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Activation and upregulation of keratinocyte and epidermal transglutaminases are associated with depletion of their substrates in psoriatic lesions

N.S. ALHARBI, I.S. ALMAMI

Department of Biology, College of Science, Qassim University, Buraydah, Al-Qassim, Saudi Arabia

Abstract. – OBJECTIVE: Psoriasis is a chronic skin disorder caused by abnormal interactions between epidermal and immune cells. Thus, the interplay between the proliferation and differentiation of epidermal components should be tightly regulated to protect against psoriasis. The differentiation process is primarily controlled by transglutaminases (TGs). However, studies on TG enzymes and their molecular alterations in psoriatic skin lesions are limited. Therefore, this study aimed to investigate TG activity and gene and protein expression in human psoriatic and normal skin tissues.

MATERIALS AND METHODS: Keratinocyte TG (TG1), and epidermal TG (TG3) activity, localization, protein levels, and gene expression in human psoriatic skin were determined by immunohistochemistry and RT-qPCR. The expression of TG substrates (loricin and involucrin - IVL) was also investigated using RT-qPCR.

RESULTS: TG1 and TG3 enzymatic activities and gene expression were significantly higher in psoriatic skin tissue than in normal skin tissue. However, both TGs were present in the same location and were equally highly expressed. Moreover, the expression of two TG substrates (loricin and involucrin) was significantly decreased compared to that in psoriatic and healthy skin samples.

CONCLUSIONS: The activation and upregulation of TG1 and TG3 result from the depletion of their substrates (loricin and involucrin), both of which play a major role in the pathogenicity of psoriatic skin tissue and are necessary for proper skin development.

Key Words:
Psoriasis, Skin, Epidermal, Transglutaminase enzymes, Transglutaminase activity, Gene expression.

Introduction

Psoriasis is a chronic inflammatory autoimmune skin disease affecting approximately 2% of the general population[1]. It is characterized by scaly red lesions that develop into plaques at many sites on the human body[2-4]. Histologically, psoriatic skin has a thickened epidermis (acanthosis) due to the amplified proliferation of keratinocytes, epidermal elongation (rete ridge formation), and thickening and retention of keratinocyte nuclei in the stratum corneum (parakeratosis)[3]. Although the exact cause of psoriasis is unknown, it is probably caused by dysregulated keratinocyte proliferation, differentiation, and infiltration of immune cells in skin lesions[3,4].

The skin is a barrier that protects from environmental influences, such as infectious agents and heat dehydration[5]. Epidermal keratinocytes originate from the basal stratum and move upward through the epidermal layers, forming a multilayered epithelium[6]. Terminal differentiation of epidermal keratinocytes involves covalently cross-linked proteins formed in the cornified cell envelope (CE)[7]. This layer is a critical skin structure containing many epidermal proteins such as keratin, loricrin, involucrin, trichohyalin, vimentin, and several small proline-rich proteins (SPRs)[8,9]. Transglutaminase (TG) uses these proteins as substrates to develop this layer by forming an N-(y-gluta-myl)-lysine isopeptide bond[10]. In addition, disruption of the molecular functions of these enzymes has been linked to many skin diseases, including lamellar ichthyosis, ichthyosis vulgaris, psoriasis, and Dariers disease[9,11].

TGs are a family of enzymes that catalyze protein cross-linking by forming covalent bonds between glutamine and lysine residues in different polypeptides. This covalent isopeptide bond is highly resistant to proteolytic enzymes and increases the stability of modified proteins[12].

The main TGs associated with skin epidermal tissue are keratinocyte TG (TG1), TG2, epidermal TG (TG3), and TG5, which are distinctly distributed within this tissue. TG1 is primarily found in the upper layers of the epidermis and hair follicles[13] and plays a specific role in terminal

Corresponding Author: Ibtesam S. Almami, MD; e-mail: I.Almami@qu.edu.sa

epidermal differentiation by acting as an anchor protein[14]. TG2, also known as tissue transglutaminase (tTG), is a glutamine gamma-glutamyl transferase expressed in almost all tissues, organ-specific cell types[15,16], and basal keratinocytes[17]. TG3 is present in the upper granular layer and hair follicles and catalyzes the assembly of intra-protein crosslinks[18]. Although the exact location of TG5 is unknown, it is thought[14] to be located in the spinous and granular layers.

TGs are considered[19-21] crucial for the formation of cutaneous barriers. Thus, defects in TG1, TG3, and TG5 have essential effects on the integrity and function of the epidermis and trigger skin diseases[13]. TG upregulation causes an irregular skin phenotype[5]. The ability of TGs to switch a specific protein in tissue on or off by modifying its post-translational structure and activity makes it an interesting topic for biomolecular research.

TG expression and activity in the normal skin epidermis have been previously investigated[5,22-24]. Under physiological conditions, both TG1 and TG3 exhibit latent transamidation activity and low expression. Their catalytic function is initiated during terminal epidermal differentiation and assembly of the cornified cell envelop[5]. This activity is tightly regulated by tazarotene-induced gene 3 (*TIG3*) in TG1[22] cells and cathepsin proteolysis in TG3[23]. Tanabe et al[24] recently showed that TG1 downregulation decreased TG3 expression in a 3D culture model of differentiated keratinocytes. These findings suggested that TG1 strongly influences epidermal formation[25]. In contrast, TG5 is expressed in the stratum spinosum and granulosum, and is induced by TG5[26]. The gene expression and activity of these enzymes correlate with the deposition and aggregation of various epidermal proteins, including loricrin, a small proline-rich protein, involucrin, keratins, and filaggrin, which are used as substrates by TGs for cross-linking during keratinocyte differentiation and CE formation[7].

