Knocking-in the R142C mutation in transglutaminase 1 disrupts the stratum corneum barrier and postnatal survival of mice

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Background: Mutations in the gene encoding transglutaminase 1 (TG1) are responsible for various types of autosomal recessive congenital ichthyosis (ARCI), such as lamellar ichthyosis (LI), congenital ichthyosiform erythroderma (CIE) and some minor variants of ARCI. A point mutation of R143C in the β-sandwich domain of TG1 has been often identified in patients with LI or CIE.

Objective: To elucidate the effect of that point mutation on skin barrier structures and functions, we generated mice with a point mutation of R142C, which corresponds to the R143C mutation in human TG1.

Methods: A mouse line with the R142C point mutation in TG1 was established using a gene targeting technique and the Cre-loxP system. The skin phenotypes were analyzed in homozygous mutant Tgm1 R142C/R142C mice.

Results: In the skin of Tgm1 R142C/R142C mice, expression of the mutant transcripts was comparable with wild-type or Tgm1 R142C/R142C mice. However, the amount of mutated protein in the skin was markedly decreased in Tgm1 R142C/R142C mice, and the TG1 activity of Tgm1 R142C/R142C keratinocytes was almost lost. Tgm1 R142C/R142C mice exhibited morphological and functional skin barrier defects and neonatal lethality. The stratum corneum of those mice lacked cornified envelopes, and loricrin, the major structural component, failed to assemble at the corneocyte cell periphery. Tgm1 R142C/R142C mice showed a marked increase in transepidermal water loss and their skin was easily permeable to toluidine blue dye. The intercellular lipid lamellar structures of the stratum corneum were irregular and the 13-nm periodic X-ray diffractions from the stratum corneum lipid molecules were lost in vivo.

Conclusion: From these results, we suggest that the R142C mutation of TG1 reduces the enzyme stability which is indispensable for development of the stratum corneum and skin barrier function and for postnatal survival of mice.

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1. Introduction

Transglutaminases (TGs) (EC 2.3.2.13) comprise a family of Ca²⁺-dependent enzymes that catalyze the cross-linking between polypeptides via γ-(γ-glutamyl) lysine bonds. The post-translational modifications by TGs enable the formation of supramolecular assemblies of protein components, which are functional in a wide variety of biological events, including blood coagulation, cell apoptosis and differentiation, tissue regeneration, and immune reactions [1]. In humans, members of the TG family are encoded by 6 different gene clusters and the genes for 8 TG isoforms and band 4.2 protein have been identified [2,3]. Of those TG family members,
the genes encoding coagulation factor XIllA, TG1, TG5 and band 4.2 are responsible for inherited human disorders, congenital factor XIllA deficiency, congenital ichthyoses, acral peeling skin syndrome, and hereditary spherocytosis, respectively.

Mutations of the gene encoding TG1 (TGM1) were first identified in affected members of pedigrees with lamellar ichthyosis (LI) [4,5], a severe form of autosomal recessive congenital ichthyosis (ARCI). LI is a disfiguring congenital skin disorder characterized by thickened large brownish hyperkeratotic scales which cover the entire body surface. Following that, mutations in TGM1 have also been found in milder forms of ARCI with fine whitish scales and generalized erythemas (erythroderma), which are clinically classified as (non-bullous) congenital ichthyosiform erythroderma (CIE), and as other variants of ARCI, including self-healing colloidon baby, acral self-healing colloidon baby, or bathing suit ichthyosis [6]. Mutations in other genes, ABCA12, ichthyin, ALOX12B and CYP4F2, have also been shown to result in ARCI [6]. Thus, ARCI is a clinically and genetically heterogeneous disease, but mutations in TGM1 are most frequently encountered in patients with ARCI.

TG1 is expressed largely in keratinized squamous epithelia [7], and in the skin, the expression of TG1 is detectable in the upper spinous and mainly granular layers of the epidermis and in hair follicles. In contrast to the other TG isozymes, TG1 is anchored to the plasma membrane through myristic and palmitic acid linked via thioester bonds at a Cys cluster near the N-terminal. The membrane localization of TG1 is required for the assembly of loricrin and other specialized proteins such as involucrin, small proline-rich proteins, elafin and cystatin A, to form the cornified envelope (CE) at the cell periphery [8]. Indeed, the CE is totally deficient in TG1 knockout (Tgm1−/−) mice [9] and an incomplete or defective CE is observed in some cases of LI [10]. The intercellular spaces between corneocytes are sealed with lipid lamellae composed of ceramides, cholesterol, and free fatty acids. The outer surface of the CE which faces the intercellular spaces lacks plasma membrane, and instead, ω-hydroxy-eryceramides covalently cross-link with the CE to form the corneocyte lipid envelope (CLE) [11]. Formation of the CLE and the maturation of regularly arranged lipid lamellae are required for the maintenance of skin barrier function. TG1 is not only essential for assembly of the CE, but also for stabilizing the lipid lamellar structures possibly via formation of the CLE [12,13].

The conserved catalytic residues containing the active site Cys residue of TG1 are essential to form a thioester intermediate with the γ-carboxamide group of a glutamate donor in TG1-catalyzed acyl transfer reactions [3]. The mutations reported in LI or in CIE are postulated to cause the loss of enzyme function, but those mutations do not necessarily occur at amino acid residues around the active site Cys residue. Mutated codons vary in cases of LI or CIE, and furthermore, the phenotype–genotype correlations are unclear [14,15].

