The Multipotential Cells of the Limbus

RICHARD A. THOFT, LEE A. WILEY and NIRMALA SUNDARRAJ

Pittsburgh, USA

Summary

Ample evidence exists that there is a centripetal movement of cells from the periphery of the cornea toward the centre. While conjunctival cells have the capacity to transdifferentiate into corneal epithelial cells, the limbal region appears to act as a barrier between the conjunctival and corneal epithelia, even after large epithelial defects are created.

The existence of limbal stem cells is suggested by the apparent role of the limbus in acting as a source of peripheral corneal cells. While specific staining of limbal cells has been reported in the rabbit, there is no positive identification of such stem cells in the human. However, in the human there is negative staining for both a keratin cytoskeleton antigen and a cell surface antigen in the limbal epithelial zone. Efforts positively to identify human limbal stem cells continue, as do efforts to culture and transplant such cells.

Only recently has the limbus been identified as a possible source of epithelial cells for replenishment of the corneal epithelial mass. This report will review the observations, both clinical and experimental, which have led to this conclusion, and will also indicate further directions for research and the possible clinical applications of limbal therapy.

In 1977, Thoft and Friend¹ emphasised the

potential interdependence of the corneal and conjunctival epithelial layers by coining the term 'the ocular surface' (Fig. 1). This phrase encourages discussing the covering of the globe and the lids as a unit, and suggests their shared characteristics rather than their differences. The ocular surface includes the epithelium from the lash line onto the back of the lids, into the fornices, and its reflection back over the globe, including that which covers the cornea. This term has found broad acceptance in reference to conditions such as drying, toxicity, and scarring which tend to affect both the conjunctival and corneal regions.

The inter-relationships between conjunctival and corneal epithelium become of increasing interest as the similarities of these two regions are emphasised. As experimental evidence has accumulated, the limbus has emerged as the source of cells for ocular surface renewal.

Fig. 1. The ocular surface is a term used to describe the continuous epithelium which begins at the lash line, extends onto the back of the lids, into the fornices, and onto the globe. It includes the conjunctival, limbal, and corneal epithelia.

Corneal Re-epithelialisation

While earlier work 40 years ago suggested that corneal defects could be healed from the conjunctiva,² it was not until some years later that Shapiro *et al.* first described the histologic transformation of conjunctival into corneal epithelium.³ The invariable change from the conjunctival to the corneal phenotype progresses through several stages following re-epithelialisation of a corneal defect. These include an histologically undifferentiated initial phase showing neither goblet cells or corneal epithelial basal cells; a phase with numerous and large goblet cells scattered throughout the epithelium; a later phase with multicentric nests of corneal cells in the midst of the evanescent conjunctival cells; and finally, a histologically normal corneal epithelium.

This conversion from one highly differentiated cell type to a second, different, highly differentiated cell type was called 'trans-differentiation'. This seems to be a better term than either 'de-differentiation' or 'metaplasia', since these latter phases have come to suggest a loss of biologic specificity.⁴ It should be emphasised that this transdifferentiation took place from conjunctival cells, since the limbal epithelium was removed along with the corneal epithelium. This observation weakens the hypothesis that corneal epithelium can originate solely from the limbus, since conjunctiva peripheral to the limbus also seems capable of transdifferentiation.

Differential Growth Characteristics of Corneal Epithelium

In a series of experiments in cell culture, Ebato *et al.* have shown that human corneal epithelium replicates at different rates, depending upon its location.^{5,6} Central corneal epithelium migrates off explants, but shows very few mitoses. As the periphery of the cornea is approached, explants show increasing vigour of the epithelium, with the highest rate of mitosis and shortest doubling time found at the limbus. While a gradient of mitotic activity was not found in earlier *in vitro* labelling experiments in whole human globes,⁷ preliminary observations by us in human corneal-scleral preparations that are

tritium-labeled *in vitro* suggest that there is increased mitotic activity in the limbal region.

X, Y, Z Hypothesis of Centripetal Corneal Epithelial Movement

The potential for transdifferentiation shown by conjunctival epithelium was consistent with the hypothesis that the corneal epithelial mass could be maintained, in part, by a centripetal migration of cells from the conjunctiva, across the limbus, and onto the central cornea.⁸

There were two clinical observations which supported this concept. The first was the movement of cysts, related to sutures, onto the donor cornea after keratoplasty, a direction which implied a centripetal movement of the cell sheet.⁹ A second study in a large population of keratoplasty patients revealed the absence of epithelial graft reactions after about one year, despite the continued occurrence of endothelial immunological reactions.¹⁰ The inference from these observations could be that none of the donor epithelium remains to serve as a locus for an immunologic reaction.

