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Collagen VII deficient mice show morphologic and histologic corneal changes that phenotypically mimic human dystrophic epidermolysis bullosa of the eye

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ABSTRACT

Background: Absence of collagen VII causes blistering of the skin, eyes and many other tissues. This disease is termed dystrophic epidermolysis bullosa (DEB). Corneal fibrosis occurs in up to 41% and vision loss in up to 64% of patients. Standard treatments are supportive and there is no cure. The hypomorphic mouse model for DEB shows production of collagen VII at 10% of wild type levels in skin and spleen, but the eyes have not been described. Our purpose is to characterize the corneas to determine if this is an appropriate model for study of ocular therapeutics.

Methods: Western blot analysis (WB) and immunohistochemistry (IHC) were performed to assess presence and location of collagen VII protein within the hypomorphic mouse cornea. Additional IHC for inflammatory and fibrotic biomarkers transforming growth factor-beta-1 (TGF-β1), alpha-smooth muscle actin (α-SMA), connective tissue growth factor (CTGF), proteinase 3, tenascin C and collagen III were performed. Clinical photographs documenting corneal opacification were assessed and scored independently by 2 examiners. Histology was then used to investigate morphologic changes.

Results: IHC and WB confirmed that hypomorphic mice produce less collagen VII production at the level of the basement membrane when compared with wild-types. IHC showed anomalous deposition of collagen III throughout the stroma. Of the 5 biomarkers tested, TGF-β1 showed the strongest and most consistently staining. Photographs documented corneal opacities only in mice older than 10 weeks, opacities were not seen in younger animals. Histology showed multiple abnormalities, including epithelial hyperplasia, ulceration, fibrosis, edema, dysplasia, neovascularization and bullae formation.

Conclusions: The collagen VII hypomorphic mouse shows reduced collagen VII production at the level of the corneal basement membrane. Corneal changes are similar to pathology seen in humans with this disease. The presence of anomalous stromal collagen III and TGF-β1 appear to be the most consistent and strongest staining biomarkers in diseased mice. This mouse appears to mimic human corneal disease. It is an appropriate model for testing of therapeutics to treat EB ocular disease.

1. Background

Collagen VII is an essential basement membrane protein that forms anchoring fibrils within the corneas of humans and rabbits (Gipson et al., 1987, 1989). Anchoring fibrils are a critical component of anchoring complexes, which attach stratified squamous epithelium to underlying tissues (Gipson et al., 1987, 1988). Deficiency of collagen VII causes spontaneous blistering of the skin, eyes, gastrointestinal and genitourinary tracts (Fine and Mellerio, 2009a, 2009b). In the eyes, patients experience epithelial defects, scarring and vision loss (Tong et al., 1999; Lin et al., 1994; Fine et al., 2004; Destro et al., 1987).

The disease caused by collagen VII deficiency is known as dystrophic epidermolysis bullosa (DEB). This rare, inherited disorder is due to a missense or non-sense mutations within the *COL7A1* gene, located on chromosome 3 (Fine et al., 2004). The national EB registry estimated the prevalence of DEB (all subtypes) from 1986 to 2002 to be 6.65–12

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per 1 million, affecting all ethnicities, with equal gender distribution (Fine, 2016; Hernandez-Martin et al., 2013).

Phenotypic disease manifestation in the eye is highly variable, ranging from mild intermittent corneal epithelial defects to severe fibrosis and opacification of the corneal stroma resulting in vision loss (Destro et al., 1987; Fine et al., 2014; Matsumoto et al., 2005a). Of the 3 DEB subtypes, severe generalized recessively inherited DEB (RDEB-SG) has the highest incidence of ocular involvement, with corneal disease reported in 35–50% of patients, corneal scarring in 24–41%, and vision loss of one or both eyes in 3–64% (Tong et al., 1999; Lin et al., 1994; Fine et al., 2014; McDonnell et al., 1989; Smith et al., 2009; El Hachem et al., 2014). Studies of patient impact have described the eye as a critical area of disease involvement for which there is currently no standardly accepted treatments other than supportive care (El Hachem et al., 2014; Azizkhan et al., 2007; Figueira et al., 2010; Gans, 1988; Matsumoto et al., 2005b).

Histologic evidence of the corneal and conjunctival changes in humans with DEB is not well documented due to a paucity of available surgical and autopsy samples. No histologic studies of corneas from DEB animal models have been reported.

In 2010, Dr. Bruckner-Tuderman published a catalogue of DEB animals that showed strains of mice and rats available for small animal model testing (Bruckner-Tuderman et al., 2010). Most experiments are conducted with collagen VII knock-out mice that survive only 7–12 days without systemic rescue by infusion, gene therapy or anti-fibrotic agents (Bruckner-Tuderman et al., 2010; Fritsch et al., 2008). The short life span of these mice decreases the likelihood that corneal disease will be seen because the mechanism for scarring is repetitive blistering and injury takes time to occur.

In an effort to study topical therapies without the influence of systemic treatments, a hypomorphic mouse that produces approximately 10% of wild-type collagen VII protein levels was developed and first described by Drs. Fritsch and Bruckner-Tuderman et al., in 2008. This murine model produces sufficient collagen VII to survive up to 20 weeks or longer without systemic rescue (Fritsch et al., 2008). This model demonstrates limb changes (contracture of the paws) similar to that seen in human patients with DEB. Its creation offers the opportunity to determine the effects of strictly topical agents. The development of topical agents is of particular interest because disease expression is highly variable. Patients with localized disease may benefit from topical therapies and thereby avoid risks associated systemic treatments.

