Facilitated Wound Healing by Activation of the Transglutaminase 1 Gene

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Transglutaminase 1 (TGase 1) is a Ca^{2+} -dependent enzyme which catalyzes ϵ -(γ -glutamyl)lysine cross-linking of substrate proteins such as involucrin and loricrin to generate the cornified envelope at the cell periphery of the stratum corneum. We have shown that disruption of the *TGase 1* gene in mice results in neonatal lethality, absence of the cornified envelope, and impaired skin barrier function. Based on the importance of TGase 1 in epidermal morphogenesis, we have now assessed its role in wound healing. In neonatal mouse skin, TGase 1 mRNA as well as keratin 6 α was induced in the epidermis at the wound edges as early as 2 hours after injury and that expression continued in the migrating epidermis until completion of re-epithelialization. The TGase 1 enzyme co-localized on the plasma membrane of migrating keratinocytes with involucrin, but not with loricrin, which suggests the premature assembly of the cornified envelope. Similar injuries to TGase 1 knockout mouse skins grafted on athymic nude mice showed substantial delays in wound healing concomitant with sustained K6 α mRNA induction. From these results, we suggest that activation of the *TGase 1* gene is essential for facilitated repair of skin injury. (*Am J Pathol* 2000, 157:1875–1882)

Transglutaminases (TGases) (EC 2.3.2.1.3) belong to a Ca^{2+} -dependent enzyme family and catalyze the formation of ϵ -(γ -glutamyl)lysine cross-links between peptide-bound glutamine residues and the primary amine group of various amines.¹ Such cross-links are generally formed in or between proteins by reaction with the ϵ -amino group of lysine residues and they act to stabilize proteins via polymerization against chemicals, proteolytic enzymes, and physical disruption. TGases have been proposed to participate in a wide variety of biological events such as

fertilization, development, differentiation, apoptosis, coagulation, and wound healing.²

Cutaneous wound healing is a complex process consisting of three overlapping phases, inflammation, proliferation, and remodeling.³ Skin injury elicits a temporary repair by formation of a clot that plugs the defect, followed by invasion of inflammatory cells and then fibroblasts and capillaries into the clot to form a contractile granulation tissue. Meanwhile, the cut epidermal edges migrate forward to cover the denuded wound surface. During those wound healing processes, TGases are activated in all layers of the skin corresponding to the epidermis, the dermis, and the panniculus carnosus.⁴ Coagulation factor XIIIa (plasma TGase) is activated by thrombin and contributes to the formation of a fibrin clot at the site of injury.⁵ Tissue TGase (TGase 2) is expressed in endothelial cells, macrophages, and skeletal muscle cells throughout the healing process.⁶ TGase 2 might be induced in response to acute-phase injury cytokines, such as transforming growth factor- β ,⁷ interleukin-6,⁸ and/or tumor necrosis factor- α ,⁹ because the *TGase 2* gene has response elements to these cytokines. TGase 2 that binds to a cell surface complex composed of plasminogen, urokinase receptor, and mannose-6-phosphate receptor, converts latent transforming growth factor- β to its active form, providing a positive amplification loop for TGase 2 expression in wound sites.¹⁰ TGase 2 and factor XIIIa may participate in the cross-linking of extracellular matrix molecules, such as fibrin/fibrinogen,^{11,12} fibronectin,¹³ collagen,¹⁴ laminin/nidogen,¹⁵ osteopontin,¹⁶ and vitronectin.¹⁷ Thus, TGases effectively construct a scaffold for migration of inflammatory and endothelial cells by stabilizing the extracellular matrix,⁶ and the end product of the process is the replacement of the fibrin matrix with granulation tissue. Even after the wound has healed, those TGase isozymes possibly promote contraction throughout the wound by cross-linking extracellular matrix molecules. In addition to the important role of TGase 2 in dermal wound healing, the enzyme is also involved in the cross-linking of anchoring fibrils at the dermo-epidermal junction via ligation of collagen VII as a potential substrate.¹⁸