We previously generated TG1 null mice, Tgm1−/− mice, which are lethal soon after birth due to severe skin barrier defects [9]. Following that study, we established a mouse line with the R142C point mutation in TG1. Surprisingly, despite the fact that this is only a one base substitution in TG1, levels of the mutant TG1 protein in the skin are very low and the enzyme activity is lost, and eventually the phenotype of mice becomes comparable to that of Tgm1−/− mice.

2. Materials and methods

2.1. Animals

All studies involving animals were designed in accordance with the International Guiding Principles for Biomedical Research.

Involving Animals published by the Council for the International Organization of Medical Science and were reviewed and approved by the Animal Use and Care Committee of the Hyogo College of Medicine and by the Animal Care and Welfare Committee of SPring-8. All mice used in this study were maintained under specific pathogen-free conditions. CAG-cre transgenic mice [16] were kindly provided by Prof. Junichi Miyazaki, Division of Stem Cell Regulation Research, Osaka University Graduate School of Medicine.

2.2. Construction of the targeting vector

A 2.5-kb BamHI–EcoRI DNA fragment containing exons 1–3 of the mouse TG1 gene (Tgm1) was isolated from a 129/SVJ genomic DNA library as described previously [9] and was inserted in a pBluescript vector (Stratagene, La Jolla, CA). The codon coding arginine 142 (CGT) of exon 3 in the insert was mutated to cysteine (TGT) using the mutagenic oligonucleotide 5′-GGAGCT-

GATTGTGCCGTGGGCACCCCTCC-3′ and a QuickChange Site-Directed Mutagenesis kit (Stratagene). The 1.8-kb BamHI–PstI fragment containing the mutation was ligated to a 5′ upstream 5.5-kb Xhol–BamHI genomic DNA fragment to form a 7.3-kb long arm. A 2.5-kb PstI–Xhol fragment containing exon 4 of the Tgm1 gene was also isolated for the short arm. Those arms were inserted into a vector containing a loxP-flanked neo cassette derived from pBS246 [17] and pMC1DTpA [18] to generate the pTG1MTRGV targeting vector, as shown in Fig. 1A.

2.3. Selection of targeted ES cell clones

Ten million ES cells established at the Department of Social and Environmental Medicine, Osaka University Graduate School of Medicine, were transfected with 25 μg of a NotI-linearized targeting vector pTG1MTRGV using electroporation. After positive selection with 150 μg/ml G418 and negative selection with expression of the diphertheria toxin A fragment (DT-A) gene, homologously recombined ES cell clones were isolated by PCR using the primers, P1 (5′-GGGACATCTCCAGACTGCTTG-GAAAGG-3′) in the PKG promoter and P2 (5′-GGGATCTCCGCT-AATATTGCCTGTCGCA-3′) in the Tgm1 gene (Fig. 1A).

2.4. Generation and genotyping of mice with the R142C mutation

ES cell clones with the heterozygous mutant Tgm1 gene were expanded and aggregated with 8 cell mouse embryos to obtain blastocysts. The blastocysts were transferred to aggregated embryos to pseudopregnant mothers to obtain chimeric animals [19]. Male chimeras derived from those clones were bred with C57BL/6 female mice. Mice carrying the mutated allele in the germline were isolated and F2 offspring generated by their interbreeding were identified by PCR analysis of tail DNA using primers P1 and P3 (5′-GGTACTGTTTACGTTGCTGTGGTTCT- TAG-3′) in exon 4 (Fig. 1A). Those primers were designed to confirm the targeted allele containing the loxp-flanked neo cassette. Those mice were back-crossed with C57BL/6 mice more than 10 generations and were used to establish a mouse strain Tgm1+/R142C-fl.ex/ne/+ with the mutated allele. Those mice were crossed with CAG-cre transgenic mice [16] to remove the neo cassette in the germline. The removal of the neo cassette was confirmed by PCR using primers P4 (5′-AGGGCTGACTGACGACCCCTCCTGTCCTTCA-3′), which is 30-bp upstream of the 5′ end of the loxp-flanked neo cassette, and P3 (Fig. 1B), and finally, a mouse line Tgm1+/R142C was established with the heterozygous R142C mutation in Tgm1 was established. For genotyping offspring of the mouse line, primers P5 (5′-GCGAATCTGTTGCTACTGTCTGGAT-3′) in exon 3 and P3 were used (Fig. 1B).
Fig. 1. (A) Structure of the mouse Tgm1 gene, targeting vector and mutated alleles encoding R142C. The diphtheria toxin A (DT-A) gene and the neo cassette (pgk-neo) were inserted in reverse orientation in the targeting vector. The vector was designed to contain Tgm1 exon 3 encoding the R142C mutation followed by a loxP-flanked neo cassette in intron 3. Closed boxes indicate the approximate positions of exons. Arrowheads flanking the neo cassette indicate the 34-bp loxP. Restriction sites for BamHI, XhoI, HindIII, PstI, EcoRI and Xhol are shown by B, Xh, H, P, E and Xh, respectively. P1–5 are PCR primers for genotyping. (B) Genotyping of mice. (Left) The removal of the neo cassette in mice was detected by a size-reduction in the PCR product from 2.7 in Tgm1+/R142C floxNeo to 1.0 kb in Tgm1+/R142C (+/R142C) which was amplified using primers P3 and P4. (Right) The genotypes of offspring generated by interbreeding of Tgm1+/R142C mice were determined by PCR using primers P3 and P5. Note that the PCR bands in lane +/R142C is half as intense as in the other lanes. (C) The heterozygous and homozygous C to T substitution in Tgm1 transcripts of the skin. (D) Expression of the Tgm1 gene in the skin; the gene expression of Tgm1 in Tgm1+/R142C and Tgm1+/R142C/R142C mice was comparable to levels in wild-type mice. (E) Western blotting of protein extracts from the skin using an anti-TG1 antibody (H-87) or an anti-β actin antibody (AC-15). TG1 protein was absent in the extract from Tgm1−/− skin, but was weakly detectable in Tgm1+/R142C/R142C skin. +/+ wild-type; +/−, Tgm1+/−; −/−, Tgm1−/−; +/R142C floxNeo, Tgm1+/R142C floxNeo; +/R142C, Tgm1+/R142C; R142C/R142C, Tgm1+/R142C/R142C for B–E.
2.5. Sequencing of Tgm1 cDNA and quantitative real-time RT-PCR (qRT-PCR)