Because all three TGs play critical roles in keratinocyte differentiation and, thus, in cornfield envelope formation, defects in their genes have also been linked[9,11,27,28] to many pathological human epidermises. For instance, a mutation in TG1 can result in a rare keratinization disorder called "lamellar ichthyosis", which is characterized by abnormal formation of the cornified cell envelope[11]. TG3 expression is upregulated in patients with celiac disease, which causes skin blistering and herpetiformis phenotype[27]. A comparative study[9] of different human epidermal skin diseases showed that TG5 indirectly affects some skin disorders, including psoriasis, ichthyosis vulgaris, and Darer's disease. Recently, TG2 upregulation was shown[28] to be associated with the development of psoriasis.

Few studies in literature have focused on TG gene expression in psoriatic skin; however, no studies have focused on their enzymatic activity and protein levels. Therefore, this study primarily aimed to investigate the activity, gene expression, and protein levels of both TG1 and TG3 in human psoriatic skin and normal skin tissue samples. The results of this study may directly affect the future design of therapeutic drugs for the treatment of psoriasis.

Materials and Methods

All oligo-primers (Table I) were purchased from Integrated DNA Technologies (Coralville, IA, USA) and diluted to a working concentration of 10 μM/μL using ddH_2O and stored at -20°C. The primers were designed using Integrated DNA Technologies tools (PrimerQuest Tool, available at: https://eu.idtdna.com/pages/tools/primerquest), and the resuspension and dilution of the primers were calculated using the Resuspension Calculator and Dilution Calculator (both available at: https://eu.idtdna.com/pages/tools).

Biological Material from Humans

Human skin samples were obtained from the Department of Dermatology at Qassim University Hospital. All studies were approved by the Medical Ethics Committee of Qassim University and were conducted in accordance with the principles of the Declaration of Helsinki.

Human skin samples were obtained from the upper arm or back of eight 20-50-year-old male patients with moderate to severe psoriasis and eight healthy individuals with no history of skin diseases as controls, using a 3.0-mm diameter punch. No treatment was administered for at least two weeks before the biopsy. The samples collected were immediately added to 1 ml of RNA-Conserve™ Solution (G-Biosciences, St. Louis, MO, USA), rested at 25°C for 2 hours, followed by overnight incubation at 4°C, and then stored at −20°C until further processing. Biopsy samples for immunohistochemistry (IHC) were fixed overnight in 10% (v/v) formaldehyde.

Histology and Immunohistochemistry

The specific TG1 and TG3 locations and distributions in the epidermal layers of human psoriatic

Table I. Different oligonucleotide primers used in this study and their accession numbers.

Gene/Accession number	FWD Set 1	REV Set 1
Loricrin		
NM_000427.3	CGAAGGAGTTGGAGGTGTTT	GGCTTCTTCCAGGTAGGTTAAG
β-actin		
NM_001101.5	GGATCAGCAAGCAGGAGTATG	AGAAAGGGTGTAACGCAACTAA
Involucrin (IVL)		
NM_005547.4	GCCCACAAAGGGAGAAGTATTG	GACACTGCGGGTGGTTATTT
TG1		
NM_000359.3	GATGGCAGCTTCAAGATTGTTT	GAGCCTTCTGGGTGCTTATAG
TG3		
NM_003245.4	TGACGAAGGCTGTGTTTCC	AGGACTGGAGATGCTGATAGT

and normal skin tissues were compared. Briefly, tissues were fixed in 10% formaldehyde and embedded in paraffin. The tissue was then sectioned into 3 µm-thick sections, deparaffinized in xylene, and finally hydrated in ethanol. Deparaffinized tissue sections were immunohistochemically analyzed using a highly sensitive IHC detection kit for mouse and rabbit primary antibodies (abx097195; Abbexa, Cambridge, UK), according to the manufacturer's instructions. Briefly, the antigens were retrieved in a sodium citrate buffer (10 mM Sodium citrate, 0.05% tween 20, pH 6.0) at 100°C for 10 min, and the tissue sections were incubated with 0.3% (v/v) H_2O_2 blocking reagent for 10 min at 25°C. After washing thrice in phosphate buffer saline (PBS)/TBS (Abbexa Ltd, Cambridge, UK) for 2 min each, the sections were incubated in a protein-blocking buffer (Abbexa Ltd, Cambridge, UK) for 10 min at 25°C to block nonspecific background staining. After washing three times in PBS/TBS for 2 min each, the sections were probed with (1:1,000, v/v) anti-TG1 (abx210724: Abbexa Ltd, Cambridge, UK) or anti-TG3 (abx103072: Abbexa Ltd, Cambridge, UK). After washing, sections were then incubated with HRP Polymer (Abbexa Ltd, Cambridge, UK) 50 µl/slide and incubated for 30 min. This was followed by washing three times with PBS/TBS for 2 min each time. Then, the sections were incubated with a mix of 50 µL DAB Chromogen and DAB Substrate (Abbexa Ltd, Cambridge, UK) for 1-10 min, rinsed four times with the buffer, counterstained with hematoxylin according to the manufacturer's instructions, and visualized under an optical microscope x10, x20, and x40. Positive IHC staining was quantified using ImageJ software (National Institute of Health, Maryland, USA).

Tissue Homogenization and Protein Isolation

The biopsy samples from human skin were processed using a Kinematica Polytron® 1300D Homogenizer (Brinkmann Instruments, Westbury, NY, USA). Proteins were extracted from human skin biopsy samples using the ProteinExt® Mammalian Total Protein Extraction Kit (DE101, Transgenbiotech, Beijing, China). The skin tissue was briefly minced, washed twice with 1 mL of pre-chilled PBS, and centrifuged at 500 × g for 5 min. After discarding the supernatant, 1 mL of ice-cold lysis buffer [total Protein Extraction Buffer (TPEB) containing a protease Inhibitor Cocktail (proteinSafe™ Protease Inhibitor Cocktail, EDTA-free (100×) (DE101, Transgenbiotech, Beijing, China)] was added to the tissue and mixed. This mixture was then transferred to a pre-chilled glass homogenizer and homogenized for 6-10 cycles. The suspension was transferred to a new 1.5 mL microfuge tube, incubated on ice for 30 min, and vortexed every 10 min. This was centrifuged at 14,000 × g at 4°C for 10 min, and the supernatant was then collected and stored at –80°C for downstream experiments. Tubes were occasionally placed on ice to avoid heating the samples during homogenization. For between-sample replacement, the homogenizer probe was rinsed three times with distilled water, once with 95% ethanol, and finally with lysis buffer (DE101, Transgenbiotech, Beijing, China). A Coomassie (Bradford) Protein Assay kit (ThermoFisher Scientific, Waltham, MA, USA) was used to measure the protein concentration in human skin samples according to the manufacturer's instructions.

