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The keratocyte: Corneal stromal cell with variable repair phenotypes

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Abstract

Keratocytes, also known as fibroblasts, are mesenchymal-derived cells of the corneal stroma. These cells are normally quiescent, but they can readily respond and transition into repair phenotypes following injury. Cytokines and other growth factors that provide autocrine signals for stimulating wound responses in resident cells are typically presented by platelets at the site of an injury. However, due to the avascular nature of the cornea many of the environmental cues are derived from the overlying epithelium. Corneal epithelial-keratocyte cell interactions have thus been extensively studied in numerous in vivo corneal wound healing settings, as well as in in vitro culture models. Exposure to the different epithelial-derived factors, as well as the integrity of the epithelial substratum, are factors known to impact the keratocyte response and determine whether corneal repair will be regenerative or fibrotic in nature. Finally, the recent identification of bone-marrow derived stem cells in the corneal stroma suggests a further complexity in the regulation of the keratocyte phenotype following injury.

Keywords

Keratocyte; Cornea; Injury; Apoptosis; Myofibroblasts; Transparency; Plasticity

Cell facts

- Keratocytes are neural-crest derived mesenchymal cells that sparsely populate the corneal stroma.
- Keratocytes are quiescent, dendritic cells that upon injury to the cornea are stimulated to either undergo apoptosis or transition into repair phenotypes.
- Structural integrity of the basement membrane is critical for minimizing the fibrotic response of keratocytes and subsequent scarring and loss of corneal clarity.
- Bone-marrow derived stem cells have been detected in the corneal stroma and may participate in the activation of the keratocytes following injury.

1. Introduction

The cornea is a highly specialized transparent tissue located at the anterior most surface of the eye. It provides two-thirds of the optical power of the eye, refracting and focusing incident

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light on the retina and plays a protective role in the eye by acting as an external barrier to infectious agents. The cornea is composed of three tissue layers: the outer stratified squamous epithelium, the inner endothelium, and the intermediate stroma (Fig. 1). The stroma makes up 90% of the corneal thickness and is comprised of a heterodimeric complex of type I and type V collagen fibers, which are arranged in bundles referred to as lamellae (Fini & Stramer, 2005). The parallel arrangement of the lamellae as well as the uniform spacing of the fibers, are thought to result in “destructive interference” of incoming light rays, thereby reducing scatter and promoting corneal transparency.

Situated between the collagen lamellae in the stroma are the keratocytes, or fibroblasts, which are a population of quiescent, mesenchymal-derived cells of the mature cornea (Hay, 1979). These cells exhibit a slow turnover and are sparsely arranged in the stroma, yet they form an interconnected cellular network with one another through dendritic processes (Muller, Pels, & Vrensen, 1995). Keratocytes also contain crystallins; highly expressed proteins that are known to contribute to the transparent nature of the cornea (Jester et al., 1999a). Upon injury, keratocytes are stimulated to either undergo cell death or to lose their quiescence and transition into repair phenotypes. These repair phenotypes can either promote regeneration or they can induce fibrotic scar formation, the latter of which is detrimental to the otherwise transparent cornea (Fini & Stramer, 2005). Recently, there has been a keen interest in the response of keratocytes to injury due to the expansion in development and application of keratorefractive surgeries for correcting vision.

2. Keratocyte origin and plasticity

The embryonic development of the cornea has been studied in numerous species, however, the chick model has been extensively used to examine the formation of the corneal stroma (Hay, 1979). Following separation of the lens from the overlying ectoderm the primitive corneal epithelium secretes an acellular primary stroma, consisting of loosely arranged collagen fibrils (Hay, 1979; Hendrix, Hay, von der Mark, & Linsenmayer, 1982). This is followed by two waves of neural crest cell migration: the first wave of mesenchymal cells forms the endothelial cell layer, and the second wave invades the primary stroma and forms the corneal keratocyte population (Hay, 1979). In contrast to the chick, other species, such as rodents, exhibit only a single influx of mesenchymal cells, which contributes to both corneal stromal and endothelial cells (Cintron, Covington, & Kublin, 1983).