Total RNA was isolated from 19.5 days postcoitum (dpc) mouse skins using an RNeasy Fibrous Tissue Kit (Qiagen, Inc., Hilden, Germany) according to the manufacturer's instructions and was subjected to sequencing of the Tgm1 cDNA or to qRT-PCR. A Primerscript II High Fidelity RT-PCR Kit (TAKARA, Tokyo, Japan) and primers Fxmtrgm1c 5′-TAAGGCAATCCATGGAAGCTTGCCTGCAGA-3′ and Fxrtgm1c 5′-CCAGGTTTAACACGTCTCACCTGAT-3′ were used for RT-PCR and the sequence of the amplified full-length Tgm1 cDNA was determined using a 3130xl Genetic Analyzer (Applied Biosystems, Life-Technologies Corp., Carlsbad, CA). For qRT-PCR, a TaqMan RNA-to-Ct Kit (Applied Biosystems) and an ABI7900HT sequence detection system (Applied Biosystems) were used. An mRNA encoding glycerolaldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal standard for qRT-PCR. The probes for qRT-PCR were obtained from Applied Biosystems Assays-on-Demand. The product numbers of the probes for Tgm1, Tgm2, Tgm3, Tgm5, Tgm6 and GAPDH are Mm00498375_m1, Mm0436980_m1, Mm00436999_m1, Mmm0551325_m1, Mm00624922_m1 and Mm99999915_g1, respectively. The relative abundance of each target transcript was assessed with regard to internal controls according to the manufacturer's instructions. Experiments were repeated at least three times, and results are expressed as fold induction of wild-type transcripts ± standard deviation (n = 3).

2.6. Western blot analysis

Total protein was isolated from 20 to 30 mg of 19.5-dpc mouse skins using an AllPrep RNA/protein kit, a TissueRuptor and a QIAshredder (Qiagen) according to the manufacturer's instructions. Proteins precipitated with acetone were re-solubilized in 9 M urea, 2% Triton X-100, 5% 2-ME, 2% lithium dodecyl sulfate and 40 mM Tris. Protein concentrations were measured using an EZQ Protein Quantitation Kit (Invitrogen Corp., Carlsbad, CA) and 10 μg of each protein sample were subjected to 5–20% gradient SDS-polyacrylamide gel electrophoresis and were electroblotted on polyvinylidene difluoride (PVDF) membranes using an iBlot™ Dry Blotting System (Invitrogen Corp.). Membranes were incubated with a rabbit anti-TG1 polyclonal antibody H-87 (1:200 dilution) (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or with a mouse anti-β actin monoclonal antibody AC-15 (1:50,000) (Sigma–Aldrich Corp., St. Louis, MO). Bound antibodies were detected using a horseradish-peroxidase-conjugated donkey anti-rabbit secondary antibody (1:1000 dilution) or a sheep anti-mouse secondary antibody (1:200 dilution) (GE Healthcare Bio-sciences Corp., Piscataway, NJ). Blotted proteins were visualized using an ECL Plus Western Blotting Detection system (GE Healthcare Bio-sciences Corp.) and were recorded using a chemiluminescence imager ImageQuant LAS4000 mini (GE Healthcare Bio-sciences Corp.) or LAS-1000 (FujiFilm Corp.).

2.7. Culture of mouse keratinocytes

The excised skins from 19.5-dpc pups were incubated while floating on PBS containing 0.1% Dispase II (Sanko Junyaku Co., Ltd., Tokyo, Japan) overnight at 4°C. Epidermal sheets were then separated from the dermis with forceps and were gently torn into pieces by gentle pipetting to generate a single cell suspension in PBS. The cells were collected by centrifugation and were seeded into 10-cm culture dishes containing CoT-07 medium (CELLTEC Advanced Cell Systems, Bern, Switzerland) supplemented with antibiotics (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B), and were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. When they became 75% confluent, cells were trypsinized and plated into 6-well plates at an initial seeding density of 1.0 × 104 cells per cm2 and when they reached 80% confluence, cells were harvested to prepare protein extracts for measurement of TG1 activity.

