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Mutations in 3 β -hydroxysteroid- δ 8, δ 7-isomerase paradoxically benefit epidermal permeability barrier homeostasis in mice

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Abstract

Inherited or acquired blockade of distal steps in the cholesterol synthetic pathway results in ichthyosis, due to reduced cholesterol production and/or the accumulation of toxic metabolic precursors, while inhibition of epidermal cholesterol synthesis compromises epidermal permeability barrier homeostasis. We showed here that 3 β -hydroxysteroid- δ 8, δ 7-isomerase-deficient mice (TD), an analog for CHILD syndrome in humans, exhibited not only lower basal transepidermal water loss rates, but also accelerated permeability barrier recovery despite the lower expression levels of mRNA for epidermal differentiation marker-related proteins and lipid synthetic enzymes. Moreover, TD mice displayed low skin surface pH, paralleled by increased expression levels of mRNA for sodium/hydrogen exchanger 1 (NHE1) and increased antimicrobial peptide expression, compared with wild-type (WT) mice, which may compensate for the decreased differentiation and lipid synthesis. Additionally, in comparison with WT controls, TD mice showed a significant reduction in ear thickness following challenges with either phorbol ester or oxazolone. However, TD mice exhibited growth retardation. Together, these results demonstrate that 3 β -hydroxysteroid- δ 8, δ 7-isomerase deficiency does not compromise epidermal permeability barrier in mice, suggesting that alterations in epidermal function depend on which step of the cholesterol synthetic pathway is interrupted. But whether these findings in mice could be mirrored in humans remains to be determined.

KEYWORDS

cholesterol, epidermal permeability barrier, hydration, ichthyosis, pH

1 | INTRODUCTION

The epidermis plays a crucial role in regulating a host of protective functions, including regulation of the cutaneous permeability barrier, antimicrobial defence and protection against UV irradiation.^{1–3} Stratum corneum lipids, including cholesterol, sphingolipids and fatty acids, are key components of the extracellular membrane

bilayers that mediate the epidermal permeability barrier.⁴ Over recent decades, much attention has been paid to the role of epidermal cholesterol metabolism in normal skin and dermatoses, including certain rare inherited disorders. Blockade of epidermal cholesterol synthesis leads to abnormal barrier function and other cutaneous abnormalities, depending on which step is affected in the cholesterol synthetic pathway (Figure S1). For example, though inhibition

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of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase induces severe abnormalities in epidermal structure and function,^{5,6} mevalonate kinase deficiency occasionally causes subcutaneous oedema and morbilliform rash.⁷ Similarly, the epidermis also appears normal in humans with Smith-Lemli-Opitz syndrome, caused by recessive mutations in $\delta(7)$ -dehydrocholesterol reductase.⁸ Moreover, skin appears normal in mice with a deficiency in either squalene synthase⁹ or 3β -hydroxysterol δ^{14} -reductase.¹⁰ In contrast, deficiency in 3β -hydroxysteroid dehydrogenase, induced by mutations in NAD(P) H steroid dehydrogenase-like protein, causes ichthyosiform skin lesions.¹¹ Patients with X-linked dominant forms of chondrodysplasia punctata (CDPX2), also named Conradi-Hünemann syndrome (OMIM: 302960), display a deficiency in 3β -hydroxysteroid- $\delta 8$, $\delta 7$ -isomerase, which converts 8(9)-cholestenol to lathosterol in the latter stages of cholesterol biosynthesis. This disorder also displays ichthyosiform skin lesions.¹²

2 | QUESTIONS ADDRESSED

Whether epidermal functions are impaired in CDPX2 patients remains unknown. In the present study, we employed the tattered (TD) mouse model, a murine analog of CDPX2, to characterize the phenotype of this disorder.

3 | EXPERIMENTAL DESIGN

3.1 | Animals and experimental protocol

All animal procedures were approved by the Animal Studies Subcommittee (IACUC) of the San Francisco Veterans Administration Health Care System and performed in accordance with their guidelines. 3β -hydroxysteroid- $\delta 8$, $\delta 7$ -isomerase-deficient mice (TD mice)¹³ were gifted from Dr Gail E. Herman at the Ohio State University College of Medicine. The TD mutation site is between *TFE3* and *DXHXS7465E* in Xp11.23, with a single-nucleotide substitution at position 454, a G→A transition, resulting in an amino acid substitution of arginine for glycine at amino acid position 107.¹³ Because mice with a homozygous mutation of β -hydroxysteroid- $\delta 8$, $\delta 7$ -isomerase died before birth, only 8-10-week-old female heterozygous mice were used in this study, whereas uninvolved littermates served as the controls. Basal stratum corneum (SC) hydration and surface pH were measured (CM 825/PH 900, Courage & Khazaka, Germany) on shaved back skin. Barrier disruption was achieved by repeated tape-stripping until transepidermal water loss (TEWL) rates increased $\geq$ threefold. TEWL rates were measured immediately, 2 and 4 h after tape-stripping, using a TM300 probe connected to an MPA5 (Courage & Khazaka, Germany). Percentage of barrier recovery rates was calculated as described previously.¹⁴

Irritant and allergic contact dermatitis were induced as previously described^{15,16} in TD and WT mice, using phorbol 12-myristate 13-acetate (TPA, Sigma-Aldrich, St. Louis, MO) and

4-ethoxymethylene-2-phenyl-2-oxazolin-5-one (oxazolone, OX, Sigma-Aldrich, St. Louis, MO), respectively. TPA (0.001% in acetone) was applied once to the inner and outer surface of the ear. Ox (0.01% in acetone) was applied once following an initial Ox sensitization. In both cases, ear thickness was measured 18 h post-treatment. Data were presented as per cent increase over pretreatment.