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In contrast to those TGase isoforms, little attention has been given to the role of TGase 1 (keratinocyte TGase) in wound healing since the report by Mansbridge and Knapp¹⁹ that TGase immunoreactivity is detectable in the epidermis 15 hours after tape-stripping and 3 days after generation of a suction blister. TGase 1 is essential for the development and maturation of the stratum corneum, especially for production of the cornified envelope at the cell periphery of the stratum corneum,²⁰ by assembly of precursor proteins such as involucrin²¹ and loricrin.²² Furthermore, TGase 1 may be necessary to maintain skin barrier function by covalent cross-linking of ω -OH ceramides with involucrin.²³ The *TGase 1* gene is one of the genes responsible for autosomal recessive lamellar ichthyosis and a variety of mutations in that gene have been identified in some families with the disorder.^{24,25} The *TGase 1* gene is expressed mainly in differentiating layers of normal stratified squamous epithelia and in inner root sheath cells,²⁶ although TGase 1 localizes not only in the stratified squamous epithelia but also concentrates at cadherin-based adherens junctions of simple epithelial cells.²⁷ In addition to these roles of TGase 1 in the skin, the unusual expression of the enzyme in the brain has been implicated in the pathogenesis of Alzheimer's disease.²⁸

Recently, we have generated transgenic mice bearing a *lacZ* gene directed by a 2.5-kb 5' upstream promoter of the human *TGase 1* gene and we have analyzed β -galactosidase expression in these mice.²⁶ In a series of studies using those *TGM1-lacZ* transgenic mice, we have noticed that β -galactosidase staining is strongly induced in the epidermis close to the skin injury. Furthermore, based on the phenotype of our TGase 1 knockout mice, which have defective epidermal development and keratinization, lack the cornified envelope, and have impaired barrier function,²⁰ we have speculated on the importance of TGase 1 during skin regeneration. In this study, we characterize *TGase 1* gene expression during wound healing of the skin and the results suggest the essential role of TGase 1 in that process.

Materials and Methods

Materials

Rat monoclonal TGase 1 antibody was kindly supplied by Dr. Hiiragi, Department of Cell Biology, Kyoto University Graduate School of Medicine, Kyoto, Japan.²⁷ Rabbit polyclonal mouse loricrin and mouse involucrin antibodies were from Covance Research Products Inc. (Richmond, CA); Cy3-conjugated anti-rat IgG and fluorescein isothiocyanate-conjugated anti-mouse IgG were from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA); fluorescein isothiocyanate-conjugated and Texas Red-conjugated anti-rabbit IgGs were from Vector Laboratories, Inc. (Burlingame, CA).

Experimental Wounding, Harvesting, and Sectioning

All studies involving animals were reviewed by the Animal Use and Care Committee of the Kyoto Prefectural University of Medicine. BDF1 neonatal mice were anesthetized and a 10-mm linear full-thickness incision was made on the dorsal skin. Wounds were harvested from three mice at 1, 2, 3, 4, 8, 12, 16, 20, 24 hours and 2, 3, 4, 5, 6 days after wounding. For skin grafting, nine normal controls and eight *TGase 1*^{-/-} neonatal mice were killed by decapitation and their dorsal skins were excised and transplanted onto athymic nude mice. Two weeks after the skin grafting, a round wound 3 mm in diameter was made at the center of each graft with a biopsy punch. Wounds made on the grafted skins were harvested for histological examinations at 48 hours and 5, 10, and 11 days after wounding.

Wound tissues were fixed in 10% formaldehyde in phosphate-buffered saline (PBS), then embedded in paraffin. Those tissues were sectioned at 4- μ m thickness and were used for hematoxylin and eosin staining and for *in situ* hybridization. For immunofluorescence, tissues were embedded in OCT compound (Miles Inc., Elkhart, IN) and were frozen at -80°C, then sectioned at 5- μ m thickness at -20°C.