Total RNA Isolation

Total RNA was isolated using GET™ Total RNA kit (786-132; G-Biosciences, St. Louis, MO,

USA). Briefly, the skin biopsy samples were homogenized in 200 µL genomic lysis buffer using a clean pestle and incubated with 5 µL Longlife™ Proteinase K suspension (786-132; G-Biosciences, St. Louis, MO, USA), at 60°C for 1 h. The sample tubes were centrifuged for 5 min at 5,000 × g, and the supernatant was transferred to a clean tube (SKU: 80-1500, Biofargo, Henrico, VA, USA). Next, 400 µL of GET Binding Buffer (786-132; G-Biosciences, St. Louis, MO, USA) were added. The mixture was then transferred to a GET Silica Column (786-132; G-Biosciences, St. Louis, MO, USA) and centrifuged at 12,000 × g for 1 min at 25°C. After discarding the flow through the column, the column was washed twice with 0.6 mL GET Washing buffer (786-132; G-Biosciences, St. Louis, MO, USA) and centrifuged at 12,000 × g for 1 min at 25°C. The column was centrifuged again at 14,000 × g for 3 min to remove residual GET Washing buffer. The column was then placed on a new nuclease-free 1.5 ml microfuge tube (SKU: 80-1500, Biofargo, Henrico, VA, USA), and the RNA was eluted by adding 50 µL of GET Elution Buffer (786-132; G-Biosciences, St. Louis, MO, USA) warmed at 60°C at the top of the membrane in the column. This mixture was incubated at room temperature for 15 min, and RNA was collected by centrifugation of the spin column at 12,000 × g for 1 min. The RNA purity and concentration were measured using a NanoDrop ND 1,000 spectrophotometer (Thermo Fisher Scientific, WM, USA).

Quantitative Real-Time Polymerase Chain Reaction (PCR)

For RT-PCR, specific primers for TG1, TG3, loricrin, IVL, and ß-actin (Table I) were designed using the PrimerQuest Tool (Integrated DNA Technologies). The primers produced amplicons spanning exon-exon junctions and included all known alternatively spliced mRNA variants. The ABScript II One-Step SYBR Green RT-qPCR Kit (RK20404; ABclonal Technology, Woburn, MA, USA) was used to reverse transcribe 300 ng of total RNA into cDNA for qPCR amplification. RT-qPCR was performed using an AiraMx Real-Time PCR System (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer's instructions. The volume of the PCR mix included SYBR Green RT-qPCR Buffer, 10 µL ABScript II Enzyme Mix, 0.8 µL forward primer (10 µM), 0.4 µL reverse primer (10 µM), 0.4 µL ROX Reference Dye (50×), 0.4 µL total RNA, and 2 µL RNase-free H_2O up to 20 µL. The thermos-cycling conditions were: reverse transcription for one cycle for 5 min at 42°C and another cycle for pre-denaturation at 95°C for 1 minute followed by 40 reaction cycles for 5 s at 95°C and 30-34 s at 60°C. The samples were run in duplicate on a qRT-PCR Thermal Cycler (AriaMx Real-time PCR System, Agilent Technology, Santa Clara, CA, US), and the experiments were repeated three times to validate the results. Data were automatically analyzed using the supplied software (AiraMx software, Agilent Technology, Santa Clara, CA, US) after setting the plate for comparative quantitation. The transcript abundance of different genes relative to that of ß-actin was calculated and used to determine the changes in mRNA expression. The results were plotted using the GraphPad Prism software (GraphPad Software, La Jolla, CA, USA).

TG1 Activity Measurement

TG1 activity was measured as described by Hitomi[5] using a specific keratinocyte transglutaminase (Tgase-1) colorimetric microassay kit: TG1-CovTest (opr0038: Covalab, Bron, France). This method was based on a colorimetric microassay for TG1 quantification. Briefly, the spermine-conjugated microtiter well plate was equilibrated with 150 µL 1× washing buffer and then 50 µL biotinylated peptide in the reaction mixture (Biotin-pepKS/$CaCl_2$) in the presence of 50 µL tissue extraction buffer (Covalab, Bron, France) for 30 min at 37°C with gentle shaking. Ethylenediamine-tetraacetic Acid (EDTA) (Covalab, Bron, France) was used as a negative control. After washing with 200 µL 1× washing buffer and 200 µL 0.1 M NaOH, 100 µL 1:2,000 freshly prepared enzyme tracer (SAV-HRB) (Covalab, Bron, France) was added and incubated for 15 min at 37°C. This was followed by three washes with 1× washing buffer. Then, 100 µL horseradish peroxidase substrate (Covalab, Bron, France) was added and incubated for 5 min at room temperature. The enzyme reaction was stopped using a 100 µL blocking reagent, and the optical density was measured at 450 nm using a microplate reader (Epoch™ 2 Microplate Spectrophotometer; BioTeK, Winooski, VT, USA). All experiments were performed in triplicate. One unit of TG activity was defined as the change in absorbance of 0.01 at 450 nm/h. The specific activity of TG1 in different samples was normalized to the protein concentration in each sample.

