Stem cell therapy in ocular surface disorders

An Essay
Submitted for partial fulfillment
Of the requirement of the
Master degree in ophthalmology

Submitted by

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Acknowledgements

First and foremost, I am most grateful to ***Allah*** *for guiding me all my way through this work, giving me a chance to know a little about one of his endless miracles in his creation. To Allah, i relate any success in my life.*

I would like to express my deepest gratitude and appreciation to Prof. ***Dr. Tarek Mohammed M. Abdallah****, Professor of Ophthalmology, Faculty of medicine, Ain Shams University, for his support, cooperation, and kind encouragement throughout this work.*

I would like also to thank ***Dr. Mohammed Saad Rashad,*** *Lecturer of Ophthalmology, Faculty of medicine, Ain Shams University, for his kind assistance, beneficial ideas, and encouraging comments to finish this work.*

Last but not least, my profound thanks to my family especially my husband for their continuous encouragement and support.

Contents

List of abbreviation

ABCG	ATP-binding cassette G
AMG	Amniotic membrane graft
AMT	Amniotic membrane transplantation
APSI	Autoimmune polyglandular syndrome
ARK	Aniridia related keratopathy
Bcrp١	Breast cancer resistant protein ١ (The formal name of ABCG٢)
bFGF	Basic fibroblast growth factor
BM	Bone marrow
BMP	Bone morphogenesis protein
BMZ	Basement membrane zone
BrdU	Bromodeoxyuridine
BSA	Body surface area
C/EBPd	CCAAT enhancer binding protein delta
CD	Cluster of Differentiation
CK	Cytokeratin
CLAU	Conjunctival limbal autograft
CLSCT	Cultivated limbal stem cell transplantation
COPs	Corneal precursors
CP	Cicatricial pemphigoid
CX	Connexin
DLK	Deep lamellar keratoplasty
DMEM	Dulbecco's modified eagle's medium
ECCE	Extracapsular cataract extraction
ECM	Extra cellular matrix
EDTA	Ethylenediaminetetraacetic acid

EEC	Ectro dactyly-ectodermal dysplasia clefting
EGF	Epidermal growth factor
eTAC	Early transient amplifying cells
EX-LAU	Ex vivo expanded limbal cell limbal autograft
FACS	Fluorescine activated cell sorting
FCS	Fetal calf serum
FGF	Fibroblast growth factor
FSPs	Focal stromal projections
Fu	Flurouracil
GDNF	glial cell-derived neurotrophic factor
GFRα	GDNF family receptor alpha
GI	Gastrointestinal
HA	Hyaluronan
HCE	Human culture epithelial cell
HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus
HLA	Human leukocytic antigen
HTLV	Human T cell lymphotropic virus
ICCE	Intracapsular cataract extraction
Ig	Immunoglobulin
IGF	Insulin like growth factor
IL	Interleukin
KAA	Keratopathy associated to aniridia
KD	Kilo Dalton
KGF	Keratinocyte growth factor
KLAL	Keratolimbal allograft
LC	Limbal crypts
LESCs	Limbal epithelial stem cells

LI	Label retaining index
LIF	Leukemia inhibitory factor
LK	Lamellar keratoplasty
Ln	Laminin
LRC	Label retaining cells
Lr-CLAL	Living-related conjunctival limbal allograft
Lr-EX-LAL	Living-related ex vivo expanded limbal cells limbal allograft
LSCD	Limbal epithelial stem cell deficiency
LSCT	Limbal stem cell transplantation
MACS	Magnetic activated cell sorting
MAP	Mitogen activated protein
MCP-١	Monocyte chemotactic protein-١
MEM	Modified Eagles′s medium
MMC	Mitomycin C
N/C	Nucleus cytoplasmic ratio
NGF	Nerve growth factor
OCP	Ocular cicatricial pemphigoid
OSD	Ocular surface disorders
PBS	Phosphate buffered saline
PDGF	Platelet-derived growth factor
PK	Penetrating keratoplasty
PV	Palisades of Vogt
PVA	polyvinyl alcohol
SC	Stem cell
Sey	Small eye
SEY	Small eye sydrome
SJS	Steven Johnson syndrome
SP	Side population

SPARC	Secreted protein acidic and rich in cysteine
SSEA-٤	Stage specific embryonal antigen ٤
TACs	Transient amplifying cells
TD	Terminally differentiated
TEN	Toxic epidermal necrolysis
TGF- β	Transforming growth factor β
TNF	Tumor necrosis factor
UV	Ultraviolet
Vs	Versus

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Introduction

Introduction

Our window to the world is provided by the cornea on the front surface of the eye. the integrity and functionality of the outermost corneal epithelium is essential for vision A population of limbal epithelial stem cells(LESC) are responsible for maintaining the epithelium throughout life by providing a constant supply of daughter cells that replenish those constantly lost from the ocular surface during wear and tear and following injury **(Notara et al, ٢٠١٠)**

Forty five million individuals worldwide are bilaterally blind and another ١٣٥ million have severely impaired vision in both eyes because of loss of corneal transparency. Treatments range from local medications to corneal transplants and more recently to stem cell theraby **(Majo et al; ٢٠٠٨)**.

The cornea, conjunctiva and limbus comprise the tissue at the ocular surface. All of them are covered by stratified squamous non-keratinized epithelium and stable tear film. The ocular surface health is insured by intimate relationship between ocular surface epithelia and preoculer tear film. Therefore, there are two types of ocular surface failure. The first one is characterized by squamous metaplasia and loss of goblet cells and mucin expression. This is consistent with unstable tear film which is the hallmark of various dry eye disorders. The second type of ocular surface failure is characterized by replacement of the normal corneal epithelium in a process called limbal stem cell deficiency **(Sangwan and Tseng, ٢٠٠١)**

Ocular conditions with abnormalities of ocular surface repair as a result of limbal stem cell deficiency include: pterygium, limbal tumours, aniridia, severe scarring

following burn, cicatricial pimphigoid, Steven Johnson syndrome, herpes simplex epithelial disease, radiation keratopathy, contact lens induced keratopathy, neuroparalytic keratitis, and drug toxicity **(Dua et al, ٢٠٠٠)**.

Stem cells are present in all self reviewing tissue and have unique properties. The epithelia of ocular surface although anatomically continuous with each other at the corneoscleral limbus, the two cell phenotypes represent quite distinct subpopulations. Stem cells for the cornea are located at the limbus. The microenvironment of the limbus is considered to be important in maintaining stemness of the stem cells. They also act as a"barrier" to conjunctival epithelial cells and prevent them from migrating on to the corneal surface **(Sangwan, ٢٠٠١)**.

In certain pathologic conditions, however, the limbal stem cells may be destroyed partially or completely resulting in varying degrees of stem cell deficiency with characteristic clinical features. These include conjunctivalization of the cornea with vasculerization, appearance of goblet cells and an irregular and unstable epithelium **(Sangwan, ٢٠٠١)**.

Partial stem cell deficiency can be managed by removing the abnormal epithelium and allowing the denuded cornea, especially the visual axis, to resurface with cells derived from the remaining intact epithelium. In total stem cell deficiency, autologus limbus from the opposite normal eye or homologous limbus from living related or cadaveric donors can be transplanted on to the affected eye. With the latter option, systemic immunosuppression is required. Amniotic membrane transplantation is a useful adjunct to the above procedures in some instances **(Dua and Azuara-Blanco, ٢٠٠٠)**.

Aim of the work

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In this essay, a review of literature of pathophysiology of ocular surface disorders with limbal stem cell deficiency. Working is attempted together with new published data concerning: Concept, techniques, and future of stem cell transplantation in this type of ocular surface disorders.

Review of Literature

Anatomy of the Ocular Surface

Anatomy of the Ocular Surface

The tissues at the ocular surface include the cornea, conjunctiva, and the intervening zone of the limbus. The primary function of the entire region is to refract and transmit light to the lens and retina. Although the cornea and its surface tear film constitute the tissue actually performing the tasks, the limbus and conjunctiva support the cornea in these important functions. **(Gipson and Surgrue, ١٩٩٤).**

The Cornea:

The cornea is highly specialized tissue that refracts and transmits light to the lens and retina. In humans, it is about twice as thick at the periphery than at the center (١ mm compared with ٠,٥ mm. **(Gipson, ١٩٩٤)**

The tissue of the cornea appears simple in composition because it is composed only of an outer stratified squamous nonkeratinized epithelium, an inner dense connective tissue stroma with its resident fibroblast-like keratocytes, and a monolayered cuboidal endothelium bordering the anterior chamber (Figure ١). Corneal transparency, avascularity, and highly ordered structure make it unique among all tissues of the body. Cells of all layers interact with and influence each others' functions. They do not act alone, but mediators (cytokines) expressed by one cell type influence cells of adjacent layers. **(Gipson and Joyce, ٢٠٠٠)**

Epithelium:

The surface of the cornea is covered by stratified squamous nonkeratinizing epithelium, which in humans, has five to seven cell layers. The epithelium is ٥٠ to ٥٢ μm thick. The corneal epithelium has functions unique to it and

functions that are common to all other epithelia of the body. Several of its unique functions include light refraction and transmittance and survival over an avasculer bed. The unique function of light refraction is brought about by its absolutely smooth, wet apical surface and its extraordinarily regular thickness. Transparency of the epithelium to light appears to be brought about by scarcity of cellular organelles and possibly by high concentrations of enzyme crystallines. **(Sax et al, ١٩٩٦)**.

The epithelium has specialized metabolic characteristics that allow it to exist over an avasculer connective tissue. **(Friend and Hassell, ١٩٩٤)**. Protection of these unique and vital functions is provided by a high density of sensory nerves that send unmyelinated endings to terminate within the suprabasal and squamous cells of the epithelium. The density of nerve endings per unit area appears to be ٣٠٠ to ٤٠٠ that of the epidermis. The epithelium also has a rapid and highly developed ability to respond to wounds **(Rozsa and Beuerman, ١٩٨٢)**

In addition to its specialized functions, the corneal epithelium has the routine housekeeping functions of all epithelia that border the outside world. The layers of cells provide a barrier to fluid loss and pathogen entrance and resist abrasive pressure by tightly adhering to one another and to the underlying connective tissue stroma. **(Gipson and Joyce, ٢٠٠٠)**

The stratified epithelium includes three or four layers of outer flat squamous cells termed squames, one to three layers of midepithelial cells termed wing cells because of their rounded cell body and lateral winglike cellular processes, and a layer of columnar basal cells (Figure ٢). The latter secrete and maintain the epithelium's basement membrane, which compared with that of the other stratified

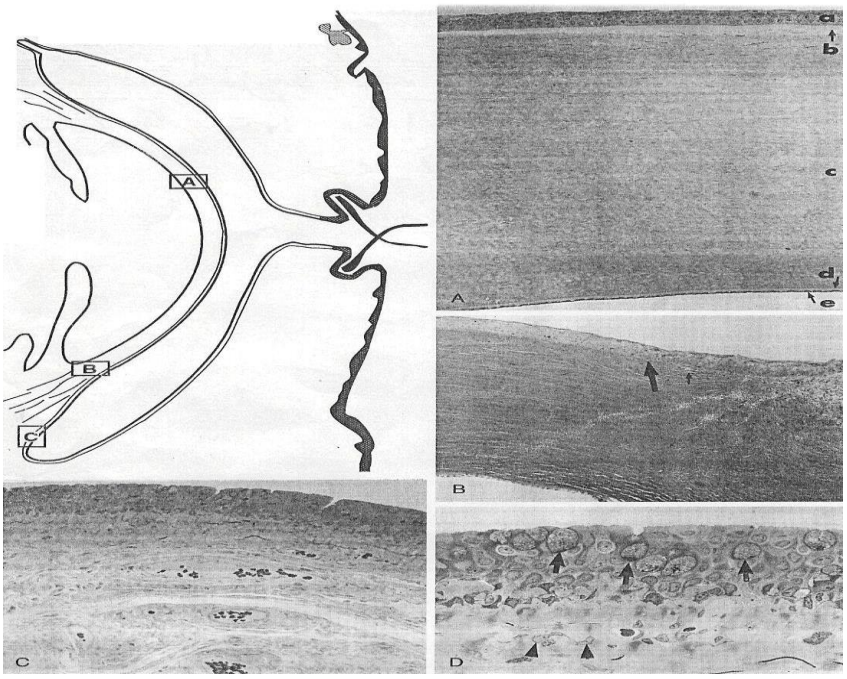

Figure (١): Diagram and light micrographs of ocular surface tissues. Boxes A through C correspond to regions in the light micrographs A through C at the right; all are the sections of human tissue, as is D, which shows a higher magnification of conjunctival epithelium. A, Section through the central cornea, a, Epithelium; b, Bowman's layer; c, Lamellar stroma; d, Descemet's membrane; e, Endothelium. B, section through the limbus. The large arrow designates the end of Bowman's layer and the small arrow the position of the first blood vessel encountered outside the corneal stroma. C, section of bulbar conjunctiva. Note the highly vasculerized connective tissue. D, section of bulbar conjunctiva demonstrating the presence of numerous goblet cells (arrows) within the stratified epithelium and the cellular nature of the connective tissue of the connective tissue of the substantia propria (arrowheads) **(Gipson and Joyce, ٢٠٠٠)**

epithelia (i.e., epidermis), is smooth or planar and nonundulating. This smooth or planar characteristic may support the regular thickness of the epithelium over the entire cornea. Like all other stratified epithelia, the epithelium of cornea is self-renewing, turning over in humans and rats in about ٥ to ٧ days **(Hanna and O'Brien, ١٩٦٠)**

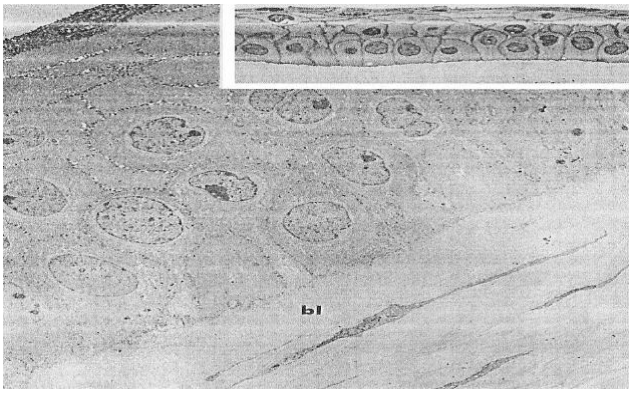

Figure (٢): Sections of corneal epithelium as seen by light (inset) electron microscopy showing superficial, wing, and basal layers and Bowman′s layer (bl). In the electron micrograph, note the surface microplicae and interdigitating cell membranes with electron dense desmosomes. Electron-lucent profiles of endoplasmic reticulum are widely scattered, primarily within basal and wing cells. Electron-dense hemidesmosomes are prominent along the basal cell membrane of the columnar basal cells adjacent to the basal lamina **(Gipson and Joyce, ٢٠٠٠)**

Basal cells are the mitotically active layers; as they divide, daughter cells begin their movement off the basement membrane toward terminal differentiation and desquamation from the apical surface. Until recently, it was thought that one daughter cell resulting from a division moved off the basal lamina, leaving one daughter cell in place to undergo mitosis again **(Lavker et al, ١٩٩١)**Recent data using bromodeoxyuridine (BrdU) labeling indicate that the two progeny of a single division move together toward the apical surface **(Beebe and Masters, ١٩٩٦)**

All cell layers of epithelium have a sparse accumulation of cytoplasmic organelles. Endoplasmic reticulum and mitochondria are sparsely distributed around the cytoplasm, with a Golgi apparatus present in supranuclear position, particularly in the basal cell layer. In the apical cell layers, Golgi cisternae and small membrane bound vesicles consistent in size and structure with Golgi associated vesicles are especially prominent **(Gipson and**

Joyce, ٢٠٠٠)

Of the three cytoplasmic filament types within all cells, keratin or intermediate filaments are the major type within the cytoplasm of cells of the corneal epithelium. On electron micrographs, the cell cytoplasm of all layers of the corneal epithelium appears full of these filaments, and keratin proteins, which polymerize to form the filaments, are among the most abundant proteins of the tissue. The keratin family of proteins that form intermediate filaments is a complex family of about ٣٠ polypeptides, which are of two classes: type I, or acidic; and type II, or neutral and basic. The intermediate filaments within ectodermally derived epithelia are formed by the pairing of two specific keratin proteins, one from each class. In the corneal epithelium, as basal cells differentiate to apical cells, two keratin pairs are expressed sequentially. First, k٥ and k١٤ are expressed in basal cells; subsequently, suprabasal cells express k٣ and k١٢ **(Kurpakus et al, ١٩٩٠)**. K١٢, a ٦٤-KDa Keratin, is believed to be cornea specific **(Schermen et al, ١٩٨٦)**

The keratin filament system within the corneal epithelium provides a structural framework for the epithelial cytoplasm. It has been suggested that the cytokeratin filaments not only increase the tensile strength of the epithelial cells but also, by keeping the nucleus and other organelles in their proper positions, dramatically affect the overall organization of the cell. A major role of the intermediate filaments of the corneal epithelium is to provide the cytoskeletal component of the system that anchors cells tightly to one another and to their substrate through the desmosome and hemidesmosome. Such tight anchorage is critical to a stratified epithelium that borders the outside world and is subject to the abrasive pressures from lid movement and eye rubbing **(Gipson and Joyce, ٢٠٠٠)**

The other two types of cytoskeletal filaments within cells are actin filaments and microtubules. Actin filaments, as with all cells are present throughout the cytoplasm of cells of the corneal epithelium. They are particularly prevalent as a network along the apical cell membranes of the epithelium, where they extend into microplicae (Figure ٣), and at the junction of the lateral membranes, where they are associated with adherens and tight junctions **(Gipson and Anderson, ١٩٧٧)**. The actin filament system is particularly important in providing the cytoskeletal connection of cell adhesion molecules, such as the integrins and cadherins, and the cytoskeletal component of adherens and tight junctions in epithelia. **(Gipson and Joyce, ٢٠٠٠)**

Composed of both α- and β-subunits of the proteins known as tubulins, microtubules are the third major cytoskeletal element within all cells. They have not been studied in detail within the fully differentiated corneal epithelium. Although they are not obvious on electron micrographs of corneal epithelia, they are obvious within the spindles of mitotic basal cells, where they provide the cytoskeletal framework for chromosome segregation. They do not appear to play a significant role in corneal epithelial wound healing, indicating that they are not required for epithelial migration and that mitosis is not required for epithelial wound coverage **(Gipson et al, ١٩٨٢)**

The intercellular junctions:

The corneal epithelium, like all other epithelia, has intercellular junctions that function not only in cell adhesion but also in cell communication and barrier formation. Four junction types are present. Desmosomes, which are present along the lateral membranes of all corneal epithelial cells, function in cell-to-cell adherence; adherens junctions,

which are present along the lateral membrane of the apical cells of the epithelium, function to maintain cell-to-cell adherence in the region of tight junctions; the tight junctions are present along with adherens junctions in apical cell lateral membranes, where they function to provide a paracelluler permeability barrier; and gap junctions, which function in cell-to-cell communication, allow intercellular passage of small molecules up to ٢٠٠٠ Da. The latter are present along lateral membranes of all cells of the epithelium. Basal cells have gap junction with a different molecular composition (connexin ٤٣) than suprabasal cells (connexin ٥٠) **(Alberts et al, ١٩٩٤)**. Molecules present along cell membranes also function in cell-to-cell adhesion. Two types of cell adhesion molecules in membranes of corneal epithelial cells outside specialized junction regions are cadherins (specifically, E-cadherin) **(Gipson, ١٩٩٤)** and several of the integrin heterodimers **(stepp et al, ١٩٩٣)**

The two surfaces of the corneal epithelium, the apical and basal surfaces, have specializations indicative of their roles in the epithelium. The apical surface is specialized to maintain the tear film and mucous layer **(Nichols, ١٩٨٥)** and, with that layer, provides the extraordinary smooth refractive surface of the cornea. To facilitate this function, the apical cell membrane has short rigid like folds, termed microplicae, that form regular undulations of the membrane when viewed in cross-section (Figure ٣). In addition, microvilli (finger-like projections of the membrane) Up to ١ µm in length are present. These two membrane specializations presumably supply an increased surface area for adherence of the mucous layer of the tear film. Scanning electron microscopic studies of the corneal surface demonstrate that apical cells scatter electrons to varying degrees. Cells that scatter electrons to a lesser degree are termed dark cells. Light cells, which scatter electrons to a greater degree, have a higher density of surface microplicae

and microvilli **(Pfister, ١٩٧٣)**

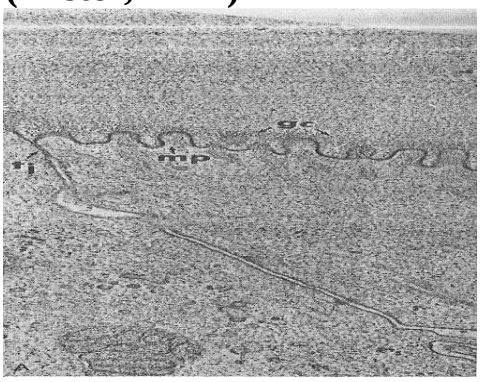

Figure (٣): micrographs showing specializations of the apical membrane of the apical cells of the ocular surface. Electron micrograph of mucin layer preserved on apical membranes of guinea pig conjunctiva. Note microplicae (mp) in cross-section and electron density of the glycocalyx (gc) regions at the tips of the micropicae. Note tight junction (ti) between adjoining cells **(Gipson and Joyce, ٢٠٠٠)**

It has been hypothesized that the dark cells with fewer surface membrane specializations represent the "oldest" cells of the ocular surface and therefore are about to desquamate **(Pfister, ١٩٧٣)**. The undulating, specialized apical membrane bears a prominent glycocalyx that is intimately associated with the tips of the microplicae and with the mucous layer of the tear film. The corneal cells express a membrane spanning mucin, designated MUCI, which is present in the apical cell membrane and is component of the glycocalyx **(Inatomi et al, ١٩٩٥)**

The basal surface of the epithelium is specialized to provide tight anchorage to the stroma **(Gipson et al, ١٩٨٧)**. A series of linked structures, termed the anchoring complex, extends from the cytoplasm of the basal cell, through the basal cell membrane, then through the basal lamina and into the anterior of Bowmen's layer at the anterior region of the stroma. The structures of the anchoring complex visible by electron microscopy include keratin filaments that insert into the hemidesmosome plaque; the hemidesmosome, which is the specialized anchoring junction on the basal

membrane; anchoring filaments, which extend from the hemidesmosome to the basement membrane; and anchoring fibrils, which extend from the basement membrane into Bowmen's layer. These anchoring fibrils form an interwining network and terminate distal to the basement membrane in anchoring plaques. The linked structures and their molecular components are shown diagrammatically in Figure (٤) (**Gipson and Joyce, ٢٠٠٠**).

