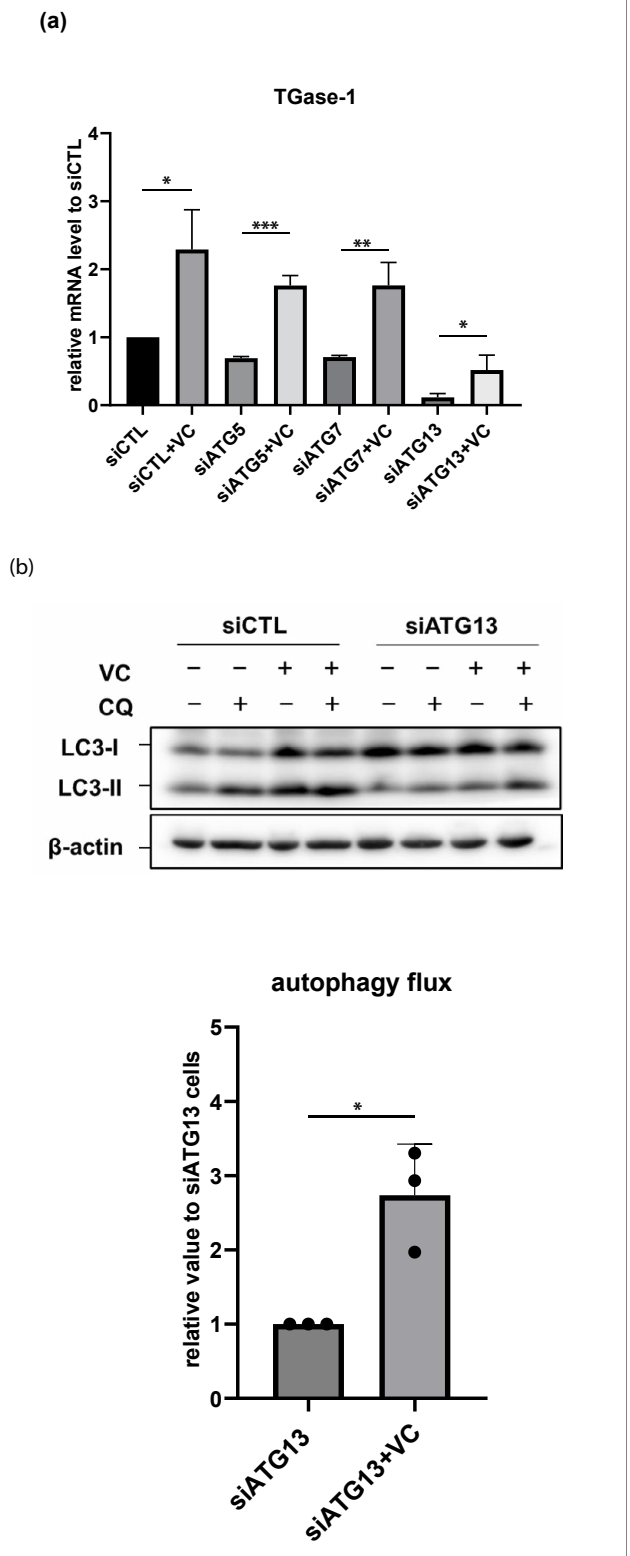

Figure 3. Vitamin C suppresses TGase-1 expression decrease caused by impaired autophagy. Expression of TGase-1 in keratinocytes in the presence of siCTL or siATG13, treated with or without sodium ascorbate, was determined by RT-PCR (a). Autophagy flux was assessed in keratinocytes in the presence of siCTL or siATG13, treated with or without sodium ascorbate (b). Data are presented as mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

remains insufficiently understood, vitamin C is likely to activate residual autophagy in these cells. In addition, TGase-1 expression decrease was significantly inhibited in ATG5, ATG7, or ATG13 knockdown cells treated with vitamin C. Taken together, vitamin C may partially control the expression through autophagy activation.

In conclusion, our study reported, for the first time, a novel finding that decreased autophagy leads to reduced TGase-1 expression in epidermal keratinocytes, potentially affecting the regulation of CE formation and compromising skin barrier function. Additionally, our findings also highlight the potential of vitamin C in modulating autophagy and rescuing decreased TGase-1 expression in cells with deficient autophagy. Collectively, autophagy may play a critical role in the regulation of TGase-1 expression, and vitamin C may be involved in maintaining skin barrier function through autophagy activation.

