Transplantation of Limbal Stem Cells

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Core Messages

- Most self renewing tissues are served by a population of stem cells
- Potency and plasticity are two important characteristics of stem cells. They may also have the potential to transdifferentiate
- Stem cells usually reside in a defined 'niche'. The corneal epithelial stem cells are believed to be located in the limbal palisades
- Clinical and laboratory evidence strongly supports the notion that corneal epithelial stem cells are located at the limbus but no marker yet exists that can positively identify a limbal stem cell
- Limbal stem cell deficiency can be congenital or acquired. Ocular surface burns, immune mediated ocular surface diseases and chronic inflammation are important causes of limbal stem cell deficiency
- The effects of limbal stem cell deficiency can range from mild, such as loss of limbal anatomy or conjunctivalisation of the peripheral cornea, to severe, such as corneal invasion by a thick fibrovascular pannus or persistent epithelial defects with stromal melts
- The diagnosis of limbal stem cell deficiency is essentially clinical, but impression cytology may help. Presence of goblet cells on the cornea is diagnostic
- Limbal stem cell deficiency can be unilateral or bilateral, partial or total
- Mild cases of partial deficiency can be treated by sequential sector conjunctival epitheliectomy
- Total unilateral cases can be treated with auto-limbal transplantation
- Bilateral cases often require allo-limbal transplantation from living related or cadaver donors
- Auto-limbal and living related donor transplantation should be avoided in the presence of active inflammation. Auto-limbal transplantation should be avoided in unilateral manifestation of a systemic disease
- Amniotic membrane transplant can be combined with any of the limbal transplant procedures
- Allografts usually require long-term systemic (and/or topical) immunosuppression
- All associated pathology such as lid malpositions, trichiasis, secondary glaucoma and cataracts should ideally be managed prior to considering limbal transplantation, as far as clinically possible
- Buccal mucosa grafts help restore some moisture to a dry ocular surface. Living tissue transplants usually do not survive in a dry environment
- Long-term outcomes of auto-limbal transplants are far better than those of allo-limbal transplants
- Ex vivo expansion of limbus derived epithelial cells as a sheet on different substrates can also be used in ocular surface reconstruction with good results but is also subject to immune rejection

3.1 Introduction

In this chapter the general characteristics of stem cells (SC) and their niche are first described. The evidence supporting the existence of SC at the corneoscleral limbus, both clinical and scientific, is then explored providing the scientific basis for the transplantation of the limbus in the management of limbal SC deficiency. A brief account of the causes and effects of SC deficiency is provided as background to the indications and different techniques of limbal SC transplantation. The surgical techniques are elaborated together with postoperative management and any adjunctive procedures that may complement limbal transplantation.

Ex vivo expanded limbal stem cells on amniotic membrane or other substrates can also be used in ocular surface reconstruction. This constitutes a distinct method of putative limbal stem cell transplantation and is the subject of another chapter in this book. This technique of preparing the tissue construct and stem cell transplantation is therefore omitted from this chapter.

3.2 Stem Cells

3.2.1 Definition

Stem cells are progenitor cells that are responsible for cellular replacement and tissue regeneration. They are the ultimate source cells from which arise almost all other cells that constitute a given organ served by the SC. SC can be found in both embryonic and adult tissues and represent only a very small proportion (0.01–10 %) of the total cell mass [1, 32, 44].

3.2.2 Characteristics of Stem Cells

Stem cells are poorly differentiated or undifferentiated, long-lived, slow cycling but highly clonogenic cells that have a high capacity for self renewal and an increased potential for error-free division [65–67]. They have the ability to proliferate indefinitely [32] and generally live for the duration of the organ(ism) in which they reside. A constant pool is maintained by different strategies of cell division. The most accepted strategy is that of asymmetric cell division whereby one daughter cell stays back in the stem cell niche and the other follows the path of proliferation and differentiation, acquiring functional characteristics of the tissue or organ. The same balance can be maintained if the two daughter cells from one SC proceed down the path of differentiation and the two daughter cells of another SC stay back in the niche as stem cells [57, 61].

The daughter cell(s) that step outside the stem cell pool are destined to divide and differentiate with the acquisition of features that characterise the specific tissue. Such a cell is called a 'transient amplifying cell' (basal corneal epithelial cells) and is less primitive than its parent stem cell. It is believed in some quarters that there exists a window of opportunity during which some of these cells ('transient cells') [54, 55] can revert to the SC pool as SC. Transient amplifying cells divide more frequently than stem cells but have a limited proliferative potential and are considered the initial step of a pathway that results in terminal differentiation. They differentiate into 'postmitotic cells' (wing cells) and finally to 'terminally differentiated cells' (superficial squamous cells). Both postmitotic and terminally differentiated cells are incapable of cell division. All cells except stem cells have a limited life span and are destined to die [45, 66, 84].

Potency and plasticity are two key attributes of SC. SC have the potential to give rise to different cell lineages. This potency is, however, not uniform and there exists amongst SC a hierarchy of potential. SC can be totipotent, pluripotent, multipotent, or unipotent. The zygote that can form the entire embryo and part of the placenta is an example of a totipotent cell. Cells of the inner cell mass from which most tissues that arise from the three germ layers can be derived, but not components of the placenta, are considered pluripotent. Most tissue specific SC are multipotent, capable of producing lineages that

can differentiate in two, three or more different cell types with functional attributes of the organ in which they reside. Some SC, in their natural environment, may have only limited potential with the ability to generate only one specific cell type. These SC are labelled unipotent SC or committed progenitors. SC of the epidermis and the corneoscleral limbus are considered to be examples of this category [1, 3, 68].

The 'plasticity' of SC refers to their ability to transdifferentiate. Some SC when relocated to a different site (tissue) can assume the role that supports the structure and function of the new site, thus aiding in regeneration, repair and maintenance of the cell population at the new site. Stem cell potential and plasticity are both more pronounced in embryonic SC compared to adult SC. Embryonic SC have virtually an unlimited potential for self-renewal and differentiation. Given the right microenvironment and the right signals, adult and embryonic SC can (theoretically) be made to follow a desired path of differentiation or propagated indefinitely, in an undifferentiated state. Herein lies the immense therapeutic potential of SC [3].

3.2.3 The Stem Cell 'Niche'

The microenvironment in which the SC reside is referred to as their 'niche' [60]. SC are usually confined to their 'niche' where the microenvironment supports and maintains the stemness of SC and affords a degree of protection. In solid organs, where cell migration commences at one point and progresses until the cells are shed at a distant point(s), the SC niche is usually located at the point of commencement. The 'niche' represents the collective influence of other local matrix cells, the extracellular matrix, its vascularity, basement membrane characteristics and prevalent growth factors and other cytokines. In the intestinal mucosa, for example, it is believed that the pericryptal fibroblasts/subepithelial myofibroblasts may serve as niche cells [60, 79] and in the epidermis, beta 1 integrin mediated adhesion to its ligand, type IV collagen, is shown to influence behaviour of epidermal SC [44]. The niche also affords protection to the all-important SC [79, 95].

Summary for the Clinician

- Stem cells:
  - Undifferentiated
  - Long lived
  - Slow cycling
  - Clonogenic
  - Asymmetric division
  - Potency: usually pluripotent or multipotent
  - Plasticity: transdifferentiation
  - Niche: SC microenvironment
- SC progeny:
  - 'Transient cells'
  - Transient amplifying cells – basal epithelium
  - Postmitotic cells – wing cells
  - Terminally differentiated cells – superficial squamous cells

3.3 Limbal Stem Cells

3.3.1 The Clinical Evidence

During corneal epithelial wound healing and normal epithelial turnover, cell migration and migration of sheets of epithelium [20] have been shown to occur in a centripetal manner from the corneoscleral limbus towards the centre of the cornea [4, 5, 51]. Large corneal epithelial wounds, where the wound edge is closer to the limbus, heal at a faster rate than smaller wounds [59]. Repeated denudation of the central corneal epithelium shows that the healing rate of the second wound is more rapid than that of the first. This suggests that rapidly dividing younger cells of the periphery have moved to more central areas after the first trauma and respond readily to the second [80].