In Situ Hybridization

pMTG1-1.1 containing a 1.1-kb mouse TGase 1 cDNA²⁶ was linearized by *Sa*I digestion and antisense TGase 1 cRNA was synthesized with T7 RNA polymerase, using a digoxigenin RNA Labeling Kit (Roche Diagnostics Corp., Basel, Switzerland). Mouse keratin 6 α (K6 α) and keratin 6 β (K6 β) cRNA probes were prepared as described previously.²⁹

In situ hybridization was performed using a method described elsewhere.²⁶ Tissue sections were deparaffinized in xylene and dehydrated through a graded ethanol series. After proteinase K digestion (18 μ g/ml), the sections were postfixed with 4% (w/v) paraformaldehyde in PBS for 10 minutes and were then treated with 0.1 mol/L triethanolamine-HCl (pH 8.0) for 1 minute. After acetylation for 10 minutes, the sections were dehydrated, air-dried, and then incubated at 50°C in a hybridization buffer composed of 50% formamide, 10 mmol/L Tris-HCl (pH 7.5), 1 mg/ml yeast tRNA (Sigma Chemical Co., St. Louis, MO), 1 $\times$ Denhardt's solution (Sigma Chemical Co.), 10% polyethylene glycol 6,000, 600 mmol/L NaCl, 0.25% sodium dodecyl sulfate, 1 mmol/L ethylenediaminetetraacetic acid (pH 8.0), and 0.2 μ g/ml of probe. After hybridization, the sections were washed at 45°C for 1 hour in 50% formamide and 2 $\times$ standard saline citrate, and were then digested with 20 μ g/ml RNase (Sigma Chemical Co.) in 10 mmol/L Tris-HCl (pH 8.0) and 500 mmol/L NaCl at 37°C for 30 minutes. Hybridized digoxigenin-labeled probes were visualized with a Nucleic Acid Detection Kit (Roche Diagnostics Corp.).

Immunofluorescence and Confocal Fluorescence Imaging

Cryosections mounted on glass slides were air-dried and rinsed with PBS containing 1% bovine serum albumin for 15 minutes, and were then incubated for 1 hour with rat *TGase 1* (1:2) and rabbit involucrin antibodies (1:600) or with rabbit loricrin antibodies (1:500). Sections were then rinsed with PBS containing 1% bovine serum albumin and 0.15% Triton X-100, followed by incubation for 1 hour with Cy3-conjugated anti-rat IgG and fluorescein isothiocyanate-conjugated anti-rabbit IgG or Texas Red-conjugated anti-rabbit IgG. A confocal laser-scanning microscope, Olympus Fluoview (Olympus Optical Co., Ltd., Tokyo, Japan) was used for immunofluorescence imaging.

Results

*Time Course of *TGase 1* Gene Expression Induced by Skin Injury*

A linear 10-mm full-thickness incision was made with a scalpel on the dorsal skin of BDF1 neonatal mice and the expression of the *TGase 1* gene in the epidermis was compared with that of the *K6 α* and *K6 β* genes. Histological changes at the wound edges were stained with hematoxylin and eosin and are shown in Figure 1 (left panels A, D, G, J, M, and P). The morphological changes noted throughout the time course were basically similar to those in adult mice reported by Croft and Tarin.³⁰ Before wounding, *TGase 1* mRNA was evident in the upper spinous and granular layers of the epidermis (Figure 1B), but *K6 α* mRNA was undetectable there (Figure 1C). One hour after wounding, no obvious induction of *TGase 1*, *K6 α* , or *K6 β* mRNA was observed. However, 2 hours after injury, *TGase 1* mRNA was focally increased in a few layers of suprabasal keratinocytes near the wound edge (Figure 1E). At that time, *K6 α* mRNA was induced in a few suprabasal spinous layers (Figure 1F). The *TGase 1* mRNA signals were accentuated in keratinocytes near the wound edge from 4 to 8 hours after wounding (Figure 1H) and at later times *TGase 1* mRNA was distributed over the epidermis around the wound. This event occurred earlier than did massive leukocyte infiltration at the injured sites, which was found from 8 hours after the injury. The strongest induction of *TGase 1* mRNA was observed in migrating keratinocytes 20 hours after incision (Figure 1K), at which time the epidermis was thickening and re-epithelialization was becoming obvious under the scab. At 48 hours, when intercellular spaces between keratinocytes were widened (Figure 1M), intense signals were still evident there, but the expression of *TGase 1* mRNA was decreasing in the epidermis away from the migrating edge (Figure 1N). Ninety-six hours after wounding, *TGase 1* mRNA expression was back to normal in the thickened re-epithelializing epidermis (Figure 1Q). At 144 hours, the re-epithelialized epidermis was arranged in regular layers and the cutaneous surface became flattened (data not shown). The induction of *K6 α*