TG3 Activity Measurement

The TG3 activity was measured as described by Trigwell et al[29]. Briefly, 96-well microtiter

plates (Maxisorp Nunc, Keighley, UK) were coated with 250 µL/well 1.0 mg·ml^{-1} casein in 100 mM Tris-HCl (pH 8.0) and incubated overnight at 4°C. The wells were washed twice with distilled water and incubated with 250 µL blocking solution (0.1% w/v bovine serum albumin in 100 mM Tris-HCl at pH 8.0) at 37°C for 1 h. The wells were re-washed, and 150 µL reaction buffer (100 mM Tris-HCl (pH 8.5), containing 5 µL β-mercaptoethanol (Sigma, Burlington, MA, USA), 10 µM Peptide E51-[Biotin] (opr0055-Biotin: Covalab UK Ltd, Cambridge, UK), and 6.7 mM $CaCl_2$ or 13.3 mM EDTA) was added to each well. The reaction was started by adding 50 µL extract or diluted standard (1 mg·ml^{-1} TG3) to each well along with negative control (100 mM Tris buffer) and allowed to proceed for 1 h at 37°C. The plate was washed, incubated with 200 µL 100 mM Tris-HCl pH 8.0 containing 1:5 ExtrAvidin® peroxidase (Sigma-Aldrich, St. Louis, MO, USA) per well at 37°C for 45 min, re-washed, and developed using 200 µL developing buffer (Sigma-Aldrich, St. Louis, MO, USA) (75 µL 10 mg·ml^{-1} TMB (and 1.5 µL 3% (v/v) hydrogen peroxide in 10 mL 100 mM sodium acetate, pH 6.0) at room temperature for 5-15 min. The reaction was terminated by adding 50 µL 5 M sulfuric acid (Sigma-Aldrich, St. Louis, MO, USA). The absorbance at 450 nm was then measured using a microplate reader, Epoch™ 2 Microplate Spectrophotometer (BioTeK, Winooski, VT, USA). All experiments were performed in triplicate. One unit of TG activity was defined as the change in absorbance of 0.01 at 450 nm/h. The specific activity of TG3 in different samples was normalized to the protein concentration in each sample.

Statistical Analysis

Data were prepared using GraphPad Prism software version 10 (DotMatics, Boston, USA) and statistically analyzed by one or two analyses of variance (ANOVA) followed by Tukey's multiple comparison tests, and an unpaired *t*-test was used in some analyses. Results represent the mean ± SEM, and $p<0.05$ was considered statistically significant.

Results

TG1 Enzymatic Activity Measurement

To investigate the role of TG1 in psoriatic skin tissue, TG1 enzymatic activity was measured in homogenized biopsy samples obtained from four healthy individuals and four patients with moderate to severe psoriasis. Data analysis indicated that TG1 activity was significantly increased (n=3, $^{**}p<0.05$, $^{**}p<0.001$, $^{****}p<0.0001$) in individual psoriatic skin samples compared to individual normal skin samples (Figure 1A). The mean difference showed that TG1 activity in psoriatic skin samples was significantly increased (n=3, $^{***}p<0.0005$) by nearly one-fold compared to that in the control samples (Figure 1B).

TG3 Enzymatic Activity Measurement

To investigate the role of TG3 in psoriatic skin tissue, TG3 enzymatic activity was measured in homogenized biopsy samples obtained from four healthy individuals and four patients with moderate to severe psoriasis. Data analysis indicated that TG3 activity was significantly increased (n=3, $^{*}p<0.05$, $^{**}p<0.001$) in individual psoriatic skin samples compared to that in individual normal skin samples (Figure 2A). The mean difference showed that TG3 activity in psoriatic skin samples was significantly increased (n=3, $^{**}p<0.001$) by more than one-fold compared to that in the control samples (Figure 2B).

TG1 and TG3 Protein Expression and Location in Normal and Psoriasis Human Skin Tissue

Both TG1 and TG3 protein levels and their localization in psoriatic and healthy skin samples were investigated immunohistochemically using TG1 polyclonal antibody against TGM1 and TG3 polyclonal antibody against TGM3. The results showed that TG1 (Figure 3A, d-e) and TG3 (Figure 4A, d-e) protein levels in almost all psoriatic skin samples were significantly (n=3, $^{****}p<0.0001$, Figures 3B and 4B) higher than those in healthy skin tissue samples (Figure 3A-3B and Figure 4A-4B). In addition, the expression of both TGs was distributed in different psoriatic skin layers and was concentrated mainly in the epidermis (from the stratum cranium to the stratum basale), whereas it was concentrated in the suprabasal layers of healthy skin.

TG1 and TG3 Gene Expression in Normal and Psoriasis Human Skin

Both TG1 and TG3 gene expression levels were measured in normal and psoriatic human skin tissues. mRNA was extracted from both biopsy samples and subjected to RT-qPCR using specific primers, along with primers for gene normalization. TG1 and TG3 (Figure 5A-5B) expression in psoriatic skin tissue samples was significantly

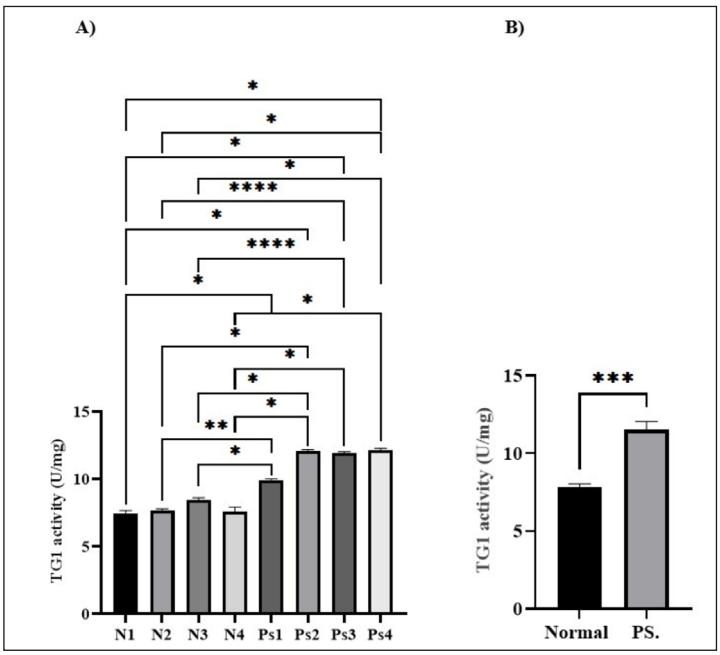

Figure 1. Keratinocyte transglutaminase (TG1) activity in normal and psoriasis human skin tissues. Proteins extracted from both normal and psoriatic human skin tissues were subjected to a specific keratinocyte transglutaminase (Tgase-1) colorimetric microassay. Data points represent the mean ± SEM TG1 specific activity of four biopsy samples. Data analysis was performed using (**A**) one-way ANOVA followed by Tukey's multiple comparisons test to compare different normal (N1–N4) and psoriasis human skin tissue (Ps1-Ps4) samples (n=3, **p<0.05, **p<0.001, ****p<0.0001) and (**B**) an unpaired t-test to compare the mean difference of normal and psoriasis human skin tissue samples (n=4). Statistical significance was set at *p<0.05.