Human corneal epithelial defects with partial limbal involvement demonstrate a preferential circumferential migration of a population of cells along the limbus, from both ends of the remaining intact limbus [21] (Fig. 3.1 A). Complete epithelial cover for the corneal surface is not established until limbal re-epithelialization is first complete, suggesting that the circumfer-

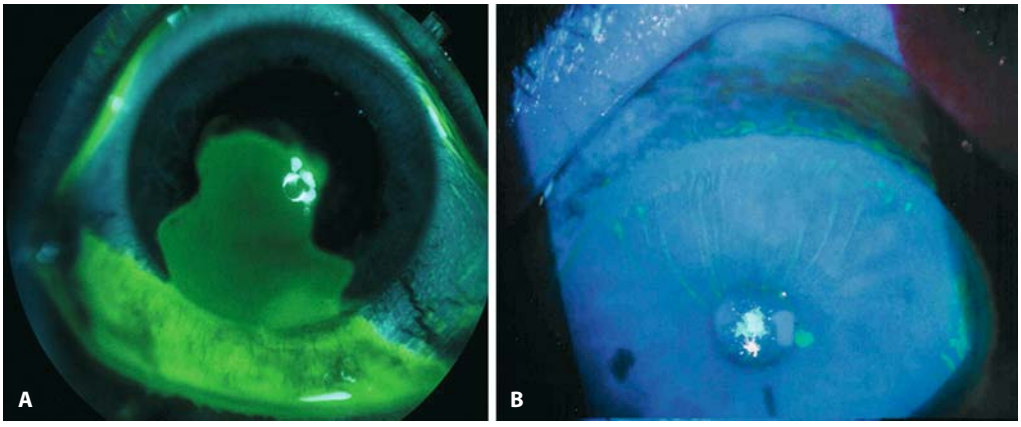

Fig. 3.1. **A** Healing of corneal epithelial wound involving the limbus showing a preferential circumferential migration of tongue-shaped sheets of limbal epithelial cells arising from either end of the remaining intact epithelium. (Slit lamp anterior segment photograph with fluorescein dye) (with permission from *Br J Ophthalmol:* Dua et al. 2001; 85:1379–1383). **B** 'Columnar keratopathy' is the name given by the author to this presentation of alternating columns of fluorescein stained epithelium and normal corneal epithelium. These correspond to the limbal palisades and represent an early sign of limbal stem cell deficiency

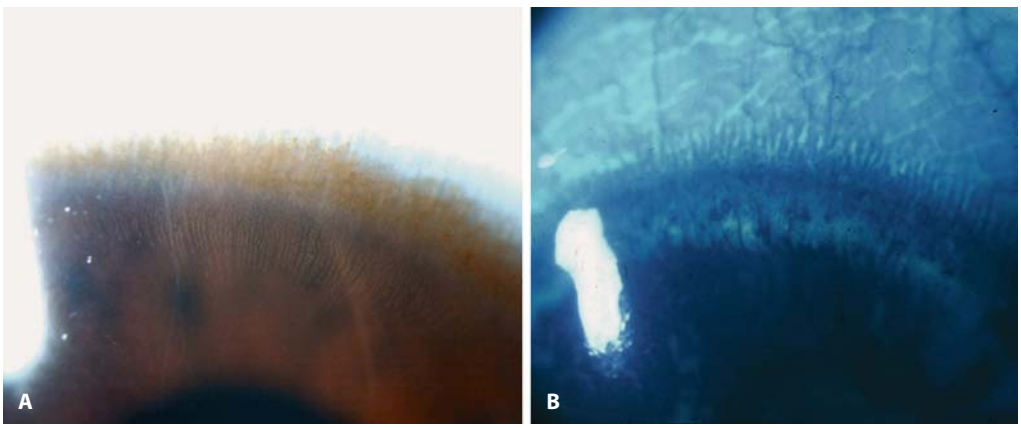

Fig. 3.2. Slit lamp photograph of the limbus showing the palisade (of Vogt) structure with: **A** pigment columns migrating into peripheral cornea and **B** fluorescein staining of columnar migration in response to a central abrasion

entially migrating population of cells probably represents in part the healing response of limbal stem cells. In patients with limbal abnormalities, alternating columns of normal and fluorescein staining cells often corresponding to limbal palisades – columnar keratopathy – have been noted to extend from the limbus towards the centre in radial or curvilinear rows [22] (Fig. 3.1B). The palisades of Vogt and the interpalisade rete ridges provide a unique structure to the limbus (Fig. 3.2). The structure of the palisades and the rete ridges, their vascularity and pigmentation are all analogous to repositories of stem cells in the monkey palm epidermis [9, 86, 88]. It has also been reported that hemidesmosomes of peripheral cells of normal and healing mouse corneas are arranged in radial rows, leading to the interpretation that this

orientation represents centripetal migration of epithelial cells [5]. Very recently, a unique anatomical structure, termed the limbal epithelial crypt [27], has been discovered at the peripheral end of the interpalisade rete ridges, numbering approximately five to seven per human cornea. This has features consistent with those of a SC repository or 'niche'.

3.3.2 The Scientific Evidence

Basic research has identified a number of characteristics that are unique to the limbal basal epithelial cells and set them apart from the rest: Mitosis rates are highest at the limbus, both in the normal physiological state and following stimulation [31, 37, 8]. Limbal epithelial cells have the greatest proliferative potential in vitro, compared to any other part of the cornea [28–30]. Limbal basal cells lack the epithelial cell differentiation cytokeratin CK3 [18, 56, 73, 101]. Impression cytology examination of the human limbus shows that, morphologically, the limbal cells are smaller, more densely packed and have a greater nucleus to cytoplasm ratio compared to adjacent corneal and conjunctival cells (H.S. Dua, unpublished observations, Fig. 3.3A, B).

Several other attributes that are unique to the limbal epithelium (Table 3.1) have also been described. These include the presence of alpha-enolase [101, 102], EGF receptors [100, 103], pigment [9], cytokeratin profile (CK3/12 negative) [7, 73], presence of vimentin [53–55], CK19 and specific basement membrane characteristics [34, 35, 85]. Vimentin and CK19 positive, CK3 negative clusters of cells with unique electron microscopic morphology have been demonstrated [54, 55]. Connexin 43 (Cx43), a gap junction protein, has been noted in human corneal but not limbal basal epithelium [12, 58, 96]. It has been proposed that absence of Cx43 segregates cells from adverse events generated in neighbouring cells and helps preservation of SC in their microenvironmental niche [96].

Zhao et al. [98] have recently reported that limbal epithelial cells cultured in the presence of mitogens express neural progenitor markers, specifically nestin. A transcriptional factor, p63 involved in morphogenesis, has been proposed to identify keratinocyte stem cells at the limbus [63], but its role as a marker of limbal SC is controversial [25, 46]. Similarly, well defined markers of haematopoietic SC, namely CD34 and

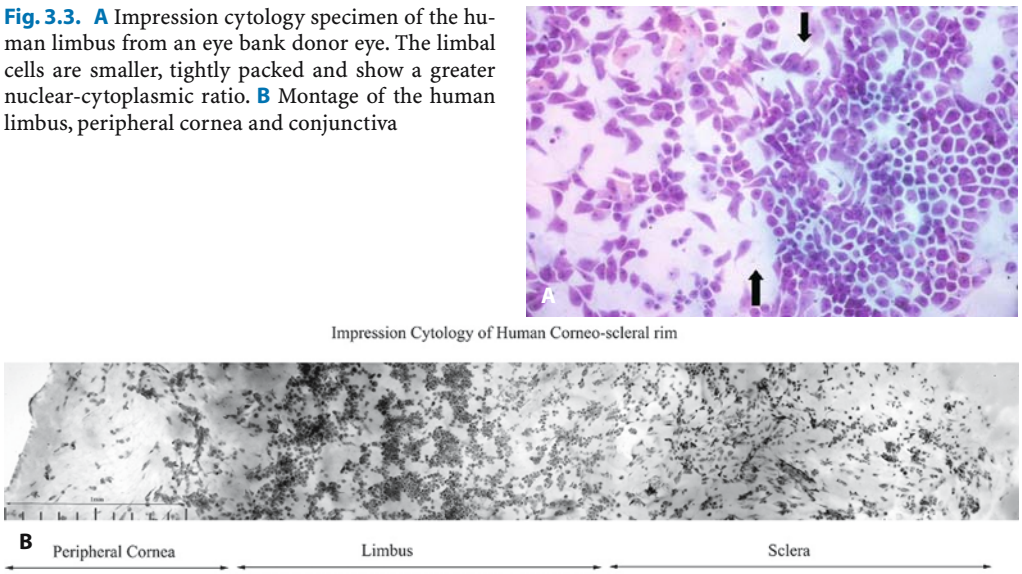

Fig. 3.3. **A** Impression cytology specimen of the human limbus from an eye bank donor eye. The limbal cells are smaller, tightly packed and show a greater nuclear-cytoplasmic ratio. **B** Montage of the human limbus, peripheral cornea and conjunctiva

