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Dry eye modulates the expression of toll-like receptors on the ocular surface

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ABSTRACT

We aimed to determine if toll-like receptor (TLR) expression is modulated in response to dry eye-associated conditions and in dry eye syndrome (DES). Primary human corneal epithelial cells (HCEC), an SV40 HCEC cell line or a normal human conjunctival epithelial cell line (IOBA-NHC) were cultured under hyperosmolar stress (HOS) (400–500 mOsm/kg) or with DES associated cytokines (IL-1α/β, TNFα or TGFβ) at concentrations ranging from 1 to 1000 ng/ml for up to 24 h. Epithelial cells were harvested from a human cornea organ culture model following 24 h of desiccation. Conjunctival impression cytology samples were harvested from subjects with DES and age and gender-matched normal subjects. TLR4, TLR5 or TLR9 mRNA or protein was examined by quantitative RT-PCR, western blotting or flow cytometry. TLR functionality was evaluated in terms of addition of TLR agonists and quantitation of secreted inflammatory cytokines by the use of ELISA and Luminex assays. In SV40 HCEC, HOS significantly increased TLR4 by 8.18 fold, decreased TLR9 by 0.58 fold, but had no effect on TLR5 mRNA expression. TLR4 and TLR9 protein were decreased by 67.7% and 72% respectively. TLR4 mRNA was also significantly up-regulated by up to 9.70 and 3.36 fold in primary HCEC and IOBA-NHC respectively. DES associated cytokines had no effect on TLR4, TLR5 and TLR9 expression. In response to desiccation, TLR4 and TLR5 mRNA were significantly up-regulated by 4.81 and 2.51 fold respectively, while TLR9 mRNA was down-regulated by 0.86 fold in HCEC. A similar trend for TLR4 and TLR9 protein was observed. TLR9 mRNA was significantly down-regulated by almost 59.5% in DES subjects. In conclusion, changes in TLR expression occur in dry eye and could have an important role in ocular surface susceptibility to inflammation and infection.

1. Introduction

Dry eye syndrome (DES) is an ocular surface condition that affects millions of individuals every year and is one of the leading causes for visits to the eye doctor. Subjects with severe DES have an increased risk for corneal ulceration and melting (Vivino et al., 2001) and ocular infection (Jhanji et al., 2009) which may result in vision loss. Although DES typically does not result in blindness, subjects often report a decreased quality of life and reduced ability to perform daily activities (Miljanovic et al., 2007) leading to loss of job productivity.

Inflammation plays a pivotal role in DES pathogenesis, and it has been demonstrated to be driven by tear film hyperosmolarity (Bron et al., 2002; Farris, 1994; Gilbard et al., 1978) and instability which stimulate an increase in pro-inflammatory cytokines (Afonso et al., 1999; Pflugfelder et al., 1999; Solomon et al., 2001) at the ocular surface, leading to the disruption of the ocular surface epithelium and exacerbation of the disease. To date, no studies have investigated if in DES there is a change in the expression of innate immune receptors that can stimulate inflammation, such as toll-like receptors (TLRs), on the ocular surface.

Toll-like receptors are a family of highly conserved glycoprotein receptors that recognize conserved motifs on pathogen associated molecular patterns on microbes and as suggested by some studies,

Abbreviations: TLR, toll-like receptor; DES, dry eye syndrome; HCEC, human corneal epithelial cells; SS, Sjögren's syndrome; HOS, hyperosmolar stress; TE, tris–EDTA buffer; PBS, phosphate-buffered saline; OD, optical density; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; OSDI, ocular surface disease index.

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host endogenous ligands (Medzhitov et al., 1997; Takeda et al., 2003). The activation of TLRs leads to the production of various pro-inflammatory cytokines and chemokines (Akira and Takeda, 2004; Takeda et al., 2003). Ten functional human TLRs have been identified (TLR1-TLR10), each binding a distinct microbial ligand. Toll-like receptor 4 (TLR4), the most extensively studied of the TLRs is activated by lipopolysaccharide (LPS) (Beutler, 2002) and endogenous host ligands such as heat shock proteins [HSP60 (Ohashi et al., 2000), HSP70 (Vabulas et al., 2002)] and hyaluronic acid fragments (Gariboldi et al., 2008). TLR5 is activated by bacterial flagellin (Hayashi et al., 2001), and TLR9 responds to the unmethylated CpG motifs found in bacterial and viral DNA (Hemmi et al., 2000; Tabeta et al., 2004). With the exception of TLR8, all TLRs are reported to be commonly expressed in the cornea and conjunctiva although some discrepancies remain regarding their subcellular localization. For a detailed review on ocular surface TLRs, the reader is referred to two review articles (Lambiase et al., 2011; Redfern and McDermott, 2010).

Studies have shown that topical application of TLR agonists on the corneal epithelium can produce extensive ocular surface inflammation (Adhikary et al., 2008; Johnson et al., 2005; Kumar et al., 2006; Zhang et al., 2003). In particular, the activation of TLR2, TLR4 and TLR9 in the murine corneal epithelium has been shown to induce sight-threatening keratitis (Johnson et al., 2005) while the application of eritoran tetrasodium, a TLR4 antagonist, can significantly inhibit corneal inflammation in response to stimulation with LPS (Sun and Pearlman, 2009) suggesting a potential therapeutic role for TLR antagonists in modulating corneal inflammation.