Following their migration, embryonic keratocytes begin to synthesize and secrete extracellular matrix components consisting of collagens types I, V, VI (Fini, 1999; Linsenmayer et al., 1983), as well as keratan sulfate, which exhibits a unique tissue-specific form of glycosylation in the cornea (Funderburgh, Mann, & Funderburgh, 2003). Between birth and eyelid opening, the number of proliferating keratocytes in the developing corneal stroma decreases dramatically from 20% of the total to nearly zero (Zieske, 2004). At the time of eyelid opening, keratocytes have withdrawn from the cell cycle, remaining in G0 rather than undergoing complete terminal differentiation (Zieske, 2004).

Upon injury to the cornea, keratocytes can transition into divergent phenotypes, which are dependent on specific environmental signals. Since keratocytes originate from a population of cranial neural crest cells, it has been postulated that some of the regenerative properties exhibited by these cells following wounding may be attributed to their stem cell-like properties, which were retained from their cell of origin. Recent experiments using quail and chick chimera grafts have shown that late-stage embryonic stromal keratocytes, when introduced into the neural crest population of earlier embryos, do not mix with the host population of crest cells. However, these cells proliferate, migrate and de-differentiate, thus contributing to a number of the neural-crest derived populations including corneal endothelial cells, keratocytes and the

musculature of the eye (Lwigale, Cressy, & Bronner-Fraser, 2005). These findings, in combination with the fact that adult keratocytes remain in G0, suggest that they may not be terminally differentiated cells but rather they may represent partially restricted precursors that can readily respond to reform the native corneal stroma and restore its transparent properties.

3. Functions: keratocyte response to corneal injury

3.1. Keratocyte cell death

One of the first observable changes in the corneal stroma following injury is death of a subpopulation of keratocytes (Wilson, Netto, & Ambrosio, 2003) (Fig. 2). In the case of corneal epithelial debridement wounds, where the epithelium is scraped away, leaving the underlying basement membrane, the keratocytes immediately beneath the basement membrane undergo apoptosis. Shortly after death, these cells are replaced by new keratocytes through mitosis of adjacent cells, and consequently no further keratocyte response occurs (Fini & Stramer, 2005; Wilson et al., 2003). This initial keratocyte cell death is a benign response, thought to have evolved in order to protect the cornea from further inflammation, and subsequent loss of transparency.

The region and extent of cell death in the corneal stroma appears to be dependent on the type of injury induced and the species (Jester, Petroll, & Cavanagh, 1999b). In a wound created by photorefractive keratectomy (PRK), the corrective surgical procedure in which corneal epithelial debridement is followed by laser ablation of the basement membrane and the corneal stroma, keratocyte apoptosis is typically observed in the superficial corneal stroma. Yet, in some species such as the rabbit, PRK can induce apoptosis that extends up to 85 μm into the stroma (Jester et al., 1999b). Laser in situ keratomileusis (LASIK) is a newer corrective procedure, in which a flap of the epithelium and basement membrane is initially peeled back by a microkeratome and laser ablation is subsequently performed on the underlying stroma. In LASIK, keratocyte death is found to occur deeper in the stroma and is usually restricted to the anterior and posterior lamellar face created by the microkeratome (Wilson et al., 2003).

Earlier studies have suggested that cytokines such as interleukin 1 (IL-1) and tumor necrosis factor alpha (TNF α), secreted from the overlying epithelium, may modulate the apoptosis of keratocytes (Wilson et al., 2003). Specifically, IL-1 is thought to induce keratocyte death by acting as a stimulator for autocrine production of the FAS ligand, an apoptotic mediator (Wilson et al., 2003). This hypothesis was based upon the fact that keratocyte apoptosis was decreased in FAS deficient mice in response to corneal epithelial injury. However, additional work has shown that when cultured keratocytes are treated with IL-1 α for 24 h they do not undergo cell death (Huang, Stramer, Fini, & Jester, 2003; West-Mays, Strissel, Sadow, & Fini, 1995). In fact, TUNEL staining in IL-1 α -treated keratocytes has not been observed until 7 days following treatment and this was correlated with a developmental transition of the cells that rendered them sensitive to IL-1 α (Huang et al., 2003). Thus, further studies are required to determine why some keratocytes are vulnerable to IL-1 α induced apoptosis while others are not and the additional factors that may regulate the apoptotic response.

