Experimental Eye Research 146 (2016) 54–59

Contents lists available at ScienceDirect

Experimental Eye Research

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

Research article

Cytokeratin expression in mouse lacrimal gland germ epithelium

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ARTICLE INFO

Article history:
Received 21 June 2015
Received in revised form
10 November 2015
Accepted in revised form 25 November 2015
Available online 30 November 2015

Keywords:
Lacrimal gland
Dry eye disease
Development
Cytokeratin

ABSTRACT

Purpose: The lacrimal gland secretes tear fluids that protect the ocular surface epithelium, and its dysfunction leads to dry eye disease (DED). The functional restoration of the lacrimal gland by engraftment of a bioengineered lacrimal gland using lacrimal gland germ epithelial cells has been proposed to cure DED in mice. Here, we investigate the expression profile of cytokeratins in the lacrimal gland germ epithelium to clarify their unique characteristics.
Methods: We performed quantitative polymerase chain reaction (Q-PCR) and immunohistochemistry (IHC) analysis to clarify the expression profile of cytokeratin in the lacrimal gland germ epithelium.
Results: The mRNA expression of keratin (KRT) 5, KRT8, KRT14, KRT15, and KRT18 in the lacrimal gland germ epithelium was increased compared with that in mouse embryonic stem cells and the lacrimal gland germ mesenchyme, as analyzed by Q-PCR. The expression level of KRT15 increased in the transition from stem cells to lacrimal gland germ epithelium, then decreased as the lacrimal gland matured. IHC revealed that the expression set of these cytokeratins in the lacrimal gland germ epithelium was different from that in the adult lacrimal gland. The expression of KRT15 was observed in the lacrimal gland germ epithelium, and it segmentalized into some of the basal cells in the intercanulated duct in mature gland.
Conclusion: We determined the expression profile of cytokeratins in the lacrimal gland epithelium, and identified KRT15 as a candidate unique cellular marker for the lacrimal gland germ epithelium.

1. Introduction

Lacrimal glands secrete tear fluid, including tear water and tear proteins, thus maintaining the homeostatic microenvironment for a healthy ocular surface (Mathers, 2000; Mishima, 1965). A shortage of tear volume caused by lacrimal gland dysfunction leads to dry eye disease (DED) (Mathers, 2000). Systemic diseases and environmental exposures including Sjögren's syndrome, ocular cicatricial pemphigoid, aging, extensive usage of a visual display, the use of contact lenses and refractive surgery have all been reported as pathological causes of DED (Uchino et al., 2011; Tsubota and Nakamori, 1993; Toda et al., 2004). DED is the most common eye disease and results in ocular surface epithelial damage, ocular discomfort, significant loss of vision and a decrease in the quality of life (Kaido et al., 2011). Regenerative therapeutic approaches to restore lacrimal gland function have recently been suggested as a future curative approach for DED (Lemp, 1987; Hirayama et al., 2013a).

One of the goals of regenerative medicine is to generate the desired cells, tissues and organs from pluripotent stem cells to restore damaged organ function (Evans and Kaufman, 1981; Martin, 1981; Thomson et al., 1998; Takahashi and Yamanaka, 2006). Current studies of cell differentiation have increased our understanding of developmental biology, which provides information regarding the sequential requirements of the transcription factors, growth factors, signaling cascades, and cell–cell interactions necessary to optimize culture conditions (Murry and Keller, 2008; Snykers et al., 2009; Zaret and Grompe, 2008). For the generation of target cells, cell characterization, including the identification of cell markers such as surface membrane proteins that define the specific cell characteristics, is indispensable to verify the commitment to a cell lineage (Lombaert and Hoffman, 2010).