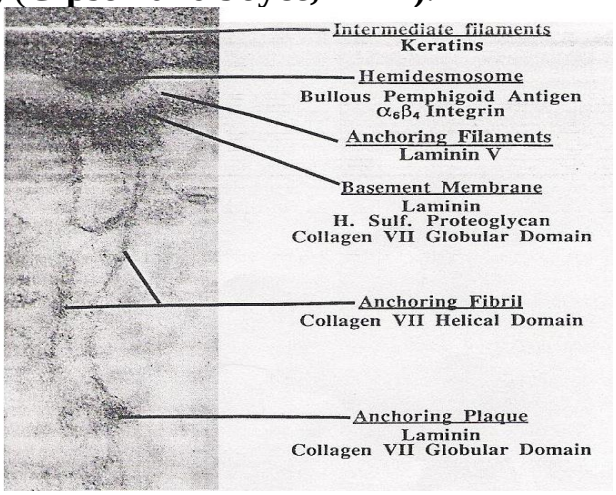

Figure (٤): Electron micrograph demonstrating adhesion complex of the corneal epithelium. The linked structures of the complex and their known molecular components are identified **(Gipson and Joyce, ٢٠٠٠)**.

Stroma:

The corneal stroma as shown in Figure (١) is the connective tissue located between the epithelial basal lamina and Descemet's membrane, the thick extracelluler matrix secreted by the endothelial monolayer. The stroma comprises about ٩٠٪ of the corneal thickness and includes both Bowmen's membrane and lamellar stroma. The major functions of the stroma are to maintain the proper curvature of the cornea as the primary lens of the eye, to provide mechanical resistance to intraocular pressure, and to transmit light into the eye without significant absorbance.

Corneal transparency is dependent on the maintenance of a low level of stromal hydration and on the orderly arrangement of collagen fibers within the stroma **(Gipson and Joyce, ٢٠٠٠)**

Bowman's Layer:

Bowman's layer (Figure ٥) is an ٨- to ١٠- µm acelluler zone of randomly arranged collagen fibrils that forms an interface between the basal lamina of the epithelium and the subjacent lamellar stroma**.** Constituents of this layer are believed to be synthesized and secreted by both epithelial cells and stromal keratocytes **(Tisdale et al, ١٩٨٨)**. Bowman's membrane contains several collagen types, including type I, V, and VII **(Gordon et al, ١٩٩٤),** and proteoglycans such as chondroitin sulfate proteoglycan **(Li et al, ١٩٩١)**. Both Bowman's membrane and lamellar stroma contain fibrils composed of collagen type I and V; however, the fibrils in Bowman's membrane are smaller in diameter (about ٢٠ nm) than those in the stroma (٢٥ to ٣٠ nm) **(Birk et al, ١٩٨٦)**. Fibril diameter appears to be regulated by the relative ratio of type V to Type I collagen—the greater the amount of type V, the smaller the fibril diameter **(Birk et al, ١٩٩٠)**

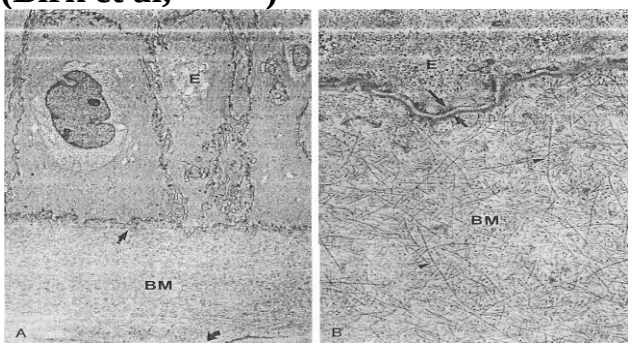

Figure (٥): A, Bowman's membrane (BM) forms an acellular interface between the basal cells of the epithelium (E) with its basement membrane (straight arrow) and the lamellar stroma (curved arrow). Note the relative thickness and felt-work like appearance of Bowman's membrane. B, The random arrangement of collagen fibrils

(arrowheads) is shown. Also note the close association of the hemidesmosomal structures (large arrow) on the basal aspect of the epithelial cells, the highly organized extracellular matrix (small arrow) of these cells and Bowman's membrane **(Gipson and Joyce, ٢٠٠٠)**

The specific function of this layer is not clearly understood, but its network of collagen fibrils may stabilize the transition between the epithelial and stromal layers, ensure adhesion of the overlying epithelial cells to the stromal matrix, and contribute to the smooth curvature of the corneal surface **(Gipson and Joyce ٢٠٠٠)**

Lamellar stroma:

The lamellar stroma is the thick collagenous layer posterior to Bowmen's membrane. Collagen type I and V are the predominant fibriller collagens in the lamellar stroma, although small amounts of other fibrillar collagens, such as type III, also may be present **(Trelstad, ١٩٧٤)**.The stroma contains collagen type XII, which cross-links fibrillar collagens, and type VI, which forms microfibriller networks **(Zimmermann, ١٩٨٦)**. Keratin sulfate proteoglycan and dermatan sulfate proteoglycan are the predominant proteoglycans within the corneal stroma **(Hassell et al, ١٩٩٢).** Lumican is the core protein of keratin sulfate proteoglycan, whereas decroin is the core protein for derrmatan sulfate proteoglycan **(Gipson and Joyce, ٢٠٠٠)**

As shown in figure (٦ A), the constituents of the lamellar stroma are organized precisely. The basic structural unit of the fibrillar collagens is tropocollagen, an asymmetric molecule about ٣٠٠ nm long and ١,٥ nm in diameter. Fibriller collagens are composed of three polypeptide chains coiled in a triple helix. These molecules polymerize to form elongated collagen fibrils with diameters of ٢٥ to ٣٠ nm. The uniformity of collagen fibril diameter appears to result from specific interactions between type V

collagen, located toward the center of the fibril, and type I collagen, on the fibril exterior. As mentioned previously, the relative ratio of type V to type I collagen appears to regulate fibril diameter. The interfibrillar distance also is highly uniform and be maintained by apposing interactions at the fibril surface. In the thick cornea, type XII collagen binds to type I on the fibril exterior and may form lateral "bridges" between fibrils, thus limiting interfibriller distance **(Olsen and McCarthy, ١٩٩٤)**.

Proteoglycans bind to the exterior surfaces of collagen fibrils. The polyanionic nature of glycosaminoglycan side chains attracts cations and water molecules and may exert a swelling pressure on the collagen fibrils, which is balanced by the interactions between collagen type I and XII. Microfibrils composed of type VI collagen also associate with type I collagen **(Kobayashi, ١٩٩٥)**, but the specific function of these fibrils is not known. Collagen fibrils are packed in parallel bundles extending from limbus to limbus, and the bundles are arranged in layers, or lamellae. The stroma of the human eye contains ٢٠٠ to ٢٥٠ lamellae. Lamellae in the middle and posterior regions of the stroma are arranged at approximate right angles, where those in the anterior stroma are arranged at less than right angles. The small diameter of the collagen fibrils and their close, regular packing creates a lattice or three-dimensional diffraction grating **(Berman, ١٩٩١)**

The lattice "theory" of Maurice **(Maurice, ١٩٥٧)** suggests that the ability of the cornea to scatter ٩٨٪ of incoming light results from equal spacing of the collagen fibers. Scattered light waves interact in an ordered fashion, eliminating destructive interference. The lamellar organization of the stroma also produces a uniform tensile strength across the cornea, withstanding intraocular pressure and maintaining appropriate corneal curvature **(Gipson and**

Joyce ٢٠٠٠)

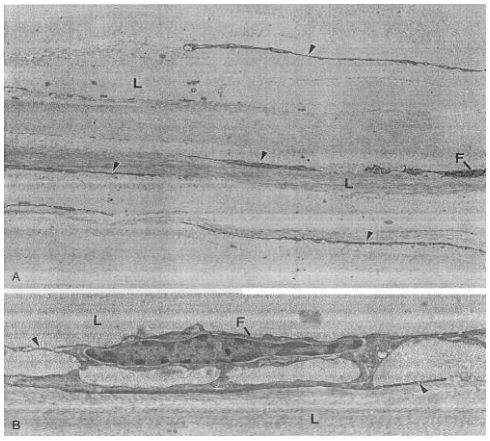

Figure (٦): Sections of corneal stroma showing collagen bundles arranged in lamellae (L), which are oriented in different angles. A, Micrograph illustrating the stacked lamellae and long, attenuated processes (arrowheads) of the stromal fibroblasts (F) located between the lamellae. B, collagen bundles in the upper lamella (L) are sectioned crosswise, whereas those in the lower lamella are sectioned at an angle. Junctions between the cytoplasmic processes of neighboring fibroblasts form a network of communicating cells **(Gipson and Joyce, ٢٠٠٠)**

The matrix components of the lamellar stroma are secreted and maintained by stromal fibroblasts, also known as keratocytes. As shown in (Figure ٦ B), these long, attenuated cells are arranged parallel to the corneal surface and are located between the collagen lamellae. The keratocyte cell body contains an elaborate rough endoplasmic reticulum and Golgi apparatus, reflecting its active synthetic function. Keratocytes extend slender cytoplasmic processes and can form gap junctions with neighboring cells, resulting in a network of communicating cells **(Watsky, ١٩٩٥)**

An ultrastructural study of human cornea demonstrated the presence in central stroma of unmylinated nerve fibers that run parallel to the collagen bundles, pass through Bowman's membrane and the basal lamina of the epithelium, and associate with sub-epithelial cells. Nerve fibers were found to invaginate stromal keratocytes as well

as corneal epithelial cells. This finding suggests that nerves may mediate information exchange between the epithelium and stroma under certain conditions, such as corneal wounding **(Gipson and Joyce, ٢٠٠٠)**

The endothelium:

The endothelium is the single layer of cells located at the posterior of the cornea; it permits the passage of nutrients from the aqueous humor into the cornea **(Klyce and Beuerman, ١٩٨٨).**

The requirement for a monolayer that is "leaky" to aqueous humor is met through the barrier function of the endothelium, formed by cell–to–cell contacts and interdigitating lateral membranes. The endothelium is the major cell layer responsible for maintaining the relatively low level of stromal hydration necessary for corneal transparency. Stromal hydration is controlled by the activity of ionic pumps in the plasma membrane of endothelial cells. The relatively high extracelluler ion concentration produced by these pumps draws water from the stroma, thus maintaining the highly organized collagen lamellar structure required for corneal transparency. The endothelium also secretes a thick basal lamina, termed Descemet's membrane, which lies between the endothelial cells and the posterior stroma. Descemet's membrane is one of the thickest basement membranes found in normal tissue; however its specific function is unknown **(Gipson and Joyce, ٢٠٠٠)**

Morphologic characteristics:

At birth, the endothelial monolayer consists of about ٤٠٠,٠٠٠ cuboidal cells. Each cell is ٤ to ٦ μm thick, is about ٢٠ μm wide, and has a surface area of about ٢٥٠ μm^2.

The average cell density at birth is about ٤٠٠٠ cells/mm² **(Svedbergh and Bill, ١٩٧٢)**. In young corneas, the endothelium forms a pattern of polygonal cells with five to seven sides **(Rao et al, ١٩٨٢)**. Scanning electron microscopy of the monolayer surface (Figure ٧) reveals the hexagonal cell shape and numerous lateral, interdigitating cellular processes **(Sherrard and Ng, ١٩٩٠)** these processes increase the area of contact between neighboring cells and resemble interlocking fingers. Occasionally, a centrally located cilium, about ٢ to ٧ µm long is present on the surfaces of peripheral cells. The function of this cilium is unclear **(Gipson and Joyce, ٢٠٠٠)**

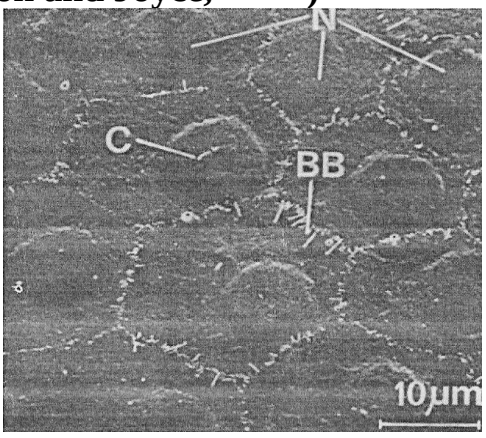

Figure (٧): Scanning electron micrograph of the surface of the corneal endothelium illustrating the hexagonal shape of the cells as well as other surface features, including nuclei (N) that bulge from the cell surface, a single cilium (C), and long lateral projections (BB) that bridge from one cell onto the body of adjacent cells **(Svedbergh and Bill, ١٩٧٢)**.

Ultrastrucural characteristics:

The ultrastuctural features of the endothelium reflect its functions. Numerous mitochondria within the cytoplasm indicate that these cells are metabolically active. The cytoplasm also contains an elaborate rough and smooth endoplasmic reticulum, numerous ribosomes, and a prominent Golgi apparatus reflective of high level of protein

synthesis. A circumferential band of actin–containing microfilaments is located beneath the apical plasma membrane at the cell periphery and most likely is involved in maintaining cell shape and mediating cellular movement **(Fujino and tanishima, ١٩٨٧)**

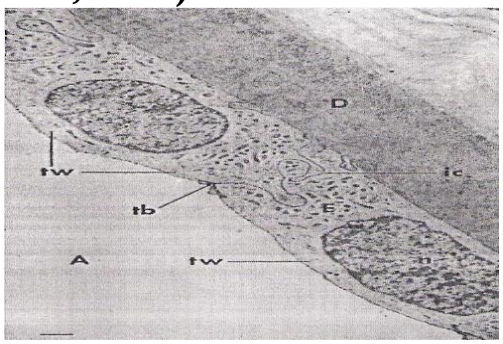

Figure (٨): Low magnification transmission electron micrograph illustrating the general orientation and ultrastuctural features of the corneal endothelium and Descemet's membrane. A band of actin-containing filaments, termed terminal web (tw), is present in the anterior aspect of the cells and excludes other cell organelles. junctional complexes located on the apical aspect of the lateral plasma membranes are visible at this low magnification as a terminal bar (tb). The intercellular border (ic) formed between adjacent cells is long and sinuous. A, anterior chamber; E, endothelium; n, nucleus; D, Descemet's membrane. Bar = ١µm **(Iwamoto and Smesler, ١٩٦٥)**.

Endothelial cells synthesize and secrete a thick basal lamina known as Descemet's membrane. Focal areas of increased electron density are present on the cytoplasmic aspect of the basal plasma membrane and may represent a form of adhesion plaque anchoring the endothelium to Descemet's membrane. Cytoplasmic processes extend from the basal aspect of the cells and penetrate Descemet's membrane, possibly contributing to increased adhesiveness of the monolayer **(Iwamoto and Smelser, ١٩٦٥)**

Focal tight junctions (maculae occludentes) on the apical aspect of the lateral membranes are small areas in which the outer leaflets of the plasma membranes of adjacent cells appear to fuse, obliterating the extracelluler

space **(Kreutziger, ١٩٧٦)**

Gap junctions formed between adjacent cells (Figure ٩) are located at all levels of the lateral plasma membrane below the tight junctional complexes **(Montcourrier and Hirsch, ١٩٨٥)** they possess a characteristic pentalaminar structure and are the site of electrical and metabolic coupling, which facilitates cell to cell communication **(Rae et al, ١٩٨٩)**

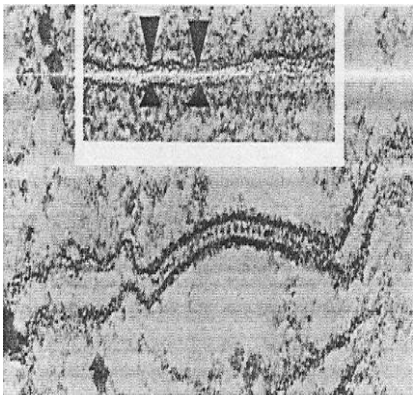

Figure (٩): Electron micrograph of gap junctional complexes illustrating the characteristic regular spacing of the connexin cross-bridges that draw adjacent plasma membranes into close apposition. Inset, Arrowheads indicate areas in which the gap between cell membranes is clearly visible **(leuenberger, ١٩٧٣).**

Barrier function:

As an avascular tissue, the cornea receives oxygen mainly from the environment through the tear film **(Weissman et al, ١٩٨١)**, but its nutritional requirements are met by the aqueous humor. As such glucose, amino acids, and vitamins needed by the epithelial cells and stromal keratocytes must traverse the corneal endothelial monolayer. This nutrient transport occurs primarily through a paracellular route; that is solutes move between the cells rather than being actively transported through them. This form of transport requires that the endothelial monolayer be leaky to substances within the aqueous humor but not permit bulk fluid flow into corneal stroma. The barrier to bulk fluid

flow from the aqueous humor to the stroma is formed primarily by the focal tight junctions of the endothelium. Experiments with molecular tracers indicate that small molecules do not penetrate the tight junctions but rather enter the intercellular spaces by leaking around them **(Montcourrier and Hirsch, ١٩٨٥)**

Gap junctions and the sinuous, elaborate interdigitation of the lateral plasma membranes together may form a secondary barrier to fluid flow **(Watsky, ١٩٩٠)**. Gap junctions narrow the width between apposing cell membranes from normal intercellular gap of ٢٥ to ٤٠ nm to about ٣ nm **(Kreutziger, ١٩٧٦)**

Pump function:

Transparency is essential for the function of the cornea as the primary lens of the eye. Transparency results from the uniformity of the tissue elements comprising the cornea and from the regularity of their spatial organization **(Mishima, ١٩٨٢)**

Precise arrangement of the collagen bundles within the corneal stroma is especially important for corneal clarity. This precise arrangement depends to a great extent on the maintenance of a relatively low level of stromal hydration. Proteoglycans associated with the collagen fibrils within the stroma bind water, producing a natural pressure gradient across the endothelial monolayer. In addition, loss of integrity of the endothelial cell layer can hydrate the stroma. The disorganization of collagen fibrils that results from stromal swelling causes light absorbance, corneal clouding, and reduced vision **(Gipson and Joyce, ٢٠٠٠)**

The endothelium maintains a low level of stromal hydration by the activity of ionic pumps. Studies suggest

that metabolic energy is needed to maintain normal corneal thickness **(Harris, ١٩٦٢)**. This energy requirement appears to be associated with the activity of specific adenosine triphosphatases (ATPases), located in the lateral plasma membrane **(McCartney, ١٩٨٧)**, that catalyze ion exchange. These ATPases, which act as the metabolic pump, are believed to function by creating a net ionic flux from the intracellular to the extracellular milieu. The osmotic gradient produced causes water to be drawn passively from the stroma to the aqueous humor **(Hodson, ١٩٧٧)**. At least two active transport systems appear to contribute to the pumping mechanism: a Na+, k+ -ATPase pump **(Lim and Fischbarg, ١٩٨١)** and a bicarbonate-dependent Mg^{2+} -ATPase pump **(Mayes and Hodson, ١٩٧٩)**

The requirement that the endothelium permit passage of nutrients into the cornea and, at the same time, maintain a barrier to the free flow of water into the stroma presents an interesting cell biologic paradox. The pump-leak hypothesis attempts to resolve this paradox, stating that the rate of leakage of water and solutes into the corneal stroma is balanced by the rate of pumping of excess water from the stroma back to the aqueous **(Maurice, ١٩٨٤)**

As long as the equilibrium suggested by this hypothesis is maintained, the corneal stroma remains relatively dehydrated, and corneal clarity is maintained. (Figure ١٠) illustrates this equilibrium. Any imbalance between the rate of fluid leak into cornea and the rate of ionic pumping of fluid out of the cornea results in corneal swelling and loss of visual acuity **(Gipson and Joyce, ٢٠٠٠)**

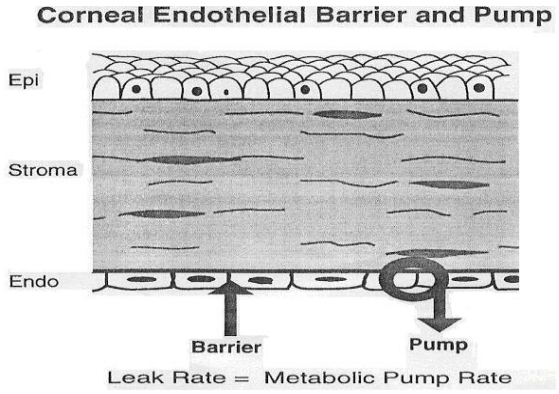

Figure (١٠): The "pump-leak" hypothesis. When the rate of fluid leakage into the stroma is balanced by the rate of fluid pumped out of the stroma, normal corneal architecture and thickness are maintained **(Waring et al, ١٩٨٢)**.