The inference is supported with experimental work by Kinoshita,¹¹ who showed that the epithelium of lamellar keratoplasties in rabbits was replaced by epithelium from the host in about a year. Also supportive of the concept of significant central sheet movement is the work of Buck,¹² who marked the epithelium and its subjacent stroma with India ink. He noted that the epithelium soon moved centrally, leaving behind the ink which remained in the stroma.

The Role of Conjunctiva in Corneal Epithelial Maintenance

While the movement of cells from the periphery of the cornea seems well established, the source of these cells is not certain. In the original X, Y, Z hypothesis it was not necessary to ascribe an origin for the cells. Because transdifferentiation from conjunctival epithelium into corneal epithelium can occur in rabbits, it was assumed that cells from the conjunctiva could simply drift across the limbus to provide either acute or chronic replacement of peripheral corneal cells.

A series of experiments was done to deter-

mine the role of conjunctiva in corneal epithelial wound healing.¹³ There was an increased mitotic response in conjunctiva peripheral to even 5 mm corneal epithelial defects. However, the increased mitotic activity lasted for only two days, even with an epithelial removal which extended outside the

limbus for 2–3 mm. For the large defects, epithelial healing was not completed before the conjunctiva peripheral to the limbus resumed its baseline level of mitotic activity. This must indicate that there is not a major drift of cells across the limbus during re-epithelialisation of the cornea, with the further implication

Fig. 2. Indirect immunofluorescence staining of normal human cornea and limbus.

(a) Reacted with a monoclonal antibody (AE5) against corneal keratin.

(b) Corresponding phase-contrast photomicrograph.

An area of staining of apical cells with AE5 is seen in the limbal region at the left of the montage. A region of approximately 2.0 mm encompassing limbal and peripheral cornea is devoid of staining. The photomicrograph to the right of the montage demonstrates dense staining of all the layers of central epithelium. Reaction with the monoclonal antibody begins in the apical cells, extending to the basal cells as central cornea is approached.

The areas in a and b, delimited by the rectangle, are shown at a greater magnification in Fig. 3.

(c) Reacted with a monoclonal antibody (HE1/11F) against an antigen associated with epithelial plasma membrane.

(d) Corresponding phase-contrast photomicrograph.

A pattern of staining similar to that of AE5 is present for HE1/11F. A 2.0 mm wide region of limbal and peripheral cornea is devoid of staining. Reaction with HE1/11F begins in the apical epithelial cells and extends to all layers of the epithelium in the central cornea.

The areas in c and d, delimited by the rectangle, are shown at a greater magnification in Fig. 4.

Fig. 3. Enlargement of the region delimited by the rectangle in Fig. 1a and b (reacted with the monoclonal antibody AE5).

that the limbal area is the customary locus for cells which do move onto the peripheral cornea.

The Limbus as the Locus of Multipotential Epithelial Stem Cells

As evidence accumulates that pinpoints the limbus as the source of some of the peripheral corneal epithelial cells, attempts continue to identify these cells. The most useful study to date is that of Schermer, Galvin, and Sun.¹⁴ They demonstrated that a 64K keratin was not found in the basal cells at the limbus in rabbits, but that it appeared in the superficial cells in that region, as well as in all the cells of the central epithelium. Their hypothesis was that the non-staining cells at the base of the limbal region were the stem cells, and that the daughters of these cells showed not only the accumulation of the 64K keratin, but also had a great capacity to divide. As these 'transient amplifying' cells move toward the centre of the cornea, they become terminally differentiated and expressed the characteristic keratin pairs of mature corneal epithelium.

A second study relating to identification of limbal stem cells was that of Bukusoglu and Zieske,¹⁵ who reported finding a specific

monoclonal antibody that specifically binds basal cells in the rabbit limbal epithelium. It is possible that this positive affinity may identify the limbal stem cells with certainty.

Human Limbal Antigen Studies

In an attempt to confirm the observations of Schermer, Galvin and Sun, we have evaluated the cells of the human limbus in corneal-scleral preparations from eye bank eyes. As shown in Fig. 2a and b, the use of AE5 (kindly supplied by Dr. Sun) shows a region of approximately 2.0 mm, encompassing the limbal and peripheral corneal epithelium, which is devoid of staining. In contrast, the central epithelium shows dense staining of all layers. Peripheral to the limbus, staining with AE5 is noted also, in an area which is certainly conjunctival. Figure 2c and d shows a similar staining pattern with a different monoclonal antibody, HE1/11F. This latter antigen has not been fully characterised, but appears to be on the cell surface rather than in the cytoplasm.