The lacrimal gland is organized according to the tubuloalveolar system, which consists of acini, ducts, myoepithelial cells and peripheral tissues such as nerves (Schechter et al., 2010). Such functional 3D architecture of the mature lacrimal gland is achieved through a epithelial–mesenchymal interaction during organogenesis (Makarenkova et al., 2000; Tsau et al., 2011). Recently, the concept of bioengineered lacrimal gland replacement therapy to cure DED has been demonstrated by the engraftment of a bioengineered lacrimal gland germ that was reconstituted by using

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http://dx.doi.org/10.1016/j.exer.2015.11.020
0014-4835/

cells from mouse embryonic lacrimal gland germs (Hirayama et al., 2013a, b). Thus, lacrimal gland germ epithelial cells may be attractive candidates for regeneration using pluripotent stem cells (Hirayama et al., 2015). These cells may be useful for bioengineered organ replacement therapy to restore lacrimal gland function; therefore, the characterization of their attributes is required.

Here, we characterized cytokeratin expression in the mouse lacrimal gland germ epithelium, and our results provided evidence for the definition of the cells that constitute the epithelium.

2. Methods

2.1. Animals

C57BL/6 mice were purchased from Japan SLC Inc. (Shizuoka, Japan). Mouse embryonic stem cells (mESCs) were kindly provided by Prof. Minoru Ko, Department of Systems Medicine, Keio University (Nishiyama et al., 2009). The care and handling of the animals were performed in accordance with the NIH guidelines. All of the experimental protocols were approved by the Animal Care and Use Committee of the Keio University (Approval No. 09167).

2.2. Preparation of samples for quantitative PCR

Lacrimal gland germs that were isolated from embryonic day-16.5 male and female mice were treated with 50 U/ml dispase (BD, Franklin Lakes, NJ, USA) for 1.5 min at room temperature. The epithelial and mesenchymal tissues were separated surgically by using 23 G needles as previously reported (Hirayama et al., 2013a; Ogawa et al., 2013; Nakao et al., 2007). Adult lacrimal glands were obtained from 7-week-old mice.

2.3. Quantitative polymerase chain reaction (Q-PCR)

Total RNA was extracted from cells using an RNeasy Mini Kit (QIAGEN, Hilden, Germany). The expression levels of the mRNAs in each RNA sample were determined using the Thermal cycler Dice Real Time System (TaKaRa Bio Inc., Otsu, Japan). QPCR was performed using a One Step SYBR PrimeScript PLUS RT-PCR Kit (TaKaRa Bio Inc.). The conditions were as follows: initial hold at 42 °C for 5 min; incubation at 95 °C for 10 s; 50 cycles at 95 °C for 5 s and then 60 °C for 31 s. The expression of mRNA was assessed by evaluating threshold cycle (CT) values. The CT values were normalized to the expression level of glyceraldehyde-3-phosphate dehydrogenase, and the relative amount of mRNA specific to each of the target genes was calculated.

2.4. Immunostaining

Immunostaining for the lacrimal gland epithelium of E16.5 mouse (E16.5 LGE) was carried out using the fixed tissues on the dish. In short, 6 h after cultivation of the E16.5 LGE in AK-03 (Ajinomoto, Tokyo, Japan), the tissue was fixed with 4% paraformaldehyde (pH 7.0) in phosphate buffered saline (PBS) for 20 min at room temperature. After two rinses with PBS, the cells were incubated with 0.1% Triton X-100 in PBS for 15 min at room temperature and then washed three times with PBS for 5 min each. For the whole lacrimal glands from 7-week-old mice (7w LG), the tissues were fixed in Mildform 10 N (Wako, Osaka, Japan) overnight at 4 °C, and frozen sections (10 μm) were prepared and immunostained. Samples were then incubated with 10% bovine serum albumin (BSA) in PBS for 30 min at room temperature, followed by primary antibody incubation for 16 h at 4 °C. The primary antibodies used were the following: rabbit anti-cytokeratin 5 (1:250, Abcam, Cambridge, UK), rabbit anti-cytokeratin 8 (1:500, Abcam), rabbit anti-cytokeratin 8 (1:500, Abcam), rabbit anti-cytokeratin 14 (1:500, Abcam), rabbit anti-cytokeratin 15 (1:250, Abcam), rabbit anti-cytokeratin 18 (1:500, Abcam), goat anti-E cadherin antibody (1:250, Abcam). The secondary antibody reactions were carried out by incubation with the corresponding species-specific antibodies (1:500, Life Technologies) for 1 h at room temperature in the dark. After four washes with PBS for 5 min each, the specimens were nuclear-stained with DAPI (Life Technologies) and observed with an IX73 inverted microscope (Olympus, Tokyo, Japan).