mRNA was found at 2 hours (Figure 1I) and the distribution of *K6 α* mRNA seemed to be optimal at 20 hours after wounding (Figure 1L). At 48 hours, *K6 α* mRNA expression was detected in the initial wound site and in the regenerating epidermis (Figure 1O) and was still evident 96 hours after the injury (Figure 1R), by which time the induction of *TGase 1* mRNA had normalized. The expression of *K6 β* mRNA began 2 hours after injury as did *K6 α* , and strong signals were observed 48 hours after the injury (data not shown). *K6 α* mRNA often extended down to the basal layer, whereas *K6 β* mRNA was primarily restricted to the suprabasal layers of the epidermis.

*Coordinated Induction of Involucrin with *TGase 1* Enzyme in Injured Epithelia*

The expression of *TGase 1* enzyme and its substrate proteins, involucrin and loricrin, during wound healing were assessed in five control and four injured neonatal mice by confocal immunofluorescence imaging. In control mice, *TGase 1* enzyme was evident in a few layers under the stratum corneum and in the inner root sheath of hair follicles (Figure 2A). Involucrin and loricrin were also located in the subcorneal layers, but loricrin was not evident in hair follicles (Figure 2, B and C). The expression of those substrate proteins was virtually unchanged 8 hours after the injury (data not shown). At 48 hours, *TGase 1* enzyme was abundantly expressed suprabasally in the thickened epidermis near the wound edge and in migrating keratinocytes (Figure 2D). The fluorescence of *TGase 1* enzyme was clearly localized at the cell periphery, suggesting membrane translocation of the enzyme (Figure 2G). At that time, the expression of involucrin was detectable in the upper layers of the thickened epidermis and in migrating keratinocytes (Figure 2, E and H). Involucrin was also observed in differentiated layers of the epidermis (Figure 2H), and almost completely colocalized with *TGase 1* enzyme at the cell periphery (Figure 2I). Some fluorescein isothiocyanate-staining of cell debris and scabs at the wound sites and the stratum corneum was nonspecific. In contrast, loricrin expression was not evident at the wound edge and migrating epidermis (Figure 2F). Even in the thickened epidermis, where the abundant expression of *TGase 1* and involucrin was observed, loricrin expression was limited to a few layers under the stratum corneum. The expression patterns of *TGase 1* enzyme, involucrin, and loricrin were unchanged on the opposite side of the wound edge examined in those samples (data not shown).

*Retardation of Wound Healing with Sustained *K6 α* Gene Expression in the *TGase 1*^{-/-} Grafted Skins*

To elucidate the contribution of *TGase 1* in wound healing, we examined the effect of *TGase 1* deficiency on epidermal regeneration. Because of the neonatal lethality of *TGase 1*^{-/-} mice,²⁰ we grafted their skins onto athymic nude mice and examined histological changes and *K6 α*

Figure 1. Induction of TGase 1 and K6 α mRNAs during wound healing in neonatal mouse skin. A 10-mm, full-thickness skin wound was made in BDF1 dorsal skin. The skin was harvested just before (A–C), 2 hours (D–F), 4 hours (G–I), 20 hours (J–L), 48 hours (M–O), and 96 hours (P–R) after the injury. The tissues were stained with H&E (A, D, G, J, M, P), or were subjected to *in situ* hybridization of TGase 1 (B, E, H, K, N, Q) and K6 α mRNA (C, F, I, L, O, R). The induction of TGase 1 and K6 α mRNAs begins in the epidermis at the wound edge as early as 2 hours after the injury (E and F). The induction of TGase 1 mRNA becomes more pronounced (H) and is evident even in migrating keratinocytes (K). The induction of TGase 1 mRNA is almost normalized until 96 hours after the injury (Q), at which time K6 α mRNA expression is still evident (R). Scale bar, 50 μ m.

mRNA expression during wound healing of those TGase 1^{-/-} skin grafts. Two weeks after transplantation, skin grafts from normal neonates resembled normal adult mouse skin (Figure 3A), where the expression of K6 α

mRNA was barely detectable in the epidermis (Figure 3B). In contrast, grafted skins from TGase 1^{-/-} neonates showed thickened epidermis with massive stratum corneum and immature hair follicles (Figure 3G). K6 α mRNA

