Ectodysplasin A regulates epithelial barrier function through sonic hedgehog signalling pathway

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Abstract

Ectodysplasin A (Eda), a member of the tumour necrosis factor superfamily, plays an important role in ectodermal organ development. An EDA mutation underlies the most common of ectodermal dysplasias, that is X-linked hypohidrotic ectodermal dysplasia (XLHED) in humans. Even though it lacks a developmental function, the role of Eda during the postnatal stage remains elusive. In this study, we found tight junctional proteins ZO-1 and claudin-1 expression is largely reduced in epidermal, corneal and lung epithelia in Eda mutant Tabby mice at different postnatal ages. These declines are associated with tail ulceration, corneal pannus formation and lung infection. Furthermore, topical application of recombinant Eda protein markedly mitigated corneal barrier dysfunction. Using cultures of a human corneal epithelial cell line and Tabby mouse skin tissue explants, Eda up-regulated expression of ZO-1 and claudin-1 through activation of the sonic hedgehog signalling pathway. We conclude that EDA gene expression contributes to the maintenance of epithelial barrier function. Such insight may help efforts to identify novel strategies for improving management of XLHED disease manifestations in a clinical setting.

Keywords: Ectodysplasin A • epithelial barrier dysfunction • X-linked hypohidrotic ectodermal dysplasia • tight junction

Introduction

Eda is a member of the tumour necrosis factor (TNF) superfamily regulating ectodermal development in various organs. In humans, germ-line EDA mutations cause a syndrome designated as XLHED characterized by the absence or malformation of multiple ectodermal appendages resulting in hypotrichosis, hypodontia, anhidrosis or hypohidrosis [1–4]. Spontaneous EDA mutant penetrance appears in other species including dog [5], cattle [6] and mouse [7].

XLHED-afflicted individuals in their youth are at increased risk for developing severe, acute pneumonia and respiratory infections [2, 8]. Respiratory infections, wheezing and recurrent sinus infections can last throughout adulthood in most cases [9, 10]. XLHED patients also experience an increased prevalence of allergic diseases [11], atopic diathesis as well as chronic dermatitis throughout their lifetime [12]. It was presumed that exocrine gland insufficiencies of the respiratory tract [8, 13, 14] and skin surface [10] could lead to chronic inflammatory and recurrent infection. However, the pathogenic mechanism underlying these changes remains unclear.

Ocular surface anomalies are another major category of XLHED-related diseases. All adult XLHED patients have corneal pathology such as pannus formation [15]. Eda mutant Tabby mice also experience dramatic increases in ocular surface inflammation and corneal pannus in their adult stage [16]. Previous studies favour the hypothesis that keratopathy in XLHED patients results from an altered tear film lipid layer caused by a meibomian gland developmental defect.
[17]. However, this possibility is not supported by the finding that transfection of the Eda-A1 gene to Tabby mice prevented corneal defects without restoring meibomian gland formation. This result instead indicates that Eda is required for maintenance of ocular surface integrity rather than meibomian gland morphogenesis [16].

The clinical manifestations of XLHED indicate that not all of the symptoms result from EDA control of mechanisms underlying morphogenesis. Instead this realization prompted suggestions that EDA may play a role in maintaining the homeostasis of organs such as skin, cornea and pulmonary tissues after the developmental stage. Clarifying the functional roles of EDA in maintaining normal tissue function still warrants further study.

We show here that epithelial tight junctional integrity is compromised in the cornea, skin and lung of Tabby mice, which was accompanied by abnormal ZO-1 and claudin-1 protein expression leading to epithelial barrier dysfunction. This defect markedly increases tissue susceptibility to surface pathogenic infiltration, which leads to chronic inflammation in these mice. Moreover, we found that the Eda protein regulates epithelial tight junction formation through activation of the sonic hedgehog (Shh) signalling pathway. Our findings provide new insights into pathophysiological mechanism of XLHED disease and identify a new Eda protein signalling pathway. Our findings provide new insights into pathophysiological mechanism of XLHED disease and identify a new Eda protein signalling pathway.