3.2. Keratocyte transition to an “activated” phenotype

In penetrating keratectomy injuries in which the basement membrane is disrupted, initial keratocyte cell death in the cornea is followed by further transition of a subpopulation of remaining keratocytes to a repair or “activated” phenotype. Approximately 6 h after injury, the activated keratocytes lose their quiescence, enter into the cell cycle and subsequently migrate to the site of injury. Their cell size and organelle content increase and they begin to exhibit many morphological characteristics of fibroblasts, including a fusiform shape, multiple nucleoli and a lack of cytoplasmic granules (Fini & Stramer, 2005). A cell culture model for

examining the activation of fibroblasts has been devised and closely recapitulates the change in phenotype of keratocytes during wounding (Fini & Stramer, 2005). In this model, keratocytes are isolated from the uninjured stroma and maintained in serum free condition in which they retain many of the in vivo characteristics of quiescent keratocytes. This includes their dendritic morphology and interconnecting pseudopodia. Addition of serum and further passaging of these cells results in their activation involving the onset of features described above. Other changes included changes in gene expression, such as an induction in fibronectin and the matrix metalloproteinase (MMP), collagenase. Changes in MMP expression are thought to be responsible for remodeling of the extracellular matrix (Fini & Stramer, 2005). Finally, repair transition is also associated with a loss in expression of the corneal crystallins, transketolase and aldehyde dehydrogenase 1A1 (Jester et al., 1999a).

The use of the in vitro culture model, as well as further confirmation with in vivo corneal wound models, has revealed that a number of growth factors and cytokines contribute to the activation of keratocytes. For example, IL-1 α , expressed by the corneal epithelium and released into the stroma upon injury to the epithelial substratum, has been identified as a required mediator of collagenase gene expression in activated fibroblasts (Fig. 2). With time and addition of serum into the culture media, the cells obtain competency for autocrine IL-1 α synthesis, and this is referred to as the "IL-1 α loop" (West-Mays et al., 1995). Expression of the IL-1 α loop is also detected in activated keratocytes isolated from the cornea 7 days following a keratectomy injury, a time at which re-epithelialization and basement membrane reformation has occurred. Thus, ongoing secretion of autocrine IL-1 α enables activated keratocytes to continue with the remodeling of the repair tissue in the absence of epithelial secreted IL-1 α . Further studies have shown that the competence of activated keratocytes to express IL-1 α is dependent on the expression of the required signaling intermediate NF κ B (Fini & Stramer, 2005).

3.3. Transition to the myofibroblast phenotype

The myofibroblast is another keratocyte phenotype observed in the corneal stroma following injury and was first identified in skin wounds (Jester et al., 1999b). These cells are a subpopulation of activated fibroblasts defined by their larger appearance and expression of alpha smooth muscle actin (α SMA). Corneal myofibroblasts, found directly within the wound, are thought to be responsible for wound contraction as well as for ECM deposition and organization during corneal repair. Transforming growth factor beta (TGF β) is a cytokine released by corneal epithelial cells that can control the behavior of repair fibroblasts. Serum-free cultured keratocytes exposed to TGF β , exhibit a transformation into myofibroblasts, which is specific for TGF β since co-treatment with blocking antibodies to TGF β has shown to inhibit this transformation. TGF β has thus been identified as the potent inducer of myofibroblast transformation in corneal keratocytes (Jester et al., 1999b). Despite this transformation additional work has revealed that activated corneal fibroblasts and myofibroblasts are not terminally differentiated phenotypes, and have the ability to transition into one another (Maltseva, Folger, Zekaria, Petridou, & Masur, 2001).