2.8. Measurement of TG1 activity

Protein extracts of cultured mouse keratinocytes were prepared using a Proteoextract Native Membrane Extraction kit (Calbiochem, San Diego, CA) according to the manufacturer's instructions. The protein concentrations of both the cytosolic and the membrane fractions were determined by the Bradford method (Bio-Rad Laboratories, Inc., Hercules, CA). The in vitro TG1 activity in each extract was measured using a microplate assay that was recently established [20], with slight modifications. The principle of this method is the measurement of cross-linking products between a biotin-labeled TG1-specific substrate peptide (pepK5: YEHHKLPSWPF, as a glutamine-donor substrate), and spermine (as a glutamine-acceptor substrate) which was previously immobilized in the microtiter wells. Briefly, 100 μl of reaction mixture, containing each extract solution and biotinylated pepK5 in 10 mM phosphate buffer, pH 8.0, 150 mM NaCl, 15 mM CaCl2 and 2.5 mM dithiothreitol, were incubated at 37°C for the indicated time intervals. Enzymatic reactions were stopped by the addition of excess EDTA at the final concentration of 50 mM. After washing with Tris-based buffered saline (10 mM Tris–HCl, pH 8.0, 150 mM NaCl), avidin-conjugated peroxidase was added and incubated for 1 h. Then, after washing as before, the amounts of incorporated biotinylated peptides were detected using streptavidin-peroxidase (Rockland Immunochemicals, Inc., Gilbertsville, PA) and the peroxidase substrate 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma–Aldrich Corp.). After color development, absorbance at 450 nm was measured using a spectrophotometer.

2.9. Hematoxylin and eosin staining and immunofluorescence

Tissues from 19.5-dpc mice were fixed in 10% formaldehyde in phosphate-buffered saline (PBS), then embedded in paraffin. Those tissues were sectioned at 4-μm thickness and deparaffinized sections were subjected to hematoxylin and eosin staining. Immunofluorescence for TG1 was performed as described previously [21]. In brief, cryosections of the skin were incubated with a rat monoclonal anti-TG1 monoclonal antibody (TG1F-1) [22] (1:1) and bound antibodies were visualized using a Cy3-conjugated donkey anti-rat IgG (H + L) antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Fluorescence images were recorded using a confocal laser scanning microscope LSM510 META (Carl Zeiss Microlmaging GmbH, Göttingen, Germany).

2.10. In situ TG assay

Cryosections prepared as described above were washed three times with PBS and blocked with 1% bovine serum albumin (BSA) in PBS for 30 min at room temperature. Blocking solutions were removed and 0.1 mM Alexa Fluor® 488 cadaverine (Invitrogen Corp.) in 100 mM Tris–HCl (pH 7.4) containing 5 mM CaCl2 was added, and sections were then incubated for 90 min at 37°C. Reactions were stopped with 25 mM EDTA in PBS for 5 min at room temperature. Fluorescence images were recorded described above.

2.11. Ultrahigh-resolution immunofluorescence microscopy

Ultrathin cryosections were prepared as described previously [23,24]. Briefly, tissues were fixed for 2 h at 22°C with 4% paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.4)
containing 5% sucrose, solidified with gelatin, infiltrated with 2.3 M sucrose, and mounted on specimen pins designed to fit a cryo-ultramicrotome (Leica, Wetzlar, Germany). The samples were then frozen and stored in liquid nitrogen until sectioned. Ultrathin cryosections (50–100 nm thick) were cut with a Leica ultramicrotome EM UC6b equipped with an FC6 cryounit and were then transferred to 2% 3-aminopropyltriethoxy-silane (Sigma–Aldrich Corp.) coated coverslips. Ultrathin cryosections were then incubated for 30 min at 37 °C with rabbit anti-mouse loricrin antibody (1:500). The blocking solution consisted of 1% BSA and 5% normal goat serum in PBS. The sections were incubated for 30 min at 37 °C with the secondary antibody Alexa Fluor® 594-labeled goat anti-rabbit IgG (10 μg/ml) (Invitrogen Corp.), and the sections were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Control sections received the same treatment, with the exception that the primary antibody was either omitted or was replaced with purified nonimmune IgG. Fluorescence and differential interference contrast (DIC) images were obtained using a BX60 microscope (Olympus, Tokyo, Japan) equipped with a Spot RT SE6 CCD camera (Diagnostic Instruments, Sterling Heights, MI) and processed with the MetaMorph image analysis system (Universal Imaging, Downingtown, PA). Figures were compiled using Photoshop CS software (Adobe Systems, San Jose, CA).

2.12. Electron microscopy

Freshly obtained skin samples from 19.5-dpc mice were fixed in 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, containing 0.05% CaCl2 overnight at 4 °C. The samples were washed with 0.1 M cacodylate buffer (pH 7.4) containing 5% sucrose, then were infiltrated with a 20% sucrose solution, embedded in Neg-50 Frozen Section Medium (Richard-Allan Scientific, Kalamazoo, MI), placed in aluminum-foil molds, and then flash-frozen in liquid nitrogen. Tissue sections (40-μm thickness) were prepared using a Microm HM 550 cryostat (Microm, Walldorf, Germany). The sections were postfixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4, containing 0.8% potassium ferrocyanide for 60 min at 4 °C. Samples were then dehydrated in a graded ethanol series and subsequently embedded in Quetol 812 (Nissin EM, Tokyo, Japan). Ultrathin sections were stained with uranyl acetate and lead citrate and were examined using a H-7500 (Hitachi High-Technologies Co., Tokyo, Japan) transmission electron microscope. To examine intercellular lipid lamellae, the protocol was modified as follows [25]: skin samples were postfixed in 0.2% ruthenium tetroxide (Strem Chemicals, Newburyport, MA) in 0.1 M cacodylate buffer, containing 0.5% potassium ferrocyanide for 30 min at 22 °C. To visualize the CLE, skin samples were immersed first in absolute pyridine for 2 h at 22 °C prior to aldehyde fixation as described above [11].