Figure 2. Expression of TGase 1, involucrin, and loricrin in injured neonatal mouse skin. Intact skins (**A–C**) or injured skins 48 hours after wounding (**D–F**) were stained with TGase 1 (**A** and **D**), involucrin (**B** and **E**), or loricrin (**C** and **F**) antibodies and were analyzed by confocal fluorescence imaging as described under Materials and Methods. **G** and **H**: A higher magnification of **D** and **E** showing the migrating edge of keratinocytes expressing TGase 1 and involucrin, respectively. **I**: A composite image of **G** over **H**, showing co-localization of TGase 1 and involucrin at the cell periphery. The **arrows** show the initial wound sites and the **arrowheads** indicate the migrating edge of the epithelium. Scale bar, 100 μ m (**A–F**); 25 μ m (**G–I**).

expression was evident in the epidermis (Figure 3H), suggesting hyperproliferation of the epidermis. An injury was then made in the center of each graft, and those tissues were harvested up to 11 days after wounding. At 48 hours after the injury, the wounds were covered with bloody crusts but no apparent difference was observed between wounds in control and in *TGase 1*^{−/−} skin. In the control grafts, epithelial spurs were elongated from the wound edges and had invaded under the scab, and finally closed 5 days after the injury (Figure 3C). At that time, K6 α mRNA remained around the wound site, but its expression was decreasing in the re-epithelialized epidermis (Figure 3D). On the other hand, at that same time, wounded *TGase 1*^{−/−} skin grafts still had large clots in the center, under which epithelial migration was apparently interrupted (Figure 3I). A strong induction of K6 α mRNA was evident in the migrating epidermis (Figure 3J). At 11 days after wounding, the control grafts had matured with

hair follicles (Figure 3E) and the K6 α mRNA expression had subsided. However, in *TGase 1*^{−/−} skin grafts, epithelial spurs had just fused in each center of the wound (Figure 3K) and a diffuse expression pattern of K6 α mRNA remained. Thus, the regeneration of the epidermis was markedly delayed and the expression of K6 α was uncontrolled in *TGase 1*^{−/−} grafted skins.

Discussion

In the present study, we provide evidence that the *TGase 1* gene is activated at very early stages of wound healing. The early induction of *TGase 1* gene expression might result from cytokines released from keratinocytes in response to cell damage or mechanical stimuli (the free edge effect), although such triggering factors are still unidentified. *TGase 1* gene induction at the wound site

Figure 3. Wound healing and induction of K6 α mRNA of control and *TGase 1*^{-/-} skins grafted on nude mice. Skins from control (A–F) and *TGase 1*^{-/-} neonates (G–L) were grafted onto nude mice. Two weeks after transplantation (A, B, G, H), an injury was made with a 3-mm diameter punch in the center of each graft. Wounded grafts were harvested at 5 (C, D, I, J) and 11 days (E, F, K, L) after the injury. The tissues were stained with H&E (A, C, E, G, I, K), or were subjected to *in situ* hybridization of K6 α mRNA (B, D, F, H, J, L). The control skin grafts mimic adult mouse skin (A) and K6 α mRNA is hardly evident (B). The wounds are already closed at day 5 (C), at which time K6 α mRNA remains around the initial wound site and is seen only weakly in the regenerated epidermis (D). The control skin grafts are completely remodeled (E) and the K6 α mRNA expression subsides (F) 11 days after wounding. On the contrary, the *TGase 1*^{-/-}-grafted skins show acanthosis with markedly thickened stratum corneum and irregular hair follicles (G), where K6 α mRNA is evident (H). At day 5, the wounded *TGase 1*^{-/-} grafts are covered with a large clot under which epithelial migration is apparently interrupted (I) and K6 α mRNA is strongly induced in the migrating epidermis (J). Arrowheads indicate the tip of the migrating epithelium. The re-epithelialization of the *TGase 1*^{-/-}-grafted skins has been completed at 11 days after wounding (K), but the expression of K6 α mRNA is still sustained (L). Scale bar, 100 μ m.

occurs earlier than the massive infiltration of leukocytes. However, cytokines or lymphokines released by those infiltrating cells, such as tumor necrosis factor- α ,^{31,32} interferon- γ ,^{33,34} interleukin-1,³⁴ and/or activin A,^{35,36} might participate in the maintenance of *TGase 1* gene expression during wound healing, because those cytokines are able to activate the *TGase 1* gene.