Materials and methods

Animals

The EDA mutant Tabby mice (C57BL/6J-A^Tabby^-; Eda^TA/J) and wild-type C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The mice were housed at the Experimental Animal Center of Xiamen University, and some were kept in a specific pathogen-free (SPF) barrier facility, while others were kept in a conventional facility (CF) with free access to water and food. All animal protocols were performed in accordance with the Association for Research in Vision and Ophthalmology (ARVO) statement for the use of Animals in Ophthalmic and Vision Research and approved by the Animal Ethical Committee of Xiamen University. All experiments were performed in accordance with the tenets set forth in the Declaration of Helsinki.

Haematoxylin & eosin staining

After the animals were sacrificed, the eye, skin and lung tissues were dissected and fixed in 4% paraformaldehyde overnight and embedded in paraffin. The tissue sections (5 μm) were deparaffinized and stained with haematoxylin for 1 min. and eosin for 2 min. After that, the sections were mounted using mounting medium and examined under a light microscope (Eclipse 50i, Nikon, Japan).

Immunostaining

Cryostat sections (6 μm) of mice eyes or skin tissues or cultured cells were fixed in 4% paraformaldehyde for 20 min. The samples were washed 3 times with PBS, followed by incubation in 0.2% Triton X-100 for 10 min. After rinsing three times with PBS for 5 min. each and pre-incubation with 2% bovine serum albumin (BSA) for 1 hr at room temperature, sections were incubated with anti-CD45 (sc-20687, 1:100, Invitrogen, Carlsbad, CA, USA), Gli-1 (sc-20687, 1:100, Invitrogen), Gil-1 (sc-20687, 1:150, Santa Cruz) primary antibodies at 4°C overnight. After three washes with PBS for 10 min. each, they were incubated with AlexaFluor 488-conjugated secondary antibody (donkey anti-rabbit, goat, rat or mouse IgG, 1:300, Life Technologies, Carlsbad, CA, USA) for 1 hr at room temperature. After three additional PBS washes for 5 min., the samples were counterstained with DAPI and then mounted for analysis under the confocal laser scanning microscope (Fluoview 1000, Olympus, Tokyo, Japan).

Western blotting

The skin, corneal epithelium, lung tissue or cultured cells were extracted with cold lysis buffer comprising 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease and phosphatase inhibitor cocktails. Equal amounts of protein extracts were subjected to electrophoresis on 8% or 10% SDS-PAGE and then electrophoretically transferred to PVDF membrane. After blocking in 1% BSA for 1 hr, the membranes were incubated with primary antibodies ZO-1 (61-7300, 1:1000, Invitrogen), claudin-1 (519,000, 1:1000, Invitrogen), Gli-1 (sc-20687, 1:500, Santa Cruz) and α-actin (A3854, 1:10,000, Sigma-Aldrich) overnight at 4°C. After three washes with Tris-buffered saline with 0.05% Tween-20 for 10 min. each, the membranes were incubated with HRP-conjugated goat or rabbit anti-mouse or rabbit IgG (1:10,000) for 1 hr. The results were visualized by enhanced chemiluminescence reagents and recorded with an imaging system (ChemiDoc XRS, Bio-Rad, Hercules, CA, USA).

RNA isolation and quantitative real-time RT-PCR analysis

Total RNA was isolated from the samples using Trizol Reagent (Invitrogen) and extracted following the manufacturer’s protocol. RNA was reverse transcribed to cDNA by the ExScript RT Reagent kit (Takara Bio, Shiga, Japan). Quantitative real-time RT-PCR (qRT-PCR) was performed with a StepOne Real-Time detection system (Applied Biosystems, Foster City, CA, USA) using an SYBR Premix Ex Taq Kit (Takara Bio, Shiga, Japan). The amplification program included an initial denaturation step at 95°C for 10 min., followed by 40 cycles of 95°C for 10 sec., 57°C for 30 sec., and 75°C for 10 sec.; then, the melting curve analysis was conducted at once from 65 to 95°C. SYBR Green fluorescence was measured after each extension step, and the specificity of amplification was evaluated by melting curve analysis. The primers were designed using Primer 3 system, and they are listed in Table S1. The results of the relative quantitative real-time PCR were analysed by the comparative threshold cycle (Ct) method and normalized to β-actin as the reference gene.