3.2 | Determination of expression levels of epidermal mRNA

Skin samples were collected for determination of mRNA levels in the epidermis, separated by brief heating.¹⁷ Expression levels of epidermal mRNA were measured by real-time, quantitative PCR (Q-PCR), as described previously.² Briefly, total RNA was isolated from mouse skin using STAT 60 (TEL-TEST, Inc, Friendswood, TX), TRIzol (Invitrogen, Carlsbad, CA), or RNeasy Mini RNA isolation kit (Qiagen, Valencia, CA). cDNA was prepared by reverse transcription with Tetro cDNA Synthesis Kit (Bioline, Taunton, MA), and mRNA expression levels were measured by Q-PCR, using SYBR Green Master Mix (Applied Biosystems (ABI), Foster City CA) on an ABI machine 7300 or 5300. Primer sequences are listed in Table S1. Relative expression of the mRNAs was compared to the housekeeping enzyme, GAPDH. Results were presented as percentage of WT control, after setting vehicle-treated sites as 100%.^{18,19}

3.3 | Electron microscopy

Skin biopsies of the back skin from untreated mice and from tape-stripped mice 2 h after treatment were fixed in 4% lanthanum in Karnovsky's solution and postfixed with either 0.25% ruthenium tetroxide or 1% aqueous osmium tetroxide, containing 1.5% potassium ferrocyanide, as described previously.²⁰ Ultrathin sections were examined using an electron microscope (Zeiss 10A, Carl Zeiss, Thornwood, NY) operated at 60 kV. Images were captured using the Digital Micrograph 3.10.0 software from Gatan, Inc (Pleasanton, CA).

3.4 | Statistics

Data are expressed as the mean $\pm$ SEM. GraphPad Prism 5 software (San Diego, CA, USA) was used for all statistical analyses. Mann-Whitney test was used to determine the statistical significances between two groups.

4 | RESULTS AND DISCUSSION

TD mice weighed less and displayed patchy hair loss in comparison with WT littermates (Figure 1A vs B). These reductions in body weight in TD mice became more evident as these mice aged (Figure 1C). Yet, the gross appearance of the skin in TD

FIGURE 1 Characteristics of tattered (TD) mice. (A and B) shows pictures of TD and WT mice, respectively. (C) shows the changes in body weight in TD vs WT mice. (D and E) depicts the basal epidermal function (expressed as % of WT mice) and permeability recovery rates after acute barrier disruption (expressed as % of recovery), respectively. Barrier disruption was achieved by repeated tape-stripping 8- to 10-week-old mice until TEWL rates increased over threefold. (F) shows changes in ear thickness following topical application of TPA (0.001%) and oxazolone (0.01%). Methods are detailed in the supplemental materials. Data are expressed as % increase over normal ears. Number of mice in each group and significances are indicated in the figures

mice appeared normal. While the levels of SC hydration were comparable between TD and WT mice, striking reductions in basal transepidermal water loss (TEWL) rates, as well as a more acidic skin surface pH were evident in TD vs WT mice (Figure 1D). Moreover, epidermal permeability barrier recovery after tape-stripping accelerated significantly in TD mice in comparison to WT mice (Figure 1E). In addition to superior epidermal functions, thresholds to exogenous inflammatory stimuli, [ie phorbol ester (TPA) and oxazolone (OX)], increased markedly in TD mice vs WT mice (Figure 1F $p < 0.05$). Together, these results demonstrate that a lack of 3β -hydroxysteroid- δ 8, δ 7-isomerase does not compromise, but rather enhances epidermal functions,

while also increasing the resistance of TD mice to inflammatory stimuli.

Because both epidermal lipid production and keratinocyte differentiation are key determinants of epidermal permeability barrier function, we next determined whether the enhanced permeability barrier of TD mice was paralleled by changes in the expression of mRNAs for epidermal lipid production and/or keratinocyte differentiation. Surprisingly, expression levels of mRNA for the rate-limiting synthetic enzymes of cholesterol and sphingolipids were markedly reduced in TD mice (Figure 2A). Moreover, mRNA levels for keratinocyte differentiation marker-related proteins were also significantly reduced in TD mice vs WT mice (Figure 2B).