Table 3.1. Differences between epithelial cells of the limbus and central cornea (CK, cytokeratin; CX, connexin; EGFR, epithelial growth factor receptor)

Limbus	Central cornea
CK 5/14+ve	CK 5/14–ve
CK 3/12–ve	CK 3/12+ve
CK 19+ve	CK 19–ve
P63+ve	P63–ve but see results in this paper
CX 43–ve	CX 43+
Vimentin+ve	Vimentin–ve
Intrinsic melanogenesis	
Cytochrome oxidase and ATpase+ve	
Alpha-enolase+ve	Alpha-enolase+ve
Beta-1-integrin+ve	Beta-1-integrin+ve
EGFR+ve (strong)	EGFR+ve
ABCG2+ve	ABCG2–ve

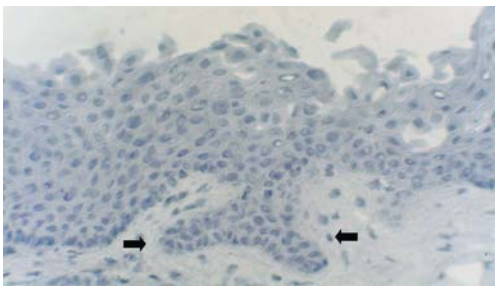

Fig. 3.4. Limbal epithelial crypt: representing a solid cord of cells extending from the undersurface of a limbal palisade. These cells are positive for the putative stem cell marker ABCG2. Haematoxylin stained cryo section, ×100

CD133, have failed to demonstrate any unique subpopulation of cells at the limbus [25, 47]. An ATP-binding cassette transporter protein, ABCG2, is believed to be a marker of a side population of cells that have the ability to efflux Hoechst 3342 dye [99]. Side population cells that contain this transporter protein are believed to be stem cells [36]. Limbus epithelial cells have been shown to express ABCG2 [94] and these may represent the subpopulation that contain the stem cells. The limbal epithelial crypt recently demonstrated by Dua et al. [27] contains cells that predominantly stain positive for ABCG2, indicating that the crypt may provide the niche for corneal epithelial SC (Fig. 3.4).

The above data strongly supports the notion that progenitor cells exist at the corneoscleral limbus. Whether these are truly SC as defined in other organ systems remains to be established. There is evidence to suggest that SC or progenitor cells for the conjunctival epithelium reside maximally in the fornices and for goblet cells and perhaps for conjunctival epithelium may also be scattered throughout the epithelial surface.

Summary for the Clinician

- **Evidence for corneal epithelial (limbal) stem cells:**
- **Clinical:**
  - **Unique palisade architecture**
  - **Centripetal migration from limbus**
  - **Circumferential migration along limbus**
  - **Pigment and other deposits migrating in columnar manner from limbus**
  - **Larger corneal epithelial wounds (closer to limbus) heal faster**
  - **Second wounds heal faster**
  - **Relative resistance of limbus epithelium to denudation**
  - **Columnar keratopathy**
  - **Limbal deficiency allows conjunctivalisation of cornea and persistent epithelial defects**
- **Scientific:**
  - **Different morphology of limbal cells**
  - **Increased hemidesmosomes at limbus basal epithelium**

- Increased mitosis rates at limbus
- Increased proliferative potential of limbal basal cells
- Absence of cytokeratin 12 in limbal basal cells
- Absence of gap junctions in limbal basal cells
- Presence of certain enzymes such as alpha-enolase and ABCG2
- Different basement characteristics at limbus compared to central cornea
- Presence of limbal epithelial crypts (niche)

3.4 Limbal Stem Cell Deficiency

3.4.1 Causes of Limbal Stem Cell Deficiency

Stem-cell deficiency can be congenital or acquired. Congenital SC deficiency occurs as a result of hereditary aplasia of limbal stem cells as occurs in aniridia and congenital erythrokeratodermia. More often though, stem cell deficiency is acquired as a result of extraneous insults that acutely or chronically destroy limbal stem cells. These include chemical or thermal injuries, ultraviolet and ionising radiation, Stevens-Johnson syndrome, advanced ocular cicatricial pemphigoid, multiple surgery or cryotherapy, contact lens wear, or extensive/chronic microbial infection such as trachoma. Keratitis associated with multiple endocrine deficiencies, neurotrophic (neural and ischaemic) keratopathy and chronic limbitis also lead eventually to SC deficiency but are less common [13, 18, 24, 33, 41, 42].

Summary for the Clinician

- Causes of limbal stem cell deficiency:
- Congenital: aniridia, erythrokeratodermia
  Acquired:
  - Chemical and thermal burns
  - Chronic inflammatory disorders
  - Progressive cicatrisation conditions – OCP, SJS
  - Prolonged contact lens wear
  - Multiple ocular surface surgery
  - Medicamentosa including preservatives
  - Idiopathic

3.4.2 Effects of Limbal Stem Cell Deficiency (Modified from Dua et al. [25])

The hallmark of limbal stem cell deficiency is 'conjunctivalisation' of the cornea and the most significant clinical manifestation is a persistent corneal epithelial defect.

The clinical symptoms of limbal deficiency may include decreased vision, photophobia, tearing, blepharospasm, and recurrent episodes of pain (epithelial breakdown), as well as a history of chronic inflammation with redness.

Depending on the extent of limbal involvement, SC deficiency can be partial or total. Partial SC deficiency may vary in extent to involve the pupillary area, when intervention is usually required, or exclude the visual axis when none or minimal intervention with topical medication may be required. Further, partial SC deficiency may vary in severity from mild, when only an abnormal epithelial sheet covers a variable area of the cornea, to severe when a part of the cornea, usually including the pupillary area, is covered by a thick fibrovascular pannus.

The clinical features of SC deficiency, from mild to severe, include the following [13, 14, 18, 21, 22, 24, 43, 64, 88]: (a) loss of limbal anatomy, (b) irregular, thin epithelium, (c) stippled fluorescein staining of the area covered by abnormal epithelium, (d) unstable tear film, (e) filaments and erosions, (f) superficial and deep vascularisation, (g) persistent epithelial defects leading to ulceration, melting and perforation, (h) fibrovascular pannus, and (i) scarring, keratinisation and calcification.

1. Loss of limbal anatomy: The normal limbal architecture with rows of palisades and the perilimbal vascular arcade is usually best defined at the superior and inferior limbus. The architecture may vary depending on age of the individual. With increasing age the definition of palisades becomes less distinct nasally and temporally. Pigmentation of the limbal palisades is a feature in some races. Alterations in limbal anatomy include con-

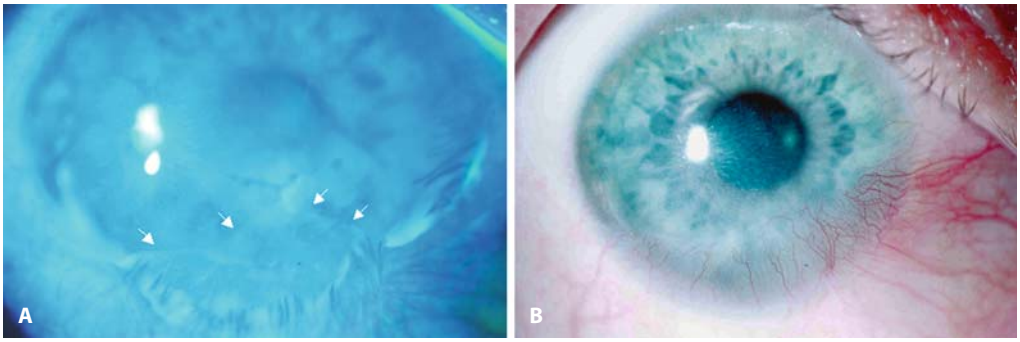

Fig. 3.5. **A** Signs of mild limbal stem cell deficiency – peripheral conjunctivalisation highlighted with fluorescein staining. The junction of corneal and conjunctival phenotypes of epithelia is marked with arrows. **B** Peripheral vascularisation with loss of limbal architecture

tiguous or patchy fluorescein staining of conjunctiva derived cells at the limbus and extending onto the peripheral cornea, segmental limbal hyperaemia indicating chronic inflammation, thickening of limbal epithelium, vascularisation of peripheral cornea and scarring (Figs. 3.1B, 3.5A, B).