higher than that in healthy skin tissue samples after ß-actin normalization (n=3; ***p<0.0005 and ****p<0.0001, respectively).

Loricrin and IVL Gene Expression in Normal and Psoriasis Human Skin

To investigate the TG substrates, which are also markers of healthy skin differentiation and development, the involvement of loricrin and IVL in epidermal terminal differentiation and skin formation was investigated. mRNA was extracted from both biopsy samples and subjected to RT-qPCR using specific primers, along with primers for gene normalization. Loricrin (Figure 6A) and IVL (Figure 6B) gene expression levels were significantly lower in psoriatic skin tissue

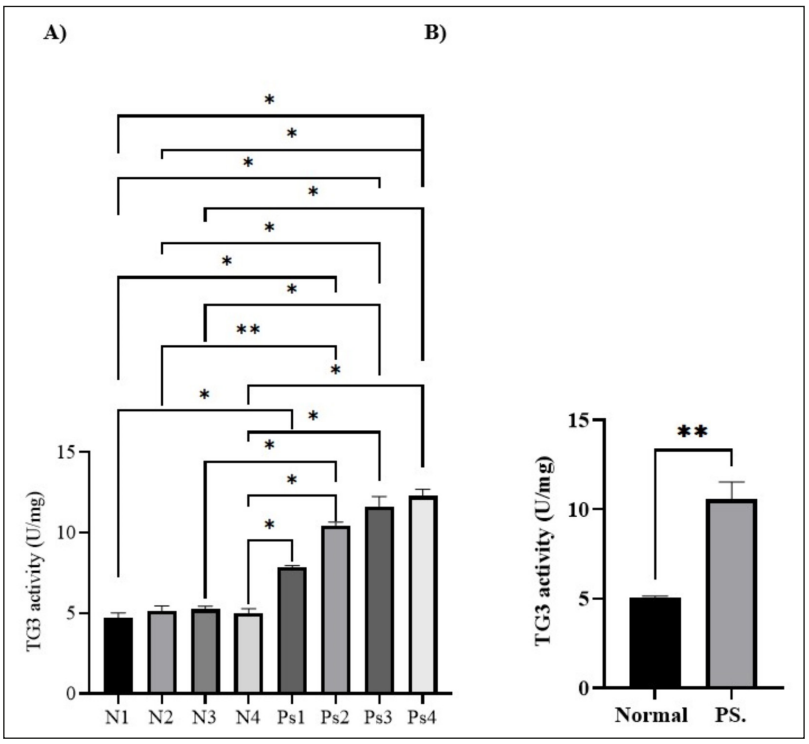

Figure 2. Epidermal transglutaminase (TG3) activity in normal and psoriasis human skin tissue. Proteins extracted from both normal and psoriatic human skin tissues were subjected to a specific epidermal TG3 colorimetric incorporation activity assay. Data points represent the mean ± SEM TG3 specific activity of four biopsy samples. Data analysis was performed using (**A**) one-way ANOVA followed by Tukey's multiple comparisons test to compare different normal healthy (N1–N4) and psoriasis human skin tissue (Ps1–Ps4) samples (n=3, *p<0.05, **p<0.001) and (**B**) an unpaired t-test to compare the mean difference of normal and psoriasis human skin tissue samples (n=4). Statistical significance was set at *p<0.05.

samples than in healthy skin tissue samples (n=3; $^{**}p < 0.005$ and $^{***}p < 0.001$, respectively).

Discussion

The results of this study reveal the roles of TG1 and TG3 in psoriasis development. TG1, TG3, and TG5 play distinct roles in the construction and development of epidermal skin layers by catalyzing their main components, proteins, or peptides[19-21]. In this study, both TG1 and TG3 activities were slightly increased in healthy skin (Figures 1 and 2). However, TG1 activity appears to be more abundant than TG3 activity in healthy skin. This explains why TG1 depletion in knockout mice causes a defective stratum corneum and early death, suggesting its essential role in the distribution of CE precursor proteins at the cell border, which cannot be compensated for by other TG isozymes[30,31]. Moreover, reduced TG1 activity is correlated with an ichthyosis-like phenotype in mouse models[32]. Interestingly, both TG1 and TG3 activities were significantly increased in psoriatic skin samples; however, a side-by-side comparison revealed that TG3 activity in psoriatic skin samples was markedly higher (n=4, $^{**}p<001$) than TG1 activity (Figure 7), suggesting that TG3 may play a role in the development of psoriatic lesions. However, we cannot rely on this because of the

