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Research article

Characterization of the mucocutaneous junction of the human eyelid margin and meibomian glands with different biomarkers

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ABSTRACT

Purpose: To investigate the morphology of the human eyelid margin and the presence of different cytokeratins, mucins and stem cell markers within the skin epithelium, mucocutaneous junction (MCJ) and palpebral conjunctiva.
Methods: Eyelids of body donors were investigated histologically and ultrastructurally as well as by immunohistochemical methods using antibodies to cytokeratins 1, 4, 7, 8, 10, 13, 14, 15, and 19; mucins MUC1, MUC4, and MUC5AC and potential stem cell markers K15, BCRP/ABCG2, integrin β1, and N-cadherin.
Results: The expression pattern of cytokeratins, mucins and stem cell markers varied across the different epithelia of the human eyelid. Within the MCJ, CK7, 15 and 19 were absent, whereas the epithelium reacted positive to antibodies to CK1, 4, 8, 10, 13 and 14. Reactivity was also observed for MUC1 and MUC4, but not for MUC5AC. No reactivity was determined for K15, BCRP/ABCG2 and integrin β1 in the area of the MCJ epithelium but a strong reactivity was present for N-cadherin.
Conclusions: The present immunohistochemical findings lead to a better characterization of the MCJ. Additionally, the knowledge of distribution of biomarkers like cytokeratins, mucins and stem cells can be useful in the investigation of MCJ disturbances which occur in several disorders of the meibomian glands and the lid epithelium in the course of dry eye syndrome and especially meibomian gland dysfunction.
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1. Introduction

Dry eye syndrome (DES) is a multifactorial disease of the tears and the ocular surface that results in symptoms of discomfort, visual disturbance and tear film instability with potential damage to the ocular surface. It is accompanied by increased osmolarity of the tear film and inflammation of the ocular surface (Anon., 2007). Although DES can result from a deficiency in the aqueous component of tears, it is more commonly associated with hyperevaporation of tears (Anon., 2007; Tong et al., 2010). The most common cause of this hyperevaporative form is considered meibomian gland dysfunction (MGD), a term used to describe a diffuse abnormality of the meibomian glands resulting in decreased tear stability, symptoms of eye irritation and ocular surface disease (Anon., 2007; Korb and Henriquez, 1980; Nelson et al., 2011; Nichols et al., 2011). Moreover, MGD can also be responsible for inflammatory conditions of the eyelids that may not manifest as classic DES. MGD can be due to changes of the gland itself or can be a consequence of changes of its orifice or the surrounding lid tissue (Hallengren et al., 1982; Mathers et al., 1991; McCulley et al., 1982; Nelson et al., 2011).

The margin of the eyelid is an essential structure for the thin spread of a stable tear film and its re-formation with every blink to achieve a thin, optically perfect tissue–air interface. This area has been termed the lid wiper (for review see Knop et al., 2011). At the human eyelid margin, the keratinized stratified squamous epithelium transitions to an epithelium of the marginal conjunctiva. This transition zone, called "mucocutaneous junction" (MCJ) is of particular interest with respect to several eye diseases including MGD and subsequently evaporative DES, as it is known that changes in the tissue occur with age (Den et al., 2006; Hykin and Bron, 1992; Sullivan et al., 2006; Yamaguchi et al., 2006) and under several pathological conditions (Pflugfelder et al., 1990; Tseng, 1985). As mentioned, the morphological changes observed in MGD are squamous metaplasia of the meibomian gland orifice (Gutgesell et al., 1982; Lee and Tseng, 1997) and presence of keratinization or displacement of the MCJ (Matsumoto et al., 2008; Tomlinson et al., 2011). A clinically observable alteration of the marginal conjunctival epithelium of the upper eyelid is

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Table 1
Primary and secondary antibodies used for immunohistochemical stainings.

Antibody	Clonality	Species	Dilution	Origin
CK1	Monoclonal	Mouse	1:20	Novocastra
CK4	Monoclonal	Mouse	1:100	Novocastra
CK7	Monoclonal	Mouse	1:50	Santa Cruz
CK8	Monoclonal	Mouse	1:25	Dako
CK10	Monoclonal	Mouse	1:50	Abcam
CK13	Monoclonal	Mouse	1:200	Novocastra
CK14	Monoclonal	Mouse	1:20	Novocastra
CK15	Monoclonal	Mouse	1:100	Milipore
CK19	Monoclonal	Mouse	1:40	Chemicon Europe
MUC1	Monoclonal	Mouse	1:100	Novocastra
MUC4	Monoclonal	Mouse	1:50	Zymed
MUC5AC	Monoclonal	Mouse	1:50	Novocastra
ABCG2 (BCRP)	Monoclonal	Mouse	1:20	Kamiya Biomedical Company
K15 (LHK15)	Monoclonal	Mouse	1:50	Abcam
N-Cadherin	Polyclonal	Rabbit	1:300	Abcam
Integrin β1	Monoclonal	Rabbit	1:250	Abcam
Secondary antibody	Clonality	Species	Dilution	Origin
Biotinylated anti-rabbit IgG	Polyclonal	Goat	1:200	Vector Laboratories
Biotinylated anti-mouse IgG	Polyclonal	Rabbit	1:200	Dako

CK = cytokeratin; MUC = mucin; ABCG2, K15, N-cadherin and integrin β1 are stem cell markers.

termed lid wiper epitheliopathy (LWE). It correlates with dry eye symptoms.