2. Irregular, thin epithelium: When the initial injury is mild and superficial or the disease process leading to stem cell deficiency is slowly progressive, loss of a segment of limbal epithelium may occur without significant damage to the substratum. A sheet of conjunctival/metaplastic epithelium consequently covers the cornea without any notable vascularisation. This epithelium is usually thin and irregular as can be seen by the pooling of fluorescein dye at the junction of the abnormal and remaining normal epithelium (Fig. 3.6, see also Fig. 3.11A) [14].
3. Stippled fluorescein staining of the area covered by abnormal epithelium: The abnormal conjunctival/metaplastic epithelium readily takes up fluorescein dye [43], allowing easy visualisation of the abnormal cells and their pattern of distribution. The abnormal fluorescein-staining 'conjunctivalised' epithelium may take on the pattern of columns, whorls or wedges with the broad base towards the limbus and the narrow curving apex toward the corneal centre (Figs. 3.5A, 3.6) [22].
4. Unstable tear film: The abnormal epithelium demonstrates a rapid tear film break up time

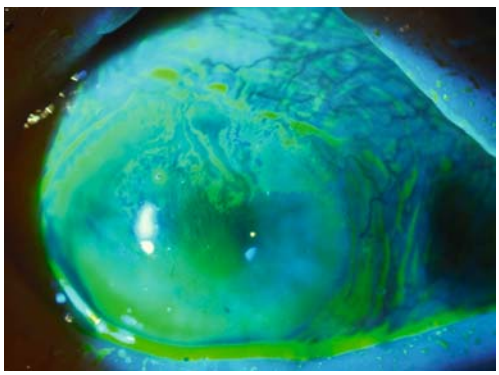

Fig. 3.6. Peripheral conjunctivalisation with pooling of dye and stippled staining of the abnormal epithelium, between 12 and 3 o'clock

over it and areas of negative and positive fluorescein staining.

5. Tags of loose epithelium, filaments with mucus and recurrent erosions are other features associated with the abnormal epithelial cover on the cornea.
6. Superficial and deep vascularisation: In moderate to severe cases of stem cell deficiency, superficial and/or deep vascularisation of the cornea occurs. It is largely restricted to the area of stem cell deficiency and may affect a segment of the limbus or the entire circumference may become involved (Fig. 3.7).
7. Persistent epithelial defects (Fig. 3.8): Chronic non-healing ulceration of the corneal epithelium or cycles of repeated breakdown

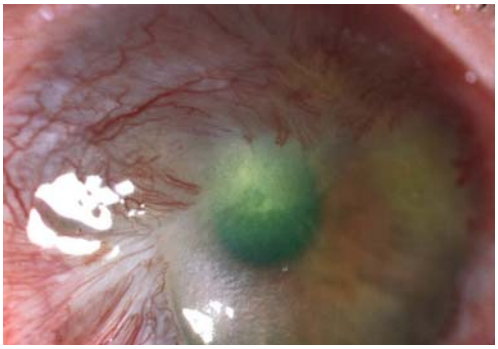

Fig. 3.7. Superficial and deep vascularisation with a fibrovascular pannus encroaching on the cornea following chemical burn in which 9.5 clock hours of the limbus and 60% of the conjunctiva were involved (clinical grade – 9.5/60%) (with permission from *Br J Ophthalmol:* Dua et al. 2001; 85:1379–1383)

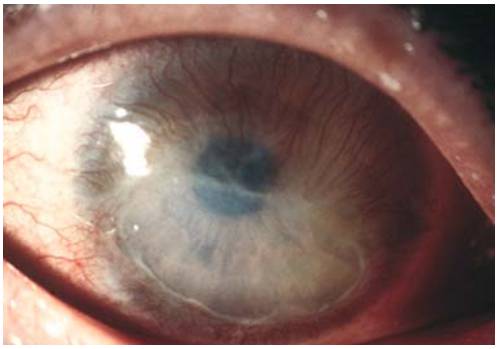

Fig. 3.8. Persistent epithelial defect and fibrovascular pannus on cornea related to total stem cell deficiency following unilateral alkali (cement) burn (clinical grade 12/65%) (with permission from *Br J Ophthalmol:* Dua HS and Azuara-Blanco A 2000; 84:273–278)

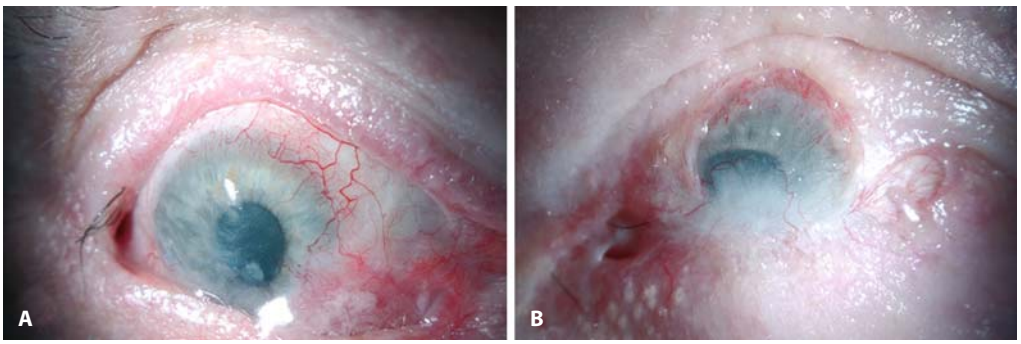

Fig. 3.9. **A** Right eye of patient with 10 clock hours of limbus and 70% conjunctival involvement following a chemical (alkali) burn. **B** Left eye of same patient with 12 clock hours of limbs and 90% conjunctival involvement (clinical grade 10/70% RE, 12/90% LE). Scarring, vascularisation, adhesions and some keratinisation are present. The lids on both sides were also severely damaged

followed by healing, associated with a chronic low grade inflammation, is a feature of limbal stem cell deficiency. These defects are liable to lead to deep stromal infiltrates that may or may not be related to infection. The edges of the epithelial defect have a distinct rolled-up or heaped appearance. Over time, progressive melting of the corneal stroma with perforation can occur.

8. Fibrovascular pannus: In moderate to severe cases of stem cell deficiency, epithelial cover of the denuded cornea is associated with encroachment of fibrovascular tissue of varying thickness (Figs. 3.7, 3.8) [49]. This tissue supports the thickened multilayered conjunctiva derived epithelium.
9. Scarring, keratinisation and calcification: The end stage of the aftermath of limbal stem cell deficiency, whatever the cause, is scarring and eventually calcification of the affected tissue. Usually by this stage the inflammation has subsided and the eye is comparatively comfortable. In patients who have associated severe dry eyes the covering epithelium becomes totally or partially keratinised (Fig. 3.9 A, B).

Summary for the Clinician

- Effects of limbal stem cell deficiency
  - Mild → severe
  - Loss of limbal anatomy
  - Conjunctival epithelial ingress onto cornea – stippled fluorescein staining
  - Columnar keratopathy
  - Unstable tear film over affected area
  - Frank conjunctivalisation
  - Corneal vascularisation – superficial and deep
  - Fibrovascular pannus covering corneal surface
  - Persistent epithelial defect
  - Stromal melting
  - Perforation, scarring, calcification
  - Keratinisation

3.4.3 Diagnosis of Stem Cell Deficiency

The diagnosis of stem cell deficiency remains essentially clinical. On slit lamp biomicroscopic examination, the conjunctivalised cornea presents a dull and irregular reflex. The epithelium is of variable thickness and translucent to opaque. Conjunctival epithelium on the cornea appears to be more permeable than corneal epithelium and takes up fluorescein stain in a stippled or punctate manner. In cases of partial conjunctivalisation of the cornea, fluorescein dye tends to pool along the junction of the sheets of corneal and conjunctival epithelial cell phenotypes. At this junction, the corneal epithelial sheet shows tiny processes or undulations that give the junction its characteristic appearance.

Loss of architecture of the limbal palisades of Vogt and vascularisation are other common features. When damage is extensive, vascularisation occurs in the form of fibrovascular pannus, which increases the thickness of the affected area of the cornea. However, the underlying corneal stroma may be considerably thinned by the initial insult of disease process.