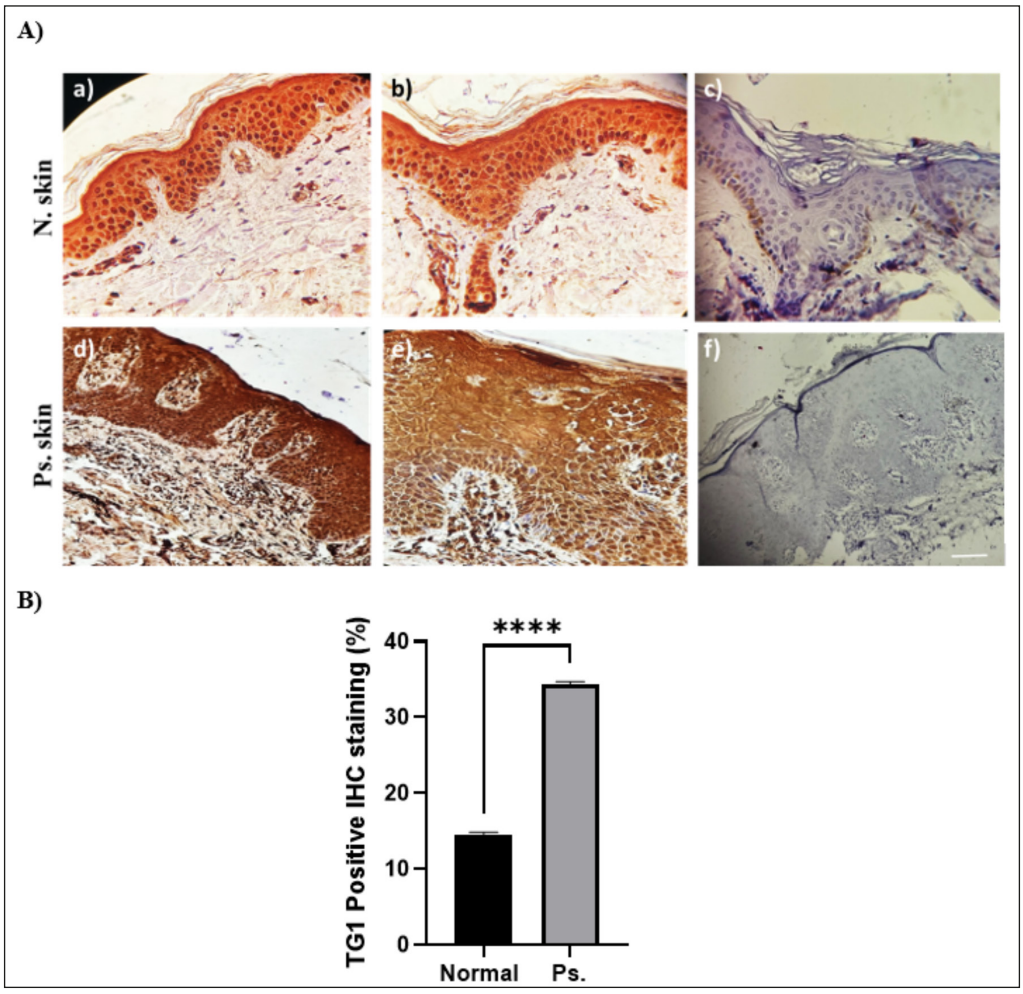

Figure 3. Keratinocyte transglutaminase (TG1) protein expression and location in normal and psoriasis human skin tissue. **A**, Immunohistochemical (IHC) staining of (a-b) normal (control) and (d-e) psoriatic skin tissue biopsy specimens for keratinocyte TG1 using a polyclonal antibody against TGM1. Negative IHC reaction for TG1 in (c) normal and (f) psoriatic skin tissue. The original magnifications of the images were 20× and 40×, respectively. Scale bar, 20 mm. **B**, Quantification of the percentage of positive TG1 IHC staining. An unpaired *t*-test was used to compare the mean difference between normal and psoriasis human skin tissue samples (n=3, $^{****}p<0.0001$). Statistical significance was set at $^{*}p<0.05$.