2.13. Cornified envelope (CE) assay

Excised dorsal skins of 19.5-dpc mice were immersed in PBS containing 10 mM EDTA at room temperature for 1 h to separate the epidermis. Epidermal sheets were heated in 2% SDS extraction buffer (0.1 mM Tris, pH 8.5, 20 mM DTT, 5 mM EDTA, 2% SDS) at 95 °C for 10 min [10]. Insoluble CEs were precipitated by centrifugation at 9000 × g for 15 min and their morphology was observed microscopically.

2.14. In situ skin permeability assay

The in situ skin permeability assay using toluidine blue was performed as reported by Hardman et al. [26]. In brief, 19.5-dpc anesthetized mice were rinsed in PBS and were then immersed successively in 25%, 50%, 75% and 100% methanol for 1 min each, and were then rehydrated in PBS and stained in 0.1% toluidine blue for 10 min at room temperature. Those animals were briefly washed in PBS and were immediately photographed.

2.15. Measurement of transepidermal water loss (TEWL)

TEWL from the dorsal skins of 19.5-dpc mice (n = 5) was examined using a Tewameter MSC100 and a probe TM300 attached with an adaptor for the measurement of mouse TEWL (Courage + Khazaka electronic GmbH, Köln, Germany), as described previously [9].
2.16. Small angle microbeam X-ray diffraction

Experiments using synchrotron radiation were reviewed and approved by the SPring-8 Proposal Review Committee (proposal nos. 2005B0734, 2006A1051 and 2006B1026). The small angle X-ray diffraction (SAXD) from the stratum corneum of 19.5-dpc mice was recorded at BL40XU (High Flux Beamline) of SPring-8 (Hyogo, Japan) as described previously [27]. A part of the dorsal skin of anesthetized pups was gently picked up with clips and an X-ray microbeam was allowed to impinge on the upper surface of the skin between the clips. The stepwise upward-shift of each mouse pup enabled the microbeam to scan from the surface to the deeper parts of the skin. A quasi-monochromatic high-flux X-ray beam from a helical undulator (λ = 0.083 nm) was focused with two mirrors laid horizontally and vertically. In the experimental hatch, an X-ray beam 5 μm in diameter was obtained behind a collimating pinhole (5 μm in diameter) and a guard pinhole (100 μm in diameter). The sample-to-detector distance was about 2.3 m. The scattering vector, \( q \), was calculated as \( (4\pi/\lambda)\sin(2\theta)/2 \) where \( 2\theta \) is the scattering angle. SAXD patterns from the stratum corneum were recorded with an X-ray image intensifier (V5445P, Hamamatsu Photonics, Hamamatsu, Japan) coupled to a CCD camera (C4880-50-24A, Hamamatsu Photonics) with 1024 \( \times \) 1024 pixels.

3. Results

3.1. Generation of knock-in mice with the R142C mutation in TG1

The mutation of Arg to Cys at codon 142 (R142C) of TG1 was introduced in ES cells using a gene targeting technique. In the targeting vector, exon 3 containing the mutated codon was placed upstream of the loxP-flanked neo cassette in the opposite direction in intron 3 of the Tgm1 gene, as shown in Fig. 1A. G418-resistant 96 ES cell clones were analyzed by PCR and 13 mutant clones carrying the targeted allele were isolated. Based on karyotyping, 5 clones were selected and used to generate chimeric animals. Finally, chimeric males derived from one of the clones were crossed with C57BL/6 mice to establish the Tgm1\(^{+/-}\)R142C/loxNeo strain which was heterozygous for the mutated allele. Mice from that strain were intercrossed with CAG-cre transgenic mice to remove the neo-cassette and the mouse strain Tgm1\(^{-/-}\)R142C with the R142C heterozygous mutation of the Tgm1 gene was established. The R142C mutation in the Tgm1 gene was confirmed by sequencing of tail genomic DNA and by full-length sequencing of reverse-transcription PCR products of TG1 mRNA isolated from the skin of mutated mice (Fig. 1C). In contrast to Tgm1\(^{+/-}\) and Tgm1\(^{-/-}\) mice, the expression of Tgm1 transcripts in the skin was not altered in Tgm1\(^{+/-}\)R142C or in Tgm1\(^{-/-}\)R142C mice (Fig. 1D). The expression of other members of the Tgm family, Tgm2, Tgm3, Tgm5 or Tgm6, were not affected in the skin of Tgm1\(^{+/-}\)R142C mice (data not shown). Western blotting of 19.5-dpc mouse skin extracts revealed that the size of the TG1 protein was not different among wild-type, Tgm1\(^{+/-}\)R142C and Tgm1\(^{-/-}\)R142C mice. However, the amount of TG1 protein in Tgm1\(^{+/-}\)R142C skin was markedly decreased (Fig. 1E).