The presence of goblet cells on impression cytology specimens taken from the corneal surface or in biopsy specimens of the fibrovascular pannus covering the cornea is pathognomonic of conjunctivalisation of the cornea (Fig. 3.10 A) [25, 69]. Biopsy specimens also demonstrate a multilayered, at times keratinised epithelium overlying dense fibrous and vascular tissue (Fig. 3.10 B). Intraepithelial lymphocytes, which are a feature of conjunctival epithelium, are also seen on conjunctivalised corneal epithelium. These are predominantly CD8+/*HML-1 + cells (cytotoxic T lymphocytes expressing the human mucosal lymphocyte antigen) [23, 25]. Features of squamous metaplasia or loss of cornea specific cytokeratins (CK 3/12) on immunohistology are other effects noted on biopsy specimens.

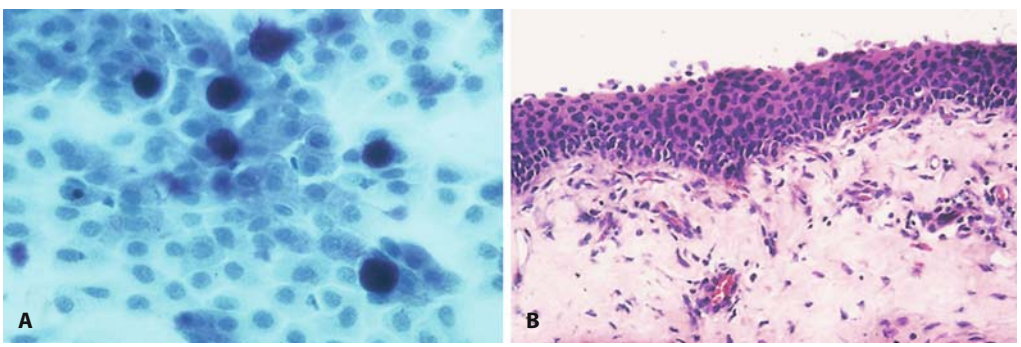

Fig. 3.10. **A** Impression cytology from surface of cornea with stem cell deficiency and a fibrovascular pannus showing goblet cells. PAS stain, ×400. **B** Biopsy of fibrovascular pannus showing multilayered epithelium, vascularisation and intraepithelial lymphocytes along the basal layers

Summary for the Clinician

- Diagnosis of limbal stem cell deficiency
  - Essentially clinical
  - Impression cytology – goblet cells on cornea pathognomic
  - Biopsy – multilayered epithelium, intraepithelial lymphocytes, vessels
  - Vimentin and CK 19 positive cells in central cornea (normally present in peripheral cornea and limbus)

3.5 Limbal Transplant Surgery

3.5.1 Principles

Management of stem cell deficiency can be considered in the following steps:

After Acute Injury. When a patient presents after an acute insult it should be ascertained whether the involvement of the limbus is partial or total. This can be done by use of fluorescein stain and slit lamp examination. If partial, appropriate medication required for the underlying cause and to control inflammation should be initiated. The eye should be examined at 24- or 48-h intervals and the process of re-epithelialisation observed. If this is occurring from the remaining intact limbal epithelium [21], this should be encouraged and any attempt at re-epithelialisation from the conjunctival epithelium should be discouraged by sequential sectoral conjunctival epitheliectomy (SSCE, see below) [14,15]. If total, allow the cornea to be covered by conjunctival epithelium, if possible, before contemplating surgical intervention. This may take several days. The guiding principle should be that corneal epithelial cover for cornea and conjunctival epithelial cover for conjunctiva is the ideal end result but conjunctival epithelial cover for cornea is preferable to no epithelial cover to cornea.

In Established Cases. The principles underlying surgical procedures involving limbal stem cells are firstly to expand the corneal epithelial sheet derived from any existing sector of limbus in the affected eye. This can be achieved by SSCE (see below) [14,15], especially if the cornea is partially covered by a layer of thin, metaplastic, conjunctivalised epithelium. If no healthy sector of limbus is available in the affected eye and if the other eye is normal with a positively documented absence of involvement in the original injury, autologous limbal transplantation should be considered. If the other eye is also affected or the underlying condition is a systemic illness such as Stevens-Johnson syndrome, allografts from a living related donor or from a cadaver donor should be considered. In the acute stage of limbal stem cell deficiency, for example acute chemical burns, auto-limbal or living related donor limbal transplants should be avoided at all costs. The chances of the transplanted material becoming caught up in the inflammatory and scarring process are high with loss of a valuable resource for future reconstruction. Use of auto or living related donor tissue, if available, should be attempted in quiet eyes. All the above procedures can be complemented with amniotic membrane transplantation. Penetrating keratoplasty may be combined with or following any of these procedures.

Limbal transplantation involves taking a lamellar strip of limbal tissue, usually with some adjacent peripheral cornea and/or conjunctiva and transplanting it to a suitably prepared bed in the host eye. Sutures are usually required to keep the donor graft in place.

3.5.2 Preoperative Considerations

All associated lid abnormalities, intraocular pressure problems and presence of cataract should ideally be dealt with prior to undertaking ocular surface restorative surgery. Symblepharon correction with amniotic membrane or buccal mucosa graft should also precede stem cell grafting. At times, if a corneal graft procedure is being contemplated at the time of stem cell grafting, it can be combined with cataract extraction and lens implantation. When an intumescent cataract is associated with raised pressure, corneal grafting may become a necessity if

a dense fibrovascular pannus or corneal scar precludes visualisation of the interior of the eye.

Patients with limbal SC deficiency and conjunctivalised corneal surface tend to manifest persistent chronic inflammation. Stem cell grafts do not perform well in the presence of inflammation and can be destroyed by the inflammatory and scarring processes. Ideally inflammation should be controlled and the eye rendered as quiet as possible with the use of topical and systemic steroids or other immunosuppressants which may become necessary in some conditions such as Stevens-Johnson syndrome and ocular cicatricial pemphigoid.

Most stem cell grafts do not survive in a dry (eye) environment. At times the injurious insult resulting in stem cell deficiency also results in a severe dry eye state. In such situations, if topical lubricants including autologous serum drops, punctal occlusion and buccal mucosa grafts do not restore adequate moisture to the ocular surface, a keratoprothesis procedure should be considered.

Summary for the Clinician

- Treatment algorithm
- General principles:
  - Manage underlying factors, e.g., chronic inflammation, contact lens wear, topical medications
  - Topical lubrication
  - All associated problems, e.g., raised pressure, conjunctival adhesions, lid malpositions, should be addressed before undertaking ocular surface reconstruction
  - Limbal transplants do not perform well in dry eyes
- In acute limbus injury:
  - If partial, i.e. some limbus is surviving – allow corneal epithelialisation to occur from limbus derived cells – SSCE
  - If total:
    a) Allow conjunctival epithelium to grow onto cornea
    b) Transplant sheet of ex vivo expanded limbal epithelial cells
    c) Avoid use of autologous or living related donor tissue until acute inflammation is well under control
- In established cases:
  - Treat eye lid problems, glaucoma and conjunctival adhesions first
  - Partial or total
  - Partial:
    a) Visual axis not involved: symptomatic, lubricants of SSCE
    b) Visual axis involved: SSCE
    c) Dense fibrovascular pannus: sector limbal transplant

    Total:
    a) Unilateral: auto-limbal transplant
    b) Ex vivo expansion of autologous limbal cells
    c) Bilateral: allo-limbal transplant
    d) Ex vivo expansion of cells (living related, living non-related, cadaver)
    e) Amniotic membrane and autologous serum drops as adjuncts
    f) Allo-transplants require systemic immunosuppression

3.6 Surgical Techniques

3.6.1 Sequential Sector Conjunctival Epitheliectomy (SSCE) [14, 15]

(Figs. 3.11, 3.12)

In cases with partial, mild to moderate conjunctivalisation of the cornea, without significant fibrovascular pannus, removal of the conjunctivalised epithelium is all that is required. This can be achieved at the slit lamp under topical anaesthesia, using a crescent blade or a surgical knife. It is important to remove all conjunctival epithelium, especially along its line of contact with the remaining corneal epithelium. Following removal of conjunctival epithelium from the corneal surface, it is important to closely monitor the patient to ensure that the denuded surface is re-epithelialised by cells derived from the remaining corneal epithelial sheet, i.e. limbal derived cells and not by conjunctival cells. This can be effected by repeatedly debriding (sequential epitheliectomy) any conjunctival epithelium that encroaches upon the limbus until

