Transglutaminase 1 Replacement Therapy Successfully Mitigates the Autosomal Recessive Congenital Ichthyosis Phenotype in Full-Thickness Skin Disease Equivalents


TO THE EDITOR
Autosomal recessive congenital ichthyosis (ARCI) disrupts normal keratinization, resulting in generalized scaling of the skin. There are presently no curative therapies available (Fleckman et al., 2013). Local protein replacement is, therefore, an encouraging approach for a more specific treatment.

ARCI refers to a heterogeneous group of rare skin keratinization disorders with an estimated prevalence of 1 in 50,000–200,000 (Dreyfus et al., 2014). The disease is characterized by notable impairments to the skin’s barrier function, resulting in frequent infections and increased transepidermal water loss. ARCI is caused by mutations in 1 of 12 identified genes involved in epidermal differentiation. The most common of these are loss of function mutations in TGM1, affecting approximately 30% of patients (Rodriguez-Pazos et al., 2009). TGM1 encodes transglutaminase 1 (TG1), a protein that plays an essential role in the formation of the cornified envelope (Eckert et al., 2005). Because animal models of severe keratinization disorders such as ARCI are not viable and animal skin poorly represents human skin (Gerber et al., 2014), the use of organotypic skin equivalents has emerged as a valid tool to investigate ARCI.

In the present study, full-thickness skin equivalents generated from fibroblasts and keratinocytes of ARCI patients with mutations in TGM1 were treated topically with TG1. Because biomacromolecules do not normally overcome the skin barrier, owing to their high molecular weight, protein delivery was mediated by use of thermoresponsive nanogels (tNG) (Cuggino et al., 2011). Proteins as large as 150 kDa have been encapsulated within tNGs and subsequently released above a thermal trigger point (Giulbudagian et al., 2018b; Witting et al. 2015). Our groups previously reported the epidermal delivery of functional TG1 using topically applied tNGs and rescue of barrier defects in TGM1 knockdown skin equivalents (Witting et al. 2015). However, whether TG1-loaded tNGs are an effective topical treatment for ARCI skin with TGM1 mutations, rather than transiently induced TGM1 knockdowns, was still unclear.

The study was approved by the Ethics Committee of the Medical University of Innsbruck, Austria, and samples were taken after obtaining written informed consent of the probands. Full-thickness skin equivalents were generated from fibroblasts plus normal keratinocytes, keratinocytes with transient TGM1 knockdowns, or keratinocytes from ARCI patients with TGM1 mutations (Figure 1). In comparison to normal equivalents, TGM1 knockout and patient equivalents both demonstrated slightly thinned stratum corneum and epidermis, with reduced cell number within the granular layer. The epidermal differentiation markers keratin 14 and 10 were distributed appropriately. TG1 activity was present in normal skin equivalents but not in those generated from patient cells or TGM1 knockout keratinocytes, in line with the inactivating mutations found in patient 1, and the absence of persistent TG1 expression in patient 2 and knockdown equivalents. Notably, knockdown equivalents demonstrated increasing TGM1 transcript levels over time (>50% after 10 days cultivation), indicating a loss of effective repression (Supplementary Figure S1 online).

To assess their biocompatibility, TG1-loaded tNGs were incubated with normal, patient 1, and patient 2 keratinocytes, as well as fibroblasts for up to 48 hours, resulting in no significant cytotoxicity at any of the tested concentrations (Figure 1b, Supplementary Figures S2 and S3 online). Concordantly, no significant cytotoxicity was observed following the application of tNGs onto skin equivalents (Figure 1g). Additionally, the ability of TG1, alone or loaded in tNGs, to enter keratinocytes was assessed by confocal microscopy. In both cases, TG1 entered the cytoplasm in a time-dependent manner (Supplementary Figure S4 online).

Notably, tNGs entered more rapidly than the TG1, which, with their lack of clear intracellular co-localization, would suggest that the tNGs and TG1 enter keratinocytes separately, concurrent with the relatively quick release of protein at temperatures ≥35°C. It should be noted, however, that previous evidence indicates TNGs are largely unable to overcome the stratum corneum of even barrier-deficient skin, suggesting that, in most cases, little or no contact will occur between them and viable epidermal cells (Giulbudagian et al., 2018a).