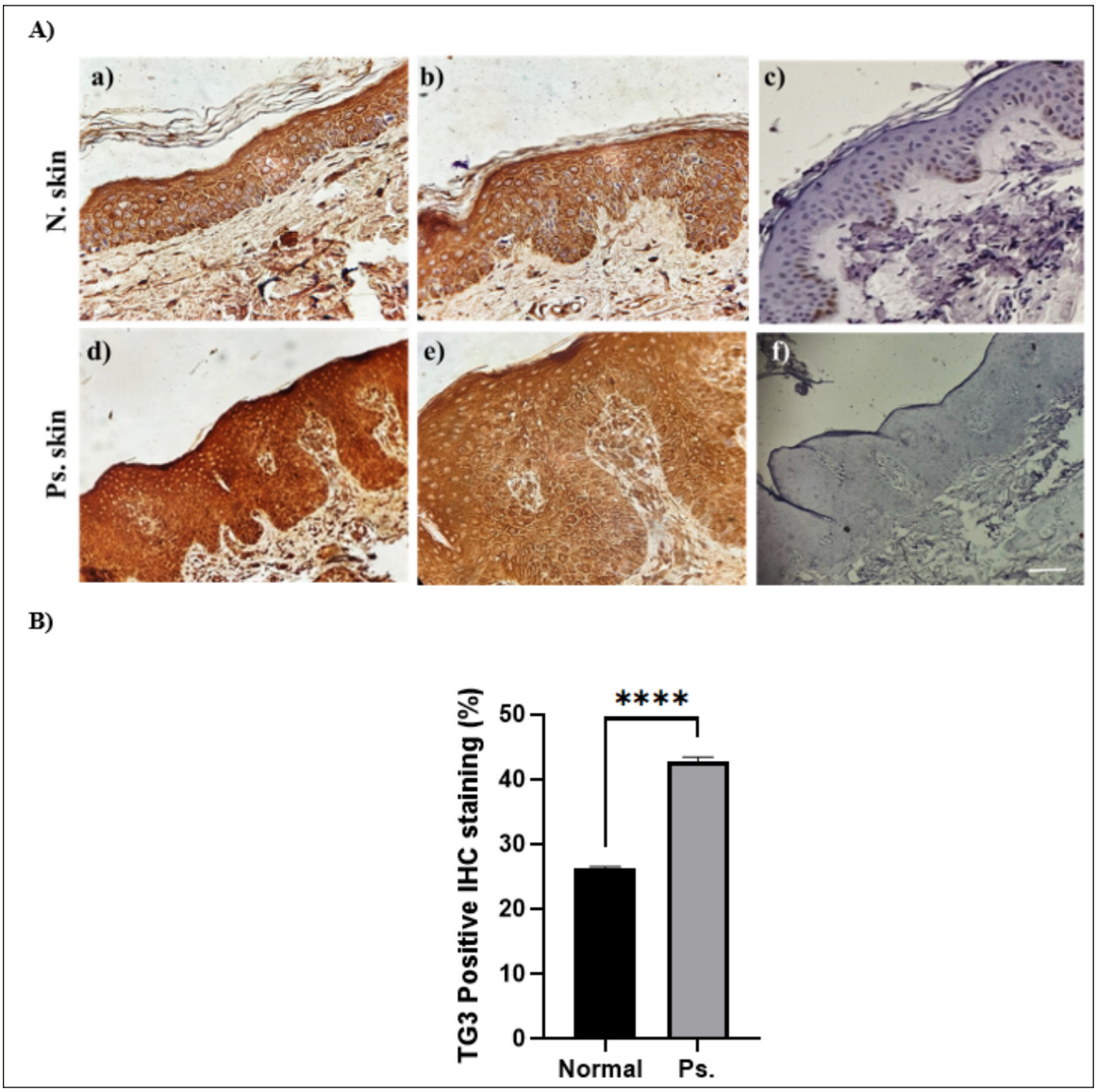

Figure 4. Epidermal transglutaminase (TG3) protein expression and location in normal vs. psoriasis human skin tissue. **A**, Immunohistochemical (IHC) staining of biopsy specimens from normal human healthy (control) (a–b) and psoriatic skin (d–e) tissues for epidermal (TGM3) using a polyclonal antibody against TGM3. Negative IHC reaction for TG1 in normal healthy tissue (c) and psoriatic skin tissue (f). The original magnifications of the images were 20×, and 40×, respectively. Scale bar, 20 mm. **B**, Quantification of the percentage of positive TG3 IHC staining. An unpaired *t*-test was used to compare the mean difference between normal and psoriasis human skin tissue samples (n=3, ****$p<0.0001$). Statistical significance was set at *$p<0.05$.

different assay conditions and substrates used to measure TGs' activity.

IHC also revealed a substantial increase in TG1 and TG3 protein levels (Figures 3 and 4), which were distributed in almost all layers of the psoriatic skin samples, mainly in the spinous and granular layers[7,14]. This was also associated with the thickening and irregular appearance of the contents of the two layers[33]. In this study, we also observed that TG1 and TG3 mRNA were overexpressed in psoriatic skin samples, which agrees with the results of a previous bioinformatics analysis of human and imiquimod-induced mice psoriatic lesions[34]. In particular, the significant increase in gene expression and activity of both TGs appears to be a compensatory mechanism resulting from hyperproliferation and defective keratinocyte differentiation. Ling et al[35] have recently reported that TG3 overexpression in imiquimod-induced psoriatic lesions in mice decreased the production

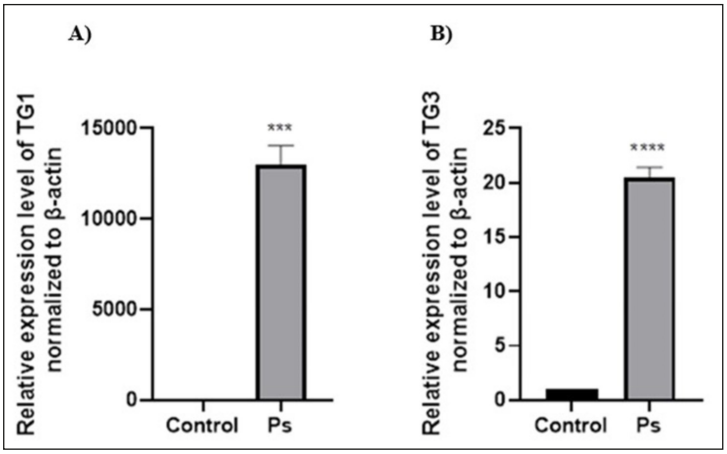

Figure 5. TG1 and TG3 gene expression in normal and psoriasis human skin tissue. Messenger RNA was extracted from normal and psoriatic skin tissue biopsy samples and subjected to RT-qPCR using (**A**) TG1- and (**B**) TG3-specific primers along with primers specific for a normalizing gene (ß-actin). Data is indicated as the mean±SEM from three independent samples and was analyzed using an unpaired *t*-test to compare the mean difference between normal and psoriasis human skin tissue (n=3; ***$p<0.0005$ and ****$p<0.0001$, respectively). Representative values of psoriatic human skin (PS.) were normalized and expressed relative to that of normal tissue (control), with a fixed value arbitrarily set to one. Statistical significance was set at *$p<0.05$.

of proinflammatory factors and elevated the inflammatory response. This suggests that TG3 is a potential therapeutic target in psoriasis.

IVL is a TG substrate and is the first precursor for cross-linking during envelope assembly[7,36]. Loricrin is the major component of the cornified envelope in the epidermis[37]. This cornified envelope precursor is expressed later during skin differentiation, whereas IVL, along with TG1 and TG3, is expressed earlier in the granular and spinous layers[38]. Our results showed that gene expression of both loricrin and IVL was markedly decreased in psoriatic skin compared to that in normal skin (Figure 6). Moreover, these two proteins play important roles in terminal differentiation and homeostatic maintenance of the epidermis[39]. Since psoriatic phenotype development is due to increased proliferation and decreased dif-