The enlarged sections (Figs. 3 and 4) show

Fig. 4. Enlargement of the region delimited by the rectangle in Fig. 1c and d (reacted with the monoclonal antibody HE1/11F).

that the limbal area is, in fact, not staining, since Bowman's membrane is seen to stop just beneath the non-staining region. While this pattern is not inconsistent with that found in rabbits, it does suggest that either there are more stem cells in the human limbus, so that they occupy the full depth of the epithelium at that region, or that even the transient amplifying cells which are thought to be the cells intermediate between stem cells and terminally differentiated cells do not produce either the AE5 or HE1/11F proteins.

The identification of a possible locus of limbal stem cells in humans encourages isolation of an antigen specific for these cells, so that production of a monoclonal antibody which can be used for histologic identification can be undertaken. Such studies will make possible not only the localisation of human limbal stem cells, but will make isolation and culture of such cells possible in tissue culture. This technology has been mastered recently for culturing the stem cells of the mouse bone marrow.¹⁶ As demonstrated in the lethally-irradiated mouse, transplantation of as few as 50 stem cells is life-saving, allowing repopulation of the host marrow. The remarkable proliferative and pleuropotential differentiation characteristics of such cells may mean that transplantation of only a few stem cells of the human limbus might make possible renewal of the entire ocular surface.

References

- ¹ Thoft RA and Friend J: Biochemical transformation of regenerating ocular surface epithelium. *Invest Ophthalmol Vis Sci* 1977, **16**: 14-20.
- ² Friendenwald JS: Growth pressure and metaplasia of conjunctival and corneal epithelium. *Doc Ophthalmol* 1951, **5**: 184-7.
- ³ Shapiro MS, Friend J, Thoft RA: Corneal re-epithelialization from the conjunctiva. *Invest Ophthalmol Vis Sci* 1981, **21**: 135-42.
- ⁴ Kinoshita S, Friend J, Thoft RA: Biphasic cell proliferation in transdifferentiation of conjunctival to corneal epithelium in rabbits. *Invest Ophthalmol Vis Sci* 1983, **24**: 1008-14.
- ⁵ Ebato B, Friend J, Thoft RA: Comparison of central and peripheral human corneal epithelium in tissue culture. *Invest Ophthalmol Vis Sci* 1987, **28**: 1450-6.
- ⁶ Ebato B, Friend J, Thoft RA: Limbal epithelium grows better in tissue culture than does peripheral corneal epithelium. *Invest Ophthalmol Vis Sci* (in press).
- ⁷ Hanna C, Bicknell DS, O'Brien JE: Cell turnover in the adult human eye. *Arch Ophthalmol* 1961, **65**: 695-8.
- ⁸ Thoft RA and Friend J: The X, Y, Z hypothesis of corneal epithelial maintenance. *Invest Ophthalmol Vis Sci* 1983, **24**: 1442-3.
- ⁹ Kaye DB: Epithelial response in penetrating keratoplasty. *Am J Ophthalmol* 1985, **89**: 381-7.
- ¹⁰ Alldredge OC and Krachmer JH: Clinical types of corneal transplant rejection. Their manifestations, frequency preoperative correlates, and treatment. *Arch Ophthalmol* 1981, **99**: 599-604.
- ¹¹ Kinoshita S, Friend J, Thoft RA: Sex chromatin of donor corneal epithelium in rabbits. *Invest Ophthalmol Vis Sci* 1981, **21**: 434-41.
- ¹² Buck RC: Measurement of centripetal migration of normal corneal epithelial cells in the mouse. *Invest Ophthalmol Vis Sci* 1985, **26**: 1296-9.
- ¹³ Danjo S, Friend J, Thoft RA: Conjunctival epithelium in healing of corneal epithelial wounds. *Invest Ophthalmol Vis Sci* 1987, **28**: 1445-9.
- ¹⁴ Schermer A, Galvin S, Sun T-T: Differentiation related expression of a major 64K corneal keratin in vivo and in culture suggest limbal location of corneal epithelial stem cells. *J Cell Biology* 1986, **103**: 49-62.
- ¹⁵ Bukusoglu C and Zieske JD: Characterization of a monoclonal antibody that specifically binds basal epithelial cells in the limbal epithelium. *Invest Ophthalmol Vis Sci* (Suppl) 1988, **29**: 192.
- ¹⁶ Spangrode GJ, Heimfeld S, Weissman IL: Purification and characterization of mouse hematopoietic stem cells. *Science* 1988, **241**: 58.