3. Results

3.1. Gene expression profile of cytokeratin in mouse lacrimal gland germ epithelium

To investigate the levels of gene expression of cytokeratin in the mouse lacrimal gland epithelium, we first performed Q-PCR analysis. We used separated epithelial and mesenchymal tissues of E16.5 mouse-lacrimal gland germs, mouse ESCs, and 7w LG for the extraction of mRNA (Fig. 1a). We analyzed the expression levels of a series of cytokeratins. Q-PCR revealed that the relative mRNA levels of cytokeratin (KRT) 5, KRT8, KRT14, KRT15, KRT18 were higher in the E16.5 lacrimal gland germ epithelium (E16.5 LGE) than in mouse ESCs (Fig. 1b). The levels of KRT5 and KRT15 mRNA were expressed at high levels in E16.5 LGE. In the comparison among the E16.5 LGE, the E16.5 lacrimal gland germ mesenchyme (E16.5 LGM) and 7w LG, the relative RNA levels of these cytokeratins (KRT 5, 8, 14, 15, 18) were higher in E16.5 LGE than in E16.5 LGM and 7W LG. Interestingly, the level of KRT15 mRNA was much higher only in E16.5 LGE (Fig. 2). These results indicated that the relative RNA levels of KRT5, KRT8, KRT14, KRT15, KRT18 were higher in E16.5 epithelium than in E16.5 mesenchyme, and KRT15 was more highly expressed in embryonic developmental stages than in mature lacrimal gland tissue. These results indicated that the expression level of KRT15 increased in the differentiation from stem cells to lacrimal gland germ epithelium, then decreased as the lacrimal

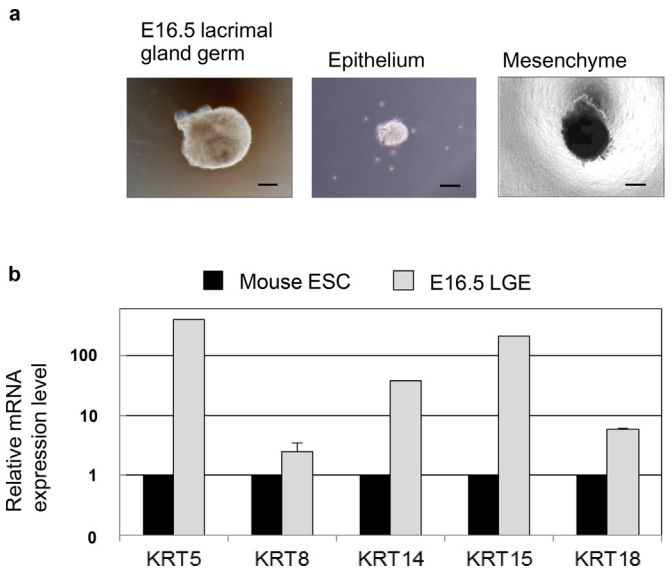

Fig. 1. mRNA expression in the lacrimal gland cells compared with that of mouse embryonic stem cells. **a.** Phase-contrast microscopic images of E16.5 mouse lacrimal gland germ. Images of whole (left), separated epithelium (center) and separated mesenchyme (right) are shown. Scale bar, 100 μm. **b.** Expression profiles of cytokeratins in E16.5 LGE and mouse ESCs. The error bars represent the mean ± standard deviation (SD) of three samples. LGE; lacrimal gland germ epithelium, ESCs; embryonic stem cells.