Evaluation of sodium fluorescein staining

One microlitre of 0.1% liquid sodium fluorescein was dropped into the conjunctival sac of mice. Ninety seconds later, corneal epithelial damage was photographed and graded with a cobalt blue filter under the slit-lamp microscope. The cornea was divided into four quadrants,
which were scored, respectively, as previously described [18]. Briefly, as follows: absent, 0; slightly punctate staining less than 30 spots, 1; punctate staining more than 30 spots, but not diffuse, 2; severe diffuse staining but no positive plaque, 3; positive fluorescein plaque, 4. The scores of each quadrant were added to arrive at a final grade.

**Measurement of epithelial permeability to carboxy fluorescein**

Corneal epithelial barrier function was evaluated based on measurements of time dependent carboxy fluorescein penetrance. Briefly, 1 µl of 0.3% carboxy fluorescein was administered to the ocular surface; 10 min. later, the eyes were washed with 1 ml PBS to remove the residual carboxy fluorescein. After that, the animals were sacrificed with an overdose of chloral hydrate anaesthesia. The corneas were excised, and each cornea was placed in a 1.5-ml tube containing 500 µl of PBS. The tubes were wrapped in aluminium foil to protect the solution from light and placed on an orbital shaker for 90 min. After 1 min. centrifugation, the supernatants were measured with a Gilford Fluoro IV fluorometer (Corning, Oberlin, OH, USA) to detect the concentration of carboxy fluorescein.

**Bacterial infection examination**

Tabby mice and the wild-type mice were sacrificed, and the eyeballs and lung tissues were harvested and washed three times with sterile PBS. After that, the corneas and lung tissues were dissected and homogenized with 500 µl PBS. After low-speed centrifugation, the supernatant was coated on LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl, 1.5% agar, pH7.0) plates in triplicate and incubated at 37°C overnight, the number of bacterial colonies was then counted.

**Statistical analysis**

Statistical analysis was performed with SPSS 16.0.0 (SPSS, Chicago, IL, USA). All summary data are reported as mean ± S.E.M. Comparisons between groups were performed by an unpaired, two-tailed Student’s t-test. P ≤ 0.05 was considered statistically significant.

**Results**

**Reduction of epithelial tight junctional protein expression in Tabby mice**

As the morphological changes in Eda-deficient Tabby mice mirror those occurring in humans, this strain is frequently used to study the underlying pathophysiological mechanisms of the XLHED syndrome [19, 20]. In mice, eyelid opening occurs around 2 weeks after birth. We harvested corneal epithelia, dorsal skin epithelia as well as their lung tissue from four- and eight-week-old Tabby mice and wild-type littersmates. Western blot results show that the corneal epithelial tight junctional ZO-1 and claudin-1 protein expression both dramatically decreased (Fig. 1A). Similarly such declines also occurred in their skin epithelium (Fig. 1B) and lung tissue (Fig. S1) at all time points when compared to wild-type littermates. Immunofluorescent staining also showed discontinuous and weaker expression patterns of ZO-1 and claudin-1 in the cornea (Fig. 1C) and skin (Fig. 1D) epithelia of Tabby mice. These results suggest that there is epithelial barrier dysfunction in both neonatal and adult Tabby mice.

**Inflammation in corneas, skin and lung tissues of Tabby mice**

In Tabby mice, ocular surface diseases occur postnatally as early as 4 weeks followed by age dependent increases in pathological severity [16]. Slit-lamp microscopy observation revealed a normal ocular surface in Tabby mice at 4 weeks. However, the corneal surface was scabrous and mildly oedematous in the central or peri-central corneal stroma in eight-week-old Tabby mice (Fig. S2A). H&E staining showed that the corneal epithelial surface was irregular in Tabby mice at 4 weeks and 8 weeks. In contrast, the epithelial surface was smooth in wild-type mice (Fig. S2B). Sporadic CD45-positive macrophages were present in the anterior part of the central corneal stroma of Tabby mice.
mice at 4 weeks. Their numbers increased and spread into the corneal epithelium at 8 weeks, while they were absent in both layers of wild-type littermates (Fig. 2A).