Cell division and monolayer repair:

Corneal endothelial cells are capable of normal division during fetal development; however, the total corneal endothelial cell reserve is limited because cell division in adult cells is limited **(Landshman et al, ١٩٨٨)**

At birth, endothelial cell density is ٣٥٠٠ to ٤٠٠٠ cells/mm^{٢}; in adults, this density is reduced to ١٤٠٠ to ٢٥٠٠ cells/mm^{٢} **(Svedbergh and Bill, ١٩٧٢)**. Cell density begins to decrease during fetal development as a result of both a rapid growth in corneal size and the limited mitotic activity that occurs after the second trimester of pregnancy **(Bahn et al, ١٩٨٦)**. Once rapid corneal growth subsides, cell density continues to decrease, but at a slower rate. Beginning at about the second year of life, decreased cell density is directly related to endothelial cell loss and the inability of the endothelium to reproduce in numbers sufficient to keep pace with this loss **(Murphy et al, ١٩٨٤)**. The overall rate of cell loss accelerates if the endothelium is injured from trauma, disease, or dystrophy **(Ikebe et al, ١٩٨٨)**.

Polymegathism (i.e., heterogeneity in cell size)

increases in the endothelium with age and as the result of damage caused by trauma, corneal infection, or disease. Cell size can become heterogeneous for several reasons. When the endothelium is injured or when cells are lost because of normal attrition, repair of the defect in the monolayer occurs mainly through enlargement and spreading of neighboring cells, causing cells to be larger in these areas **(Honda et al, ١٩٨٢)**. In addition, the number of multinucleated cells and cells with greater than the normal diploid DNA content increase with age, producing a population of very large cells. Increased heterogeneity in cell shape (i.e., pleomorphism) also occurs with age or trauma **(Schultz et al, ١٩٨٤)**

As the number of cells within the monolayer decreases and the cell enlarge, there is a decrease in the percentage of hexagonal cells within the monolayer. As polymegathism and pleomorphism increase, the endothelial monolayer can become destabilized. It is well known that a regular hexagonal pattern provides the "cellular packing" with an optimal cell-to-membrane ratio. Irregular cell sizes and shapes can increase surface tension within the monolayer, producing decreased geometric and architectural stability **(Gipson and Joyce, ٢٠٠٠)**.

When cell numbers are reduced as the result of aging or trauma and the remaining cells become larger and more pleomorphic, the ability to maintain or restore normal barrier and pump function can be compromised **(Carlson et al, ١٩٨٨)**. With decreased monolayer stability, permeability increases and the cornea can swell. Decompensation (i.e. loss of monolayer integrity and function) can occur when cell density fall below ٣٠٠ to ٤٠٠ cells/mm^{٢} or when the mean cell size reaches ٣٠٠٠-٣٥٠٠ μm^{٢} (**Landshman et al, ١٩٨٨**). Because of the stressed state of the endothelial monolayer under these conditions, the leak rate of fluid into the stroma becomes greater than the pump rate of fluid flow

out of the stroma, producing stromal edema and corneal clouding. Transplantation is the normal resource for reestablishing corneal clarity and visual outcome after decompensation of the corneal endothelium **(Gipson and Joyce, ٢٠٠٠)**.

Investigators are reexamining the relative proliferative capacity of corneal endothelial cells. These cells appear to divide in vivo, but at a very slow rate **(Singh, ١٩٨٦)**. Limited proliferation also has been observed in cells adjacent to the site of injury to the endothelial monolayer **(Laing et al, ١٩٨٤)**. Addition of growth factors, such as epidermal growth factors, basic fibroblast growth factors, acidic fibroblast growth factors, to wounded endothelium in organ culture can increase DNA synthesis in cells at the wound edge **(Couch et al, ١٩٨٧)**.

Descemet's Membrane:

Descemet's membrane is the thick extracellular matrix synthesized and secreted by the corneal endothelium (Figure ١١). In adults, this matrix consists of two layers. An anterior banded layer is formed during fetal development and consists of highly organized collagen lamellae and proteoglycans. A posterior amorphous layer is synthesized after birth and is less organized than the fetal layer. Adult Descemet's membrane contains fibronectin, laminin, type IV and type VIII collagen, heparan sulfate, and dermatan sulfate proteoglycan **(Sawada et al, ١٩٩٠)**

Corneal endothelial cells slowly synthesize and secrete basement membrane material throughout life. In young adults, the posterior layer measures about ٢ µm but increases to about ١٠ µm in older adults. The positive correlation between age and Descemet's membrane thickness suggests that there is little, if any, destruction of

previously formed basement membrane material **(Murphy et al, ١٩٨٤)**

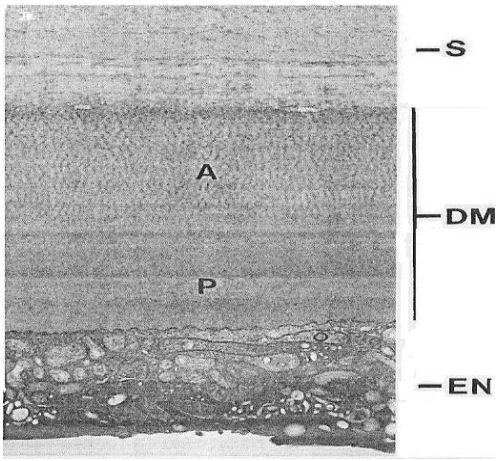

Figure (١١): Micrograph illustrating Descemet's membrane (DM) located between the posterior aspect of the corneal stroma (S) and the underlying endothelium (EN). Two regions of Descemet's membrane are apparent in adult corneas. The anterior "banded" region (A) is secreted by he endothelial cells during fetal development and is more highly organized than the posterior "amorphous" region (P), which is secreted after birth. The posterior region increases in thickness with age as a result of continued synthesis of its constituents by the endothelium throughout life **(Gipson and Joyce, ٢٠٠٠)**

Limbus

The various anatomic definitions of the limbus include the anatomist's limbus, the pathologist's limbus **(Jakobiec and Ozanics, ١٩٨٢)**. The various definitions and various angles of lines drawn on sections or diagrams of cross-section of the region indicate that there are no definite reliable boundaries to the zone. The broadest definition of the limbus is the zone between a line drawn between the termini of Bowman's layer and descemet's membrane, which forms the anterior border, and a line that passes parallel but ١ mm posterior to the anterior line, passing through the posterior end of Schlemm's canal. In this definition, both Schlemm's canal and the trabecular meshwork are within the limbus. This section reviews aspects of the limbus relevant to ocular surface function,

specifically the superficial region, including the epithelium and loose connective tissue overlying the interface of the connective tissue at the corneoscleral junction (Figure ١B). the limbal region has been termed the transition zone between cornea, conjunctiva, and sclera, and although that may be apt description, the specialized characteristics of the limbal epithelium and its immediate subjacent connective tissue indicate that the region has specialized functions supporting the cornea and that it may be a barrier to conjunctival overgrowth of the cornea **(Gipson and Joyce, ٢٠٠٠)**

Epithelium:

The epithelium of the limbus has many features common to corneal epithelium. It is a stratified squamous nonkeratinizing epithelium but has several more cell layers than corneal epithelium **(Srinivasan et al, ١٩٨٢)**

Cell junctions in the limbus are similar to those in the cornea, and the apical and basal specializations present in the limbus are the same as those in the cornea. The basal cells of the limbal epithelium appear unique and are believed to be stem cells for maintenance of the corneal epithelium **(Gipson and Sugrue, ١٩٩٤)**

The cells appear smaller and less columnar and have more cytoplasmic organelles. The basal cells sit on a basement membrane that is not flat and planar like that of the cornea; peglike interdigitations of the epithelium and stroma are present (Figure ١٢) **(Hogan et al, ١٩٧١)**.

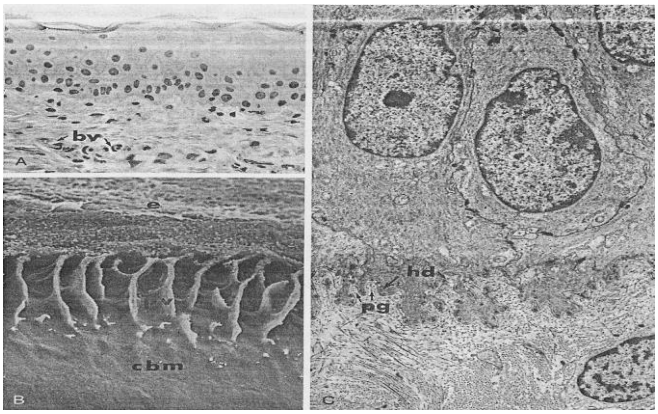

Figure (١٢): Micrographs of the limbal epithelium and subjacent connective tissue. The basal cells of the region are smaller and less columnar than those of the cornea. A, Light micrograph showing the absence of Bowman′s layer and the cellular stroma and blood vessels (bv). B, scanning electron micrograph of palisades of Vogt (pv); limbal epithelium was removed with ethyl-enediaminetetraacetic acid to demonstrate the folds or ridges in the connective tissue. Remnant epithelium (e) and denuded corneal basement membrane (cbm) are labeled. C, Electron micrograph of basal cells of limbal region. Note their small size and the hemidesmosomes (hd) present along the undulating basement membrane. Small, retelike pegs (pg) of stromal matrix extend into the epithelium (**Gipson and Joyce, ٢٠٠٠**)

Other unique characteristics of the basal cell layer include long H-thymidine label retention time, indicating slower passage through the cell cycle than basal cells of the cornea and conjunctiva **(Lavker et al, ١٩٩١)**; differences in keratin expression compared with suprabasal cells of the limbus and cells of the corneal epithelium **(Schermen et al, ١٩٨٦)**; and enhanced presence of certain metabolic enzymes and proteins, including α-enolase, cytochrome oxidase, Na^+, K^+ - ATPase, carbonic anhydrase, metallothionein, and glucose transporter **(Zeiske et al, ١٩٩٢)**.

Another characteristic of the region is that ocular surface tumors occur primarily in the limbal area and rarely are found on the cornea. Taken together, these data and those from experiments demonstrating centripetal migration of cells from the limbal region into the cornea over time indicate that the limbal basal cells are the stem cells of the corneal epithelium. Further evidence that these basal cells

are important to maintenance of the corneal epithelium comes from clinical data that demonstrate the effectiveness of limbal transplantation in the treatment of persistent, non-healing corneal problems. In addition, these basal cells are protected by pigmentation and are present within deep crypts in the limbal connective tissue, termed the palisades of Vogt (Figure ١٢ B) **(Gipson and Joyce, ٢٠٠٠)**

Connective tissue:

The connective tissue underlying the limbal epithelium is loose and less organized than the corneal stroma, and Bowman's layer is not present. Although the molecular composition of the two matrices appear to correspond, an exception is absence of the keratan sulfate proteoglycan (lumican) **(SundarRaj et al, ١٩٨٦)**

Cellular elements within the limbal stroma are more diverse than in the corneal stroma. In addition to fibroblasts, melanocytes, mast cells, lymphocytes, and plasma cells occur routinely. A major difference between the limbal stroma and that of the cornea is the presence of blood and lymphatic vessels that loop into the area of the limbal stroma. These vessels include capillaries, small arterioles and venules, and large lymphatics. Bundles of unmylinated nerves also are present. The palisades of Vogt, large folds of matrix, are a unique characteristic of this area (Figure ١٢ B). The outward folds of connective tissue are large enough to accommodate small blood vessels, lymphatics and nerves, and crypts of limbal epithelium reach down into the palisades of Vogt. The deep housing of limbal epithelium in the folds not only may protect the stem cell population but also may increase the surface area for accommodating a large cell population and increase exposure to vascularly derived nutrients and effector molecules. In addition to the large macroscopic folds of palisades of Vogt, tiny rete

(peglike folds or outpockets of stroma) begin in the peripheral stroma and extend through the limbal region into the conjunctiva (Figure ١٢ C). These rete may increase the surface area of the basal cell membrane of basal cells and may provide for additional anchoring strength in a region where hemidesmosomes are not as extensive **(Gipson, ١٩٨٩)**

Conjunctiva

General characteristics and description of regions:

The conjunctiva is the mucous membrane that covers the inner surfaces of the upper and lower lids and extends to the limbus on the surface of the globe. The two major functions of this tissue, besides connecting the lids to the globe, are provision of mucus for the tear film and protection of the ocular surface from pathogens through immune tissue. The ducts of the lacrimal, accessory lacrimal, and meibomian glands enter the conjunctival epithelium and deliver their respective products to the tear film. Three regions within the conjunctiva are recognized: the palpebral or tarsal region, which lines the inner surface of the lids; the fornical region, which lines the upper and lower surfaces of the recess or cul-de-sac known as the fornix; and the bulbar region, which lines the surface of the sclera between the fornix and the limbus. The conjunctiva has two structural components throughout all regions: the surface epithelium and the substantia propria (Figure ١٣). **(Gipson and Joyce, ٢٠٠٠)**

Epithelium:

Conjunctival epithelium is unique among stratified non- keratinizing epithelia in that it has goblet cells intercalated within it (Figure ١٣). The goblet cells are the

major producers of mucin for the tear film. Reports of the number of cell layers in the stratified epithelium vary, especially regarding fornical and bulbar areas. These variations may result from different degrees of stretch on the tissue at time of fixation for histologic study. Cell layers of the palpebral conjunctival epithelium do not vary as much, perhaps because the substantia propria is not as loose and contractile at fixation. Reports have varied from ٢ or ٣ cell layers to ١٠ to ١٢, the latter number of layers being present at the lid margin near the junction with the epidermis covering the external lid **(Gipson and Joyce, ٢٠٠٠)**

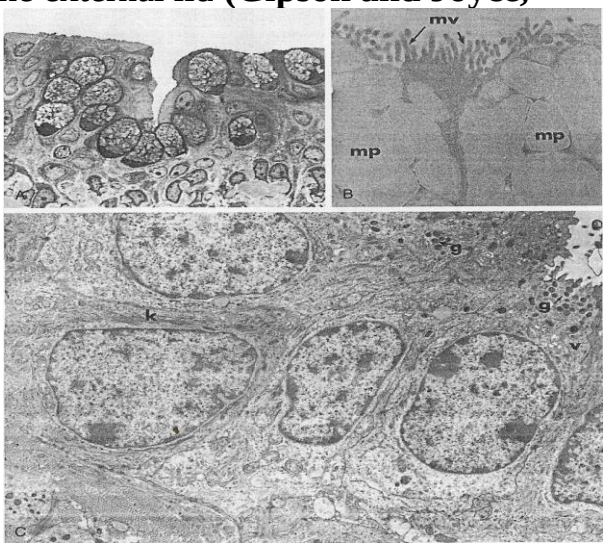

Figure (١٣): Micrographs demonstrating regions of bulbar conjunctiva. In the bulbar region, particularly in the nasal zone, goblet cells are dense. They can occur in crypts or groups, which have the appearance of acini, as demonstrated in the light micrograph A. B, Electron micrograph of the apical region of two adjacent goblet cells. Note the microvilli (mv) on the surfaces of cells and the fibrillar pattern in the mucin packets (mp). C, Low-magnification electron micrograph of non-goblet cells in the conjunctiva. Note the vesicles (v) and granules (g) in the apical region of cells and clumping of keratin filaments into bundles (k) **(Gipson and Joyce, ٢٠٠٠)**

Compared with cells of the corneal epithelium, the stratified cells of the conjunctiva have more cytoplasmic organelles. Keratin filaments in these cells are not as dispersed as those in corneal cell cytoplasm and often appear in bundles. Keratin proteins expressed by stratified

conjunctival epithelial cells also are different, with the keratin pairs K٤ and K١٣, and K٣ and K١٩. K٧ is expressed by goblet cells **(Krenzer and Freddo, ١٩٩٧)**. Cell-to-cell junctions and cell- to-substrate junctions appear similar in corneal, limbal, and conjunctival epithelia, except that gap junction proteins in the conjunctiva have not been characterized **(Griener et al, ١٩٨٥)**

The apical cells of the stratified epithelium have numerous small vesicles within their cytoplasm (Figure ١٣). It has been proposed that these vesicles (which bind Alcain blue and periodic acid−Schiff stains, indicating a highly glycosylaed content) deliver mucins onto the ocular surface and thus represent a second source of mucus for the tear film. Reports indicate that the stratified epithelium is expressing a membrane-spanning mucin designated MUCI **(Inatomi et al, ١٩٩٥)** and a second large mucin designated MUC٤ **(Inatomi et al, ١٩٩٦)**.

The goblet cells that are intercalated within the stratified epithelium of the human conjunctiva occur as individual cells. In humans, there is a regional variation in goblet cell distribution pattern and density of goblet cells **(Gipson and Tisdale, ١٩٩٧)**, the highest density being in palpebral region near the tear drainage punctum and in the midfornix. In some regions, especially the temporal bulbar conjunctiva, goblet cell density is so great that the cells appear to be clustered and arranged in acini **(Kessing, ١٩٦٨)**. Goblet cells of the conjunctiva are plump and lack the goblet “stem" a thin cytoplasmic extension to the basement membrane that is obvious in intestinal goblet cells. Mucin packets that fill the cytoplasm of goblet cells appear electron lucent; however, a fine filamentous network can be discerned within the packets (Figure ١٣ B). Studies have demonstrated that a major mucin gene expressed by the conjunctival goblet cell is the large gel-forming mucin

MUC٥AC. Tight junctions appear to be present between goblet cells and adjacent stratified cells (Figure ١٣) **(Inatomi et al, ١٩٩٦)**

The connective tissue of the substantia propria of the conjunctiva is similar to that of the superficial limbus; immune cells are especially abundant in its loose and highly vascularized connective tissue. Lymphocytes, mast cells, plasma cells, and neutrophils are common cell types in its matrix **(Srinivasan et al, ١٩٨٢)**. In fact, the substantia propria has been described as having two layers: an inner fibrous layer and an outer lymphoid layer. Although the lymphoid layer has dense accumulations of lymphocytes, these do not form lymph nodules. The accumulation of the lymphoid tissue, in addition to the phagocytic abilities of the conjunctival epithelium, demonstrates the function of the tissue in dealing with infectious agents **(Jakobiec and Iwamoto, ١٩٨٢)**

Stem cells of adult cornea

Stem cells usually reside in a microenvironment that provides external factors necessary for maintaining stem cell properties and functions, often referred to as ‘‘stemness’’ **(Parker et al, ٢٠٠٤)**. Progenitor cells are similar to stem cells in most aspects except they do not renew themselves and thus their population becomes terminally differentiated after a limited, though sometimes enormous number of cell divisions. Although not a consensus yet, stem and progenitor cells are sometimes collectively termed precursors, indicating that both can be expanded under appropriate circumstances **(Takacs et al, ٢٠٠٩)**.