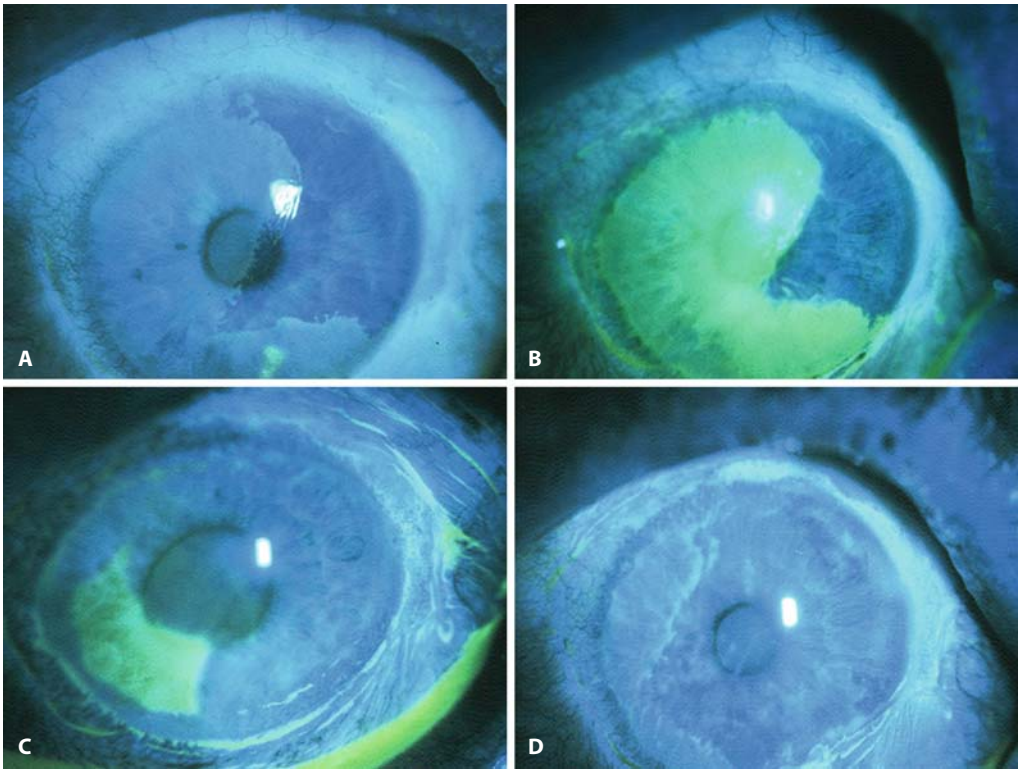

Fig. 3.11 A–D. Sequential sector conjunctival epitheliectomy (SSCE, H.S. Dua). **A** Conjunctivalisation of the cornea involving the visual axis following chemical injury. The demarcation between the two phenotypes of cells is clearly visible. **B** Appearance immediately after removing the abnormal epithelium (epitheliectomy). **C** The corneal epithelial sheet is migrating across the surface but the conjunctival epithelium too has started to re-encroach on the cornea. **D** After complete healing, the visual axis is now covered by healthy corneal epithelium. A new line of contact between conjunctival and corneal epithelium is established (fluorescein stained anterior segment photographs). The patient's vision improved from 3/18 to 6/9 (with permission from *Br J Ophthalmol:* Dua HS 1998; 82:1407–1411)

the limbus and corneal surface is re-populated by limbal epithelium derived cells.

In cases where only 1 or 2 clock hours of limbal epithelium is surviving, it may be appropriate to attempt re-epithelialisation of the visual axis only, with limbal derived cells. An area corresponding to the visual axis is debrided off its conjunctival epithelial cover and re-epithelialisation with limbal derived cells is achieved. This has the theoretical advantage of not overstressing the small remaining sector of limbal 'stem' cells. This technique of SSCE can also be usefully combined with limbal transplant to allow cells derived from transplanted limbal tissue (auto or allo) to re-populate the host corneal surface without 'contamination' from conjunctival epithelium (see below).

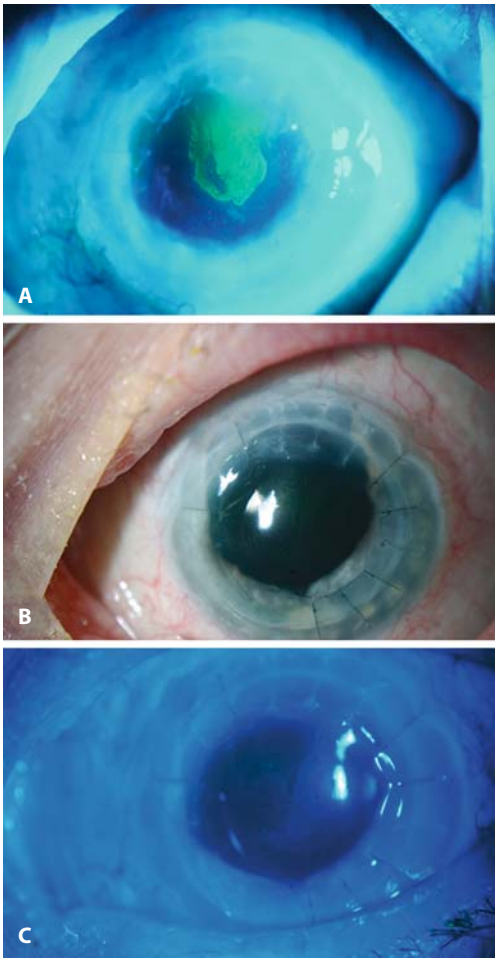

Fig. 3.12. A Conjunctivalisation of the superior cornea involving the visual axis. **B, C** After SSCE without and with fluorescein stain, respectively. The visual axis is now clear

3.6.2 Auto-limbal Transplantation

(Figs. 3.13, 3.14)

In patients where total stem cell deficiency affects only one eye, an auto-limbal transplant procedure is the ideal option [6, 19, 42, 48, 49, 88, 89]. It is important, however, to be absolutely certain that the donor eye was not involved at the time of the initial injury. In unilateral manifestations of systemic diseases, harvesting tissue from the apparently normal eye is not recommended.

The surgical technique consists of the following steps (the author's [19] modified technique is described): (a) a 16-mm Flieringa ring is sutured in place when the procedure is to be combined with a corneal graft (and lens extraction with implant). A 360° peritomy is first performed in the recipient eye. (b) The fibrovascular pannus covering the corneal surface is dissected off at a suitable plane. Any bleeding points are individually cauterised with light diathermy. (c) The donor tissue consisting of corneal-limbal-conjunctival explants is harvested from the contralateral normal eye. Two explants, corresponding to 2 clock hours (11–1 o'clock and 5–7 o'clock) and consisting of a very narrow strip (1 mm or less) of peripheral cornea, limbus and 3 mm of bulbar conjunctiva, are harvested. The conjunctival area to be removed is marked with a surgical marker pen. The conjunctiva is incised superficially with a pair of scissors and dissected in a superficial plane up to the limbus. An angled bevelled blade

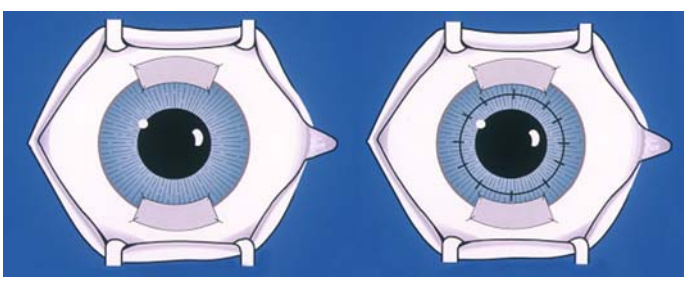

Fig. 3.13 A, B. Diagrammatic representation of autologous limbal transplantation. **A** Positioning of explants on recipient limbus at the 12 and 6 o'clock positions without or with (**B**) a corneal graft (with permission from *Br J Ophthalmol:* Dua HS, Azuara-Blanco A 2000; 84:273–278)