A mouse model for penetrating keratectomy has recently been developed and demonstrated that following a disturbance to the basement membrane, TGF β 2 is released by the corneal epithelium into the corneal stroma (Fig. 2). Once in the stroma, TGF β 2 induces the transformation of keratocytes into myofibroblasts (Stramer, Zieske, Jung, Austin, & Fini, 2003). Organotypic cultures have further shown that when corneal epithelial cells are cultured on an artificial stromal surface, where they are unable to synthesize and deposit a basement membrane, these cells release TGF β 2 into the stroma, where keratocytes are stimulated to transition into the myofibroblast phenotype (Stramer et al., 2003). When basement membrane synthesis is stimulated in this model, the fibrotic phenotype does not occur and TGF β 2 remains in the epithelium (Fig. 2). Further evidence in support of the notion that the basement membrane

controls the fibrotic response includes mouse mutants with compromised basement membranes, such as the *Le-AP-2 α* mice, a recently developed conditional knockout of the transcription factor *AP-2 α* (Dwivedi et al., 2005). In the *Le-AP-2 α* mice, the basement membrane exhibits intermittent breaks, beneath which TGF β 2 is detected in the stroma, and α SMA immunoreactivity is evident, indicative of transformation of the resident keratocytes into myofibroblasts (Fig. 3). These mice also exhibited aberrant keratocyte proliferation and immunoreactivity to phospho-Smad2, a downstream signaling molecule of TGF β .

The presence of myofibroblasts in the cornea in vivo following PRK, has also been confirmed and has been correlated with a dramatic increase in the level of stromal haze (Jester et al., 1999b). Decreased myofibroblast reflectivity, as the cells begin to disappear, also correlates with reduced haze in the rabbit PRK model. The appearance of these cells in vivo can also be attributed to TGF β since topical treatment of rabbit PRK wounds with TGF β blocking antibodies revealed a dramatic reduction in myofibroblast appearance as well as a decrease in corneal haze. Together, these data show that the basement membrane plays a vital role in maintaining corneal homeostasis and minimizing the fibrotic response by controlling the release of TGF β 2 into the stroma.

4. Stem cells in the corneal stroma

Recent attention has been focused on the plasticity of bone marrow (BM) derived stem cells. These cells are reported to have extensive differentiation capacities in a variety of tissues, including those of the eye. Specifically in the cornea, “wandering cells” have been detected in the stroma that are believed to be BM derived antigen presenting stem cells from different lineages such as dendritic cells and macrophages (Nakamura, Kurosaka, Bissen-Miyajima, & Tsubota, 2001). It has been postulated that the “wandering cells” within the stroma could be important in releasing factors that aid in the activation of the keratocytes following injury (Fini & Stramer, 2005; Hennessy, Korbling, & Estrov, 2004). Current studies have also shown the presence of additional cell types in the adult corneal stroma that express stem cell markers, have the ability to divide extensively and generate adult keratocytes (Du, Funderburgh, Mann, SundarRaj, & Funderburgh, 2005). These keratocyte progenitor cells express the ocular development gene *Pax6*, which is not expressed by the resident stromal keratocytes (Funderburgh, Du, Mann, SundarRaj, & Funderburgh, 2005). Further investigation of these potential stem cells in the corneal stroma will undoubtedly reveal further complexity in the regulation of activated keratocyte phenotypes and may have important implications for cell based therapies in corneal disease.