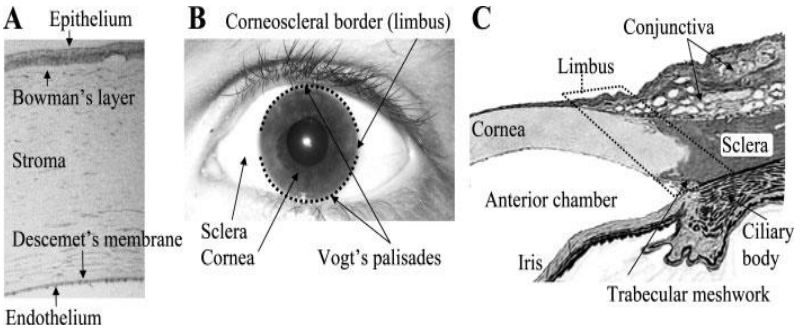

Figure (١٤): Localization of corneal stem cells. A: Histological section and tissue layers of the cornea. B: The corneal limbus is localized to the corneoscleral border. The upper and lower regions most protected by the eyelids contain the Vogt's palisades that apparently host most of the corneal epithelial stem cells. C: Cross-section of the corneoscleral transition. The corneal epithelium is contiguous with the conjunctiva, the corneal stroma transits into the sclera, whereas the corneal endothelium is linked with the trabecular meshwork. These transitional zones together contain the majority of stem cells in the adult cornea. **(Takacs et al, ٢٠٠٩)**

In the adult cornea, stem cells reside in the limbal area. The existence of epithelial stem cells in the limbus has been proposed in ١٩٧١ by Davanger and Evensen and the investigation of limbal epithelial stem cells (LESCs) greatly progressed since then. However, data suggesting the existence of stromal and endothelial stem cells in the cornea have only been published recently **(Takacs et al, ٢٠٠٩)**. A summary of the major stem cell types found in the adult cornea is provided in Table (١).

Table (١): major stem cell types found in the adult cornea **(Takaces et al, ٢٠٠٩)**

Stem cell (SC) type	Developmental origin	Localization	Isolation/Culture	Differentiation potential
Limbal epithelial stem cells (LESCs)	Ectoderm	Basal layer of limbal epithelium, palisades of Vogt	Explant cultures of corneal limbus or single limbal epithelial cells on feeder ٣T٣ cells	Hair follicle cells, neuronal cells, possible mesenchymal transition in limbal explants
Stromal stem cells	Neural crest derived mesenchyme	Central and peripheral corneal stroma, mostly the peripheral part	Side population of single stromal cells	Chondrocytes, neuronal cells
Stromal fibroblast like cells	Not known	Stroma of limbal explants	SSEA-٤$^{+}$ cells isolated by MACS	Neuronal cells, corneal epithelial cells, myocytes chondrocytes, , osteoblasts, adipocytes, pancreatic cells, hepatocytes
Stromal mesenchymal cells	Not known	De-epithelialized limbal explants	Sponteneous spindle cell outgrowth of limbal explants	Adipocytes, Osteocytes
Neural crest derived corneal stem cells (COPs)	Neural crest	Mouse corneal stroma	Sphere forming assay	Keratocytes, chondrocytes, adipocytes, neural cells
Putative endothelial stem cells	Neural crest	Peripheral endothelium	Sphere forming assay, IHC detection in corneal buttons	Corneal endothelial cells, express mesenchymal and neuronal proteins

SSEA-٤: stage specific embryonic antigen ٤
COPs: corneal precursors
FACS: fluorescine activated cell sorting
MACS: magnetic activated cell sorting
IHC: Immunohistochemistry

Pathophysiology of Ocular Surface Wound Healing

and

Stem Cell Concept

Pathophysiology of Ocular Surface Wound Healing and Stem Cell Concept

The corneal epithelium is exposed to a continuously changing external environment and serves as the frontal barrier of the entire eyeball. For clear vision, it is important that the surface of the corneal epithelium remain smooth, so that light is uniformly refracted, and that an active repair process resurfaces defects. Epithelial wound healing depends on a complex interaction of various cellular components that interact via a network of interactive, signaling molecules. Cell-cell and cell-matrix interactions play important roles in the maintenance of the stratified structure of the corneal epithelium. Any damage to the corneal epithelium elicits a series of events that help in covering the area of injury **(Agrawal and Tsai, ٢٠٠٣)**.

Interaction

The corneal epithelial wound healing response is brought about by a complex cascade of events involving cytokine (soluble factors)-mediated interactions between the epithelial cells, keratocytes of the corneal stroma, corneal nerves, lacrimal glands, and cells of the immune system. This interaction of the various components is crucial to restoring the function of the epithelium as a barrier and as the cornea's refractive surface. The level of interaction is dependent on the inciting injury. For example, lamellar, incision, and surface scrape injuries are followed by typical wound healing responses that are similar in some respects, but different in others **(Wilson et al, ٢٠٠١)**. Many cytokines and receptors modulate the process. Activation of these systems also attracts immune cells that function to eliminate debris and microbes that may reach the injured

surface and access the corneal stroma. Thus it is an orchestrated response of these components that efficiently restores corneal structure and function in most situations **(Agrawal and Tsai, ٢٠٠٣)**.

"Cross-talk"

The epithelial-mesenchymal interactions play a vital role in wound healing. These interactions can be mediated by signals transmitted from the mesenchyme to the epithelium or vice versa in a reciprocal manner. Such signals can be extracellular matrix components, cell membrane-associated molecules, and cytokines. These three types of signals are not mutually exclusive. The action of one may be dependent on or mediated by the expression of the other. Four patterns of cytokine dialogue have been studied **(Tseng, ١٩٩٦)**. Three of them constitute the basic network of the potential epithelial-mesenchymal cytokine dialogue system (Table ٢).

Table (٢): Basic network of epithelial-mesenchymal cytokine dialogue **(Agrawal and Tsai, ٢٠٠٣)**

Type	Cytokines	Function
I	TGF-α, IL-١β, PDGF-١β	Expressed by epithelial cells to converse with fibroblasts
II	IGF-١, TGF-β١, TGF-β٢, LIF, bFGF	Cross-talking between he epithelium and fibroblasts
III	KGF, HGF	Expressed by fibroblasts to simulate proliferation and migration

Importance of corneal epithelial renewal to function and transparency

In addition to the healing response to injury, a renewal process is needed to maintain the protective function of the epithelium. This process is important to the two major properties of corneal epithelium needed for normal vision. The first is the formation of a smooth refractive surface via interaction with tear film. The second is to form a tight protective junctional barrier. This barrier prevents alterations in net fluid transport from the corneal stroma and prevents penetration by pathogens. Wounding of the corneal surface epithelium leads to breakdown of the tight junctional integrity due to loss of the outer limiting epithelium. This loss can lead to a breakdown of cell membrane permeability and selectivity. This is not restored until after epithelial migration from the periphery has resulted in resurfacing of the denuded corneal epithelium. During this period of injury the cornea thus becomes susceptible to infection by microbes and the attendant consequences. The alteration in the healing process caused by such invasion can cause derangement of cytokine-mediated control of the healing. This in turn decreases the endothelial fluid transport, and increases stromal hydration, causing corneal opacity **(Agrawal and Tsai, ٢٠٠٣)**.

The normal renewal of the corneal epithelium is an ongoing process. First the surprabasal cells in the limbal and corneal epithelia are terminally differentiated and appear to have exited the cell cycle. At the other end are the basal cells of the corneal epithelium where approximately ٤٠٪ of the cells are actively proliferating **(Francesconi et al, ٢٠٠٠)** Between these two extremes are the basal cells of the limbal epithelium, where approximately ٢٠٪ of the cells are actively cycling. This group of cells contains a mix of slow-

cycling stem cells and cycling transient amplifying cells **(Zeiske, ١٩٩٤)**. The superficial cells are specialised to form tight adhesions between one another and to the basal lamellae. This provides the epithelial barrier function. As the superficial cells are lost, the underlying basal cells first move into differentiating wing cells and then migrate upwards as superficial cells. The formation of the tight epithelial barrier is of paramount importance as a physical barrier to noxious agents. The corneal epithelium is thus well maintained through a process of mitosis, migration, and shedding. The last step of superficial cell desquamation is aided by eyelid blinking **(Ren and Wilson, ١٩٩٦)**. The interaction of the superficial cells with the tear film is supported by the presence of numerous microvilli and microplicae, which facilitate transport of metabolites and tear film adhesion. This allows formation of a smooth refractive surface for the cornea **(Arffa, ١٩٩١)**.

Phases of wound healing

The healing of corneal epithelium can be divided into three distinct but continuous phases: (١) sliding of superficial cells to cover the denuded surface; (٢) cell proliferation and stratification; and (٣) reassembly of adhesion structures. These steps are preceded by the Lag phase during which the epithelial cells alter their metabolic status **(Agrawal and Tsai, ٢٠٠٣)**.

Lag phase

The time between wounding and the onset of cell migration is the lag phase. This phase sees a great deal of cellular reorganization and protein synthesis. Several cytoskeletal proteins **(Zieske and Gipson, ١٩٨٦)** such as, vinculin, actin, talin, and integrin are synthesized, as are other cell surface receptors (e.g. hyaluronan [HA] receptor

CD٤٤). Cell surface glycoprotines and glycolipids are also synthesized **(Panjwani et al, ١٩٩٠)**.

Integrin α٦β٤ is an integral membrane glycoprotein which anchors extracellular proteins to cytoskeletal components. It is a heterodimer composed of α and β subunits that is specific for binding to laminin in the corneal basement membrane. In its normal distribution it is located at the base of the basal cells. It is responsible for the adhesion of these cells to the underlying basement membrane. During the lag phase this integrin dissociates from the desmosomes and hemidesmosome and distributes evenly on the cellular surface. Thereafter this serves as an adherens molecule to the adjacent extracellular matrix (ECM) **(Clark and Brugge, ١٩٩٥)**. It is also believed that these surface glycoproteins are responsible for a bidirectional signaling between the ECM and the cytoskeleton **(Schoenwaelder and Burridge, ١٩٩٩)**.

Cell migration

Once the lag phase remodeling of the epithelial cells is complete, cells adjacent to the wound commence migration to re-establish the integrity of the ocular surface epithelium. It has been postulated that the basal and wing cells participate in the formation of the leading edge **(Gipson and Surgue, ١٩٩٤)**. Re-epithelialisation does not occur one layer at a time. It is now understood that there is a gradient of cells from opposite directions that meet in the center of the wound. The multiple layers of cells extending over the regenerating surface result from migration of epithelial cells that originated adjacent to the wound margin. In case of the suprabasal cells some of the cells have been shown to have "stalks" extending to the basement membrane **(Lavker et al, ١٩٩١)** indicating that the second

layer too comprises of displaced basal cells. It is thus postulated that progressive movement of both, basal and suprabasal cell layers that are adjacent to the wounded surface, resurfaces the corneal epithelial wound **(Zagon et al, ١٩٩٩)**.

After about ٥ hours of wounding, cells begin to migrate at a constant rate of ٦٠ - ٨٠ mm/hr until wound closure is completed. The process of cell migration is achieved by synthesis of an elaborate cytoplasmic array of actin - rich stress fibers. Blocking these stress fibres is known to inhibit epithelial cell migration and adhesion. Proparcaine, a topical anaesthetic, inhibits corneal epithelial migration partly through alteration of the actin cytoskeleton **(Mastuda et al, ١٩٨٥)**.

Cell proliferation and differentiation

Flattening and elongation of cells during migration covers the wound area. Cell proliferation then occurs to repopulate the wound area. The migratory and proliferative responses are compartmentalized in that limbal and peripheral epithelial cells exhibit an enhanced proliferative rate following wounding, while cells at the leading edge of migrating epithelium do not proliferate. Corneal epithelial debridement stimulates a ٤,٥-fold and ٣,٢-fold increase in cell proliferation in limbal and peripheral corneal epithelium respectively **(Zieske and Gipson, ٢٠٠٠)**.

The amount of cellular proliferation is demand dependent. The corneal epithelium meets this demand in various ways. In the normal situation, the stem cells located in the limbus, cycle infrequently with a relatively long cell cycle time. Upon division, stem cells give rise to regularly cycling transient amplifying (TA) cells located in the peripheral and central corneal epithelium. Young TA cells

with multiple division capacity are preferentially located in the peripheral cornea, whereas the more mature TA cells having little proliferative reserve reside in the central cornea. These mature TA cells may divide only once before becoming terminally differentiated **(Agrawal and Tsai, ٢٠٠٣)**

All the cell proliferation and differentiation even during the wound-healing phase is similar to that which occurs during normal homeostasis. During normal homeostasis, continuous centripetal movement of peripheral corneal epithelium toward the visual axis maintains corneal epithelial mass. This balances the cellular loss resulting from anterior movement of basal epithelial cells to the surface and into the tear film. This is the classic X, Y, Z hypothesis first proposed by Thoft and Friend **(Thoft and Friend, ١٩٨٣)**. This has been further supported by the mathematical model demonstrating that the rate of exfoliation of epithelial cells is consistent with their production from the limbal cells **(Sharma and Coles, ١٩٨٩)**. The half-time of corneal epithelial replacement is ٩ weeks, while the time required for ٩٥-٩٩٪ replacement is ٩-١٢ months **(Agrawal and Tsai, ٢٠٠٣)**.

Basement membrane changes

Events immediately following injury

Inflammatory cells (polymorphonuclear neutrophils) bind to the surface of the exposed basement membrane after debridement wounding **(Wagoner et al, ١٩٨٤)**. These release proteases capable of degrading the basement membrane. The tear film may also play a role, as it contains proteases, especially after corneal wounding **(Cejkova, ١٩٩٨)**. In addition, corneal epithelial cells release gelatinase B, matrilysin **(Lu et al, ١٩٩٩)**, and metalloproteinases

capable of degrading components of the basement membrane. Protease digestion of the basement membrane over a period of time after injury alters both its structure and function **(Agrawal and Tsai, ٢٠٠٣)**.

Basement membrane disassembly can affect re-epithelialisation in one or more of the following ways:

١.Exposure of leading epithelial cells to underlying stromal extracellular matrix (ECM): This induces new integrin expression and activation in migrating epithelial cells.**(pilcher et al, ١٩٩٧)**

٢. Modification of intracellular signaling pathways in migrating epithelial cells: Cytokines like Transforming growth factor β (TGF-β) and basic Fibroblast growth factor (bFGF) are found in the basement membrane (BM) after injury and are held there by binding to molecules in the matrix. Thus partial disassembly of BM releases molecules involved in modulation of cell proliferation, cell differentiation, and/or apoptosis **(Dabin and Courtois, ١٩٩١)**.

٣. Formation of a stable adhesion complex: Studies on both animal and human corneas show that the structure and composition of the epithelial basement membrane affects the adhesion of the cells sitting on it **(Iglesia and Stepp, ٢٠٠٠)**.

Reassembly of basement membrane

Basement membrane functions as a dynamic structure of the tissue morphology, differentiation, and maintenance. Remodeling of the basement membrane thus constitutes an integral part of the corneal epithelial wound healing process. Migrating epithelial cells synthesize and deposit laminin-١

beneath themselves within ٢٤ hours after photoablation **(Suzuki et al, ٢٠٠٠)**. Laminin-١ is a major constituent of the basement membrane and regulates various cell processes, including adhesion, proliferation, and differentiation **(Jiang et al, ١٩٩٩)**. The interaction of Laminin-١ and ٥ with $\alpha_6\beta_4$ integrin can mediate cell migration. Expression of connexin and desmoglein ١ and ٢ increases at approximately the same time as that of laminin-١. The observation that the re-establishment of basement membrane coincides with the formation of two of the four types of intercellular junctions suggests that the basement membrane may affect the expression of junctional adhesion proteins in corneal epithelial cells **(Torok and Mader, ١٩٩٦)**.

Integrins

The integrins are a family of cell surface glycoproteins that mediate several cellular adhesive functions. The primary function of integrins is adhesion of cells to the extracellular matrix. These interactions influence many aspects of cell behaviour including cell morphology, adhesion, migration as well as cellular proliferation and differentiation. They are fundamentally important in forming a connection between the extracellular matrix on the exterior and the cytoskeleton on the interior. In fact, they have been so named for the perceived integration of the cell surface with the cytoskeleton. The integrins function in bidirectional transduction of signals across the plasma membrane **(Hynes, ١٩٨٧)**.

Growth Factors and Cytokines

Cytokines are multipotential (glyco) proteins, which act non-enzymatically in picomolar to nanomolar concentrations to facilitate intracellular communications. It

is now known that cytokines, including growth factors, are very much involved with corneal tissue remodeling. Recent evidence also suggests that programmed cell death (apoptosis), also plays an important role during wound healing **(Agrawal and Tsai, ٢٠٠٣)**.

EGF, KGF, and HGF

A number of different growth factors are involved in the regulation of corneal wound healing. Among them, epidermal growth factor (EGF), keratinocyte growth factor (KGF), and hepatocyte growth factor (HGF) are strong mitogens of corneal epithelial cells **(Agrawal and Tsai, ٢٠٠٣)**.

EGF exists in various body fluids. In the eye it exists in tears (main source lacrimal galnd) **(Watanabe et al, ١٩٩٣)** and to increase corneal epithelial cell proliferation both in-vitro and in vivo. When epithelial erosions occur suddenly, EGF already present in tears can act immediately on the corneal epithelium to promote cell proliferation. KGF and HGF specifically promote proliferation in cells of epithelial origin in a paracrine fashion **(sotozono et al, ١٩٩٤)**. Like EGF, KGF stimulates corneal epithelial cell growth both in vivo and in vitro. HGF mRNA is also expressed in cultured human stromal fibroblasts, and its receptor mRNA in corneal epithelial cells. Both KGF and HGF play important roles in corneal epithelial cell growth as paracrine stimulators from stromal fibroblasts **(Wilson et al, ١٩٩٣)**.

TGF-β

The transforming growth factor-β (TGF-β) family of proteins comprises three closely related isoforms in mammal - TGF-β ١, -β ٢, and -β ٣. Various isoforms have been detected in the human tears and in human corneal

epithelium. TGF-β s have multifunctional regulatory activity, and are known to be intimately associated with the regulation of wound healing. It has been shown that TGF-β antagonises the actions of EGF on corneal epithelial cells, and that both TGF-β ١ and -β ٢ inhibit the corneal epithelial cell proliferation promoted by KGF and HGF and weakly inhibit the corneal epithelial cell proliferation promoted by EGF. It is now thought that TGF-β ١ or -β ٢ may be activated at the leading edge of the epithelium, where it might antagonise the proliferative effects of EGF, KGF, and/or HGF. Also, it is possible that TGF-β ١ and -β ٢ may play important roles as negative modulators against cell proliferation in the wound healing process **(Honma et al, ١٩٩٧)**.

Inflammatory cytokines in corneal wound healing

Cytokines play an important role during corneal wound healing. Various cytokines are up-regulated after injury to the corneal epithelium. Of particular interest are Interleukin ١a (IL-١a) and IL٦. The levels of these are significantly up-regulated after injury. More importantly, the levels of IL-١a and IL-٦ correlate with the severity of injury **(Sotozono et al, ١٩٩٧)**. This increase in the levels of IL-١ and IL-٦ in injured cornea can initiate the cascade of events that constitute epithelial wound healing. IL-٦ for example stimulates epithelial migration in the cornea by up-regulation of integrin **(Nishida et al, ١٩٩٢)** and the IL-١ accelerates epithelial wound closure in vitro and acts synergistically with EGF **(Boisjoly et al, ١٩٩٣)**. It is also possible that increased expression of IL-١ in the cornea may act in conjunction with EGF in tears to stimulate epithelial wound healing, and may also induce KGF and HGF expression in corneal fibroblasts **(Li and Tseng, ١٩٩٧)**, causing epithelial cell proliferation **(Malecaze et al, ١٩٩٧)**.

It is noteworthy that all the effects of IL-١ are not beneficial. IL-١ induces the matrix metalloproteinase (MMP) family of enzymes that may cause corneal stromal melting **(Girad et al, ١٩٩١)** and IL-١ released from injured corneal epithelium mediates keratocytes apoptosis. IL-١ also induces IL-٦ production and this has a role in inflammation because it induces lymphocyte differentiation. In addition, IL-١ induces the corneal epithelial and stromal cells to express IL-٨, a strong chemotactic factor of neutrophils **(Neilson et al, ١٩٩٤)** as well as a strong angiogenic factor as such, IL-١, IL-٦, and IL-٨ expression in injured cornea can induce stromal melting, cell infiltration, and neovascularization **(Koch et al, ١٩٩٢)**.