FIGURE 2 Comparison of epidermal mRNA expression and morphology in TD Mice vs WT controls. Skin samples were collected from mice at 8- to 10-week old. Epidermal RNA was isolated for determination of mRNA levels. (A and B) shows expression levels of mRNA for lipid synthetic enzymes and differentiation marker-related proteins, respectively. Number of mice in each group and significances are indicated in the figures. (C and D) is EM pictures of WT and TD epidermis, respectively. E is EM pictures of WT epidermis 2 h after acute barrier disruption while (F) is EM pictures of TD epidermis 2 h after acute barrier disruption. Arrows indicate the membrane bilayers in the lower stratum corneum. Scale bars = 0.2 μm . FAS, Fatty acid synthase; HMGC_oA, 3-hydroxy-3-methyl-glutaryl-CoA; SPT1, serine palmitoyltransferase 1

Our prior studies demonstrated that the mouse cathelicidin antimicrobial peptide (mCAMP) is crucial for epidermal permeability barrier homeostasis.²¹ Hence, we next determined whether the enhanced epidermal permeability barrier homeostasis in TD mice was accompanied by elevations in epidermal antimicrobial peptide mRNA expression. Indeed, expression levels of mRNA for CAMP (an analog of LL-37) and mouse β defensin 3 (mBD3, an analog of human β defensin 2), increased by over 70% and 20%, respectively, in TD vs WT mice (Figure 3). Taken together, these results show that deficiency in 3β -hydroxysteroid- δ 8, δ 7-isomerase leads to reductions in expression levels of epidermal mRNA for lipid production and

differentiation, while increasing expression levels of epidermal antimicrobial peptide mRNA, the latter potentially contributing to the enhanced epidermal permeability barrier homeostasis in TD mice.

As stated above, TD mice displayed a more acidic stratum corneum pH (≈ 0.3 pH unit lower than WT mice), possibly contributing to accelerating maturation of extracellular lamellar membranes, which requires lipid processing enzymes, whose optimal activity occurs in a pH range of 4-5.5. The reduced skin surface pH in TD mice can be partially attributable to elevation in the expression of mRNA for sodium/hydrogen exchanger 1 (NHE1) (increased by 62% over the wild type, $p = 0.0307$), a key regulator of the skin surface pH.

FIGURE 3 Comparison of expression levels of epidermal mRNA for antimicrobial peptides in TD mice vs WT controls. Data are expression as percentage of wild-type controls, setting expression levels in the wild-type controls as 100%. Unpaired t test was used to determine the significance between TD mice and wild-type controls. Significance and number of samples are indicated in the figure

Hence, we next determined whether the more acidic SC pH in TD mice is accompanied by accelerated maturation of lipid membrane bilayers. Although lamellar membrane structures were similar in TD vs WT mice under basal conditions (Figure 2C vs D), SC membrane structures appeared to mature more rapidly in TD than in WT mice 2 h after acute barrier disruption (Figure 2E vs F). Thus, accelerated barrier recovery in TD mice could be due, at least in part, to an acceleration of membrane maturation.

The mechanisms by which 3β -hydroxysteroid- δ 8, δ 7-isomerase deficiency enhances epidermal function are unclear. In fact, the reductions in epidermal mRNA levels for lipid synthetic enzymes and differentiation-marker proteins should instead predict sub-optimal epidermal differentiation and barrier function. One explanation could be that both the differentiation-related protein and lipid synthetic enzyme levels are far higher than needed to support the basal functions. If so, such reductions may not suffice to provoke functional changes. Pertinently, we have shown that simultaneous inhibition of cholesterol and sphingolipid synthesis did not alter the kinetics of permeability barrier homeostasis,²² and either loricrin-deficient or young filaggrin mutant mice exhibit normal permeability barrier function under basal conditions.^{23,24} The normal barrier in loricrin-deficient mice could be attributable to upregulation of other cornified envelope components such as small proline-rich proteins.²³ Certainly, the lower pH-induced acceleration in membrane bilayer formation could enhance the epidermal permeability and antimicrobial barriers. Therefore, it is less surprising that 3β -hydroxysteroid- δ 8, δ 7-isomerase deficiency does not compromise epidermal permeability barrier, despite reduced expression of epidermal mRNA for lipid synthetic enzymes and differentiation-marker proteins in TD mice.

5 | CONCLUSIONS

Mice with 3β -hydroxysteroid- δ 8, δ 7-isomerase deficiency display both competent epidermal function and increased inflammatory

threshold although these mice appear smaller, suggesting that alterations in cutaneous function depend on which step of the cholesterol synthesis pathway is affected. More acidic SC and increased CAMP and mouse β defensin 3 leads to enhanced lipid bilayer processing and may compensate for decreased epidermal differentiation.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

PME originated the concept, designed experiments and drafted the manuscript. MQM designed experiments, analysed the data and drafted the manuscript. ED, GM, DC and DL performed experiments. PME, TMM and MQM interpreted the data. MQM, PME and TMM critically reviewed the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

Figure S1. Cholesterol biosynthetic pathway and its associated disorders.

Table S1. Primer sequences.

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