Experimental Eye Research 153 (2016) 1–7

Contents lists available at ScienceDirect

Experimental Eye Research

journal homepage: www.elsevier.com/locate/yexer

Research article

Differential effect of rebamipide on transmembrane mucin biosynthesis in stratified ocular surface epithelial cells

Yuichi Uchino, Ashley M. Woodward, Pablo Argüeso*

Schepens Eye Research Institute and Massachusetts Eye and Ear, Department of Ophthalmology, Harvard Medical School, Boston, MA 02114, USA

ARTICLE INFO

Article history:
Received 25 March 2016
Received in revised form
29 September 2016
Accepted in revised form 6 October 2016
Available online 8 October 2016

Keywords:
Ocular surface
Transmembrane mucins
Rebamipide
Stratified epithelium
Notch signaling

ABSTRACT

Mucins are a group of highly glycosylated glycoproteins responsible for the protection of wet-surfaced epithelia. Recent data indicate that transmembrane mucins differ in their contribution to the protective function of the ocular surface, with MUC16 being the most effective barrier on the apical surface glycocalyx. Here, we investigated the role of the mucoprotective drug rebamipide in the regulation of transmembrane mucin biosynthesis using stratified cultures of human corneal and conjunctival epithelial cells. We find that the addition of rebamipide to corneal, but not conjunctival, epithelial cells increased MUC16 protein biosynthesis. Rebamipide did not affect the levels of MUC1, 4 and 20 compared to control. In these experiments, rebamipide had no effect on the expression levels of Notch intracellular domains, suggesting that the rebamipide-induced increase in MUC16 biosynthesis in differentiated corneal cultures is not regulated by Notch signaling. Overall these findings indicate that rebamipide induces the differential upregulation of MUC16 in stratified cultures of human corneal epithelial cells, which may have implications to the proper restoration of barrier function in ocular surface disease.

1. Introduction

The maintenance of a wet-surfaced epithelium in the most anterior optical surface of the eye is crucial to provide refractive power and to allow the passage of light onto the retina (Gipson, 2007). To achieve optimal vision, the stratified, non-keratinized ocular surface epithelia must overcome the continuous exposure to allergens, debris, pathogens, desiccation, and injury. This is achieved in part by the presence of a protective glycocalyx that localizes on the tips of microplicae in the most apical portion of the corneal and conjunctival epithelia.

Transmembrane mucins are a major component of the ocular surface apical glycocalyx. They are defined by the presence of central tandem repeats of amino acids rich in serine and threonine, and their extensive glycosylation, which comprises up to 90% of the mass of the mature molecule and contributes to the hydrophilic character of the ocular surface (Argueso, 2013; Bansil et al., 1995; Guzman-Aranguez and Argueso, 2010). The stratified epithelia of the cornea and conjunctiva produce the transmembrane mucins MUC1, 4, 16 and 20 (Mantelli et al., 2013; Woodward and Argueso, 2014). MUC1, 4, and 16 are present along the plasma membranes of the superficial cell layer of the stratified epithelium, whereas MUC20 is predominant along the plasma membranes of intermediate cell layers with limited expression on the apical glycocalyx of superficial cells (Woodward and Argueso, 2014). Members of the mucin family differ considerably in size, with MUC16 and its 22,152 amino acids being the largest of all of them (Mantelli et al., 2013). Direct comparisons of their functions *in vitro* have recently demonstrated distinct differences between MUC1 and MUC16, revealing that MUC16 promotes the trans and paracellular barriers, whereas MUC1 does not (Gipson et al., 2014).