Cytokine networks during corneal wound healing

The interaction of various cytokines that leads to a network-like action is crucial to the final outcome of wound healing. This cytokine network governs proliferation of corneal cells, and degradation of denatured collagens. It acts as a defense against foreign organisms such as bacteria and fungi (Figure ١٥). However, the excess of these cytokines that are needed for corneal wound healing can also severely damage the cornea. They can induce corneal ulceration, melting, and neovascularisation. It is speculated that the degraded corneal collagens induce expression of inflammatory cytokines, and that the suppression of stromal degradation by the matrix metalloproteinase inhibitor results in a curtailed cytokine expression. The initial expression of the inflammatory cytokines immediately after corneal injury induces a "secondary" injury **(Sotozono, ٢٠٠٠)**. It is possible that some amount of corneal infections, immune reactions, and inflammatory reactions, occur due to the cascade of the inflammatory cytokines. Thus, specific

therapies designed to control the cytokine network could well form the basis for treatment of a variety of corneal problems **(Agrawal and Tsai, ٢٠٠٣)**.

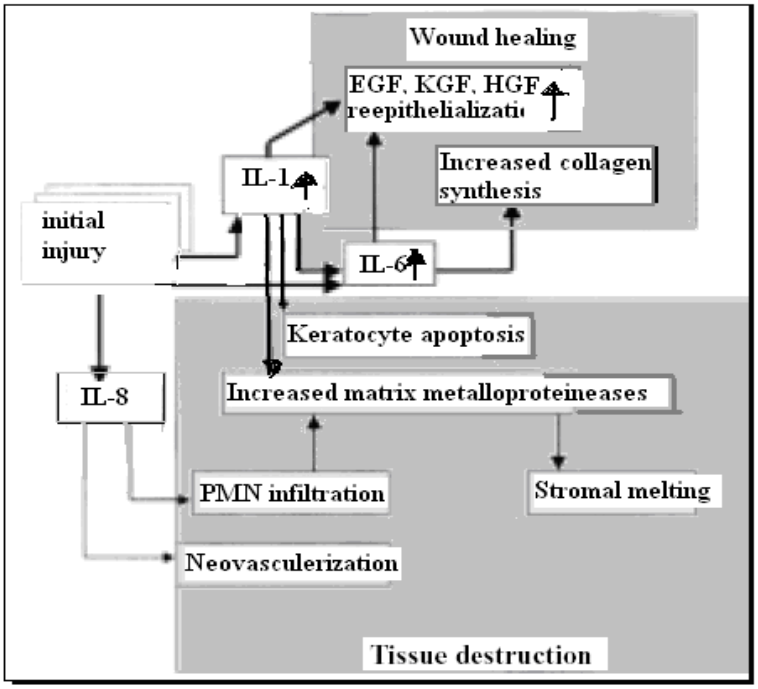

Figure (١٥): Hypothesis of cytokine network during cornea injury **(Agrawal and Tsai, ٢٠٠٣)**

Extracellular Matrix and Corneal Epithelial Wound Healing

There are two main modalities in which the extracellular matrix (ECM) can affect cell behavior. One of these is through harboring growth factors or growth factor-binding proteins. Rather than passively sequestering such factors, we now realize that the ECM plays an active role in their mobilization. Indeed, remodeling enzymes are pivotal in decisions to release matrix-bound growth factors and thereby control differentiation. Additionally, the cell-ECM interactions can directly regulate cell behavior, either

through receptor-mediated signaling or by modulating the cellular response to growth factors. In terms of cell differentiation they need to interact with specific types of matrix protein so that appropriate intracellular signal transduction cascades are triggered **(Agrawal and Tsai, ٢٠٠٣)**.

Matrix Metalloproteinases (MMPs) play an important role in ECM remodeling. Most MMPs are secreted from cells as zymogens and become activated only following cleavage of their amino-terminal pro-domains. In order that MMP proteolysis does not lead to widespread destruction of the ECM, activation is closely orchestrated adjacent to the cell surface **(Stetler-Stevenson, ١٩٩٩)**.

Inflammatory Cells and Their Function

Beginning approximately ١٢-٢٤ hours after injury to the cornea there is an influx of inflammatory cells into the corneal stroma. These inflammatory cells are thought to move in from the limbal blood vessels and possibly from the tear film. A surprisingly large type of inflammatory cells can be detected using immunocytochemistry and electron microscopy. The signals that draw these inflammatory cells into the cornea are most likely, soluble cytokines or chemokines released following injury. Corneal fibroblasts secrete monocyte chemotactic protein-١ (MCP-١) in vitro when stimulated by IL-١ or TNF a. Interestingly this protein is expressed in vivo only after epithelial scrape injury or some other injury to the overlying epithelium **(Tran et al, ١٩٩٦)**.

It seems likely that one important function of these inflammatory cells is scavenging of cellular components released during programmed cell death. Thus, apoptotic bodies that are distributed throughout the stroma are

engulfed by some these inflammatory cells. If the corneal injury is associated with tissue invasion by microorganisms then these cells function to eliminate these pathogens. These inflammatory cells may also be responsible for some of the fibroblastic appearing cells that repopulate the stroma after epithelial injury **(Mohan et al, ٢٠٠١)**.

After the immediate healing of the injured area, the inflammatory cells are gradually eliminated from the site. The exact mechanism is poorly characterized. It is likely that the majority of inflammatory cells eventually undergo apoptosis since this is the process through which these cells are eliminated in many other organs **(Agrawal and Tsai, ٢٠٠٣)**.

Role of Limbal Epithelial Stem Cells in Homeostasis of Corneal Epithelium

Corneal integrity and therefore function is dependent upon the self-renewing properties of the corneal epithelium. The prevailing hypothesis is that this renewal relies on a small population of putative stem cells located in the basal region of the limbus. These putative stem cells are primitive and can divide symmetrically to self renew and asymmetrically to produce daughter transit amplifying cells (TAC) that migrate centripetally to populate the basal layer of the corneal epithelium **(Tseng**, **١٩٨٩).**The TAC divide and migrate superficially, progressively becoming more differentiated, eventually becoming post-mitotic terminally differentiated (TD) cells (**Sun et al, ٢٠٠٦)**

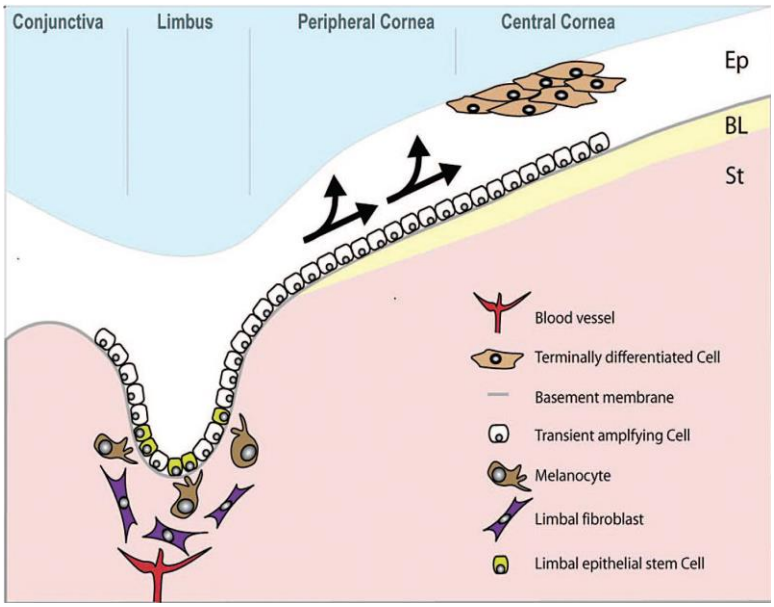

Figure (١٦): The human limbus: Limbal epithelial stem cells reside in the basal layer of the epithelium (Ep), which undulates at the limbus. Daughter transient amplifying cells (TACs) divide and migrate towards the central cornea (arrowed) to replenish the epithelium, which rests on Bowman's layer (BL). The stroma (St) of the limbal epithelial stem cell niche is populated with fibroblasts and melanocytes and also has a blood supply **(Secker and Daniels, ٢٠٠٩)**

Once fully differentiated TD squamous cells are shed from the ocular surface during normal wear and tear and this in turn stimulates the cycle of cell division, migration and differentiation **(Beebe and Masters, ١٩٩٦).** Thoft and Friend developed the 'The X, Y, Z hypothesis of corneal epithelial maintenance'. This hypothesis proposed that the addition of the proliferation of basal cells (X) and the centripetal migration of cells (Y) was equal to epithelial cell loss from the corneal surface (Z). However, they were unable to rule out the involvement of the neighboring bulbar conjunctiva **(Thoft and Friend, ١٩٨٣)**. Later, mathematical analysis indicated that the corneal epithelial cell mass could be renewed by cells from the limbal epithelium alone **(Sharma and Coles, ١٩٨٩)**. Furthermore, a fine balance between cell proliferation, differentiation, migration and

apoptosis is necessary. A variety of cytokines have been shown to play important roles in the maintenance and wound healing of the cornea. These factors are supplied in part by the adjacent tear film and the aqueous humour **(Welge-Lussen et al, ٢٠٠١).** Other growth factors are produced by keratocytes in the supporting stroma **(West-Mays and Dwivedi, ٢٠٠٦)** and by the corneal epithelial cells themselves **(Rolando and Zierhut, ٢٠٠١)**

Limbal epithelial stem cells

Throughout life, our self-renewing tissues rely upon populations of stem cells / progenitors to replenish themselves throughout life following normal wear and tear and injury. The corneal epithelium on the front surface of the eye is no exception as dead squamous cells are constantly sloughed from the corneal epithelium during blinking. At the corneo-scleral junction in an area known as the limbus, there is a population of limbal epithelial stem cells (LESCs). LESCs share common features with other adult somatic stem cells including small size **(Romano et al, ٢٠٠٣)** and high nuclear to cytoplasmic ratio **(Barrandon and Green, ١٩٨٧)**. They also lack expression of differentiation markers such as cytokeratins ٣ and ١٢ **(Kurpakus et al, ١٩٩٠)**. LESCs are slow cycling during homeostasis and therefore retain DNA labels for long time periods, however in the event of injury they can become highly proliferative **(Lavker and Sun, ٢٠٠٣)**. To replenish the stem cell pool, stem cells have the ability to divide asymmetrically (see Figure ١٧). Expression of C/EBPδ in a subset of LESC both in vivo and in vitro has recently been suggested to be involved in the regulation of self-renewal and LESC cell cycle length **(Barbaro et al, ٢٠٠٧)**.

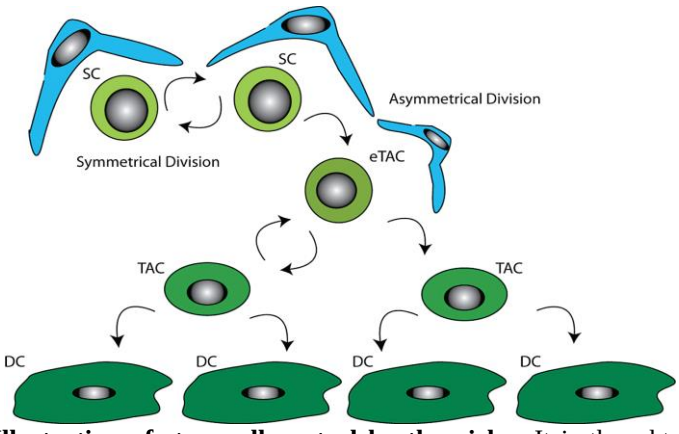

Figure (١٧): Illustration of stem cell control by the niche: It is thought LESC undergo asymmetric cell division producing a stem cell, which remains in the stem cell niche to repopulate the stem cell pool, and a daughter early transient amplifying cell (eTAC). This more differentiated eTAC is removed from the stem cell niche and is able to divide further producing transient amplifying cells (TAC), eventually giving rise to terminally differentiated cells (DC). The Double arrows represent the self-renewing capability of the stem cells. The supporting niche cells (blue) surround the stem cells (light green) **(Secker and Daniels, ٢٠٠٩)**

Evidence for stem cells in the corneal limbus

The first experimental indication of the presence of stem cells in the limbus was the observation of pigment (melanin) movement from the limbus to towards an epithelial defect following wounding of rabbit corneas **(Mann, ١٩٤٤)**.

Davanger and Evenson later observed a similar centripetal migration of pigment from limbus to central cornea in humans. Hence they proposed that the limbal Palisades of Vogt (PV) were the source of LESC **(Huang and Tseng, ١٩٩١)**.

Following lamellar keratoplasty, this centripetal migration was also observed in the rabbit as host epithelium was gradually replaced with donor epithelium **(Kinoshita et al, ١٩٨١)**. Furthermore, the complete removal of the limbus

results in impaired corneal function, neovascularization and conjunctival ingrowth **(Huang and Tseng, ١٩٩١)**.

Stem cells may be identified by the retention of DNA labels as they are slow cycling and only divide occasionally **(Bickenbach, ١٩٨١)**. Assuming stem cell division during the labeling period, stem cell exposure to DNA precursors such as tritiated thymidine or bromodeoxyuridine followed by chase periods of up to ٨ weeks labels the slow cycling cells (presumed to be stem cells). The more differentiated and more rapidly dividing daughter transit amplifying cells (TAC) undergo dilution of the label through multiple divisions. Through the use of tritiated thymidine, Cotsarelis et al, found slow cycling label retaining cells (LRCs) in the limbal basal epithelial region of the mouse cornea and postulated that up to ١٠٪ of limbal basal cells were stem cells **(Cotsarelis et al, ١٩٨٩)**. Phenotypically this population of cells appears to be more primitive in nature as they remain small and round **(Romano et al, ٢٠٠٣)**.

Limbal basal cells exhibit higher proliferative potential when compared to peripheral and central cornea both in vitro and in vivo. Large epithelial wounds in rabbits heal faster than smaller central defects. This implies that the proliferative capacity of the peripheral cornea is greater than that of the central **(Lavker et al, ١٩٩١)**. In the human, limbal explant cultures have greater proliferative potential when compared to central explants **(Ebato et al, ١٩٨٨)**. Furthermore, LESC proliferation is resistant to inhibition by tumor-promoting phorbol esters **(Lavker et al, ١٩٩٨)**. Based upon the methods of characterization used to identify features of stem cells isolated and cultured from human epidermis **(Barrandon and Green, ١٩٨٧)**, similar clonogenicity studies on cells isolated from the limbus produced large holoclone colonies (stem cell derived) with extended cell generation number. The less clonogenic

meroclones and paraclones were found elsewhere in the cornea **(Pellegrini et al, ١٩٩٩)**.

Clinical evidence also points toward the limbus as a depository for a stem cell population. During homeostasis, the limbal epithelial cells are thought to act as a barrier preventing conjunctival epithelial cells from encroaching upon the cornea **(Tseng, ١٩٨٩)**. During LESC failure (to be discussed later), the conjunctiva can invade the cornea causing chronic inflammation, painful corneal opacity and neovascularisation. Further clinical evidence pointing to the location of LESC at the limbus was demonstrated by Kenyon and Tseng, who transplanted two limbal explants taken from the contralateral healthy eye of patients on the damaged eye. This resulted in re-epithelisation of the cornea and regression of persistent epithelial defects and neovascularisation **(Kenyon and Tseng, ١٩٨٩)**.

The LESC niche

The stem cell niche, or microenvironment consisting of cellular and extracellular components, is hypothesized to prevent stem cell differentiation and thus regulates their fate **(Watt and Hogan, ٢٠٠٠)**.When a stem cell divides asymmetrically; one daughter may leave the niche to enter a differentiation pathway under the influence of environmental stimuli. The limbus differs from cornea both anatomically and functionally and hence could differentially determine stem cell fate **(Secker and Daniels, ٢٠٠٩).**

Within the limbal region of the cornea, the LESC niche is thought to be located within the palisades of Vogt (PV), an undulating region of increased surface area. The palisades are highly pigmented with melanocytes **(Higa et al, ٢٠٠٥)** and are infiltrated with Langerhan's cells **(Baum,**

١٩٧٠) and T-lymphocytes **(Vantrappen et al, ١٩٨٥)**. The melanin pigmentation is thought to shield LESCs from damaging ultraviolet light and the resultant generation of reactive oxygen species **(Shimmura and Tsubota, ١٩٩٧)**. The deep undulations of the Palisades of Vogt at the limbus provide LESC with an environment that protect them from shearing forces **(Gipson, ١٩٨٩)**. Furthermore the crypts described by Shortt et al., predominantly occur on the superior and inferior cornea where they are normally covered by the eye lids **(Shortt et al, ٢٠٠٧a)**. This may reflect the evolution of a protective environment for LESCs in humans. Basement membrane components also differ, with the limbal region containing laminin-١, ٥ and α٢β٢ chains not found in the cornea. Furthermore, type IV collagen α١, α٢ and α٥ chains are found in the limbal region whereas α٣ and α٥ are located in the cornea **(Tuori and Unsitalo, ١٩٩٦)**.

The basement membrane beneath the LESC may also act to sequester and therefore modulate growth factors and cytokines involved in LESC regulation and function **(Klenkler and Sheardown, ٢٠٠٤)**. Although the surface of the cornea is exposed to atmospheric oxygen, the LESC niche lies beneath a number of cell layers where the oxygen tension is likely to be lower. Interestingly, hypoxic in vitro conditions have been found to produce larger, less differentiated limbal epithelial cell colonies suggesting that low oxygen levels may induce selective proliferation of undifferentiated cells **(Miyashita et al, ٢٠٠٧)**.

The limbal niche is vascularised and highly innervated **(Lawrenson and Ruskell, ١٩٩١)** unlike the avascular cornea and therefore is a potential source of nutrients and growth factors for LESC. Limbal fibroblasts in the underlying stroma are heterogeneous and express secreted

protein acidic and rich in cysteine (SPARC) that may contribute to LESC adhesion **(Shimmura et al, ٢٠٠٦)**.

It is now clear that the palisades are only one component of the limbal niche architecture. Our group has identified two novel, specialized candidate niche structures, limbal crypts (LCs) and focal stromal projections (FSPs), which are found more abundantly in superior and inferior limbal quadrants. Cells lining the edges and bases of the LCs and edges and tips of the FSPs express high levels of the putative stem cell markers P٦٣a and ABCG٢ and have a small cytoplasmic to nuclear ratio, indicative of a stem cell population **(Shortt et al, ٢٠٠٧a)**

Putative positive and negative LESC markers

There are many attempts to prospectively identify LESC using a specific marker. As yet no single, reliable marker has been found. However, the expression of a combination of several features seems to allow for greater specificity **(Secker and Daniels, ٢٠٠٩)**

Putative markers can either be positive (present) or negative (absent). Limbal basal cells lack differentiation markers such as the ٦٤ kDa, cytokeratin ٣ (CK٣) that is present in all other layers of the corneal epithelium and the suprabasal layers of the limbal epithelium **(Schermen et al, ١٩٨٦)**. The corneal specific ٥٥ KD protein, cytokeratin ١٢ (CK١٢) is also expressed in a similar pattern **(Chaloin-Dufau et al, ١٩٩٠)**. Furthermore, connexin ٤٣ **(Shortt et al, ٢٠٠٧a)** and involucrin **(Chen et al, ٢٠٠٤)**, both markers of cells destined for differentiation, are also absent.

P٦٣:

The transcription factor p٦٣ is required for formation of epidermis and has been proposed as a putative positive LESC marker **(Pellegrini et al, ٢٠٠١)**. In vitro, p٦٣ was found to be expressed in limbal epithelial cell derived Limbal epithelial stem cells of the cornea holoclones with little or no expression in meroclones and paraclones. In vivo, p٦٣ was located in the limbal basal epithelium. However, since these initial observations a number of reports have suggested that p٦٣ is not sufficiently specific to act as an LESC marker as it has also been localised to basal cells of the peripheral and central cornea in humans **(Chen et al, ٢٠٠٤)**

ABCG٢:

Many types of organ-specific stem cells, including LESC have been recently shown to exhibit a side population (SP) phenotype. The SP cells are able to efflux Hoechst ٣٣٣٤٢ dye through the ATP-binding cassette transporter Bcrp١/ABCG٢. ABCG٢ has therefore been proposed to be a universal marker for stem cells **(Watanabe et al, ٢٠٠٤)**. In putative LESCs, this protein has been immunolocalised to the cell membrane and cytoplasm of a population of limbal basal cells and a few suprabasal cells **(Chen et al, ٢٠٠٤)**. Furthermore, ABCG٢ positive cells produce higher colony forming efficiency values in vitro than their negative counterparts **(De Paiva et al, ٢٠٠٥)**.

Integrin α٩:

Clusters of cells expressing the integrin α٩ have been localised to the limbal basal epithelium **(Stepp et al, ١٩٩٥)**. However, upregulation of α٩ in wounded murine corneas have since indicated this integrin to be associated with TACs **(Stepp and Zhu, ١٩٩٧)**.