Finally, patient 1 skin equivalents were topically treated with TG1, either in solution or loaded in tNGs, four times over 8 days. Untreated patient 1 equivalents demonstrated decreased barrier function, shown by the significant increases in their

Abbreviations: ARCI, autosomal recessive congenital ichthyosis; TG1, transglutaminase 1; tNG, thermoresponsive nanogels
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Figure 1. Characterization of full-thickness skin equivalents: Cryosections of (a) normal, (b) TGM1 knockdown, (c) ARCI patient 1, and (d) ARCI patient 2 skin equivalents. From left to right, images show hematoxylin and eosin, keratin 14 (green), keratin 10 (green), TG1 (green), and TG1 activity staining (counterstaining with DAPI in blue). Scale bars = 50 μm. The dashed, yellow line indicates the epidermal–dermal junction.

The viability of normal human keratinocytes derived from healthy subject (black), patient 1 (light gray), or patient 2 (dark gray) following (e) 24-hour or (f) 48-hour incubation with TG1, tNGs, and TG1-loaded tNGs as assessed by MTT assay. (g) Viability of normal skin equivalents following full treatment regimen with TG1-loaded tNGs (5 μg/cm² TG1, 500 μg/cm² tNG; applied four times over 8 days) as assessed by MTT assay; results are expressed as % untreated control. Statistical differences were assessed by one-way analysis of variance with Dunnett’s correction for multiple comparisons (n = 3). SDS served as positive control. ARCI, autosomal recessive congenital ichthyosis; H&E, hematoxylin and eosin; tNG, thermoresponsive nanogel; TG1, transglutaminase 1.
Figure 2. Skin-barrier function and TG1 activity of skin equivalents following TG1-loaded tNG treatment. Apparent permeabilities of normal or patient 1 skin equivalents, untreated (white) or treated with either unloaded tNGs (dashed white bars), TG1 in PBS (gray), or TG1 loaded in tNGs (dashed gray bars).

(a) The addition of unloaded tNGs did not alter the significant difference in $P_{app}$ values between normal (wt) and patient 1 (TG1Δ) skin equivalents.

(b) A significant difference is seen between TG1Δ equivalents treated with the vehicle control and 10 $\mu$g/cm² TG1 in the tNG treatment group, but not the PBS.

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apparent permeabilities to testosterone compared to normal equivalents (Figure 2a). Following full treatment regimens with TG1-loaded tNGs, significant decreases in apparent permeabilities—indicating improved barrier function—correlating to TG1 dose were seen (Figure 2a, 2d, Supplementary Figure S5 online). Importantly, permeation was almost unaffected by the application of unloaded tNG or TG1 dissolved in phosphate buffered saline only (Figure 2b, 2c). Activity staining confirmed the delivery of functional TG1 into viable epidermal layers (Figure 2e), and the distribution of activity was comparable to normal equivalents. Improvement of barrier activity was further confirmed by permeability tests with Lucifer yellow (Figure 2f) and N-hydroxy-sulfosuccinimide-LC-biotin (Supplementary Figure S6 online). Compared to equivalents with normal keratinocytes, a 59-fold increase was seen in the amount of Lucifer yellow fully passing through patient 1 equivalents. Similarly, 39-fold and 43-fold increases were respectively seen in patient 1 equivalents treated with unloaded tNG and TG1 dissolved in phosphate buffered saline. However, following treatment with TG1-loaded tNGs, full Lucifer yellow penetration was only 1.2-fold that of the control, clearly corroborating the role of TG1-loaded tNGs in the reconstitution of patient equivalent barrier function. It is highly likely that the majority of TG1 penetrating into the viable epidermis did so independently of the tNGs because they do not overcome the stratum corneum (Giulbudagian et al., 2018a).

This study aimed to further characterize the therapeutic potential of TG1-loaded tNGs in ARCI skin, as well as to better understand their mechanism of action, based on a previous proof-of-principle study demonstrating epidermal delivery of TG1 following topical application of TG1-loaded tNGs (Witting et al., 2015). Overall, these data verify that topical protein substitution could mitigate or even reverse the ARCI disease phenotype. Notably, Aufenvenne et al. (2013) previously demonstrated that topical applications of TG1 mixed with cationic liposomes successfully delivered the functional protein to skin equivalents, formed from TG1 mutant ARCI patient cells, grafted onto humanized mice. In contrast to our system, no changes to barrier function were observed upon treatment, likely a result of their model; unlike the typical ARCI phenotype, the grafted animals demonstrated compact hyperkeratosis and transepidermal water loss levels close to non-ARCI controls.