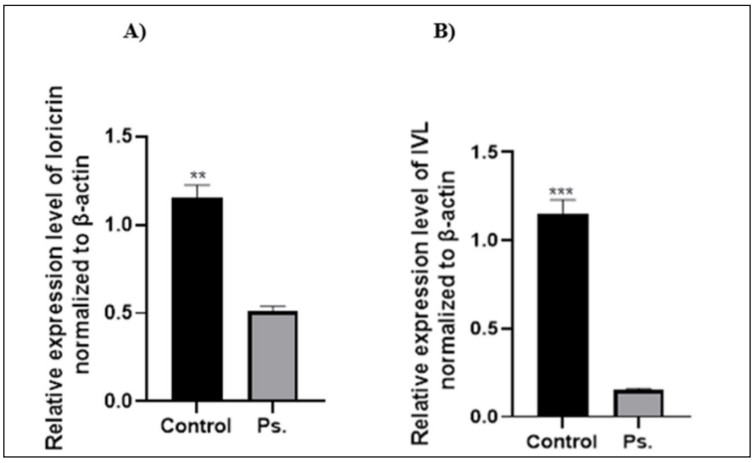

Figure 6. Loricrin and involucrin (IVL) gene expression in normal and psoriasis human skin tissue. Messenger RNA was extracted from normal and psoriatic skin tissue biopsy samples and subjected to RT-qPCR using (**A**) loricrin- and (**B**) IVL-specific primers along with primers specific for normalizing gen (ß-actin). Data is indicated as the mean±SEM from three independent experiments and was analyzed using an unpaired *t*-test to compare the mean difference between normal and psoriasis human skin tissue (n=3; **$p<0.005$ and ***$p<0.001$, respectively). The represented values of psoriasis human skin (PS.) were normalized and expressed relative to that of normal tissue (control), with a fixed value arbitrarily set to one. Statistical significance was set at *$p<0.05$.

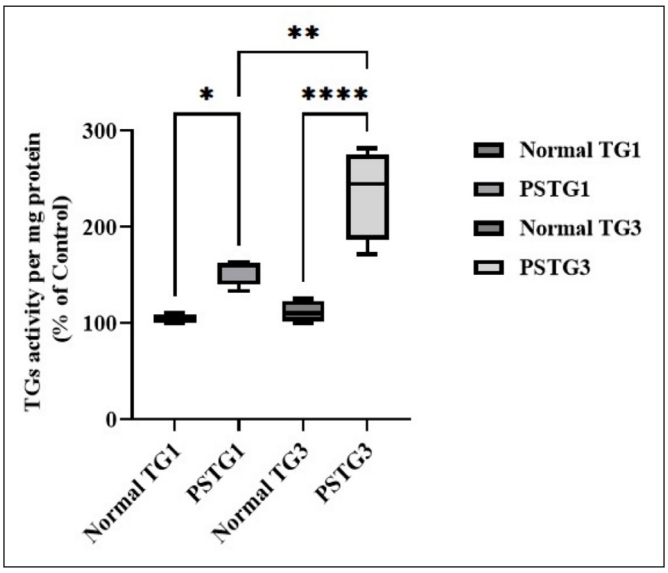

Figure 7. The compression between keratinocyte transglutaminase (TG1) and epidermal transglutaminase (TG3) activity in normal *vs.* psoriasis human skin tissue. Proteins extracted from both normal and psoriatic human skin tissues were subjected to a specific keratinocyte transglutaminase (Tgase-1) colorimetric microassay and an epidermal transglutaminase 3 colorimetric incorporation activity assay. Data points represent the mean ± SEM TG1- and TG3-specific activity from four biopsy samples. Data analysis was performed using two-way ANOVA followed by Tukey's multiple comparisons test to compare different normal) and psoriasis human skin tissue samples (n=4). Statistical significance was set at $*p<0.05$. $*p<0.03$, $**p<0.002$, $****p<0.0001$.

ferentiation of keratinocytes[40,41], the impairment of these two genes is reasonable. Several studies[42] have indicated that loricrin and IVL are downregulated in patients with psoriasis. It was believed[42] that impairment of these skin barrier proteins is associated with tumor necrosis factor-α (TNF-α) upregulation and some cytokines, such as IL17A and IL22[39,43,44]. Other studies[39,45] have suggested that the upregulation of other CE components, such as Lce1, SPRRP2D, and SPRRP2H, compensates for the counterbalancing loricrin downregulation. In contrast, other studies[46,47] have suggested that IVL, but not loricrin, is upregulated in patients with psoriasis because of the induction of PKC and cytokines such as IL-1, IFN-γ, and IL17A. In a healthy epidermis, lamellar granules are usually secreted immediately before cornification. In the psoriatic epidermis and accelerated cornfield envelope formation[48], lamellar granules failed to complete their secretion on the apical surface[49]. This explains why the depletion of these two precursor proteins terminated keratinocyte differentiation at the spinous layer level.

Conclusions

The significant increase in TG1 and TG3 activity, protein levels, and gene expression may explain psoriatic skin lesion-characterizing hyperplasia. However, our results indicated that TG3 could be responsible for the disease than TG1 because of its activity compared to TG1. It cannot be ruled out that both TGs play a role in the development of psoriatic lesions. Moreover, the increase in TG1 and TG3 gene expression may be due to the dysregulation of their substrates, loricrin, and IVL. Further studies are needed to test the hypothesis that induction of these two precursor proteins may help reduce TGs' activity and gene expression, thus attenuating skin lesion progression in patients with psoriasis. Alternatively, the use of specific transglutaminase inhibitors may provide further insight into the role of these substrates in psoriasis development. Thus, the results of this study may directly affect the future design of therapeutic drugs for the treatment of psoriasis.

Conflict of Interest

The authors declare that they have no conflict of interests.

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ORCID ID

Ibtesam Almami: 0000-0001-7876-560X.

Ethics Approval

All studies were approved by the Committee of Research Ethics of the Deanship for Scientific Research at Qassim University (number 21-06-09) and were conducted according to the principles of the Declaration of Helsinki. All subjects gave their informed consent for inclusion before they participated in the study.

Informed Consent

All subjects gave their informed consent for inclusion before they participated in the study.

Data Availability

All data are available upon reasonable request.

Authors' Contributions

Norah S. Alharbi: Conceptualization, methodology, data analysis, and writing of the first draft. Ibtesam Almami: Supervision, conceptualization, methodology, data analysis, writing, reviewing, and editing. All the authors have read and agreed to the published version of the manuscript.

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