3.2. Enzyme activity of TG1 from Tgm1\(^{+/-}\)R142C keratinocytes

We recently established an isozyme-specific assay system using the preferred substrate peptides for various TGs [20,28]. This system enables the sensitive detection of TG enzymatic activity in an isozyme-specific manner. In this assay, the K5 peptide, which is a representative favorable substrate sequence for TG1, was incorporated into spermine-conjugated microtiter wells by the catalytic reaction of TG1. As shown in Fig. 2, in cell extracts from wild-type mouse keratinocytes, a time-dependent incorporation of K5 peptides was observed both in the cytosolic and the membrane fractions, indicating the presence of TG1 activity. Based on the protein concentrations, TG1 activity was abundant in the membrane fraction, which is consistent with a previous study [29]. Although the K5 peptide cross-reacts with spermine also due to the catalytic activity of TG2 (tissue TG) according to a previous

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**Fig. 3.** Macroscopic appearance (A) and histology (B–E) of 19.5-dpc wild-type (+/+) and Tgm1\(^{+/-}\)R142C/R142C mice (R142C/R142C). (B) Hematoxylin and eosin staining of wild-type and Tgm1\(^{+/-}\)R142C/R142C skin; the cornified layers are homogeneous and granular layers are thickened in Tgm1\(^{+/-}\)R142C/R142C skin. (C) Immunofluorescence of TG1 using an anti-TG1 antibody (TG1F-1); the upper spinous and granular layers of the epidermis are positive for TG1 in wild-type mice (left), but are negative in the Tgm1\(^{+/-}\)R142C/R142C epidermis. (D) In situ TG activity using Alexa Fluor® 488 cadaverine. TG activity is evident on the cell membrane in the upper layers of wild-type epidermis (left). This activity is almost lost in the Tgm1\(^{+/-}\)R142C/R142C epidermis (right). (E) Ultrahigh-resolution immunofluorescence microscopy of loricrin. Loricrin is located at the cell periphery of the granular and cornified layers in wild-type epidermis (left). In contrast, loricrin is distributed as fine granules or aggregates in the upper layers of the Tgm1\(^{+/-}\)R142C/R142C epidermis (right). Bar: 50 μm in B–D; 10 μm in E.
study [28], there was no significant incorporation of the T26 peptide, which is a favorable substrate peptide for TG2 (Fig. 2, open symbols at 30 min and 90 min). Therefore, the reaction product formed by the K5 peptide is derived from the TG1 activity of keratinocytes. As expected, in the case of extracts from Tgm1+/− mouse keratinocytes, enzymatic activity was not observed in either the cytosolic or the membrane fractions. This result also indicates that there were no detectable enzymatic activities from other isozymes of TGs. The cell extract from Tgm1R142C/R142C keratinocytes revealed much less activity than that from wild-type keratinocytes in both the cytosolic and the membrane fractions. In the cytosolic fraction, there was a slight increase in the incorporation rate when compared to Tgm1+/− keratinocytes.

3.3. Macroscopic appearance of Tgm1R142C/R142C mice

The appearance and growth of Tgm1+/R142C mice were indistinguishable from wild-type mice, whereas Tgm1R142C/R142C mice were born alive but were smaller and less active than their littermates with wild-type or Tgm1+/R142C genotypes. Tgm1R142C/R142C neonates could not feed and died within a few hours after birth. The skin of 19.5-dpc mice was slightly erythematous and wet like a mucous membrane. The skin surface became tense with time and died out (Fig. 3A, right). Those skin phenotypes of the mutant mice closely resembled Tgm1+/− mice in appearance. Genotype analysis of more than 100 offspring from matings of heterozygous mutant mice showed a normal Mendelian frequency, indicating no embryonic lethality of the homozygous mutant mice.

3.4. Histology of Tgm1R142C/R142C skin

The stratum corneum of Tgm1R142C/R142C mice was dense and homogeneous. The lower layers of the stratum corneum were parakeratotic with flattened nuclei (Fig. 3B, right). In contrast to wild-type mice (Fig. 3B, left), keratohyalin granules were fine and densely arranged in the granular layers. In the dermis, capillaries were dilated in Tgm1R142C/R142C mice. Immunofluorescence using a TG1 antibody [22] clearly revealed the membranous localization of the enzyme in the upper spinous and granular layers of wild-type mice (Fig. 3C, left), whereas TG1 immunoreactivity was virtually lost in Tgm1R142C/R142C mice (Fig. 3C, right). The activity of TG enzymes was visualized at the cell peripheries of the upper spinous and granular layers of wild-type mice using an in situ TG assay with an Alexa Fluor® 488 cadaverine as a substrate (Fig. 3D, left). However, the fluorescence signal was almost absent in the epidermis of Tgm1R142C/R142C mice (Fig. 3D, right). The localization of loricin, a major protein component of the CE, was assessed by ultrahigh-resolution immunofluorescence microscopy. Loricin was clearly localized along the cell peripheries of the granular and cornified layers in wild-type mice (Fig. 3E, left). However, in Tgm1R142C/R142C mice, loricin was not localized at the cell periphery but instead was distributed as fine aggregates in the cytoplasm of those layers (Fig. 3E, right). The reduced staining of loricin in Tgm1R142C/R142C mice might be due to uncross-linked loricin which is readily lost by solubilization.