The Tabby mice exhibited pink skin 1 week after birth, which was maintained throughout their life (Fig. S2C). H&E staining showed mildly increased cellularity of their inter-follicular connective tissue compared with wild-type littermates (Fig. S2D). CD45-positive cells dramatically increased in the skin of four- and eight-week-old Tabby mice (Fig. 2B), indicating chronic inflammation of the skin. We also detected IL-1β and Dmkn chemokine gene expression. qRT-PCR results showed that both chemokines very markedly increased in the corneal (Fig. 2C and D) and skin (Fig. 2E and F) epithelial layers of four-week-old Tabby mice with larger rises at 8 weeks.

There was sporadic hyperaemia in one-week-old Tabby mice lung tissue along with dramatic increases in cell infiltration within the pulmonary alveoli. Immunostaining revealed numerous CD45-positive macrophages in the lung tissue of Tabby mice, while they were not present in the wild-type littermates (Fig. S3), indicating pulmonary inflammation in the neonatal Tabby mice.

**Corneal and pulmonary bacterial infection accompany epithelial barrier dysfunction in Tabby mice**

Intact corneal tight junctions are crucial for maintaining barrier function against pathogenic infiltration and preventing dysregulated immune response activation. As corneal epithelial tight junctional ZO-1 and claudin-1 protein expression declined in Tabby mice, we hypothesized that compromise of its protective barrier function would increase the possibility of bacterial infection. Accordingly, we then harvested corneal tissues from eight-week-old Tabby mice, homogenized and suspended them in PBS. Bacterial culture of the suspension from Tabby mice resulted in more bacterial colonies than on plates containing cultures obtained from wild-type mice (Fig. 3A). This difference was statistically significant (Fig. 3B). Furthermore, corneal epithelial gene expression of lysozyme 1 (Fig. 3C) and lysozyme 2 (Fig. 3D) both dramatically increased in four- and eight-week-old Tabby mice compared with the wild-type littermates, indicating more bacterial infection in Tabby mice. Similarly, bacterial culture of lung tissue suspensions from one-week-old Tabby mice was also positive while there was no bacterial colony formation from wild-type littermate mice (Fig. S4). These differences indicate that the corneal and pulmonary inflammation was at least partly because of bacterial infection in Tabby mice.

To further investigate the susceptibility of Tabby mice to bacterial infection, we bred different groups of 16-week-old Tabby mice in either CF or SPF environments and compared the incidence of corneal pannus (Fig. 3E) and tail ulcer formation (Fig. 3F) maintained in these two different environments. The results show that the morbidity of the corneal pannus was about 63.2% for those housed in the CF while it was only 15.9% in the SPF environment (Fig. 3G). Similarly, the morbidity of tail ulcer was 13.5% in the CF environment, while it was only 2.4% in SPF environment (Fig. 3H). These differences in
morbidity strongly suggest that epithelial barrier dysfunction is associated with increases in corneal bacterial infection and skin ulcer formation in Tabby mice.

Recombinant Eda protein rescues corneal epithelial barrier dysfunction in Tabby mice

The Eda protein is essential for meibomian gland development [20]. Interestingly, we found that the Eda protein is highly expressed in the meibomian gland and is present in human tear samples. However, its cognate receptor (Edar) is highly expressed in the corneal epithelium, but at a lower density in the meibomian gland (unpublished observation). As Eda is a secretory protein, we proposed that it could be secreted from the meibomian gland and targeted for controlling ocular surface epithelial function. One possibility is that corneal epithelial tight junctional compromise may result from an Eda deficiency because of the absence of meibomian glands in Tabby mice. To test whether exogenous Eda application could rescue corneal barrier dysfunction, 5 µl of a 20 ng/ml mouse recombinant Eda protein solution was administered 3 times per day to the ocular surface of four-week-old wild-type and Tabby mice. After 3 weeks, the corneal surface fluorescein dye staining pattern clearly decreased more in Tabby mice relative to what occurred in Tabby mice treated instead with the PBS vehicle (Fig. 4A), which was confirmed by fluorescein score determination (Fig. 4B). Additional validation of this difference was obtained based on measurements in corneal epithelial carboxy fluorescein (CF) permeation. There was greater infiltration of CF in the Tabby mice, which declined after Eda treatment (Fig. 4C and D). On the other hand, tight junctional ZO-1 and claudin-1 protein expression was significantly up-regulated by Eda protein application in the Tabby mice (Fig. 4E). Whole mount staining of corneal epithelia also documented ZO-1 and claudin-1 expression rescue in Eda treated Tabby mice (Fig. 4F). These results support the notion that corneal epithelial barrier dysfunction stems from the absence of Eda in Tabby mice.