TLR expression has also been shown to be increased in dry eye and its most severe form, Sjögren's syndrome (SS), an autoimmune disorder that causes functional impairment of the salivary and lacrimal glands. In the parotid gland in subjects with SS, TLR7 and TLR9 were expressed throughout the gland on the epithelial islands, lymphocytes, and ductal epithelial cells, while in control subjects, TLR7 and TLR9 expression was limited to the ductal epithelial cells (Zheng et al., 2010). In an SS mouse model, TLR4 and TLR5 mRNA was up-regulated in the cornea and TLR4 was up-regulated in the lacrimal gland (Christopherson PL, 2005). Together these data suggest that TLRs may be involved in the pathogenesis of dry eye inflammation. Considering this, TLR expression was examined in subjects with DES and in various ocular surface cells in response to dry eye associated conditions, such as hyperosmolar stress (HOS), desiccation and cytokines. This study focuses on TLR4, TLR5 and TLR9 which are known to be expressed by ocular surface cells and have been implicated in ocular surface inflammation (Adhikary et al., 2008; Johnson et al., 2005; Kumar et al., 2006; Sun and Pearlman, 2009; Zhang et al., 2003).

2. Methods

2.1. Cell cultures

Primary human corneal epithelial cell (HCEC) cultures were prepared from human corneas unsuitable for transplantation obtained from eye banks within 3–5 days of death with a mean age and standard deviation of 71.5 ± 8.9 years. The tissue was obtained in accordance with the guidelines of the Declaration of Helsinki regarding research involving human tissue. Cells were isolated as previously described (Redfern et al., 2011) and were maintained in EpiLife medium (Invitrogen; Portland, OR). Normal human conjunctival (IOBA NHC) epithelial cells (Diebold et al., 2003) were cultured in DMEM-F12 (1:1 vol/vol), containing 10% fetal bovine serum (FBS) as previously described (Narayanan et al., 2006b). SV40-transformed HCEC were a gift from Dr. Kaoru Araki-Sasaki (Tane Memorial Eye Hospital, Osaka, Japan). The cells were maintained in SHEM (DMEM-Ham's F12, 1:1 vol/vol) supplemented with 10% FBS as previously described (Redfern et al., 2011). All cultured cells were maintained at 37 °C in 5% CO_2.

2.2. Cell treatment

Cells were cultured to 60–70% confluence, washed three times with phosphate buffered saline (PBS) to remove floating dead cells as well as residual serum and growth factors and placed in supplement-free (primary HCEC) or serum-free (cell lines) media (SFM) overnight. Cells were cultured for an additional 24 h in SFM or SFM with either osmolarity ranging from 400 to 500 mOsm/kg, which was achieved by adding various amounts of sodium chloride (Li et al., 2006); or with 1–1000 ng/ml of IL-1α, IL-1β, TNFα or TGFβ for up to 24 h. In some samples treated with HOS, the hyperosmolar media was removed; the cells were washed three times with phosphate buffered saline (PBS), and cultured with normal growth media for an additional 6 or 24 h. At the end of the incubation period, the cells were either harvested in RLT lysis buffer (Qiagen; Valencia, CA) or pelleted, snap frozen and stored at −80 °C until RNA extraction or western blotting for either TLR mRNA and protein or human beta defensin (hBD)-2 mRNA expression.

2.3. Bacterial DNA

Pseudomonas aeruginosa 19660 (PA 19660) was grown in Difco nutrient broth (BD, Franklin Lakes, NJ) at 37 °C to stationary phase. Bacteria were diluted to $OD_{260nm} = 0.2$ in phosphate-buffered saline (PBS), determined to be 1×10^7 cfu/ml. Bacterial DNA was prepared suspending the bacteria in 100 mM NaCl-10 mM Tris-HCl-25 mM EDTA (pH = 8.0) with proteinase K (0.2 mg/ml) and sodium dodecyl sulfate (0.5%) and shaking at 150 rpm, 50 °C overnight. DNA was precipitated from the lysate with iso-propanol, washed with 70% ethanol, air dried, dissolved in Tris–EDTA (TE) buffer and stored at 4 °C.

2.4. Cytokines secretion

SV40 HCEC were seeded in 12-well plates at a density of 2×10^5 cells/well and allowed to adhere for 24 h. Following adherence, cells were washed two times with PBS and SHEM complete media was replaced by serum-free SHEM for 18 h. Cells were then incubated in iso-osmolar (374-377mOs/kg) or hyperosmolar (450mOs/kg) media in the presence of TLR4 agonist, LPS (1ug/ml) or TLR9 agonist, PA 19660 DNA (10 μg/ml) for 24 h. After treatment, the supernatant from each well was transferred into a 1.5 ml Eppendorf tube. All supernatant samples were snap frozen in liquid nitrogen and stored at −80 °C for later quantitation of IL-8 by an ELISA assay (BioLegend, San Diego, CA); in addition supernatant samples were interrogated for a panel of 13 inflammatory cytokines (which included IL-6) by the use of a Luminex assay (EMD Millipore, Billerica, MA) following the manufacturer's instructions.

2.5. Desiccation organ culture model

Human corneas were obtained from eye banks within 3–5 days of death. The mean age and standard deviation of the donors was 59 ± 1.7 years. Corneas with intact epithelium were stabilized with an agar mold as previously described (McDermott et al., 2001), then placed epithelial side up into 35 mm culture dishes which were filled with M199 media up to the limbal conjunctiva (desiccation model) and maintained as previously described (McDermott et al., 2001) or completely submerged (control). After 24 h, the epithelium was collected for analysis of TLR mRNA and protein expression.