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Conflict of Interest

The authors declare no competing interests.

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References

1. Proksch E, Brandner JM, Jensen JM. The skin: an indispensable barrier. Exp Dermatol. 2008; 17: 1063-72.
2. Candi E, Schmidt R, Melino G. The cornified envelope: a model of cell death in the skin. Nat Rev Mol Cell Biol. 2005; 6: 328-40.
3. Hitomi K. Transglutaminases in skin epidermis. Eur J Dermatol. 2005;15: 313-9.
4. Matsuki M, Yamashita F, Ishida-Yamamoto A, et al. Defective stratum corneum and early neonatal death in mice lacking the gene for transglutaminase 1 (keratinocyte transglutaminase). Proc Natl Acad Sci U S A. 1998; 95: 1044-9.
5. Huber M, Rettler I, Bernasconi K et al. Mutations of keratinocyte transglutaminase in lamellar ichthyosis. Science. 1995; 267: 525-8.
6. Pullar JM, Carr AC, Vissers MCM. The Roles of Vitamin C in Skin Health. Nutrients. 2017; 9: 866.
7. Wang K, Jiang H, Li W, et al. Role of Vitamin C in Skin Diseases. Front Physiol. 2018; 9: 819.
8. Al-Niaimi F, Chiang NYZ. Topical Vitamin C and the Skin: Mechanisms of Action and Clinical Applications. J Clin Aesthet Dermatol. 2017; 10: 14-17.
9. Ponec M, Weerheim A, Kempenaar J, et al. The formation of competent barrier lipids in reconstructed human epidermis requires the presence of vitamin C. J Invest Dermatol. 1997; 109: 348-55.
10. Savini I, Catani MV, Rossi A, et al. Characterization of keratinocyte differentiation induced by ascorbic acid: protein kinase C involvement and vitamin C homeostasis. J Invest Dermatol. 2002; 118: 372-9.
11. Yoshimori T. Autophagy: a regulated bulk degradation process inside cells. Biochem Biophys Res Commun. 2004; 313: 453-8.
12. Vargas JNS, Hamasaki M, Kawabata T, et al. The mechanisms and roles of selective autophagy in mammals. Nat Rev Mol Cell Biol. 2023; 24: 167-185.
13. Mizushima N, Komatsu M. Autophagy: renovation of cells and tissues. Cell. 2011; 147: 728-41.
14. Deruy E, Gosselin K, Vercamer C, et al. MnSOD upregulation induces autophagic programmed cell death in senescent keratinocytes. PLoS One. 2010; 5: e12712.
15. Gosselin K, Deruy E, Martien S, et al. Senescent keratinocytes die by autophagic programmed cell death. Am J Pathol. 2009; 174: 423-35.
16. Qiang L, Wu C, Ming M, et al. Autophagy controls p38 activation to promote cell survival under genotoxic stress. J Biol Chem. 2013; 288: 1603-11.
17. Murase D, Hachiya A, Takano K, et al. Autophagy has a significant role in determining skin color by regulating melanosome degradation in keratinocytes. J Invest Dermatol. 2013; 133: 2416-2424.
18. Yang Z, Zeng B, Pan Y, et al. Autophagy participates in isoliquiritigenin-induced melanin degradation in human epidermal keratinocytes through PI3K/AKT/mTOR signaling. Biomed Pharmacother. 2018; 97: 248-254.
19. Kim JY, Kim J, Ahn Y, et al. Autophagy induction can regulate skin pigmentation by causing melanosome degradation in keratinocytes and melanocytes. Pigment Cell Melanoma Res. 2020; 33: 403-415.
20. Moriyama M, Moriyama H, Uda J, et al. BNIP3 plays crucial roles in the differentiation and maintenance of epidermal keratinocytes. J Invest Dermatol. 2014; 134: 1627-1635.
21. Akinduro O, Sully K, Patel A, et al. Autophagy and Nucleophagy during Epidermal Differentiation. J Invest Dermatol. 2016; 136: 1460-1470.
22. Monteleon CL, Agnihotri T, Dahal A, et al. Lysosomes Support the Degradation, Signaling, and Mitochondrial Metabolism Necessary for Human Epidermal Differentiation. J Invest Dermatol. 2018; 138: 1945-1954.