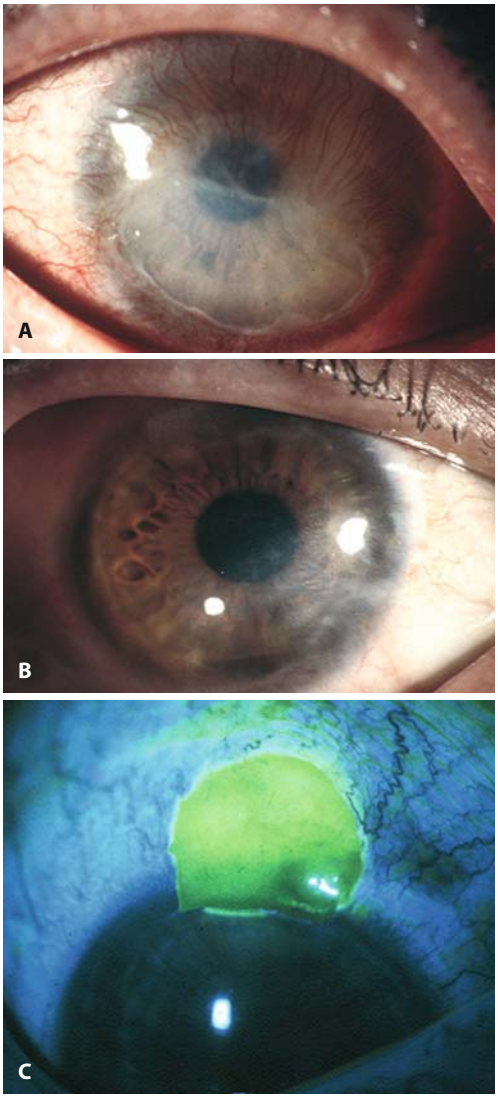

Fig. 3.14 A–C. Auto-limbal transplantation. **A** Preoperative persistent epithelial defect following an alkali (cement) burn as shown in Fig. 3.8. **B** Postoperative status after auto-limbal transplants at the 6 and 12 o'clock positions. The patient's eye is stable over 5 years postoperatively (with permission from *Br J Ophthalmol:* Dua HS, Azuara Blanco A 2000; 84:273–278. **C** Donor site for autologous limbal transplant, stained with fluorescein. Note that the central edge of the removed tissue needs to extend just central to the limbal vascular arcade

is used to (lamellar) dissect the corresponding limbal area extending into peripheral cornea to just inside (central) to the vascular arcade. (d) Suitable beds may be prepared at the superior and inferior limbus of the recipient eye by using the excised explants as templates to mark the area to be prepared. This is not always essential. (e) The donor tissue is then sutured onto the recipient eye with two interrupted 10-0 nylon sutures at the corneal margin and two along the scleral edge of the explant. Care should be taken not to bury the knots in the explant tissue as this could strip the explants off when attempting to remove the sutures in the postoperative period. At times the knots may be left unburied to facilitate removal. The conjunctiva of the recipient eye is then approximated to the donor conjunctiva with interrupted 8-0 Vicryl sutures (absorbable), taking a bite into episclera. (f) When a penetrating keratoplasty is also required, this is performed after the limbal explants are first sutured in place. (g) A bandage contact lens is placed on the cornea and subconjunctival antibiotics and corticosteroids are injected at the end of the procedure.

Adjunctive use of amniotic membrane can be made either as a graft to provide a suitable bed for limbal explant derived epithelial cells to grow on the cornea and/or as a patch to prevent conjunctival epithelial cells from extending onto the cornea and admixing with the limbal explant derived cells (see below).

3.6.3 Allo-limbal Transplantation

3.6.3.1 Living Related Donor

When a living related donor, who is tissue matched to the recipient, is available, tissue is harvested from one donor eye and used on the recipient eye exactly as described above for auto-limbal transplantation [10, 70].

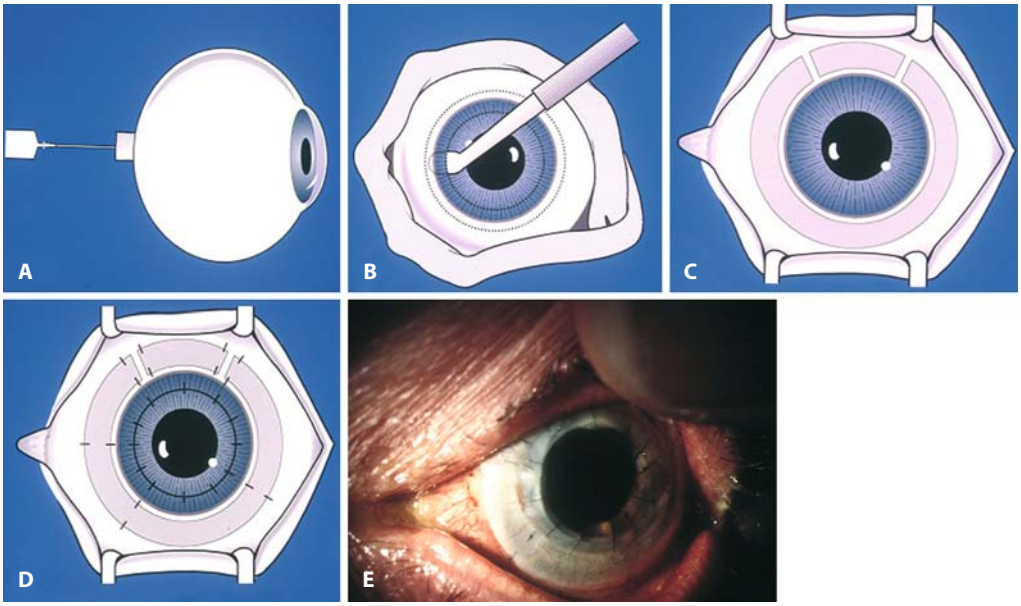

Fig. 3.15 A–E. Diagrammatic representation of allo-limbal transplantation. **A** Injection of air to firm the donor globe. **B** Harvesting the limbal circumference from the donor globe. **C** Positioning of explants on recipient limbus without **C** or with **D** a corneal graft. As the donor explant is placed slightly peripheral to the recipient limbus, more than one donor may be required as shown. **E** Combined allo-limbal transplant and corneal graft (with permission from *Br J Ophthalmol:* Dua HS, Azuara-Blanco A 1999; 83:414–419

3.6.3.2 Cadaver Donor

In most instances, limbal tissue is obtained from cadaver donor eyes [16, 41, 42, 48, 81–83, 88, 89, 91]. In such an event, tissue matching is not usually practical. In the author's protocol, a pair of 'fresh' donor eyes is used within 48 h of death. Donor eye retrieval should be done within 24 h of death and surgery within the next 24 h. Donor age of less than 50 years is preferred. 'Fresh' and 'young' donor eyes are preferred because the success of the procedure depends on the transplantation of healthy limbal stem cells.

The surgical technique consists of the following steps (the author's technique [16] is described) (Fig. 3.15): Donor limbus tissue is prepared before the patient is anaesthetised. (a) The donor eyeball is inflated with air (1–2 ml), injected through the stump of the optic nerve, to make the globe firm. (b) The globe is fixed on a Tudor Thomas stand. A vacuum (or manual) trephine with a diameter 3 mm smaller than the corneal diameter (i.e., average of vertical and horizontal corneal diameter) is used to trephine the central donor cornea to one-fourth to one-fifth of the stromal depth (approximately 150 µm). Proper centration is important to ensure that a uniform width of peripheral cornea is obtained. (c) Superficial lamellar dissection of the peripheral cornea is then carried out using an angle bevelled blade, and extended into the sclerocorneal junction and 1 mm beyond, into sclera. Approximately 1–2 mm of donor conjunctiva, if present, is maintained. The dissected tissue is divided at one point and excision completed with a curved scissors, by cutting along the outer circumference of the dissected tissue. The limbal tissue to be grafted thus consists of an open ring of peripheral corneal and limbal epithelium (and conjunctival epithelium at places), and superficial corneal, limbal and scleral stroma. (d) Preparation of the recipient eye is similar to that described for auto-limbal transplantation except that a 'bed' is not prepared to receive the limbal

ring explant. The 'open ring' of donor tissue is placed on the host limbus and sutured with interrupted 10-0 nylon sutures at the corneal and scleral margin. Six to eight sutures are first passed along the inner (corneal) edge of the donor tissue and partial thickness of host corneal stroma. A similar number of sutures are then passed directly opposite to the inner sutures, along the outer (scleral) edge of the donor tissue. These are anchored to the superficial sclera of the host. The tension on these sutures determines the final tension on the inner sutures. The knots are trimmed and buried. (e) This method invariably leaves a small gap (approximately 5–8 mm) between the two ends of the donor tissue ring (superiorly). This is filled with a piece of donor limbal tissue, cut to size, harvested from the other eye of the same donor. This piece usually requires a couple of additional sutures along either edge. (f) The host conjunctiva is approximated to the scleral edge of the transplanted limbal ring with interrupted 8-0 Vicryl sutures (absorbable). (g) A penetrating keratoplasty if required at the time of surgery is performed after the limbal ring is sutured in place. The donor graft for penetrating keratoplasty (usually 7–7.5 mm) is obtained from the central cornea of the donor whole globe. (h) A bandage contact lens is placed on the cornea and subconjunctival antibiotics and corticosteroids are injected at the end of the procedure.