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References

- Cintron C, Covington H, Kublin CL. Morphogenesis of rabbit corneal stroma. *Invest Ophthalmol Vis Sci* 1983;24(5):543–556. [PubMed: 6841000]
- Du Y, Funderburgh ML, Mann MM, SundarRaj N, Funderburgh JL. Multipotent stem cells in human corneal stroma. *Stem Cells* 2005;23(9):1266–1275. [PubMed: 16051989]
- Dwivedi DJ, Pontoriero GF, Ashery-Padan R, Sullivan S, Williams T, West-Mays JA. Targeted deletion of AP-2 α leads to disruption in corneal epithelial cell integrity and defects in the corneal stroma. *Invest Ophthalmol Vis Sci* 2005;46(10):3623–3630. [PubMed: 16186342]
- Fini ME. Keratocyte and fibroblast phenotypes in the repairing cornea. *Prog Retin Eye Res* 1999;18(4): 529–551. [PubMed: 10217482]

- Finis ME, Stramer BM. How the cornea heals: cornea-specific repair mechanisms affecting surgical outcomes. *Cornea* 2005;24(8):S2–S11. [PubMed: 16227819]
- Funderburgh JL, Mann MM, Funderburgh ML. Keratocyte phenotype mediates proteoglycan structure: a role for fibroblasts in corneal fibrosis. *J Biol Chem* 2003;278(46):45629–45637. [PubMed: 12933807]
- Funderburgh ML, Du Y, Mann MM, SundarRaj N, Funderburgh JL. PAX6 expression identifies progenitor cells for corneal keratocytes. *Faseb J* 2005;19(10):1371–1373. [PubMed: 15901670]
- Hay ED. Development of the vertebrate cornea. *Int Rev Cytol* 1979;63:263–322. [PubMed: 395131]
- Hendrix MJ, Hay ED, von der Mark K, Linsenmayer TF. Immunohistochemical localization of collagen types I and II in the developing chick cornea and tibia by electron microscopy. *Invest Ophthalmol Vis Sci* 1982;22(3):359–375. [PubMed: 7037675]
- Hennessy B, Korbly M, Estrov Z. Circulating stem cells and tissue repair. *Panminerva Med* 2004;46(1):1–11. [PubMed: 15238877]
- Huang J, Stramer B, Finis ME, Jester JV. IL-1 α induces human corneal fibroblast but not cultured corneal keratocyte apoptosis through a metalloproteinase and TGF- β regulated cascade. *Invest Ophthalmol Vis Sci* 2003;44:872. ARVO-E abstract
- Jester JV, Moller-Pedersen T, Huang J, Sax CM, Kays WT, Cavanagh HD, Petroll WM, Piatigorsky J. The cellular basis of corneal transparency: evidence for ‘corneal crystallins’. *J Cell Sci* 1999;112(Pt 5):613–622. [PubMed: 9973596]
- Jester JV, Petroll WM, Cavanagh HD. Corneal stromal wound healing in refractive surgery: the role of myofibroblasts. *Prog Retin Eye Res* 1999;18(3):311–356. [PubMed: 10192516]
- Linsenmayer TF, Fitch JM, Schmid TM, Zak NB, Gibney E, Sanderson RD, Mayne R. Monoclonal antibodies against chicken type V collagen: production, specificity, and use for immunocytochemical localization in embryonic cornea and other organs. *J Cell Biol* 1983;96(1):124–132. [PubMed: 6186670]
- Lwigale PY, Cressy PA, Bronner-Fraser M. Corneal keratocytes retain neural crest progenitor cell properties. *Dev Biol* 2005;288(1):284–293. [PubMed: 16263107]
- Maltseva O, Folger P, Zekaria D, Petridou S, Masur SK. Fibroblast growth factor reversal of the corneal myofibroblast phenotype. *Invest Ophthalmol Vis Sci* 2001;42(11):2490–2495. [PubMed: 11581188]
- Muller LJ, Pels L, Vrensen GF. Novel aspects of the ultrastructural organization of human corneal keratocytes. *Invest Ophthalmol Vis Sci* 1995;36(13):2557–2567. [PubMed: 7499078]
- Nakamura K, Kurosaka D, Bissen-Miyajima H, Tsubota K. Intact corneal epithelium is essential for the prevention of stromal haze after laser assisted in situ keratomileusis. *Br J Ophthalmol* 2001;85(2):209–213. [PubMed: 11159488]
- Stramer BM, Zieske JD, Jung JC, Austin JS, Finis ME. Molecular mechanisms controlling the fibrotic repair phenotype in cornea: implications for surgical outcomes. *Invest Ophthalmol Vis Sci* 2003;44(10):4237–4246. [PubMed: 14507867]
- West-Mays JA, Strissel KJ, Sadow PM, Finis ME. Competence for collagenase gene expression by tissue fibroblasts requires activation of an interleukin 1 α autocrine loop. *Proc Natl Acad Sci USA* 1995;92(15):6768–6772. [PubMed: 7624317]
- Wilson SE, Netto M, Ambrosio R Jr. Corneal cells: chatty in development, homeostasis, wound healing, and disease. *Am J Ophthalmol* 2003;136(3):530–536. [PubMed: 12967809]
- Zieske JD. Corneal development associated with eyelid opening. *Int J Dev Biol* 2004;48(8–9):903–911. [PubMed: 15558481]