Electron microscopy revealed that the stratum corneum, which was compact and dense in wild-type mice (Fig. 4A), was markedly thickened and piled-up in Tgm1R142C/R142C mice (Fig. 4B). The granular layers were thickened with keratohyalin granules of various sizes (Fig. 4B) and the CE (marginal band) at the cell periphery of each corneocyte was almost missing in Tgm1R142C/R142C mice (Fig. 4C). The intercellular lipid lamellar structures in Tgm1R142C/R142C mice were examined using specimens fixed with ruthenium tetroxide (Fig. 4D). Lipid lamellae sealing the intercellular spaces among the corneocytes are regularly arranged in wild-type mice [13]. However, in Tgm1R142C/R142C mice, the intercellular spaces were uneven and the arrangements of the intercellular lamellae were substantially disturbed. Furthermore, CLEs, the
outermost layers of the intercellular lamellae, were also incomplete in the stratum corneum in Tgm1<sup>R142C/R142C</sup> mice (Fig. 4E). The histology of Tgm1<sup>+/R142C</sup> mice was not different from wild-type mice (data not shown).

3.5. Defective skin barrier of Tgm1<sup>R142C/R142C</sup> mice

The maturation of the CE was examined using a CE assay developed by Jeon et al. [10]. As shown in Fig. 5A, intact CEs were abundant in specimens from wild-type mice, while such insoluble envelopes were completely absent in specimens from Tgm1<sup>R142C/R142C</sup> mice. Thus, the maturation of the CE was incomplete in Tgm1<sup>R142C/R142C</sup> mice. The skin barrier function was assessed by an in situ skin permeability assay and by measurement of TEWL. When 19.5-dpc mice were immersed in toluidine blue, the skin of wild-type mice was not permeable to toluidine blue and the mice were not stained (Fig. 5B, left). In contrast, the entire skin of Tgm1<sup>R142C/R142C</sup> mice was completely stained dark blue (Fig. 5B, right). TEWL from the skin surface was assessed in 19.5-dpc mice. TEWL was maintained at very low levels in wild-type mice (Fig. 5C) and in Tgm1<sup>+/R142C</sup> mice (data not shown). However, TEWL was markedly increased in Tgm1<sup>R142C/R142C</sup> mice (Fig. 5C). These results suggest that a severe skin barrier impairment occurs in Tgm1<sup>R142C/R142C</sup> mice.

3.6. Small-angle X-ray diffraction (SAXD) of the stratum corneum of living Tgm1<sup>R142C/R142C</sup> mice

Lamellar structures of lipids at the molecular level were assessed by analyzing SAXD patterns from wild-type mice. Broad diffraction peaks were evident in SAXD patterns from the stratum corneum of wild-type mice, which were recorded 10 μm from the surface of the skin. Fig. 6 shows SAXD patterns obtained from the stratum corneum of living mouse skins. The SAXD profiles in the range below Q ≈ 0.2 nm<sup>−1</sup> were cut off by the X-ray beam stop. Diffraction peaks were seen beyond this range in the meridional direction (the direction perpendicular to the skin surface) which indicates that the planes of the lamellar structures of lipids are parallel to the skin surface. In wild-type mice, three distinct peaks were recorded in the meridional intensity distribution (Fig. 6). We infer that these peaks resemble those from a long lamellar periodicity of about 13 nm that was previously observed in mouse stratum corneum [30], although the present peaks are broader and the position of the third peak deviates from that in the previous report [30]. In addition, a broad peak might lie behind the peak from the long lamellar structure at Q ≈ 1.0 nm<sup>−1</sup>, which is due to the short lamellar structure with 6-nm periodicity [30,31]. The diffraction patterns from Tgm1<sup>+/R142C</sup> mice were similar to those from wild-type mice (data not shown). On the other hand, one broad peak related to the structure with 6-nm periodicity was recorded from the skin of Tgm1<sup>R142C/R142C</sup> mice (Fig. 6, right). This peak was broad in directions both along and across the meridian, indicating that the lipids are much less ordered than in wild-type mice. These results suggest that the periodicity and molecular orientation of the lipid lamellae are totally disturbed in the stratum corneum of Tgm1<sup>R142C/R142C</sup> mice.

4. Discussion

A variety of TG1 mutations have been identified in ARCI, but little is known about correlations between genotypes and clinical symptoms even in the major types of ARCI, LI and CIE [6]. The severity of ichthyosis is not necessarily consistent between unrelated pedigrees or among affected members of families with the same TG1 mutations [14,15]. Regardless of the heterogeneity in the clinical manifestations of ARCI, at least typical severe LI often develops in patients with the R143C mutation which is prevalent in the Finnish population [32]. In this study, the impact of the mutation in TG1 was characterized in mice. We previously generated Tgm1<sup>−/−</sup> mice by gene targeting and showed that those knockout mice are lethal soon after birth [9]. LI patients with the point mutation of R143C in TG1 (corresponding to R142C in mice) can survive and therefore we were expecting that mice with the same mutation would be viable and would show a phenotype resembling LI. However, unexpectedly, knocking-in the homozygous R142C mutation of TG1 in mice resulted in a phenotype almost identical to Tgm1<sup>−/−</sup> mice with neonatal lethality and the impairment of skin barrier structures and functions.