Diseases in which transmembrane mucins are altered have been described at the ocular surface and include non-autoimmune and autoimmune dry eye (Mantelli and Argueso, 2008). Abnormalities in the glycosylation of transmembrane mucins have been identified, and several authors have reported changes in the distribution of mucin-type carbohydrates in the epithelial glycocalyx of patients with dry eye (Guzman-Aranguez and Argueso, 2010). These involve reduction in the binding of the H185 antibody to an O-acetyl sialic acid epitope on MUC16 on the apical cell surface of stratified cells (Argueso and Sumiyoshi, 2006; Danjo et al., 1998), and release of galectin-3, a multimeric protein that binds transmembrane mucin carbohydrates in the epithelial glycocalyx, into the tear film (Uchino et al., 2015). The association of transmembrane mucin

* Corresponding author. Schepens Eye Research Institute, 20 Staniford Street, Boston, MA 02114, USA.
E-mail address: pablo_argueso@meei.harvard.edu (P. Argüeso).

http://dx.doi.org/10.1016/j.exer.2016.10.007
0014-4835/

protein biosynthesis and dry eye remains debated. Studies have shown that subjects with Sjögren's syndrome display a significant increase in MUC16 (Caffery et al., 2008), and elevation in MUC1 and MUC16 has been observed in postmenopausal women with non-Sjögren's dry eye (Gipson et al., 2011). Others, however, have failed to reveal compelling evidence supporting a significant relationship, except for a marginal reduction in MUC16 expression in symptomatic dry eye (Srinivasan et al., 2013). It has been hypothesized that the potential loss of MUC16 from discrete apical areas on the ocular surface glycocalyx is a local phenomenon that is not within the range of current methods of detection (Gipson et al., 2011).

Rebamipide, an amino acid analog of 2(1H)-quinolinone, is a cytoprotective agent originally marketed as an oral drug for the treatment of gastric mucosal disorders and gastritis. It has been shown to increase mucus secretion (Iijima et al., 2009), prevent the delay of wound repair (Watanabe et al., 1998), and protect against reactive oxygen metabolite-mediated cell damage (Hahm et al., 1997), in gastric epithelial cells. Recently, rebamipide has been developed as an ophthalmic solution for the treatment of dry eye disease (Kinoshita et al., 2014). Topical administration of rebamipide has been shown to exert a potent anti-inflammatory action in a murine model of primary Sjögren's syndrome (Arakaki et al., 2014) and to increase the amount of glycoconjugate biosynthesis and barrier function in a rabbit model of ocular surface epithelial damage (Urashima et al., 2004). Studies using monolayer cell culture systems have shown that rebamipide increases MUC1 and MUC4 expression and biosynthesis in human corneal epithelial cells (Itoh et al., 2014; Takeji et al., 2012). Interestingly, the effect of rebamipide on MUC16 in these systems has produced inconsistent data, which has been attributed to the failure of cells grown as monolayers to properly differentiate (Itoh et al., 2014).

Critical to the maintenance of a wet-surface epithelium at the ocular surface is the highly conserved Notch signaling pathway (Nicolas et al., 2003; Zhang et al., 2013). Notch signaling is an important factor in controlling cell fate decisions and terminal differentiation in multicellular organisms. Early studies on the function of Notch signaling in mucosal epithelia revealed that Notch signaling is involved in the maintenance of postmitotic, mucin-producing cells (Nicolas et al., 2003; van Es et al., 2005). The presence of Notch1, Notch2 and Notch3 has been demonstrated at the ocular surface, where they play pivotal roles in maintaining barrier function and regulating MUC16 biosynthesis during corneal and conjunctival epithelial cell differentiation (Ma et al., 2007; Movahedan et al., 2013; Xiong et al., 2011). Here, we investigated whether rebamipide modulates transmembrane mucin biosynthesis and promotes the activation of Notch signaling in an *in vitro* model of stratified human corneal and conjunctival epithelia. Our results indicate that rebamipide promotes the sole upregulation of MUC16 biosynthesis through a Notch-independent mechanism in corneal epithelial cells.