Eda regulates epithelial barrier function through the sonic hedgehog signalling pathway

Eda mediates its function through binding Edar and activates downstream signal cascades [21]. Different studies indicate that Eda-Edar signalling is mediated for the most part, if not totally, by the IκB kinase-dependent canonical NF-κB signalling pathway [22, 23]. It was also shown that bone morphogenetic protein (BMP) activity could be suppressed while the sonic hedgehog (Shh) signalling pathway was instead up-regulated by the Eda-linked pathway during ectodermal organogenesis [24–28]. Previous reports suggested that the Shh pathway regulates tight junctional protein expression [29, 30]. This was validated by showing that the stomach epithelial tight junction protein expression pattern was disrupted in Shh knockout mice [31]. Based on those observations, we hypothesized that Eda regulates the
tight junction protein expression through the Shh signalling pathway. To test this hypothesis, skin explants from one-week-old wild-type and Tabby mice were cultured in the absence or presence of Eda (0.1 μg/ml), Shh (0.5 μg/ml) and a Shh inhibitor cyclopamine (20 μM). After a 24-hrs or 48-hrs culture, total mRNA and protein harvested from the explants were analysed by qRT-PCR and Western blot, respectively. The results show that the baseline of Shh expression was significantly lower in Tabby mice compared with that in wild-type mice. Addition of Eda significantly increased the expression of Shh in Tabby mice skin explants, and this rise could be inhibited by cyclopamine (Fig. 5A). However, both Eda and Shh medium supplementation did not up-regulate Shh gene expression on wild-type mice skin explants (Fig. 5A). We also monitored GLI-1 gene expression, a downstream mediator in the Shh signalling pathway. GLI-1 was also significantly down-regulated in Tabby mice. Eda and Shh application did not increase GLI-1 expression in wild-type mice; however, both Eda and Shh supplementation up-regulated GLI-1 mRNA expression in Tabby mice (Fig. 5B). Western blot results further confirmed that Eda up-regulated GLI-1 protein expression as well as tight junctional proteins ZO-1 and claudin-1, and this up-regulation was blocked by cyclopamine (Fig. 5C).

To further validate the contribution made by Eda in regulating epithelial tight junctional protein expression and integrity, we constructed a pcDNA3.1-EDA plasmid and transfected it into a human corneal epithelial (HCE) cell line whose EDA mRNA expression level was very low. One week after transfection, qRT-PCR results showed that the EDA plasmid was successfully transfected as EDA expression dramatically increased in these EDA transfected cells (Fig. 6B). Moreover, both SHH and GLI-1 gene expression were up-regulated in these EDA transfected cells (Fig. 6B).

To further document the functional role of Eda, we also applied human Eda recombinant protein to the HCE cells. Eda protein application dramatically up-regulated SHH gene expression, whereas cyclopamine had no effect on this response (Fig. 6C). Similarly,
GLI-1 gene expression was also up-regulated by Eda, while cyclopamine markedly decreased this rise (Fig. 6D). Western blot results showed that Eda dose dependently up-regulated ZO-1, claudin-1 and Gli-1 expression, and such an effect could be blocked by cyclopamine (Fig. 6E). Immunofluorescent staining further confirmed that ZO-1 and claudin-1 were strongly enhanced in HCE cells after Eda treatment, while cyclopamine remarkably inhibited the expression of these proteins. Meanwhile, Gli-1 nuclear expression was dramatically induced by Eda and could be blocked by cyclopamine (Fig. 6F).