Expression levels of the Tgm1 gene in Tgm1<sup>R142C/R142C</sup> skin are not lower than in wild-type or in Tgm1<sup>−/−</sup> skin, and therefore, the base substitution from C to T at the first nucleotide of codon 142 does not affect the stability of Tgm1 mRNA. However, protein
levels of TG1 in the skin are markedly reduced in Tgm1<sup>R142C/R142C</sup> mice. The in situ TG assay revealed that the activity of TG is almost lost in the epidermis of Tgm1<sup>R142C/R142C</sup> mice in vivo. The substantial reduction of TG1 activity in the membrane fraction of keratinocytes derived from the mutant mice suggests that the decrease in the mutant TG1 enzyme causes the phenotype similar to Tgm1<sup>-/-</sup> mice.

Because the crystal structure of TG1 has not yet been elucidated, molecular modeling of TG1 has been used to predict conformational changes associated with the loss of enzyme activity. Human TG1 presumably has a globular structure consisting of five domains, the unique N-terminal peptide (M1 to R92) containing the Cys cluster for membrane anchoring, and four successive domains, the β-sandwich (S94 to F246), the α/β-catalytic core (N247-R572), the β-barrel 1 (G573-R688) and the β-barrel 2 (T689-A816). Except for the N-terminal peptide, the other domain structures are highly homologous to TG3 [33]. Most of the single missense mutations in TG1 occur at residues contributing to hydrogen bonding in the same domain or across a domain interface. Besides the frame-shift or premature termination mutations, those point mutations would affect enzyme activity possibly by modification of the active site or Ca<sup>2+</sup>-binding sites, protein misfolding, domain rearrangements and/or instability and/or impairment of interactions between substrate proteins. The R143 residue is located between β-sandwich domains 2 and 3 of TG1 and is completely conserved among other members of the TG family, TG1-7, Xllla and P4.2 protein. As predicted by Candi et al. [34], that residue is far from the catalytic core and from the three Ca<sup>2+</sup> binding sites, but it participates in hydrogen bonds that bind the D253 residue in the linker of the catalytic core domain and the N-terminus of the β-sandwich domain. Although how the R142C mutation affects the three-dimensional structure of mouse TG1 is still unknown, the mutation might disrupt the topology of those two domains, which would result in the inactivation of the TG1 enzyme. Laiho et al. assumed that the R143C mutation might lead to premature degradation of the enzyme due to a misfolding of the protein [32]. Our study shows that the mutant mouse TG1 with R142C is so unstable in vivo that the enzyme activity remains very low, and the effect of the loss of TG1 activity in mouse skin is almost comparable to that in the null condition of the enzyme.

The function of the skin barrier is evaluated as the outside-inside or the inside-outside barrier. Mutant mice are easily permeable to toluidine blue from outside of the skin and the TEWL from the skin surface is very high. The defects in both barriers are possibly due to the immature structures of the stratum corneum in the mutant mice. Indeed, the assembly of loricrin at the corneocyte
cell periphery is incomplete and formation of the CE (marginal band), which is a protein barrier of the stratum corneum, is totally impaired. Lipid lamellar structures sealing the intercellular spaces in the layers are also irregular and poorly formed in Tgm1R142C/R142C mice. This suggests the impaired organization of intercellular lipid molecules in the layers. In this study, we succeeded in recording SAXD patterns from living stratum corneum using microbeam X-ray generated by SPring-8. This technique avoids the chemical modification of molecules, such as the cross-linking of molecules during the fixation of tissues, which is used to prepare specimens for electron microscopy, and makes it possible to characterize the intact molecular organization of the intercellular lipids. Indeed, SAXD reveals that stratum corneum intercellular lipid molecules of wild-type mice form lamellar sheets with about 13-nm periodicity. In contrast, in Tgm1R142C/R142C mice, those lamellar structures are disrupted and the broad peak due to the short (6 nm) lamellar structure becomes evident. Similar data were obtained from Tgm1−/− mice (data not shown). It has been reported that α-hydroxyacidemolecules that are partly covalently bound to CE play an important role in the formation of the long lamellar structure [35], while the formation of the short lamellar structure seems to be different, since this structure is basically composed of the arrangement of water and lipid layers [31]. These facts suggest that the orientation of intercellular lipid molecules in the long lamellar structure is random in those mutant mice with the loss of TG1 activity. Easily deformable cellular architecture due to decreases in physical strength of corneocytes with defective CE may be a factor for the disordered lipid molecules in the long lamellar structure in Tgm1R142C/R142C mice. On the other hand, the short lamellar structure which is not attached to the CE may remain in the mutant mice, but the broad distribution of the diffraction suggests that the orientation of lipid molecules constituting the 6-nm lamellar structure are also irregularly arranged. Those findings obtained from living pups by SAXD coincide with the ultrastructure of the defective intercellular lamellae and the loss of CLE in the stratum corneum of Tgm1R142C/R142C mice.

In humans, patients with the same mutations in TG1 developing Li or CIE which is rarely lethal in the perinatal period. Why mice with the R142C mutation are neonatally lethal is unknown. One reason for the neonatal lethality of the knock-in mice might possibly be the defective skin barrier function which is undoubtedly involved directly in the maintenance of postnatal life in mice. However, the functional disturbance of TG1 in other cell types, including bile duct epithelia, blood vessels and kidneys have not been excluded in this study. Possible compensatory or alternative mechanisms for the defective tissue functions including skin barrier functions caused by this particular mutation might not be sufficient for postnatal survival in mice. Mice with the R142C point mutation will be useful as a model to develop new therapeutic strategies for ARCI by recovery of or compensation for the decreased activity of mutated TG1.

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