N-cadherin:

N-cadherin is an important mediator of cell-cell adhesion and may play a key role in the maintenance of haemopoietic stem cells by facilitating adhesion to osteoblasts in the bone marrow niche **(Calvi et al, ٢٠٠٣)**. Hayashi and coworkers found expression of N-cadherin in a subpopulation of limbal epithelial basal cells and in adjacent melancytes implying N-cadherin plays an important role in interactions between LESC and their corresponding niche cells **(Hayashi et al, ٢٠٠٧)**.

Neural markers:

Even though the limbal epithelium is derived from the surface ectoderm a number of neural stem cell markers have been suggested as LESC markers. Recent in depth immunological studies of neurotrophic factors and their receptors in the human has found NGF, glial cell-derived neurotrophic factor (GDNF) and their corresponding receptors TrkA and GDNF family receptor alpha (GFRα)-١ to be exclusively expressed in the limbus **(Qi et al, ٢٠٠٨)**.

Notch ١:

Notch ١ is a ligand-activated transmembrane receptor that has been shown to maintain progenitor cells in a number of tissues. The role of Notch signalling in the cornea is unclear. However, cell clusters in the palisades of Vogt have been found with some co-localisation with ABCG٢ **(Thomas et al, ٢٠٠٧)**.

C/EBPδ and Bmi١:

The cell cycle arrest transcription factor C/EBPδ has also been implicated in the regulation of LESC self-renewal. Limbal epithelial basal cells that express C/EBPδ co-express Bmi١ (which is involved in stem cell self renewal) and Np٦٣α **(Barbaro et al, ٢٠٠٧)**.

Bmi-١ is a member of the polycomb gene family, and was shown to be essential for the self-renewal of haemopoietic and neural stem cells **(Molofsky et al, ٢٠٠٣)**. In ٢٠٠٦, it was shown that Bmi-١ mRNA was expressed in the slow cycling side population cells of the limbal epithelium and was barely detectable in the central cornea, and it was suggested that Bmi-١ should be used as a LESC marker **(Umemoto et al, ٢٠٠٦)**. **In ٢٠٠٧, Barbaro et al.** showed that C/EBPd, together with Bmi-١ and DNp٦٣a identifies resting limbal stem cells, but DNp٦٣a continued to be expressed in proliferating basal epithelial cells (activated stem cells and TACs **(Molofsky et al., ٢٠٠٣)**.

Musashi-١:

The RNA binding protein, Musashi-١ is produced in the developing and adult eye **(Raji et al, ٢٠٠٧)** and has recently been found in putative LESCs co-cultured with amniotic epithelial cells as feeders **(Chen et al, ٢٠٠٧)**.

Limbal Stem Transplantation in Management of Limbal Stem Cell Deficiency

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Preoperative staging of disease severity

Diseases that affect the ocular surface are multifactorial and present different stages of severity. Choice of treatment and visual prognosis are dependent upon a wide variety of factors. The most important features to consider in evaluating these patients include the degree of limbal stem cell (SC) loss, the extent of conjunctival disease, and presence and etiology of conjunctival inflammation. Other contributing factors include tear film abnormalities, the presence or absence of keratinization, eyelid abnormalities, laterality of disease, and the general health and age of the patient. The additional need for subsequent penetrating or lamellar keratoplasty will also affect the likelihood of successful ocular surface and visual rehabilitation **(Schwartz et al, ٢٠٠٢)**

Because so many factors will determine not only a patient's symptomatology, but also the choice of treatment and prognosis for success, it is important to establish a preoperative staging system that defines disease severity based on these critical factors. If we are going to recommend appropriate treatment and suitably evaluate outcomes, it is imperative that patients from one staging group be compared to patients within the same group **(Tseng et al, ١٩٩٨)**.

A practical approach at staging sever ocular surface diseases (OSD) is outlined in table (٧) **(Schwartz et al, ٢٠٠٢)**.

Table (٧): Classification of ocular surface disease based on number of lost stem cells and presence or absence of conjunctival inflammation **(Schwartz et al, ٢٠٠٢)**

	Normal conjunctiva (Stage a)	**Previously inflamed conjunctiva (Stage b)**	**Inflamed conjunctiva (Stage c)**
Partial stem cell deficiency (Stage I)	Iatrogenic, CIN, contact lens (Stage Ia)	History of chemical or thermal injury (Stage Ib)	Mild SJS, OCP, recurrent chemical injury (Stage Ic)
Total/Subtotal cell deficiency (Stage II)	Aniridia, severe contact lens and iatrogenic (Stage IIa)	History of severe chemical and thermal injury (Stage IIb)	Sever SJS, OCP, recurrent chemical or thermal injury (Stage IIc)

CIN: conjunctival intraepithelial neoplasia

It is imperative to consider these preoperative factors in order to compare the efficacy of one surgical management with another appropriately **(Tsubota et al, ١٩٩٩)**.

Preoperative factors for staging disease severity

Laterality of Disease:

The most significant preoperative factor of visual function and quality of life is laterality of disease. Patients with unilateral disease, no matter how severe in the affected eye, have the opportunity for normal vision in the fellow eye. These patients, therefore, suffer less morbidity from their disease than do patients with bilateral disease and often do not need to undergo surgery to restore driving or

reading vision. Many patients with monocular disease will opt to undergo surgery to restore binocular vision for treatment of a chronically painful or cosmetically unacceptable diseased eye. For these patients, the normal fellow eye serves as a potential source of stem cells for transplantation to the diseased one, thereby potentially eliminating the need for an allograft. One autograft is preferred over an allograft for two important reasons. First, patients undergoing autograft do not need to be placed on systemic immunosuppression and thus avoid the potential serious complications associated with these medications. Second, autograft patients do not carry the risk of graft rejection, which is the principal cause of limbal stem cell graft failure **(Schwartz et al, ٢٠٠٢)**.

Extent of Limbal Stem Cell Deficiency:

Severity of disease is dependent upon the extent of limbal stem cell deficiency. Patients with partial loss of the limbal stem cells typically have a better prognosis than those with total loss. If there are residual normal stem cells, there is an opportunity for ocular surface rehabilitation without the need for transplantation. Patients with partial stem cell deficiency may be treated successfully with medical management or sequential conjunctival epitheliectomy with or without amniotic membrane transplantation. Patients with more extensive stem cell deficiency cannot populate their corneal surfaces with remaining stem cells and most often require limbal stem cell transplantation to restore a healthy ocular surface. In this way, patients with sectoral epitheliopathy from iatrogenic limbal stem cell deficiency or partial chemical injury have treatment options unavailable to the aniridic patient with total limbal stem cell deficiency, and thus have a better chance for ocular surface stabilization with less invasive treatment **(Schwartz et al, ٢٠٠٢)**.

Extent of conjunctival disease:

The most severe forms of OSD occur when limbal stem cell disease occurs in combination with conjunctival disease. A normally functioning conjunctiva is essential for a properly functioning limbus and corneal surface. Therefore, in patients with combined limbal and conjunctival disease, all of the problems encountered in limbal deficiency will be present and compounded by the conjunctival, epithelial and goblet cell deficiency, mucin tear deficiency, subepithelial fibrosis, symblepharon, ankyloblepharon, foreshortening of conjunctival fornix, and in the most severe cases, keratinization of the entire surface are all manifestations of conjunctival disease. Each will worsen the symptomatology, prognosis, and likelihood for surgical success in sever OSD patients **(Schwartz et al, ٢٠٠٢)**.

Etiology of conjunctival inflammation:

The next important contributing factor is the nature of patient's conjunctival disease. A strong distinction exists between patient with abnormal conjunctiva from prior inflammation and patients who currently have active inflammation. Patients with active conjunctival inflammation have more immune mediators and cells in their ocular surfaces. These agents can exacerbate post operative inflammation after stem cell transplantation, thus increasing the risk for transplant failure. If possible, preoperative management of these patients includes postponement of surgery until inflammation can be controlled through topical and systemic medications. Postponing surgery is especially important for alkali and acid-injured patients whose conjunctival inflammation will decrease with time, especially if treated with a regimen of

anti-inflammatory medications **(Schwartz et al, ٢٠٠٢)**, unlike Patients with active autoimmune disease, such as OCP and SJS may have persistent inflammation despite immunosuppresion. This inflammation likely leads to progression of LSCD with further loss of stem cells from direct injury from inflammatory agents. In addition it is this inflammation that places these patients in the worst prognostic group, because the inflamed eye does not provide a stable environment for the transplanted stem cells **(Foster et al, ١٩٩٨)**.

Need for penetrating keratoplasty:

From experience, roughly half of patients undergoing limbal stem cell transplantation for severe OSD will need no further surgical procedure. However, half of patients will need to undergo penetrating or lamellar keratoplasty for visual rehabilitation. The presence of corneal graft potentially increases the demand for limbal stem cells to repopulate the corneal surface. This extrademand may very well stress a marginally functional transplanted stem cell population, further placing the patient at risk of chronic epitheliopathy **(Schwartz et al, ٢٠٠٢)**.

If a corneal graft is needed and the endothelium is normal, lamellar keratoplasty is preferable to penetrating keratoplasty for patients who have had limbal stem cell transplantation previously. Performing penetrating keratoplasty adds the additional risk for endothelial rejection which, in some cases, can be more difficult to control than limbal stem cell rejection and can ultimately lead to loss of patient′s vision **(Schwartz et al, ٢٠٠٢)**.

Management of LSCD

The goal of treatment for severe LSCD is to re-establish the anatomic and physiologic environment of the ocular surface by reconstruction of the corneal and conjunctival epithelium **(Santos et al, ٢٠٠٥)**.

Importance of pre-operative management

The main objective before transplanting limbal stem cells is to prepare their new "home" and to provide the best opportunity for graft survival. In particular, survival of limbal stem cells depends in part on the limbal niche that is influenced by tear film and vascularity and innervation at the limbus **(Desousa et al, ٢٠٠٩)**. Several issues need to be addressed before stem cell transplantation, including optimizing lids and the tear film, controlling inflammation, and the management of glaucoma.

Patients with ocular surface disease often have multiple factors affecting the surface, and a mild abnormality that may be tolerated in a normal eye can compromise the outcome of surgery. Hence, a low threshold to treat adnexal abnormalities before stem cell transplantation is recommended. A pre-operative systematic assessment of the adnexa, including tear film condition, eyelid position, lagophthalmos, and fornix depth is mandatory **(Di Pascuale et al, ٢٠٠٥)**. Overall, the health and function of eyelids, fornices, and tear film should be optimized before stem cell grafting to ensure the best chance of epithelial healing **(Liang et al, ٢٠٠٩)**. In cases of severe conjunctival disease and symblepharon, a source of goblet cells is required for conjunctival surface and fornix reconstruction. A variety of donor sites are available for autologous mucous membrane transplantation to the ocular and eyelid surface, including buccal, labial, hard palate, nasal turbinate, and septal mucosa **(Weinberg et al, ٢٠٠٧)**.

Ocular surface inflammation should be suppressed pre-operatively as much as possible. The eye needs to be quiet for at least ٣ months before surgery to enhance the chances of survival for transplanting stem cells. We therefore recommend topical and systemic immunosuppression several months prior to stem cell transplantation in patients with significant underlying inflammatory disease, such as atopic disease or Stevens-Johnson syndrome **(Holland and Schwartz, ٢٠٠٤)**.

The presence and severity of glaucoma can have a significant impact on the outcome. A rise in intraocular pressure is often seen following limbal transplantation, which may be attributed to the use of steroids. Additionally, multiple topical medications are toxic to the transplanted epithelial surface. Hence, there is a lower threshold in managing glaucoma in such patients. We recommend the early placement of a tube shunt in patients on more than one topical medication **(Holland and Schwartz, ٢٠٠٤)**.

Asymptomatic patients with partial and peripheral conjunctivalisation of the corneal surface may not require intervention. Corneal and conjunctival epithelial cell phenotypes have been known to co-exist on the corneal surface for prolonged periods without significant extension of the conjunctivalised area or any transdifferentiation of conjunctival epithelium into cells of corneal phenotype **(Dua, ١٩٩٥)**.

If the visual axis or most of the corneal surface is covered with conjunctiva-like epithelium, mechanical debridement of conjunctival epithelium can allow adequate corneal epithelial healing to occur from the remaining intact limbal epithelium. Scraping is done with a surgical blade under topical anesthesia at the slit lamp. Any tendency of conjunctiva-like epithelium to re-encroach on to corneal

surface is prevented by re-scraping **(Coster et al, ١٩٩٥)**. This procedure can be employed to improve visual function and reduce symptoms even when as little as two clock hours of normal limbus and peripheral cornea remain. When visual improvement is the aim of treatment, the objective should be to achieve normal corneal epithelial cover over the visual axis. This occurs when only two clock hours or less of limbus are surviving, may stretch the capacity of the remaining limbus and could eventually lead to epithelial breakdown **(Burman and Sangwan, ٢٠٠٨)**.

Mechanical debridement of conjunctiva-like epithelium and encouraging the denuded area to be resurfaced with corneal epithelial cells is a valid, simple and effective alternative to limbal transplantation in patients with partial limbal stem cell deficiency mechanical debridement can also be used to prevent migration of conjunctival epithelium on to the cornea in acute situations, with partial corneal and limbal epithelial loss. Close observation of patients with thermal, chemical or mechanical epithelial loss involving the limbus, will allow one to detect the advancing conjunctival epithelial sheet and prevent it from extending on to and beyond the limbus **(Coster et al, ١٩٩٥)**.

Tseng and colleagues (١٩٩٨) have successfully used amniotic membrane transplantation (AMT) and mechanical debridement and advocate its use to treat patients with partial stem cell deficiency **(Tseng et al, ١٩٩٨)**.

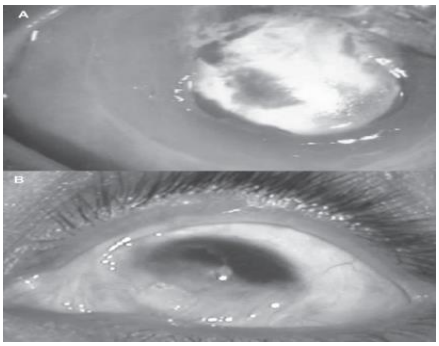


Figure (٢٢): Slit lamp photo of total (A) and partial (B) limbal stem cell deficiency due to chemical injury **(Burman and Sangwan, ٢٠٠٨)**.

Surgical techniques for limbal transplantation

Numerous techniques to replace limbal stem cells have been described **(Dogru and Tsubota, ٢٠٠٥)** with the common goal of ocular surface restoration. Holland and Schwartz have published nomenclature and classification for ocular surface procedures **(Holland and Schwartz, ١٩٩٦)**. It is based on the source of the donor tissue, the carrier tissue employed, and whether conjunctival or limbal tissue is transplanted **(Holland and Schwartz, ٢٠٠٤)**. More recently, ex vivo expanded limbal stem cells or oral mucosa cells have also been used successfully to reconstruct the ocular surface **(Higa and Shimazaki, ٢٠٠٨)**.

Table (٨): Classification of stem cell transplantation procedures **(Holland and Schwartz, ١٩٩٦)**

Procedure	Abbreviation	Donor	Transplanted tissue
Conjunctival limbal autograft	CLAU	Fellow eye	Limbus/ conjunctiva
Living-related conjunctival limbal allograft	Lr-CLAL	Living related	Limbus/ conjunctiva
Keratolimbal allograft	KLAL	Cadaveric	Limbus/cornea
Ex vivo expanded limbal cell limbal autograft	EX-LAU	Fellow eye	Ex vivo expanded
Living-related ex vivo expanded limbal cells limbal allograft	Lr-EX-LAL	Living related	Ex vivo expanded

Conjunctival limbal autograft

In unilateral LSCD, the healthy fellow eye is the most suitable source of limbal stem cell. This technique was actually a modification of Thoft's conjunctival transplant procedure on extending the grafts of bulbar conjunctiva ٠,٥ mm onto the clear cornea to obtain limbal stem cells **(Kenyon and Tseng, ١٩٨٩)**. Harvesting begins in the conjunctiva, including ٤-٥ mm of conjunctival tissue, moving anteriorly to remove a partial-thickness limbal epithelium of about one-third thickness. Preparation of the recipient eye begins with ٣٦٠° peritomy and sharp and blunt dissection of the fibrovascular pannus over the cornea and securing the transplanting block at the ٦ and ١٢ o' clock positions **(Holland and Schwartz, ٢٠٠٤)**. We prefer to use two blocks of tissue, each ٢' clock hours in circumferential extension. Recent update on the technique is using fibrin glue instead of sutures to secure the transplant **(Bakhtiari and Djalilian, ٢٠١٠)**.

The main concern with this procedure is inducing stem cell deficiency in the fellow eye. No complications in the fellow eyes were reported in Kenyon and Tseng's series and the risk to the donor eye appears extremely low if the donor eye is truly healthy with no long-term contact lens usage or subclinical exposure to original trauma and less than ٦' clock hours of limbal tissue is removed **(Kenyon and Tseng, ١٩٨٩)**. Clearly, CLAU is not an option for patients with bilateral disease.

Although CLAU is an autograft and there is no risk of immunologic rejection, like all forms of stem cell transplantations, it must be considered only after adequate control of ocular inflammation to provide a better

environment for transplanting cells **(Bakhtiari and Djalilian, ٢٠١٠)**.

Living related conjunctival limbal allograft (lr CLAL)

The lr-CLAL technique is similar to CLAU. However, the source of stem cell is a living relative instead of the fellow eye. HLA typing on all potential donors is helpful in finding more compatible tissue to transplant. Potential donors with long-term contact lens usage and glaucoma, who may eventually require trabeculectomy, should be excluded **(Kim and Djalilian, ٢٠٠٣)**. Serologic testing of potential donors for syphillis, hepatitis B and C, and human immunodeficiency virus infection should be performed to avoid risk of transmission to the recipient **(Bachtiari and Djalilian, ٢٠١٠)**.

This procedure provides conjunctival and limbal stem cells to the host with some degree of histocompatiblity. As discussed above, damage to the donor's eye is very unlikely, but should be considered. In addition, the risk of rejection exists and patients require systemic immunosuppression therapy **(Bachtiari and Djalilian, ٢٠١٠)**.

Lr-CLAL provides healthy conjunctival tissue in addition to stem cells, but it does not cover ٣٦٠° of the limbus and leaves gaps in areas that may allow conjunctivalization of the surface in total stem cell deficiency. Hence, lr-CLAL may result in better outcome for patients with partial stem cell deficiency. In the most severe cases of ocular surface disease, if the patient has extensive conjuctival disease and total LSCD, combined KLAL and lr-CLAL, called the “Cincinnati procedure,” maximizes the advantages inherent in both procedures. Introduced by Holland et al, the Cincinnati procedure begins with recipient eye preparation as for standard KLAL.

Conjunctival tissue is placed superiorly and inferiorly and keratolimbal tissue is used to fill in the gaps nasally and temporally **(Holland and Schwartz, ٢٠٠٤)**. Systemic immunosuppression is required and these patients may be at higher risk for immunologic rejection because two different types of antigenic tissues are used **(Pauklin et al, ٢٠٠٩)**.

Keratolimbal allograft

KLAL uses peripheral cornea as the carrier for allogenic cadaveric stem cells. In ١٩٨٤, Thoft introduced keratoepithelioplasty by harvesting a rim of cadaveric cornea and transplanting it to a recipient after total superficial keratectomy **(Kim et al, ٢٠٠٣)**. In ١٩٩٤, Tsai and Tseng used a whole globe and they harvested an annular ring of limbal tissue and termed the procedure human allograft limbal transplantation **(Tsai and Tseng, ١٩٩٤)**.

In ١٩٩٥, Tsubota et al. used stored corneoscleral rim for limbal stem cell transplantation and termed their procedure limbal allograft transplantation **(Tsubota et al, ١٩٩٥)**. In ١٩٩٦, Holland modified Tsubota's technique using two stored corneoscleral rims. In this procedure, the central cornea is removed with a ٧,٥٠-mm trephine **(Holland, ١٩٩٦)**. The rim is bisected and excess peripheral tissue is removed. Then, lamellar dissection to remove the posterior two-thirds of the stroma along with Descemet's membrane and endothelium is performed. The host eye surface is prepared by performing ٣٦٠° conjunctival peritomy and releasing areas of symblepharon. Superficial keratectomy is performed to peel off pannus and conjunctivalized tissue, creating as smooth a surface as possible. Amniotic membrane can be transplanted at this time. Amniotic membrane has been shown to reduce inflammation and scarring and facilitate epithelial wave movement **(Gomes et al, ٢٠٠٣)**.