2. Material and methods

2.1. Reagents and antibodies

Rebamipide, 2-(4-chlorobenzoylamino)-3-[2(1H)-quinolinon-4yl-]-propionic acid, was provided by Otsuka Pharmaceutical Co., Ltd. (Tokyo, Japan). A stock solution of rebamipide (1 mM) was prepared in Dulbecco's modified Eagle's medium–nutrient mixture F12 (DMEM/F12) (Mediatech Inc., Manassas, VA), and diluted to the required concentrations in DMEM/F12 before use. Mouse anti-human MUC1 (clone 214D4), mouse anti-human MUC4 (clone 8G-7) and mouse anti-human MUC16 (clone M11) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY), Santa Cruz Biotechnology (Dallas, TX), and Neomarkers (Fremont, CA), respectively. Rabbit anti-human MUC20 C-terminus (clone RB13033) antibody was purchased from Abgent (San Diego, CA). Rabbit anti-human Notch1 (clone H-131), Notch2 (clone 25–255), Notch3 (clone M-134) and GAPDH (FL-335) antibodies were purchased from Santa Cruz Biotechnology.

2.2. Cell culture

Telomerase-immortalized human corneal and conjunctival epithelial cells were plated at a seeding density of 5×10^4 cells/cm^2 in six- or twelve-well plates (Costar Corning, Corning, NY) and maintained at 37 °C in 5% CO_2 (Gipson et al., 2003). Cultures were grown to confluence in a medium optimized for the proliferation of keratinocytes (keratinocyte serum-free medium, K-SFM; Life Technologies; Carlsbad, CA). After reaching confluence, cells were switched to DMEM/F12 medium supplemented with 10 ng/mL epidermal growth factor (EGF) and 10% calf serum (Life Technologies) for 7 days to promote cell stratification and differentiation (Gipson et al., 2003; Xiong et al., 2011).

2.3. Rebamipide treatment and collection of cell lysates

Stratified cells were incubated with increasing concentrations of rebamipide (0.01–100 μM) in DMEM/F12 for 24 h. Cultures were then washed three times with phosphate-buffered saline (PBS), pH 7.5, and the cell lysates were extracted using radioimmunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% NP40, 0.5% deoxycholate, 0.1% SDS) supplemented with Complete™ Protease Inhibitor Cocktail (Roche Diagnostics, Indianapolis, IN). After homogenization with a pellet pestle, the cell lysates were centrifuged at 12,000 g for 45 min at 4 °C, and the protein concentration of the supernatant determined using the Pierce BCA™ Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL).

2.4. Electrophoresis and immunoblot analysis

For analysis of MUC1, MUC4 and MUC16, cell lysates (20 μg total protein) were resolved by agarose gel electrophoresis (1%, wt/vol) and transferred onto nitrocellulose membranes (Bio-Rad, Hercules, CA) by vacuum blotting. For analysis of MUC20, Notch1, Notch2, Notch3 and GAPDH, cell lysates (20–40 μg total protein) were resolved by 10% SDS-PAGE and transferred onto nitrocellulose membranes by electroblotting.

Nonspecific binding to the membranes was blocked by incubation with 5% (wt/vol) nonfat dry milk in either Tris buffered saline containing 0.1% Tween-20 (T-TBS) or PBS for 2 h at room temperature. Membranes were then incubated with primary antibodies diluted in 5% nonfat dry milk in T-TBS (MUC16, 1:2000; MUC20, 1:2000; Notch1, 1:200; Notch2, 1:200; Notch3, 1:200) or PBS (MUC1, 1:2000; MUC4, 1:2000) overnight at 4 °C, followed by the appropriate secondary antibodies coupled to horseradish peroxidase (Santa Cruz Biotechnology). Peroxidase activity was detected using the SuperSignal™ West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) for 5 min and imaged using the Syngene G-Box (Syngene, Frederick, MD). When weak or no signals were detected using the West Pico Chemiluminescent Substrate, membranes were subsequently incubated with the more sensitive SuperSignal™ West Femto Chemiluminescent Substrate (Thermo Fisher Scientific). Band intensities were quantified by densitometry (ImageJ software; National Institutes of Health, Bethesda, MD; in the public domain, available at http://rsbweb.nih.gov/ij/).