The morphological features of the lid wiper have recently been described in detail and previous work on this region has been extensively reviewed (Knop et al., 2010, 2011). Nevertheless, a deeper knowledge of the lid margin would appear to be important for a better understanding of DES and LWE. To gain further insight, the aim of our present study was to characterize the lid margin using a selection of different biomarkers, i.e. cytokeratins, mucins and stem cell markers. We also performed electron microscopy of the MCJ.

2. Materials and methods

2.1. Collection of human upper eyelid samples

Eight upper and eight lower eyelids (3 male, 5 female, age range = 50–76 years) were obtained from cadavers donated to the Department of Anatomy and Cell Biology, Martin Luther University Halle-Wittenberg, Halle, Germany. Sample tissues were dissected from the cadavers within in a time period of 4 up to 12 h post-mortem. All samples were free of recent trauma, eye or nasal infections or known diseases involving or affecting ocular surface function, and were used in accordance with the Declaration of Helsinki.

2.2. Fixation and preparation of the tissue

After dissection, tissue samples of the eight cadavers were fixed in 4% paraformaldehyde for at least 48 h. 6 of them were subsequently paraffin embedded. Tissue samples of two cadavers (from two upper and two lower eyelids, 1 male, 1 female) were additionally fixed in 2.5% glutaraldehyde for electron microscopic studies and were embedded in Epon resin.

2.3. Immunohistochemistry

Reactivity with 17 antibodies to cytokeratins CK1, CK4, CK7, CK8, CK10, CK13, CK15 and CK19; mucin peptide core epitopes MUC1, MUC4 and MUC5AC and the epitopes of ABCG2, K15, N-cadherin and integrin β1 (Table 1) was followed in tissue sections from the eight upper and eight lower eyelids.

Paraffin embedded tissue was sectioned in 6 μm sections with a microtome. The sections were placed on poly-L-lysine-coated slides, deparaffinized in xylene and rehydrated in a decreasing alcohol series. After washing in aqua dest. twice for 5 min each, the sections were heated in citrate buffer (pH 6.0) at sub-boiling temperature for 10 min. Afterwards, the sections were cooled for at least 1 h, washed in aqua dest. three times for 5 min and air-dried at room temperature. Endogenous peroxidases were inactivated by covering the sections with 3% hydrogen peroxide for 10 min. Afterwards the slides were washed with aqua dest. twice for 5 min and were incubated with TBST blocking buffer (=TBST + 5% normal serum of same species as secondary antibody) for 5 min in a humidified chamber at room temperature for 1 h to prevent nonspecific staining. The sections were then incubated with the primary antibodies diluted in PBS buffer (PBS with 2% [wt/vol] bovine serum albumin [BSA; Merck, Darmstadt, Germany] and 0.2% [vol/vol] Triton-X 100) overnight in a humidified chamber at 4 °C. The antibodies were used as listed in Table 1. The sections were rinsed with TBST three times for 5 min each and then with the biotinylated secondary antibody (Table 1) diluted in blocking solution for 30 min at room temperature. After rinsing with TBST three times for 5 min, the sections were rinsed with peroxidase-labeled ABC reagent (avidin:biotinylated enzyme complex) for 30 min at room temperature. Afterwards, sections were rinsed with TBST 3 times for 5 min. DAB substrate (freshly made just before use: 1 ml TBST + 20 μl, DAB + 2 μl 3% H_2O_2) was applied to each section for 3–5 min and staining was monitored closely under the light microscope until the desired color intensity was reached. Sections were immediately immersed in aqua dest. After counterstaining in hemalum for 1–2 min, sections were rinsed with running tap water for 1–2 min and were dehydrated by an increasing alcohol series and two times with xylene for 10 min each. Finally, sections were mounted in permount medium Entellan.

In each case, two negative control sections were prepared by treating with the secondary antibody or the primary antibody only. Control sections were also incubated with nonimmune IgG to determine possible nonspecific binding of IgG. The slides were examined and photographed with a Biorevo BZ-9000 fluorescence microscope (Keyence; Higashi-Nakajima, Osaka, Japan).

2.4. Histologic and ultrastructural analysis

From the upper and lower eyelids of 2 body donors fixed in 2.5% glutaraldehyde and embedded in Epon, 1 μm semi-thin sagittal sections were cut with a microtome (Ultracut E; Reichert Jung, Vienna, Austria) and subsequently stained with toluidine blue. Semi-thin

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