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Evaluation of Eph receptor and ephrin expression within the human cornea and limbus☆

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ABSTRACT

Eph receptor tyrosine kinases and their ligands, the ephrins, regulate the development and maintenance of multiple organs but little is known about their potential role within the cornea. The purpose of this study was to perform a thorough investigation of Eph/ephrin expression within the human cornea including the limbal stem cell niche. Initially, immunohistochemistry was performed on human donor eyes to determine the spatial distribution of Eph receptors and ephrins in the cornea and limbus. Patterns of *Eph/ephrin* gene expression in (1) immortalised human corneal endothelial (B4G12) or corneal epithelial (HCE-T) cell lines, and (2) primary cultures of epithelial or stromal cells established from the corneal limbus of cadaveric eye tissue were then assessed by reverse transcription (RT) PCR. Limbal epithelial or stromal cells from primary cultures were also assessed for evidence of Eph/ephrin-reactivity by immunofluorescence. Immunoreactivity for ephrinA1 and EphB4 was detected in the corneal endothelium of donor eyes. EphB4 was also consistently detected in the limbal and corneal epithelium and in cells located in the stroma of the peripheral cornea. Expression of multiple *Eph/ephrin* genes was detected in immortalised corneal epithelial and endothelial cell lines. Evidence of *Eph/ephrin* gene expression was also demonstrated in primary cultures of human limbal stromal (*EphB4, B6; ephrinA5*) and epithelial cells (*EphA1, A2; ephrinA5, B2*) using both RT-PCR and immunofluorescence. The expression of Eph receptors and ephrins within the human cornea and limbus is much wider than previously appreciated and suggests multiple potential roles for these molecules in the maintenance of normal corneal architecture.

1. Introduction

The optical properties of the cornea are dependent upon maintenance of a highly ordered tissue structure. Thus, conditions or diseases that disrupt normal corneal structure are invariably associated with vision loss (Cameron, 1983; Chang et al., 2001; Rabinowitz, 1998). The molecular mechanisms that establish and maintain normal corneal structure are, however, still not completely understood. This knowledge gap has lead us to consider the potential role of the Eph family of receptor tyrosine kinases and their ephrin ligands, since these molecules play important roles in establishing and maintaining tissue architecture within many organs (Egea and Klein, 2007; Pasquale, 2005). Eph receptors are the largest family of receptor tyrosine kinases but in contrast to other receptor tyrosine kinases which bind to soluble ligands, the ephrin ligands for Ephs are typically bound to the cell membrane of neighbouring cells (Davis et al., 1994). Two sub-families of Eph/ephrin molecules have been identified based upon shared structural or ligand binding properties. Generally, EphA receptors (EphA1-9) preferentially recognise ephrinA ligands that are bound to the cell surface via a glycosylphosphatidylinositol (GPI) anchor. Conversely, EphB receptors (EphB1–B4, B6) bind to transmembrane ephrinB ligands (ephrinB1–B3). Each mechanism allows for precise cell–cell signalling in a spatiotemporal manner. Activation of Eph/ephrin signalling pathways has been implicated

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in a variety of developmental and pathological processes via effects on tissue morphogenesis, cell migration, angiogenesis, cellular differentiation and tumour formation (Hafner et al., 2004; Kullander and Klein, 2002; Ruoslahti, 1999; Surawska et al., 2004).

The eye has been previously examined as a model of potential Eph/ephrin interactions, with the overwhelming majority of these studies focussed on the retina. For example, it has been reported that EphAs induce the collapse of growth cones and axon retraction in cultures of retinal ganglion cells (Knoll and Drescher, 2004). Likewise, there is convincing evidence that complimentary gradients of EphA receptors and ephrinA ligands are critical to the formation of topographic maps for the retina and superior colliculus (Knoll et al., 2001). Of the relatively few investigations conducted into Eph/ephrin molecules in the cornea, the majority have been concerned with aberrant growth of blood vessels (Kojima et al., 2007a,b). For example, EphB1-Fc chimeras induce neovascularisation in a mouse corneal micropocket assay (Huynh-Do et al., 2002). Likewise, immunohistochemical studies have provided evidence that EphB1 and ephrinB1 play important roles in regulating fibroblast growth factor (FGF) induced corneal angiogenesis (Kojima et al., 2007b). In addition, it has been shown that elevated *ephrinB2* expression enhances the growth of blood vessels over the mouse cornea *in vivo* (Maekawa et al., 2003). Recently, evidence has emerged that EphA2 suppresses corneal epithelial cell migration when stimulated by ephrinA1 ligand (Kaplan et al., 2012). Nevertheless, there remains a general paucity of data available regarding the expression patterns and potential functions of Eph/ephrin molecules within the cornea. The purpose of this investigation was therefore to perform a comprehensive study of Eph/ephrin expression within the human cornea. Data concerning the spatial distribution of Eph receptors and ephrin ligands within the cornea and limbus of donor eyes was assessed by immunohistochemistry. Patterns of *Eph/ephrin* gene expression were then determined in primary cultures of limbal stromal cells and epithelial cells as well as immortalised ocular cell lines. The results of this study demonstrate a much wider expression profile for Eph/ephrins within the anterior segment of the eye than has been previously appreciated. In particular novel findings are reported with respect to the corneal endothelium which may have important implications for future studies of this tissue.