The prepared limbal grafts are secured to the eye using ١٠-٠ nylon sutures, trying to match donor's and recipient's limbus **(Kim et al, ٢٠٠٣)**. More recently, fibrin glue was employed to secure KLAL blocks in place. This will add intra- and postoperative patient comfort and may result in a smoother ocular surface postoperatively **(presented at Eye Bank Association of American Meeting, San Francisco, ٢٠٠٩)**

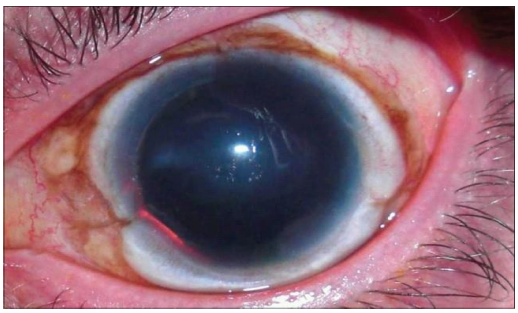


Figure (٢٣): Patient with aniridia ٦ months after keratolimbal allograft using fibrin glue **(Bachtiari and Djalilian, ٢٠١٠)**

KLAL does not provide conjunctival tissue and therefore it is the procedure of choice for patients with primary limbal involvement with minimal conjunctival involvement, such as aniridia. Patients with total LSCD and conjunctival involvement may benefit more from lr-CLAL combined with KLAL **(Kim et al, ٢٠٠٣)**.

Kim et al. reported ٨٩ patients with a follow-up of ٤,٧٠ years. Kim et al. found that ٧٣٪ of the patients had stable ocular surface at the last follow-up and subsequent keratoplasties were successful in ٦٥٪ of the patients. Nearly all of the patients received triple immunosuppressive therapy, initial patients with oral prednisone, cyclosporine, and azathioprine and, more recently, with prednisone, tacrolimus, and mycofenolate mofetil **(Kim et al, ٢٠٠٣)**.

Cultivated limbal stem cell transplantation

Kenyon and Tseng (١٩٨٩) proposed the procedure of conjunctival limbal autograft taken from the healthy fellow eye in patients with unilateral total LSCD. The procedure involves the transplantation of two large free grafts, each spanning from ٥–٧ mm in limbal arc length, removed from a healthy eye **(Kenyon and Tseng ١٩٨٩)**.

Reported complications in donor eyes have included localized haze in a patient with contact lens-induced keratopathy, pseudopterygium, filamentary keratitis, microperforation during surgery, abnormal epithelium, and corneal depression. Such a concern occurs when obtaining two such grafts from a donor eye with undiagnosed partial LSCD. Ex vivo expansion of limbal epithelial stem cells has been developed to circumvent potential complications related to conjunctival limbal autograft transplantation. The aim of differentiating these epithelial stem cells ex vivo is to help develop an equivalent of the human cornea using tissue culture techniques. Transplantation of cultivated limbal epithelium is currently the most successful alternative to surface reconstruction in patients with unilateral disease and offers a therapeutic chance to patients with severe bilateral disease **(Sangwan et al, ٢٠٠٦)**.

The various protocols for cultivation of limbal epithelium differ in the use of intact versus epithelially denuded amniotic membrane, suspension of epithelial cells rather than explants, co-cultivation of ٣T٣ fibroblast feeder layers, and air-lifting prior to transplantation. Currently no study has been conducted to compare these cultivation variables in order to determine which one is vital in achieving effective expansion of limbal epithelial progenitor cells **(Espana et al, ٢٠٠٣)**.

Pellegrini and colleagues in ١٩٩٧ first reported successful reconstruction of the ocular surface in LSCD. A ١ mm^2 biopsy sample from the healthy fellow eye was treated with trypsin for ٣ hrs. The cells were plated on lethally irradiated ٣T٣-J٢ cells and cultured in ٥٪ carbon dioxide in Dulbecco-Vogt Eagle's and Ham's F١٢ media. Grafts were prepared from confluent secondary cultures which were released from the plastic dish by neutral protease Dispase II and either mounted on petrolatum gauze or a soft contact lens **(Pellegrini et al, ١٩٩٧)**.

The next advancement in this technique was the use of human amniotic membrane as a substrate for in vitro epithelial cell culture. **Koizumi and colleagues (٢٠٠٠a)** first cultivated rabbit limbal epithelium on human amniotic membrane (HAM) in vitro and then, after transplantation onto rabbit ocular surface, confirmed the viability of the transplanted cultivated epithelium in vivo. Subsequently they showed that denuded HAM was better than intact cellular HAM for corneal epithelial cell culture **(Koizumi et al, ٢٠٠٠b)**. They adopted the culture system using epithelially denuded HAM with an underlying layer of lethally irradiated mouse ٣T٣ fibroblasts. Their results showed that the expanded epithelium resembled a corneal phenotype with respect to the expression of K٣ keratin **(Koizumi et al, ٢٠٠١a)**.

Grueterich and colleagues (٢٠٠٢b) demonstrated that culturing limbal explants on an intact amniotic membrane without the use of a ٣T٣ feeder layer resulted in a limbal epithelial phenotype. **Koizumi and colleagues (٢٠٠٢)** demonstrated that both cell suspension and explant culture methods produced a healthy epithelial cell layer, with cells from the former being morphologically more superior.

Currently most investigators prefer the explant culture technique The benefits of using explants are that they are easy to prepare and there is no danger of damaging the corneal epithelium through enzyme treatment **(Shimazaki et al, ٢٠٠٢)**.

The general principles of culturing the cells by explant culture technique involve the following steps:

- harvesting the limbal tissue (from the contralateral healthy eye in case of a unilateral LSCD, or from donor corneas for bilateral LSCD);
- selecting the appropriate carrier: a sheet of multi-layered epithelium, human amniotic membrane, collagen shields, or contact lens;
- preparation of human corneal epithelial medium; and
- explant cultures **(Sangwan ٢٠٠١).**

Confirmation of growth can be done by various methods including-direct observation, whole mount stained preparation, histopathology, immunohistochemistry, thymidine incorporation and by flow cytometry using markers for cell cycle **(Vemuganti and Balasubramanian, ٢٠٠٢)**.

Although a number of investigators have included various reconstruction techniques through the use of autogenous conjunctiva, mucous membrane grafts, collagen lattices, synthetic implants, and cell-suspension cultures, the most widely accepted universal substratum for explant cultures is the HAM **(Grueterich et al, ٢٠٠٣)**. It was recognized and successfully reintroduced by **Kim and Tseng (١٩٩٥)** for corneal surface reconstruction in rabbits. Amniotic membrane is the innermost layer of the fetal or placental membrane and consists of an epithelial monolayer, a thick basement membrane, and an avascular stroma. Preserved HAM can be used as biological substrate without

viable and active proliferative cells. It is thus nonimmunogenic and therefore does not require immunosupression when used as a graft for transplantation **(Burman and Sangwan, ٢٠٠٨)**.

Amniotic membrane serving as stem cell niche

The role of the devitalized epithelium is not yet fully understood. It has been shown that native, intact amniotic membrane (AM) epithelium contains higher levels of epidermal growth factor, keratinocyte growth factor, hepatocyte growth factor, and basic fibroblast growth factor compared with epithelially denuded AM **(Grueterich et al, ٢٠٠٢b)**.

However, **Koizumi and colleagues (٢٠٠٠b)** indicated that denuded AM promotes better corneal epithelial cell colonization than intact AM does and that corneal cells from the limbal epithelium colonize denuded AM more readily than epithelial cells from the central cornea. Corneal cells colonize intact HAM much less quickly than denuded amnion. Moreover, migrating limbal SC on denuded HAM have a smooth, uniform leading edge compared with the irregular raised edges of sheets grown on intact HAM. Morphologic observations further strengthen the support for epithelium grown on denuded HAM. The basal cells grown on bare amniotic membrane are nicely columnar, and the more superficial cells seem fairly well differentiated into wing cells and surface cells. In contrast slowly growing epithelial cells on intact HAM do not take the appearance of normal corneal epithelium. However, expanded epithelium from limbal explants on intact AM adopts a limbal epithelial phenotype whereas that expanded on epithelially denuded HAM reveals a corneal epithelial phenotype **(Grueterich et al, ٢٠٠٢b)**.

Wei and colleagues (٢٠٠٧) have further demonstrated that during ex vivo expansion on intact HAM some limbal epithelial progenitor cells migrate onto intact AM whereas some also invade the limbal stroma, very likely undergoing epithelial-mesenchymal transition. The HAM's basement membrane contains type IV collagen, Ln-١, Ln-٥, fibronectin, and collagen VII. The collagen IV subchain α٢ is identical to that of the conjunctival BM. Ln play an important role in corneal epithelial cell adhesion. Overall the basement membrane facilitates migration of epithelial cells, reinforces adhesion of basal epithelial cells, promotes epithelial differentiation, and prevents epithelial apoptosis, that is, programmed cell death **(Lee et al, ٢٠٠٠)**.

The AM stroma also contains a number of growth factors, various antiangiogenic and anti-inflammatory proteins, and natural inhibitors to various proteases **(Sato et al, ١٩٩٨)**. In view of all of the above-mentioned properties of the AM, there is little doubt that it provides an ideal stromal niche desirable for stem cell expansion **(Burman and Sangwan, ٢٠٠٨)**.

Limbal cell culture: Explant culture technique

Many investigators have adopted the explant culture technique with variations in substrate, media, feeder cells, and airlifting **(Shimazaki et al, ٢٠٠٢)**. To elaborately describe the details of each protocol is beyond the scope of this article. Therefore the modifications that the authors have incorporated in their procedure have been described with respect to reports from previous investigators.

Limbal biopsy

After informed consent is obtained from the patients or guardians. Limbal biopsy is performed on the healthy

contralateral eye or a healthy area of the ipsilateral eye. The procedure includes careful dissection of a ١x٢ mm^{٢} piece of limbal epithelium with ١ mm into clear corneal stromal tissue at the limbus under strict aseptic conditions. A conjunctival peritomy is made ٢–٣ mm from the limbus and dissection of the conjunctiva is carried forwards ١ mm beyond the limbal arcade. The harvested tissue should exclude the Tenon's capsule and should include the palisades of Vogt. Post-operatively topical antibiotic is used four times day for two weeks. Topical steroids are used in tapering doses for ٤ to ٦ weeks or until complete healing of donor site **(Burman and Sangwan, ٢٠٠٨)**

AMG procurement, processing, and preservation

Amniotic membrane is obtained from prospective donors undergoing caesarean section, who are negative for communicable diseases including HIV, hepatitis, and syphilis. Different protocols exist for the processing and storage **(Shimazaki et al, ١٩٩٧)**. According to **Kim and Tseng (١٩٩٥)**, the amnion is separated from the chorion by blunt dissection, and washed with balanced salt solution containing a cocktail of antibiotics (٥٠ µg/ml penicillin, ٥٠ µg/ml streptomycin, ١٠٠ µg/ml of neomycin, as well as ٢,٥ µg/ml of amphotericin B) under sterile conditions. The separated membranes are cut in different sizes ٣x٤ mm^{٢}) and placed on nitrocellulose paper strips with the epithelial side up. Dulbecco Modified Eagles' Medium/glycerol (١:١) is used for cryopreservation and the tissues are frozen at –٨٠ °C degrees until further use **(Kruse et al, ٢٠٠٠)**. Just before use, the amniotic membrane is thawed at ٣٧ °C for ٣٠ min, washed ٣ times with sterile PBS and placed on a sterile cut glass slide. The amniotic epithelium is removed by digestion with ٠,١٪ trypsin-EDTA at ٣٧ °C, followed by scraping. Under sterile conditions, the membrane is inspected under the microscope for complete removal of

cells. The de-epithelized membranes are then spread on a glass slide (used as culture inserts) in a Petri plate tucking the edges for a uniform surface **(Sangwan et al, ٢٠٠٦)**.

Human corneal epithelial cell growth medium

We use a modified human corneal epithelial cell (HCE) culture medium, prepared using ٩,٧ g/l Modified Eagle Medium (MEM) with addition of ١٦,٢ g/l Ham F١٢ serum, ٠,٠١mg/l epidermal growth factor, ٠,٢٥ mg/l insulin, ٠,١ mg/l cholera toxin, and hydrocortisone. The medium is filtered with ٠,٢٢ mm membrane filters using a vacuum pump. This is supplemented with autologous serum or ١٠٪ fetal calf serum (FCS) at the time of use **(Burman and Sangwan, ٢٠٠٨)**.

Explant culture technique

Koizumi and colleagues (٢٠٠١b) have used ٣T٣ feeder cells along with airlifting for epithelial stratification. **Sangwan and colleagues (٢٠٠٦)** follow a submerged explant culture system without the use of any feeder cell layer or airlifting. Limbal tissue procured by biopsy is shredded into ٤–٦ fragments, and placed on the de-epithelized membranes separately and allowed to settle down by overnight incubation at ٣٧ °C with ٥٪ CO_2 and ٩٥٪ air. The medium is changed on alternate days for ١٠–١٤ days with daily monitoring of cell growth under phase-contrast microscope. In four days polygonal cells could be seen growing from the edges of the explant.

Whole mount preparation

After confirming confluent growth of a monolayer from the explanted tissue over ١–٢ weeks, the growth is terminated by replacing the medium with ١٠٪ buffered

formalin. The whole mount preparation, stained with hematoxylin and eosin, could be seen as bluish rings of stained areas around the original explanted tissue. When observed under the microscope, the cultured cells appear as a monolayer of large polygonal cells with an epithelial appearance. The membrane with the cultured limbal tissue and the cultured cells is fixed in formalin and processed for routine histopathology with paraffin embedding. The sections are cut at ٤–٥ cm and after deparaffinization, stained with hematoxylin and eosin stain and periodic acid-Schiffs stain. In contrast to the cuboidal epithelium of the normal amniotic membrane the cultured cells form an epithelium of ١–٢ layered cells over the amniotic membrane. Immunostaining on the formalin-fixed, paraffin embedded sections can be done using prediluted antibodies to cytokeratin ٣ (K٣) and cytokeratin ١٩ (K١٩) to confirm the corneal phenotype of the cultured cells **(Vemuganti et al, ٢٠٠٤)**.

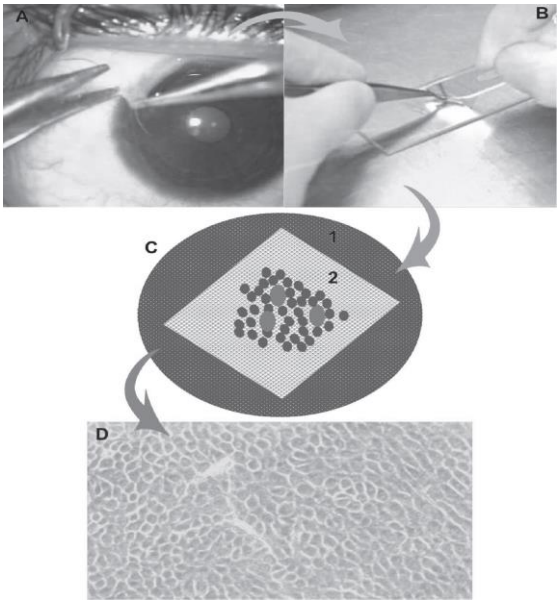


Figure (٢٤): Schematic diagram showing the steps in cultivation of limbal epithelial stem cells. **A**: Technique of limbal biopsy (See text for details). **B**: Processing of tissue in the laboratory and making the explant culture. **C**: Petridish, glass slide (white) with de-epithelialized human amniotic membrane with explants (red dots) with growing cells around it (blue dots). **D**: Monolayer of cells (١٠–١٤ days old) under phase contrast microscope **(Burman and Sangwan, ٢٠٠٨)**.

Cell suspension culture of limbal epithelial cell

Preparation of AM and ٣T٣ fibroblast cells:

As described in detail in previous reports, as regard AMG processing and preservation to obtain denuded AM. Confluent ٣T٣ fibroblasts were inactivated by incubation in ٤ μg/ml mitomycin C (MMC) for ٢ hours at ٣٧ °C under ٥٪ CO_2, and then trypsinized and plated onto plastic dishes **(Schwab et al, ٢٠٠٠)**.

Cell suspension:

The whole limbal ring was cut into two to three pieces, and these were incubated at ٣٧ °C for one hour with ١,٢ IU dispase. The corneal limbal epithelium including the limbal stem cells were suspended in ٣ ml medium, seeded onto three pieces of denuded AM spread on the bottom of the culture inserts, and co-cultured with MMC-inactivated ٣T٣ fibroblasts. The culture was submerged in the medium for ٢ weeks and then exposed to air by lowering the medium level (airlifting) for ٢ weeks to promote corneal epithelial differentiation. The culture medium used was Dulbecco's modified Eagle's (DMEM) medium and Ham's F١٢ (١:١ mixture) **(Koizumi et al, ٢٠٠٢)**.

Surgical technique

The surgery is performed ١٠–١٤ days after the limbal biopsy. Following strict aseptic precautions, under local or general anesthesia, a drop of ١:١٠٠٠ epinephrine is instilled into the conjunctival cul de sac to ensure hemostasis. The fibrovascular pannus is dissected off the corneal surface starting ٢–٣ mm beyond the limbus using a conjunctival spring scissors until the limbus and beyond. A plane of dissection is usually noted in patients who have undergone

previous ocular surface reconstruction with AM, facilitating the excision **(Burman and Sangwan, ٢٠٠٨)**.

Symblepharon release is carried out at the same time and AM is used to reconstruct the ocular surface. Fornix reconstruction is also performed where appropriate **(Burman and Sangwan, ٢٠٠٨)**.

The AM, with its monolayer of cultured limbal epithelial cells, is then transferred to the ocular surface and anchored in place at the limbus with interrupted ١٠–٠ monofilament nylon sutures placed circumferentially. The knots are trimmed and buried. The peripheral skirt of the AM is anchored to the conjunctiva with ٨–٠ vicryl sutures. Subconjunctival dexamethasone is given at the end of surgery. Some authors recommend mitomycin C subconjunctivally to prevent recurrence of symblepharon and a bandage contact lens may be inserted **(Burman and Sangwan, ٢٠٠٨)**.

In some patients with chemical injury, corneal scarring, and who may have undergone prior attempts at surface reconstruction, difficulties may be encountered in suturing the donor corneal tissue to the thin recipient bed. In such cases, cultured LSC transplantation may be combined with either a lamellar keratoplasty or deep anterior lamellar keratoplasty. Preoperative anterior segment interferometry or intraoperative pachymetry after pannus resection may indicate the residual stromal thickness and aid the decision for lamellar keratoplasty (LK) or deep anterior lamellar keratoplasty (DALK) **(Burman and Sangwan, ٢٠٠٨)**.

In cases with anterior to mid stromal scarring and thinning, a lamellar keratoplasty may be done using a crescent blade or an automated keratome. The lamellar donor corneal tissue is sutured to the recipient bed with

interrupted ١٠–٠ monofilament nylon sutures and the AM with cultivated limbal epithelium is placed on the entire de-epithelized surface. Alternatively, a deep anterior lamellar keratoplasty may be performed to provide a smooth interface **(Burman and Sangwan, ٢٠٠٨)**.

Penetrating keratoplasty (PK) after limbal stem cell transplantation (LSCT)

A penetrating keratoplasty is usually planned ٣ months post operatively once the ocular surface has stabilised for visual loss related to corneal scarring in the visual axis. The recipient cornea is trephined in the centre with a disposable trephine and a donor tissue ٠,٥ mm larger is anchored to the recipient bed with interrupted ١٠–٠ monofilament sutures. A lensectomy, vitrectomy, synechiolysis, and/or intraocular lens implantation may be carried out if deemed necessary. In the immediate post-operative period, it is imperative to watch for signs of epithelial breakdown and allograft rejection. It is worthwhile to perform a primary tarsorrhaphy in such cases **(Sangwan et al, ٢٠٠٥)**.

PK is performed three months post CLSCT as it not only ensures a stable ocular surface but also decreases the risk of corneal graft rejection by controlling inflammation. A second hypothesis is decreased sensitization by decreasing antigen presentation as the cultivated epithelium is devoid of dendritic cells. The early outcome of PK following cultivated limbal epithelial transplantation is very promising with ٨٧٪ of grafts remaining clear at mean follow up of ٨,٣ months post PK and ٢٥٪ developing corneal allograft rejection **(Sangwan et al, ٢٠٠٥)**.

Postoperative management

Postoperative management of patients who undergo limbal stem cell transplantation is one of the most important factors that determine the success rate and outcome. Postoperatively, topical antibiotic is used until the surface is completely epithelialized. Topical steroids are used to reduce inflammation and topical cyclosporine or tacrolimus may be added to the regimen as required **(Sloper et al, ٢٠٠١)**. The health of the ocular surface should be optimized with the use of nonpreserved artificial tears, punctal occlusion, bandage lens, tarsorrhaphy, and trichiasis removal. Any factor that destabilizes the ocular surface needs to be addressed aggressively and quickly **(Bakhtiari and Djalilian, ٢٠١٠)**.