In this paper we describe the corneas of the collagen VII hypomorphic mouse (C7Hypo). The specific primary aim of this observational study is to determine if the C7Hypo mouse model shows corneal changes that reflect those seen in human patients with DEB. We also aim to identify markers of inflammation and corneal fibrosis that may be modified in future therapeutic studies.

2. Materials and methods

All animal experiments were carried out in accordance with the EU directive 2010/63/EU for animal experiments

Corneal tissue: A total of 42 eyes from 23 mice, ages 6–30 weeks, were collected immediately following euthanasia and generously gifted from Drs. Nyström and Bruckner-Tuderman from the University of Freiburg, Germany. Most animals had been treated with topical anti-fibrotic agents on the paws only; none received systemic treatments. Of the 42 eyes sent, eight eyes were sent fresh frozen at −80C and used for WB analysis; fifteen eyes were embedded in optimal cutting temperature cryocompound (OCT) for immunohistochemistry; nineteen eyes were fixed in 10% formalin.

Photography: Immediately following euthanasia, 38 eyes of 19 mice age 6–17 weeks were photographed using an iPhone 6 camera (Apple, Inc). Slit lamp photography was not possible in the department of dermatology where eyes were harvested. These images were independently scored by 2 independent investigators according to the following validated scale: grade 0 = clear, no visible opacity; grade 1 = mild haze; grade 2 = moderate haze/opacification peripherally; grade 3 = moderate haze affecting the central cornea; grade 4 = whitened cornea or severe opacification throughout the cornea or cornea perforation (Suryawanshi et al., 2013).

Antibodies and proteins: Anti-collagen III and anti α-SMA antibodies (Ab) were purchased from Abcam (Cambridge, MA). Anti-collagen VII Ab (Millipore, Billerica, MA), Donkey derived Alexa Fluor 488-conjugated anti-rabbit IgG, and Alexa Fluor 595 conjugated anti rabbit IgG were purchased from (Invitrogen, Carlsbad, CA). Anti-TGF-β1 Ab were purchased from R&D Systems (Cat #MAB240, Minneapolis, MN).

Western blotting: Both corneas of 2 C7Hypo mice were carefully dissected from fresh frozen eyes and snap frozen in Liquid nitrogen and crushed using a pestle. The tissue was added directly to 50 μl of 4X sample loading buffer (pH 6.8 containing 8% SDS, 0.04% bromophenol blue, 5% 2-mercaptoethanol, 8 M urea and 40% glycerol) and boiled for 20 min. Immediately the samples were centrifuged at 13000 rpm for 1 min, then sonicated for about additional 30 min. A 20 μl sample of 2 corneas combined was loaded onto a 7.5% gradient sodium dodecyl sulfate polyacrylamide gel, and electrophoresis was performed, followed by transfer onto a nitrocellulose membrane. The membrane was blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE) for 1 h at RT the membrane and incubated with anti-collagen VII antibodies1:2000 dilution in Odyssey blocking buffer (giftfrom Dr. Nyström) overnight at +4C. Fluorescent conjugated secondary anti rabbit IgG antibodies were used at 1:5000 dilution. For The Loading Control, 20 μl of the same sample was loaded and run in a 4–12% gradient gel and probed with beta actin (Sigma) to ensure equal protein loading in all lanes. This was repeated twice with 2 additional 20 μl samples. Band intensity was quantified densitometrically using imagej.

Immunofluorescence localization: Fifteen eyes of 8 C7Hypo mice were embedded in OCT (Leica Microsystem, Nussloch, Germany) and stored at −80C until sectioning. Then, 10 micron cryo sections were cut with a Leica CM 3050 cryostat (Leica Microsystem, Nussloch, Germany). Tissue sections were placed on glass slides, air dried and fixed with acetone for 10 min at room temperature (RT). The sections were blocked with 10% donkey serum or 3% BSA in PBS for 1 h at RT and then the primary antibody, diluted in PBS containing 0.1% tween 20 (PBST) was applied and incubation over night at +4C. The slides were washed 3 times for 10 min with PBST and incubated with donkey derived secondary antibody Alexa 488 conjugated anti-rabbit IgG and Alexa 595 conjugated anti-rabbit IgG for 2 h at RT. After washing three times with PBST the slides were subsequently mounted with Vector mounting medium containing 4, 6-diamidino-2-phenylindole (DAPI) (Vector laboratories Inc. Burlingame, CA) and examined with EVOS fl –digital inverted fluorescence microscope (Westover Scientifics Inc., Mill Creek, WA). Positive controls were wild type (WT) murine corneas scarred by pseudomonas infection. Negative controls were untreated WT murine corneas.

Histology: Nineteen eyes of 11 C7Hypo mice were fixed and received in buffered 10% formalin and processed by the Tufts Medical Center Histology and Pathology core facility. Paraffin embedded sections were processed using H & E staining; all slides were carefully reviewed. Each cornea was sectioned to provide 5 slides through the center of the cornea and examined under 40X high power magnification with an experienced ocular pathologist.

3. Results

3.1. Corneal opacification by external examination reflects incidence of human disease

The mean age of photographed mice was 10.68 weeks, ranging from 8 to 17 weeks. Of the 19 mice examined, 10 were age 10 weeks and younger, and 9 were older than 10 weeks. Opacification scores were independently graded by both the PI and the senior corneal associate