2. Materials and methods

2.1. Ocular tissue

Human ocular tissue was used after acquiring donor consent and ethics approvals from the Princess Alexandra Hospital and the Queensland University of Technology (ref: 0800000807). The tissue was received as whole eyes, intact corneoscleral caps (the cornea and 1–2 mm of scleral rim), or as corneoscleral rims produced during penetrating keratoplasty. Caps were provided by the Queensland Eye Bank, Queensland Health. Rims were collected post-surgery from the Queensland Eye Centre. The ocular tissue was from both male and female donors aged between 65 and 85 years and was fixed in formalin immediately following receipt within two days of death for use in immunohistochemistry or stored in Optisol (Bausch & Lomb, Inc. Rochester, NY, USA) for between one and four days before being used to establish primary cell cultures.

2.2. Cell culture

2.2.1. Immortalised human ocular cell lines

Profiles of *Eph/ephrin* gene expression were characterised in immortalised human cell lines derived from the corneal epithelium (HCE-T; Riken Cell Bank) or corneal endothelium (B4G12; a gift from Dr Monika Valtink) and cultured as described previously (Harkin et al., 2011; Madden et al., 2011). An immortalised retinal pigment epithelial cell line (ARPE-19; ATCC Cat No. CRL-2302) was also included in this study as a example of a cell line derived from ocular tissue known to express *Eph/ephrins* (Dunn et al., 1996; Tian et al., 2005). HCE-T cell cultures were propagated in DMEM High Glucose (Invitrogen, Mulgrave, VIC, Australia) supplemented with 10% foetal bovine serum (FBS; Thermo Scientific, Scoresby, VIC, Australia), 1% penicillin/streptomycin (PS) and 400 μM L-glutamine (both Invitrogen. The B4G12 cell line was cultured in Opti-MEM-I media (Invitrogen) supplemented with 8% FBS, 5 ng/mL EGF, 20 ng/mL NGF, 100 μg/mL bovine pituitary extract, 20 μg/mL ascorbic acid, 200 μg/mL $CaCl_2$ and 0.08% chondroitin sulphate (all from Invitrogen) (Madden et al., 2011). ARPE-19 cells were cultured in a 1:1 mixture of DMEM/F12 [ATCC; Cat. No. 30-2006] supplemented with 2.5 mM L-glutamine, 1.5 g/L sodium bicarbonate and 10% FBS [ATCC; Cat. No. 30-2020]. Immortalised cell lines were seeded (1×10^5 cells) into T75 culture flasks (Iwaki, Crown, Scientific, Minto, NSW, Australia) and fed with fresh media every 3 days until approximately 80–90% confluency was achieved. Cultures were incubated at 37 °C with 5% CO_2/95% atmospheric air for the duration of the culture period.

2.2.2. Primary human limbal cell cultures

2.2.2.1. Human limbal epithelial (HLE) cells in 3T3 co-culture. HLE cells for this study were established with or without serum supplement and murine 3T3 fibroblasts (ATCC; CCL-92, Manassas, VA, USA) as feeder cells. Isolation and phenotyping of human HLE cells was performed as described previously (Ainscough et al., 2011). HLE cell cultures (passage p0 or p1) obtained from human donors ($n = 3$) were established in T75 culture flasks in DMEM supplemented with 10% FBS as described previously (Ainscough et al., 2011). Murine 3T3 fibroblasts required for co-culture with HLE cells were also initially propagated in DMEM supplemented with 10% FBS. Immediately prior to co-culture with HLE cells, 3T3 fibroblasts were gamma-irradiated (2×25 gray) to inhibit further proliferation. Primary HLE cell cultures were established in T75 culture flasks by seeding freshly isolated cells (1×10^5 cells) in the presence of 2×10^6 irradiated 3T3 feeder cells and provided with fresh media every 3 days until approximately 80–90% confluency was achieved. Cell cultures were incubated at 37 °C with 5% CO_2/95% atmospheric air. For control purposes, cultures of murine 3T3 fibroblasts without HLE cells were also established.

2.2.2.2. Human limbal epithelial cells (3T3, serum-free). HLE cell cultures were also established in serum-free medium in the absence of 3T3 cells. Specifically, HLE cell cultures ($n = 3$) were established using Defined Keratinocyte Serum-Free Medium (DK-SFM; Invitrogen) containing 1% PS solution (Bray et al., 2011). Primary HLE serum-free cell monocultures were established in T75 culture flasks by seeding freshly isolated cells (1×10^5 cells) that were provided with fresh media every 3 days for 14 days. Cell cultures were incubated at 37 °C with 5% CO_2/95% atmospheric air.

2.2.2.3. Human limbal stromal (HLS) cells. HLS cells were isolated from human donors ($n = 3$). Isolation and phenotyping of human HLS cells was performed as described previously (Ainscough et al., 2011). Briefly, HLS cultures prepared by methods used in the current study displayed a range of phenotype markers for fibroblasts and myofibroblasts (CD90, vimentin, alpha smooth muscle actin) but were negative for markers of neuronal cells (CD271), blood cells (CD45), endothelium (CD141; CD34) or epithelial cells (CD141). HLS cultures (passage (p) 0 or p1) were propagated in DMEM/F12 + GlutaMAX (Invitrogen) supplemented with 10% FBS