Adjunctive use of amniotic membrane can be made either as a graft to provide a suitable bed for limbal explant derived epithelial cells to grow on the cornea and/or as a patch to prevent conjunctival epithelial cells from extending onto the cornea and admixing with the limbal explant derived cells (see below).

3.6.4 Adjunctive Surgery

3.6.4.1 Amniotic Membrane Grafts

The amniotic membrane serves as a useful adjunct to stem cell grafting [2, 17, 26, 50, 77, 90]. It is commonly deployed to provide a suitable substratum for the transplanted limbal graft derived epithelial cells to migrate on and form adhesion complexes. After excision of the fibrovascular tissue, if the underlying host bed is found to be irregular and scarred, use of a 9- or 10-mm disc of amniotic membrane, epithelial side up, can provide a suitable substratum for the transplanted limbal derived epithelial cells to migrate upon. The amniotic membrane can also be deployed as a biological bandage to the

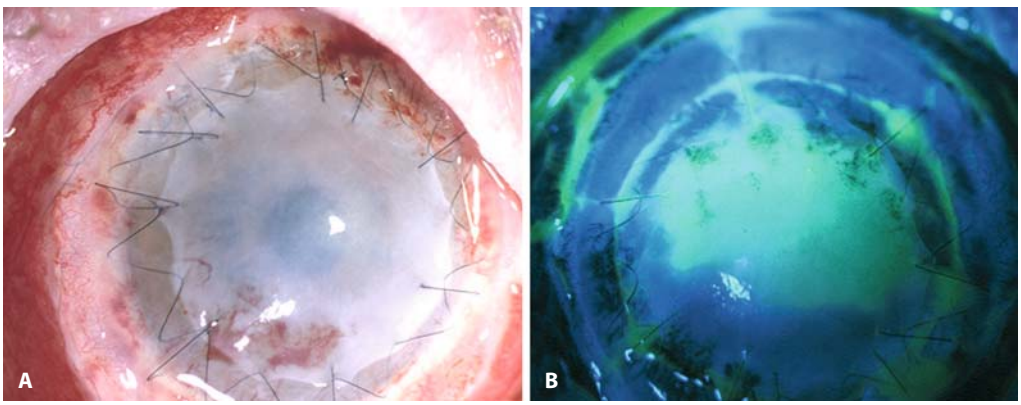

Fig. 3.16 A, B. Use of double amniotic membrane to prevent admixture of conjunctival and corneal epithelium on the corneal surface. **A** The inner membrane disc is sutured with the epithelial surface up to act as a graft and substrate for the cells to grow on, and the outer membrane, epithelial side down, acts as a patch. **B** Regenerating cells from the peritomised conjunctiva are seen growing on the outer membrane. In the absence of the outer membrane (patch) these would have encroached on the corneal surface

denuded corneal stroma, allowing epithelialisation to occur beneath it whilst trapping inflammatory cells and downregulating inflammation and scarring at the same time. Two amniotic membranes, one inner one serving as a graft and one outer membrane serving as a patch, can be simultaneously applied. The outer membrane is sutured such that its edges are tucked under the peritomised conjunctiva. Conjunctiva derived epithelium then grows on the outer membrane and is prevented from admixing with limbus-derived epithelium that is spreading onto the corneal surface. This technique was developed by the author and is regularly employed [26] (Fig. 3.16). It avoids the need for SSCE postoperatively. The outer membrane falls off or can be removed in 10–14 days. For further details on amniotic membrane, see Chap. 2 on amniotic membrane transplantation.

3.6.4.2 Ex Vivo Expansion of Limbal Cells

Limbal 'stem cell' transplantation can also be carried out by ex vivo expansion of limbal epithelial cells, either directly or on a substrate of fibrin, collagen or amniotic membrane [26, 62, 74, 75, 87].

3.6.4.3 Corneal Grafts

Lamellar or full thickness corneal grafts can be combined with auto- or allo-limbal transplantation. This may be necessary when the cornea damage is severe and when it is considered that the host corneal bed will not support a healthy epithelium despite use of an amniotic membrane. In general terms, a definitive corneal graft for visual purposes should be deferred until ocular surface epithelial integrity has been restored by limbal transplantation. However, in a recent study using limbal tissue remaining after keratoplasty from organ cultured corneoscleral discs, we have shown that such tissue (where the death to enucleation time and the time lapse between enucleation to placement in organ culture is short and where the donor is relatively young) retains good proliferative capacity for up 30 days in storage (V. Shanmuganathan, submitted to Br J Ophthalmol 2005). This offers the opportunity to carry HLA typing and matching and also allows for depletion of antigen presenting Langerhans cells. It should therefore be possible to use organ culture preserved corneoscleral discs for simultaneous allo-limbal transplant and keratoplasty with reduced risk of immune mediated rejection.

3.6.5 Postoperative Treatment

Topical preservative-free antibiotic drops such as chloramphenicol 0.5% are used four times a day for the first month. Topical preservative-free steroid drops such as prednisolone acetate 1% are used four times a day for the first 8–12 weeks, and slowly tapered during the ensuing weeks. A low dose of topical corticosteroids (one drop per day) is maintained unless elevation of intraocular pressure occurs. Autologous serum eyedrops (20%) [52, 92, 93] are given hourly until the epithelialsation is complete, usually in 7–10 days. Preservative free artificial tears are then instituted. It is important to closely monitor the re-epithelialisation process until completed. Any attempt by conjunctiva derived cells to encroach onto the corneal surface should be thwarted by SSCE, until the periphery (limbus) of the host cornea is re-epithelialised by limbus derived cells.

All patients undergoing allo-limbal transplantation also need systemic immunosuppression. Besides steroids, azathioprine, cyclosporin A, rapamycin, mycophenolate mofetil and tacrolimus (FK506, Prograf) have been used [11, 72, 78, 97]. Theoretically, immunosuppression should be continued almost indefinitely. The author has used cyclosporin A and of late FK506 up to 18 months postoperatively [78]. Attempts to reduce or stop the drug have resulted in limbal and/or corneal graft rejection episodes. Fortunately, the dose required to prevent or control rejection episodes is very low (2–8 mg/day, maintaining a blood trough level of 1–12 µg/l). Serious side effects, though they occur, are not very common, but require constant monitoring of patients and measures of kidney and liver functions. It is good practice to involve a clinical

immunologist or a physician versed in immunosuppressive therapies in the management and monitoring of these patients.

Successful stem cell grafting is a team effort involving the corneal and oculoplastic surgeons in close cooperation with the clinical immunologist. Often multiple surgical procedures are required and visual outcome, though useful from the patients' viewpoint, may be limited. The threat of limbal graft rejection is real and considerable. The all-important question of the duration of systemic immunosuppression remains to be answered. Not all long-term DNA tracking studies on recipient eyes have been able to show presence of donor derived cells even in the presence of a healthy corneal surface [38–40, 71, 76]. This would suggest that restoration of a normal surface and 'microenvironment' may allow host stem cells, either surviving limbal stem cells or bone marrow derived stem cells, to repopulate the surface. In this situation long-term immunosuppression would not be a necessity. On the other hand, long-term follow-up studies have also demonstrated that the outcome of allo-limbal transplant is not as good as that of auto-limbal transplant. This may reflect chronic 'immune mediated' damage and attrition of the transplanted limbal stem cells or the relative 'freshness' of auto grafts, conferring upon them a survival advantage.

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