In summary, topical TG1 replacement therapy is a highly promising therapeutic avenue for ARCI patients with disease-causing TG1 mutations. The work here indicates TG1 delivery to the intercellular spaces between keratinocytes, and possibly their intraacellular environments, can produce therapeutic improvements to the skin-barrier function of the ARCI phenotype. It is hypothesized that increasing the concentration or enzymatic activity of TG1 within the tNG will result in improved therapeutic efficacy and is the likely starting point for future development. The ability of tNGs to encapsulate a wide variety of proteins and deliver these past the stratum corneum of barrier-deficient skin makes them a promising platform technology to treat a range of inflammatory and monogenic skin diseases.

**CONFLICT OF INTEREST**

The authors state no conflict of interest.

**SUPPLEMENTARY MATERIAL**

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2018.11.002.

**REFERENCES**


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TO THE EDITOR

Epidermodysplasia verruciformis (EV), an autosomal recessive genodermatosis, is characterized by persistent cutaneous infections of human papilloma viruses (HPVs) of the β-genus (de Jong et al., 2018a; Huang et al., 2018). The early clinical manifestations include thin, tinea versicolor-like lesions and flat warts that develop to protruding tumors. Cutaneous malignancies, particularly squamous cell carcinomas, can develop on these lesions, primarily on sun-exposed areas. EV is considered typical, or classic, when the β-HPV infection in the skin is an isolated clinical feature and there is no evidence of compromised T-cell-mediated immunity. In atypical, or nonclassic, forms of EV, the cutaneous lesions are associated with widespread viral, bacterial, or fungal infections and development of hematologic malignancies due to compromised T-cell immunity (Youssefian et al., 2019).

The genetic basis of β-HPV infection in patients with the typical form of EV was initially shown to consist of biallelic mutations in the TMC6 and TMC8 genes, which encode EVER1 and EVER2, respectively (Ramoz et al., 2002; Youssefian et al., 2018). Quite recently, mutations in a third gene, CIB1, which encodes CIB1, with a ubiquitous pattern of expression and pleiotropic functions, have been identified in other patients with typical EV (de Jong et al., 2018b). CIB1, EVER1, and EVER2 form a complex that serves physiologically as a restriction factor in the skin, limiting infections by β-HPVs, which are widespread and asymptomatic in the general population. Thus, the human CIB1-EVER1-EVER2 complex governs innate, keratinocyte-intrinsic immunity to β-HPVs (de Jong et al., 2018b).

In this study, we report a consanguineous family with three affected individuals with the typical form of EV with a splice junction mutation in CIB1. The consequences of the mutation were investigated with RNA sequencing (RNA-seq) and differential gene expression heatmap analysis, which showed a complex splicing pattern of CIB1 pre-mRNA leading to loss of function and nonsense-mediated mRNA decay.

The proband, a 42-year-old female of Iranian origin with Persian ethnicity, was seen in a dermatology clinic for cutaneous malignancies. However, she has multiple basal cell and squamous cell carcinomas, which have metastasized to the bones of the lower extremities, necessitating amputation. The proband was the second of three siblings, all healthy at birth but who developed similar cutaneous lesions (Figure 1b). The proband’s older brother (IV-1) died at the age of 18 years of meningitis, and the proband’s younger sister (IV-3) died of complications of metastatic skin cancer after completion of our study. The clinically healthy parents are first cousins once removed (Figure 1b).

Histopathology of the flat warts of the skin of the proband showed hyperkeratosis and acanthosis, with keratinocytes displaying coarse keratohyalin granules, perinuclear halo, and blue-gray pallor, characteristic of EV (Figure 1c). The proband also had multiple hyperpigmented plaques with verrucous surface and ulcerations and scaling with raised pearly borders. Excisional biopsy samples from these lesions showed the presence of islands and broad anastomosing bands of malignant cells in the dermis with palisading basaloid cells and squamous differentiation and areas of keratinization, consistent with basosquamous carcinoma (Figure 1d). PCR-based HPV