Discussion

Eda mRNA is expressed in various organs and tissues, including the heart, kidney, pancreas, brain, lung, liver, skeletal muscle,
teeth, as well as the skin during both embryonic development and adulthood [1, 32]. Even though it is known that the Eda gene has a developmental function in controlling the morphogenesis of various ectodermal structures such as hair, teeth, nails and exocrine glands, little is known about its postnatal function. In the current study, for the first time, we found tight junction proteins such as ZO-1 and claudin-1 were dramatically down-regulated in Eda mutant Tabby mice, resulting in epithelial barrier dysfunction in various tissues such as skin, cornea and lung. We further proved that it is Eda that makes an essential contribution to maintaining surface epithelial homeostasis through regulation of tight junction formation.

Epithelial apical tight junctional integrity plays a critical role in providing an epithelial barrier function against pathogenic infiltration. Its intactness is also needed for cell–cell communication and establishing cell polarity as well as regulating paracellular movement of ions and macromolecules [33]. Disruption of the tight junction is characteristic of a number of diseases including dermatitis, pathogenic infection and cancer [34]. It was proposed long ago that genetically impaired epidermal barrier formation may be the primary cause of the rapid increase in the prevalence of atopic dermatitis and respiratory atopy [35]. Recently, defective expression of epidermal ZO-1 and claudin-1 was observed in the skin of patients with atopic dermatitis [36], where such pathology is a risk factor for viral infection and allergen sensitization [37, 38]. Our results clearly demonstrate that compromise of tight junctional integrity is associated with marked increases in vulnerability to bacterial infection and chronic...
inflammation in Tabby mice. Losses in junctional compaction may help explain why XLHED patients are at a greater risk of suffering from pneumonia, respiratory infections and are also more frequently afflicted with allergic diseases, chronic dermatitis and ocular surface inflammation than the general population.

Although the Eda-Edar signalling pathway was intensively studied, most studies focused on the effect of Eda linked signalling pathway activation on morphogenesis [3]. In our study, we found that Eda regulates ZO-1 and claudin-1 tight junctional protein expression through activation of Shh signalling pathway using in vivo and in vitro methodology. It was reported that the stomach epithelial cell–cell junction was disrupted in Shh conditional knockout mice [31], which also supports the notion that Shh is situated downstream from Eda in this signalling pathway.

Recombinant Eda protein injection into pregnant Tabby mouse could reverse the XLHED phenotype in the offspring [39]. Similarly administration of Fc-Eda to dog models of XLHED during their first 2 weeks of life also rescued developmental abnormalities [40, 41]. Animals treated with recombinant Eda protein in prenatal or early postnatal stage showed dramatically decreased, if not all, clinical signs such as eye and airway infections with improved lacrimation, meibomian gland and bronchial glands formation [40, 41]. Therefore, the ocular surface defect and pulmonary infection in EDA mutant animals may synergistically resulted from both secretory glands defect and the absence of biological function of EDA. In other words, normal secretory glands in the ocular surface and pulmonary tissues may compensate the deficiency of EDA. However, this interpretation does not fit previous study which showed transfection of the Eda-A1 gene to Tabby mice prevented corneal defects without restoring meibomian gland formation [16]. To further address this contradiction, inducible Eda knockout mice should be generated to knock down EDA gene in the adult stage, allowing us to investigate the function of Eda during the adult stage while eliminating any involvement of morphogenesis.

Currently, phase 2 neonate clinical trials are being conducted using humanized Fc-Eda-A1 protein, EDI200 to treat XLHED (www.clinicaltrials.gov, NCT01775462). There is optimism that EDI200 treatment will correct the developmental disorders of XLHED in humans. We tested if recombinant Eda protein application to the ocular surface of postnatal Tabby mice reverses declines in epithelial barrier tightness. This procedure largely rescued ZO-1 and claudin-1 tight junction protein expression. Our results point to the possibility...
that repeated exogenous Eda treatment may be necessary to sustain this protein at high enough levels to maintain normal tight junction barrier function from childhood into adulthood in XLHED patients. In summary, Eda gene expression provides essential support for the maintenance of epithelial tight junctional integrity during postnatal stage. This effect is of importance in hindering pathogenic infiltration and protecting tissues from incurring declines in their normal function. As such control by Eda gene expression of barrier function is mediated through the Shh signalling pathway, this finding pinpoints another potential drug target for treating XLHED-related diseases.

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