Fig. 1. Morphology of the cornea. Hematoxylin and eosin stained cross-sections of the adult mouse cornea showing the three layers of the cornea, the stratified corneal epithelium (CE), the collagenous stroma (S) and the endothelium (E). The keratocytes (arrowheads) are situated between the collagen lamellae in the stroma.

Fig. 2.

Cellular interactions during corneal repair. (A) Upon corneal epithelial injury, IL-1 α is released from the injured epithelium into the stroma. IL-1 α induces some of the underlying stromal keratocytes to undergo cell death, while others are induced to proliferate, secrete MMPs, and transition from a quiescent to an activated phenotype. Due to the absence of a basement membrane, corneal epithelial cells also secrete TGF β 2 into the underlying stroma inducing a subpopulation of keratocytes to undergo transformation into myofibroblasts that secrete ECM. (B) The return of the basement membrane inhibits the release of TGF β 2 into the stroma and the myofibroblast phenotype is no longer observed. The activated keratocytes continue to secrete autocrine IL-1 α and remodel the ECM. *Abbreviations:* MMPs, matrix metalloproteinase; TGF β 2, transforming growth factor beta; ECM, extracellular matrix; IL-1 α , interleukin 1 alpha.

Fig. 3.

Markers of fibroblast activation are evident in the *Le-AP-2α* corneal stroma. (A) The proliferating status of the corneal stromal cells was determined by immunostaining for proliferating cell nuclear antigen (PCNA). Little to no proliferative response was observed in the stroma of the wild-type cornea. In contrast, stromal fibroblasts (arrowhead) in the *Le-AP-2α* mutant cornea exhibited immunoreactivity for PCNA, particularly in regions where the basement membrane was absent. (B) The activated response was further examined by immunofluorescent localization of α -smooth muscle actin (α -SMA) using a CY3-conjugated secondary antibody (red). Cell nuclei were stained with DAPI (blue) to delineate the location of cells. The wild-type corneas did not exhibit any α -SMA protein expression, whereas some fibroblasts beneath the epithelium in the *Le-AP-2α* mutant corneas showed immunoreactivity to α -SMA. (C) Transforming growth factor- β 2 (TGF- β 2) was immunolocalized using a FITC-conjugated secondary antibody (green). In wild-type mice TGF- β 2 staining was absent in the corneal stroma and only evident in the corneal epithelium. In the *Le-AP-2α* mutant cornea, both the corneal epithelium and stroma (arrowhead) stained positively for TGF- β 2 (green). (D) Immunostaining for phospho-Smad2 (p-Smad2), a downstream effector of TGF- β 2 signaling, showed that in wild-type mice, p-Smad2 was localized only in the epithelium, whereas in the

Le-AP-2α mutant cornea both the stroma (arrowhead) and epithelium showed immunoreactivity to p-Smad2. *Abbreviations:* CE, corneal epithelium; S, stroma.