The regulation of *TGase 1* and K6 genes differ from each other in normal interfollicular epidermis because the *TGase 1* gene is primarily expressed at late stages of

keratinization, whereas K6 gene activation is not evident except for limited epithelia.^{26,37} The machinery for K6 α and β gene activation may not be identical to each other because the induction of those mRNAs do not necessarily co-localize in the wounded epidermis. However, the following evidence suggests that regulatory systems common to *TGase 1* and those K6 genes are activated in the wound healing process: 1) up-regulation of the *TGase 1* gene occurs as early as the K6 genes in injured neonatal mouse skin; 2) the time course of *TGase 1* gene expression overlaps that of the K6 genes during wound healing; and 4) the 5' upstream promoter of those genes are responsive to treatment with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate in the epidermis of *TGM1-LacZ* and *K6-LacZ* transgenic mice.^{26,37,38} The Jun transcription factor might mediate signals for induction of those genes,^{39,40} but further study of the complex mechanisms involved in gene regulation is necessary to explore systems orchestrating epidermal regeneration.

The expression of K16, a type I partner for the K6 protein, leads to retraction of tonofilaments in keratinocytes and allows cell movement for re-epithelialization during wound healing.⁴¹ There is no doubt that the abundant generation of *TGase 1* enzyme is required to prepare for the rapid remodeling of the stratum corneum, but there are no obvious reasons for its expression in the leading edges of migrating keratinocytes. The expression of *TGase 1* enzyme occurs with involucrin expression in migrating keratinocytes, and eventually they are co-localized on the plasma membrane to form premature cornified envelopes. In contrast, loricrin is not expressed in the leading edges and its expression is restricted to the granular layers behind the migrating epidermis. Those premature cornified envelopes might provide mechanical strength to migrating epidermal cells dissecting the wound between the collagenous dermis and the fibrin eschar. During the process, leading-edge keratinocytes release tissue plasminogen activator,⁴² urokinase-type PA,⁴³ and various matrix metalloproteinases⁴⁴ that degrade the extracellular matrix for the dissection. Stabilized membrane structures reinforced by protein cross-linking might protect keratinocytes themselves from proteolytic damage. Similar premature expression of the *TGase 1* gene^{45,46} and involucrin⁴⁷ have been found in hyperplastic psoriatic epidermis, where it is evident that thinner premature cornified envelopes are generated.⁴⁸ Analysis of the physical and biochemical nature of the prematurely cross-linked cornified envelope will be necessary to elucidate the role of *TGase 1* in wound healing as well as in psoriasis.

We also demonstrate the substantial delay in epidermal migration with sustained expression of the K6 α gene during wound repair of *TGase 1*^{-/-} grafted skins. The evidence suggests that *TGase 1* is essential to early wound healing of the injured skin. Neonatal *TGase 1*^{-/-} mice show severely impaired skin barrier function,²⁰ but it is unlikely that the aberrant skin barrier function directly affects epidermal regeneration, because skin barrier function has been restored to normal levels in grafted *TGase 1*^{-/-} skins, possibly by the massive thick covering of scales even though the cornified envelope is lacking

(unpublished data). Hiiragi and colleagues²⁷ have shown that *TGase 1* is concentrated in adherence junctions in simple epithelial cells. The minor expression of *TGase 1* in simple epithelia, including endothelial cells other than keratinocytes, might play a role in wound healing. However, *TGase 1* expression is not evident in any components of neonatal mouse skin other than the epidermis and inner root sheaths.

In conclusion, *TGase 1* is important not only for development and maturation of the epidermis, but also for the progression of cutaneous regeneration. The *TGase 1* gene is an early responder gene to acute cutaneous injury, and is possibly involved in major changes in keratinocyte cytoarchitecture for migration into the wound site.

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