Transplantation of an allograft poses the risk of rejection even in HLA-matched recipients. Therefore, all allografts such as KLAL and lr-CLAL need prolonged systemic immunosuppression, which could span their lifetime **(Espana et al, ٢٠٠٤)**. The goal of immunosuppression is to eliminate eye inflammation and prevent allograft rejection. Topical immunosuppressants are usually insufficient in controlling allograft rejection after KLAL. In a series by Kim et al., the success rate after KLAL was ٨٧٪ in patients receiving systemic immunosuppression vs. ٢٩٪ in patients treated with only topical immunosuppression **(Kim et al, ٢٠٠٣)**.

We prefer combined immunosuppressive therapy, including steroids, tacrolimus, and mycophenolate mofetil, as summarized in Table (٦). Combined systemic immunosuppression based on mycophenolate mofetil and tacrolimus seems to be more effective and safer than cyclosporine A alone **(Boratynska et al, ٢٠٠٦)**. Tseng et al. also showed the role of combined immunosuppression

with tacrolimus and mycophenolate mofetil on long-term maintenance of functional graft **(Liang et al, ٢٠٠٩)**. Alloway et al. reported that KLAL patients on mycofenolate mofetil and tacrolimus had significantly fewer adverse systemic events compared to age-matched renal transplant patients. In general, it is recommended to co-manage the patient with an organ transplant immunologist to minimize the risk of adverse effects **(Alloway et al, ٢٠٠٧)**

Table (٩): Immunosuppressive regimen after limbal stem cell allograft transplantation **(Bakhtiari and Djalilian, ٢٠١٠)**

Medication	Dosage and Duration
Corticosteroids	
Topical	Qd-qid, indefinitely
Oral	٠,٥-١ mg/Kg/d, taper over ٣-٤ months
Cyclosporin A	
Topical	٠,٠٥٪ qid, indefinitely
Oral	٣ mg/Kg/d, ١٢-١٨ months
Or	
Tacrolimus	٣-٤ mg q١٢h, ١٢-١٨ months
Azathioprine	١٠٠ mg/d, ١٨-٢٤ months
Or	
Mycophenolate	١٠٠٠mg bid, ١٨-٢٤ months

Outcome of cultivated LSCT

Schwab and colleagues (٢٠٠٠) reported a successful outcome, defined as restoration or improvement of vision, along with maintenance of corneal re-epithelialization and absence or recurrence of surface disease, obtained in ٦ of the ١٠ patients with autologous procedures and in all ٤ of the allogenic procedures **(Tsai et al ٢٠٠٠)**. Follow up ranged ١٦–١٩ months with a mean of ١٣ months. They concluded that both amniotic membrane and corneal epithelial stem cells present within the bio-engineered graft were necessary for successful repair.

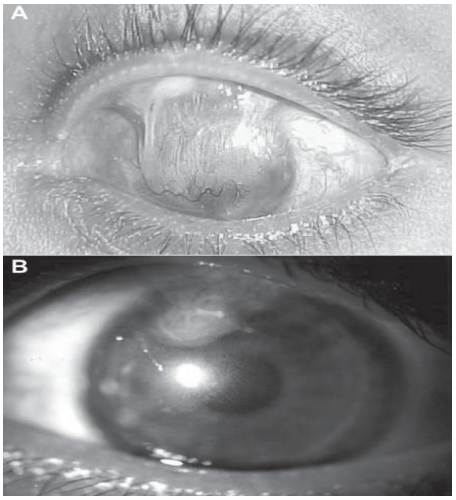


Figure (٢٥): A. Slit lamp photograph of a patient with total limbal stem cell deficiency with extensive symblephara following alkali injury. **B**. One year after autologus cultivated limbal stem cell transplantation shows clear central cornea and no recurrence of symblephara **(Burman and Sangwan, ٢٠٠٨)**.

New Technologies in Limbal Epithelial Stem Cell Transplantation

New Technologies in Limbal Epithelial Stem Cell Transplantation

Modification of amniotic membrane

The method used by the majority of research groups to treat LESC deficiency with stem cells is by transplantation of these expanded sheets using amniotic membrane (AM) as a substrate **(Shortt et al, ٢٠٠٩)**. AM is the inner layer of the sac which surrounds the embryo during gestation and consists of an single layer of epithelial cells resting on a basement membrane and underlying layers of connective tissue **(von Versen Hőynck et al, ٢٠٠٤a)** . AM's anti-inflammatory, antiangiogenic, anti-microbial and anti-viral properties justify its widespread use as a topical bandage for surgical wounds, ulcers or burns **(Gomes et al, ٢٠٠٥)**.

However, there are a number of drawbacks associated with its use as a substrate in corneal repair including challenges in the maintenance of a reliable supply of membranes, considerable variation amongst donors,
costly donor screening which does not completely eliminate the risk of viral agent transmission and ultimately lack of optimal transparency which is obviously a major issue when dealing with repair of the cornea. Bearing these aspects in mind, many have attempted to develop an alternative to AM as a carrier substance for stem cells in corneal repair. A superior alternative to AM must fulfill many of the following criteria to become a viable option for replacement. It must be optically transparent, strong enough to withstand manipulation in culture and suturing, irrigation and handling in surgery, the material must be flexible enough to take the shape of the eye and lie flat on the surface, it must be cytocompatible and able to be produced with consistent quality, preferably at high speed and low

cost. Taking all these factors into consideration it is easy to see why it has so far proved such a challenge to find a feasible alternative to AM (Figure ٢٦) **(Levis and Daniels, ٢٠٠٩)**.

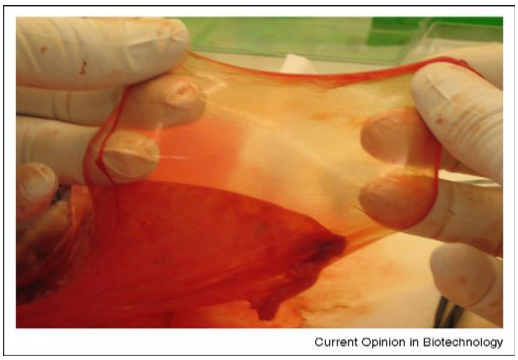


Figure (٢٦): Amniotic membrane **(Levis and Daniels, ٢٠٠٩)**

As AM possesses many favourable properties for use in this context, attempts have been made to modify the AM itself. Lyophilized AM has a much longer shelf life than cryopreserved, native AM and is safer and easier to store as it can be sterilized by gamma irradiation **(Jang et al, ٢٠٠٦)** or by peracetic acid **(von Versen-Hőynck et al, ٢٠٠٤b)**. However, these manufacturing processes can alter some of the characteristics of AM and so researchers have tried to treat the AM to protect it through the freeze–drying process. Nakamura et al. incubated AM with a non-reducing disaccharide, trehalose, and found that it significantly improved the quality of freeze–dried AM **(Nakamura et al, ٢٠٠٨)**.

The same group has also developed a fibrin glue coated AM which eliminates the requirement for sutures when transplanting the graft onto the surface of the eye **(Sekiyama et al, ٢٠٠٧)**. Uchino et al. combined the stability of a polymer with the biocompatibility of a natural material to create a hybrid for use in corneal reconstruction. They cross-linked a polyvinyl alcohol (PVA) hydrogel with

AM to produce a scaffold that was able to support a stratified epithelium and enhance in vivo stability **(Uchino et al, ٢٠٠٧)**.

Alternative substrates for epithelial cell transfer

The transfer of sheets of epithelial cells, grown on AM, onto the surface of the eye is a tricky procedure as the membranes have a tendency to fold and crease very easily. As a result, alternative carriers have been used, such as petrolatum gauze or contact lenses, where cells are grown as usual in culture dishes then released using enzymes and placed on the lens or gauze for transferral **(Pellegrini et al, ١٩٩٧)**.

Okano's group in Japan developed an ingenious system that eliminates the requirement for enzymes to detach cell sheets from tissue culture plastic before transplantation, retaining important cell–cell and cell–matrix interactions **(Yang et al, ٢٠٠٥)**. They covalently bonded a temperature responsive polymer, poly (N-isopropylacrylamide), to culture dishes. Under normal culture conditions of ٣٧˚C, the dish surface is hydrophobic and cells can attach, however, when the temperature is reduced below ٣٢˚C it becomes hydrophilic forming a hydration layer between the cells and the surface to allow spontaneous detachment **(Okano et al, ١٩٩٣)**.

To completely eliminate the need to detach cells from the growth surface, attempts have been made to grow human LESCs directly on contact lenses for subsequent transferral to the eye. One study by Deshpande et al. used a plasma polymer coated contact lens to aid cell attachment and whilst rabbit limbal epithelial cells attached and could subsequently be transferred to a rabbit corneal organ culture model, human limbal epithelial cells required detachment

using enzymes **(Deshpande et al, ٢٠٠٩)**. However, another research group was able to successfully culture human epithelial cells from a tissue explant on a non-coated contact lens and then transfer the cells to the eye of three patients with LESC deficiency to restore a transparent corneal epithelium **(Di Girolamo et al, ٢٠٠٩)**.

A fibrin sealant, produced from combining fibrinogen and thrombin, has been used as a substrate for LESC growth and is particularly useful in this context as it is a quickly degradable, natural substrate **(Higa et al, ٢٠٠٧)**. Another distinct advantage of this method is that both constituents of the cross-linked fibrin gels can be of human origin, providing the potential for an autologous bioengineered tissue. However, the use of fibrin gels may not be appropriate when a population of stem cells must be maintained as it has been shown to affect cells by causing differentiation. Han et al. reported that all cells cultivated in a fibrin gel stained positively for cytokeratin ٣, a differentiated epithelial cell marker **(Han et al, ٢٠٠٢)**.

Silk fibroin is a structural protein obtained from the cocoon of the silkworm Bombyx mori. It is a particularly useful material in corneal bioengineering as it displays a non-immunogenic response on implantation in vivo, is mechanically robust, transparent, easy to handle and has controlled degradation rates **(Lawrence et al, ٢٠٠٨)**. It has also been shown to support the growth of limbal epithelial cells in serum-free conditions to the same extent as tissue culture plastic **(Chirila et al, ٢٠٠٨)**. Nanopatterning technology allows surface modification of the silk fibroin, which could provide guidance to the migration and alignment of cells seeded on and in the corneal scaffolds. However, an important point to consider is the cost of such a natural material, which will be considerably greater than the cost of synthetic materials **(Levis and Daniels, ٢٠٠٩)**.

Collagen-based substrates

Epithelial sheets are typically transplanted directly onto the corneal stroma, whose major constituent is collagen, therefore the use of collagen as a substrate for corneal repair appears an obvious choice. Particularly as collagen has a number of favourable properties when used as a cellular substrate; it is biocompatible, has low immunogenicity, is naturally remodeled by cells and is relatively inexpensive to produce **(Neel et al, ٢٠٠٦)**.

However, hydrated collagen gels contain a large proportion of water and so are inherently weak. Collagen can be cross-linked using different methods to produce a stable hydrogel, which increases its resistance to enzymatic degradation **(Liu et al, ٢٠٠٦)**. Griffith et al. produced a whole human corneal equivalent by cross-linking a collagen–chondroitin sulfate substrate with glutaraldehyde and seeding epithelial cells on top, endothelial cells below and stromal cells within the substrate **(Griffith et al, ١٩٩٩)**. In this case, hydrogels were developed as a complete corneal replacement, but they have also been used to develop a replacement to AM. A collagen type III scaffold was cross-linked using a water soluble carbodiimide that facilitates cross linking of the collagen but is not incorporated into the final product **(Dravida et al, ٢٠٠٨)**. The refractive index, transmission and backscatter properties were similar to that of native cornea and LESCs were able to stratify and express putative stem cell and differentiated cell type markers in a similar fashion to cells on AM. Although cross-linking does enhance the mechanical properties of collagen, major drawbacks include the cytotoxicity of the crosslinker, reduced biomimetic qualities of the scaffold and prevention of cell-based scaffold remodeling **(Neel et al, ٢٠٠٦)**.

One type of collagen substrate that has superior optical properties is the collagen vitrigel membrane. A vitrigel is a gel in a stable state produced via a three-stage sequence of gelation, vitrification and rehydration. This process creates a transparent, rigid, glass like material from an opaque, soft gel after low-temperature evaporation of fluid. After the vitrification process the gel is rehydrated to provide a thin (٢٠–٥٠ mm), transparent membrane with enhanced mechanical properties **(McIntosh Ambrose et al, ٢٠٠٩)**.

McIntosh Ambrose et al. were able to culture human limbal epithelial, bovine fibroblast and rabbit endothelial cells on the surface of the vitrified membranes. They found that limbal epithelial cells expressed markers of both differentiated corneal epithelial cells (cytokeratin ٣, cytokeratin ١٢ and connexin ٤٣) and putative stem cells (P٦٣ and ABCG٢) suggesting a mixed population of cells. Although vitrigel membranes do boast many of the required properties for replacement of AM there is one major limitation of this procedure; cells cannot be seeded within the gel due to the lengthy (two weeks) dehydration process. This would eliminate the possibility of more closely replicating the native cornea by seeding epithelial cells on top of a fibroblast containing collagen stroma **(McIntosh Ambrose et al, ٢٠٠٩)**.

Collagen hydrogels are formed as fluid and cells are trapped in an unordered fashion in a random network of fibrils. This process may affect the mechanical and optical properties of the final product, however, cell organization within a collagen matrix can be influenced in a number of ways to improve the properties of the construct. Karamichos et al. demonstrated that corneal fibroblasts align and compact collagen parallel to the axis of greatest extracellular matrix stiffness. In this study, the cellular

collagen matrices were fully or partially constrained by attachment to immobilized plastic bars and after ٢٤ hours it was noted that cells were aligned parallel to the long axis in the anisotropic region of constrained matrices but randomly aligned in unconstrained (isotropic) matrices. In addition the collagen density and cell/collagen co-alignment were higher in the constrained matrices **(Karamichos et al, ٢٠٠٧)**.

An alternative way to align the fibrils is by exposing neutralized collagen to a magnetic field. By using a series of gelation–rotation–gelation cycles through a horizontal magnetic field, Torbet et al. were able to produce a scaffold of orthogonal lamellae composed of aligned collagen fibrils **(Torbet et al, ٢٠٠٧)**. Fibroblasts were seen to align by contact guidance along the fibrils and within the bulk of the orthogonal complex. Both of these methods produce collagen scaffolds that offer a much more accurate representation of the ordered nature of the native cornea, however, neither of these studies explored the behaviour of epithelial cells on the gels, data which would be necessary to assess their potential as a replacement for AM **(Levis and Daniels, ٢٠٠٩)**.

Electrospinning is a process that is commonly used to produce synthetic polymer nanofibres but its usefulness in recreating the microstructure of collagen fibres in the cornea has recently been explored. The process uses an electric charge to draw very fine fibres from a liquid and does not require the use of coagulation chemistry or high temperatures to produce the solid threads. A number of researchers have produced electrospun collagen fibres from solutions that are combined with synthetic polymers but many of the polymers or solvents are cytotoxic and so not appropriate for use in cellular applications **(Buttafoco et al, ٢٠٠٦)**.

However, Wray and Orwin have very recently produced aligned ٣٠–٥٠ nm collagen type I fibres using a less toxic solvent. They also showed that corneal fibroblasts elongated along the axis of fibre alignment, responding to changes in microstructure and organization of the matrix environment **(Wray and Orwin, ٢٠٠٩).** This method appears to provide a viable scaffold material for corneal stroma replacement but again, further investigations need to be undertaken to determine how LESCs would react to this material **(Levis and Daniels, ٢٠٠٩)**.

Serum eye drops

Commercially available artificial tears have focused on alleviating the biomechanical trauma caused by dry eye states. Attempts to develop a biological tear substitute that has lubricating, and nutrient properties promoting ocular surface renewal and immunological defense have been limited. Isolated reports of single compound topical agents such as Vitamin A **(Gilbard et al, ١٩٨٩),** EGF **(Elliott, ١٩٨٠)** and albumin **(Shimmura et al, ٢٠٠٣)** have show some in vitro and in vivo efficacy, but long-term clinical applications have not been developed. Newer lubricants such as those containing carboxymethylcellulose, boast improved ocular surface retention and epithelial proliferation, whereas sodium hyaluronate preparations exploit the property that it is a naturally occurring extracellular matrix glycosaminoglycan, which plays an important role in wound healing, inflammation and lubrication. There is evidence that hyaluronate is cytoprotective, promotes BM hemidesmosome formation, and has improved surface retention in inflamed eyes due to specific ligand binding to exaggerated CD٤٤ (a transmembrane cell surface adhesion molecule) expression

on the ocular surface during inflammation **(Gomes et al, ٢٠٠٤).**

To date, there is no true commercially available tear substitute possessing both lubricating and nutrient properties. During the last decade, however, illicit occupancy of this hiatus by autologous serum eye drops has gained popularity and acceptance, specifically as an adjunctive treatment for complex, often immune-mediated, ocular surface disorders where the production and quality of the tear film has been compromised. The beneficial effect of natural tear substitutes made with autologous serum was first evaluated in patients with keratoconjunctivitis sicca in ١٩٨٤, during the search for a preservative-free tear substitute **(Fox et al, ١٩٨٣)**. Fifteen years later, Tsubota et al. revived interest in the epitheliotrophic potential of autologous serum drops, showing beneficial effects in Sjőgren's syndrome **(Tsubota et al, ١٩٩٩)**.

Serum contains a large number of biological substances that are present in tears (Table ١٠) although some substances are present in lower or higher concentrations. These epitheliotrophic factors are likely to be responsible for the therapeutic effect of serum drops observed in patients with ocular surface disorders over and above conventional commercially available lubricants **(Rauz and Saw, ٢٠١٠)**.

Table (١٠): Comparison of the constituents of natural tears and peripheral blood serum **(Rauz and Saw, ٢٠١٠)**

Parameter	Tears	Serum
pH	٧,٤	٧,٤
Osmolality	٢٩٨	٢٩٦
EGF (ng/ml)	٠,٢-٣,٠	٠,٥
TGF-b (ng/ml)	٢–١٠	٦–٣٣
NGF (pg/ml)	٤٦٨,٣	٥٤,٠
IGF (ng/ml)	٠,٣١	١٠٥
PDGF (ng/ml)	١,٣٣	١٥,٤
Albumin (mg/ml)	٠,٠٢٣	٥٣
Substance P (pg/ml)	١٥٧	٧٠,٩
Vitamin A (mg/ml)	٠,٠٢	٤٦
Lysozyme (mg/ml)	١,٤	٦
Surface IgA (µg/ml)	١,١٩٠	٢
Fibronectin (µg/ml)	٢١	٢٠٥
Lactoferrin (ng/ml)	١,٦٥٠	٢٦٦

EGF, epidermal growth factor
TGF, transforming growth factor
NGF, nerve growth factor
IGF, insulin-like growth factor
PDGF, platelet-derived growth factor

There is no universal consensus on how autologous serum eye drops should be prepared. Patients are required to be of reasonably good health, with no significant cardiovascular or cerebrovascular disease, and free of bacterial infection. Anaemia (Hb ١١ g/dl) is a relative contraindication. Patients are screened (as for volunteer blood donations) for hepatitis B and C (HBsAg, anti-HBc, nucleic acid technology/testing), HIV I & II, human T cell lymphotropic virus, HTLV I & II and syphilis. One full

blood donation produces ~١٥٠ bottles of autologous serum eye drops with an arbitrary shelf life of ٦ months from the date of manufacture. Access to autologous serum eye drops is restricted in the UK, largely due to cost **(Rauz and Saw, ٢٠١٠)**.

Review of the literature indicates a large variation in protocols for preparation of autologous serum, including variations in production parameters (clotting phase, centrifugal force, duration of centrifugation, dilution, diluent), storage (containers, temperature, duration) and application (number of daily applications). This diversity of production is recognized to affect the concentrations of the biological constituents within the serum **(Geerling et al. ٢٠٠٤)**. Additionally, there are no randomized controlled trials to identify which of the published protocols have maximal epitheliotrophic support or whether optimal parameters have yet to be defined **(Rauz and Saw, ٢٠١٠)**.

The number of complications reported in patients receiving serum eye drops is small; most authors report no complications. A few isolated cases of microbial keratitis, bacterial conjunctivitis, increased discomfort or epitheliopathy, and eyelid eczema have been described, however these reports are confounded as the complications described may easily be attributed to the natural course of the disease being treated **(Geerling et al, ٢٠٠٤)**. Other potential disadvantages of serum eye drops are the limited stability which necessitates storage at –٢٠˚C to optimally preserve protein activity, and the risk of infection for patients and others handling the serum **(Rauz and Saw, ٢٠١٠)**.

Despite the limitations in production, there is substantial evidence that in vitro corneal epithelial cells remain viable, are less prone to apoptosis, and proliferate

and migrate more effectively in the presence of peripheral blood serum than with conventional artificial tears such as hypromellose (**Liu et al, ٢٠٠٦)**. Clinically, serum eye drops are effective in treating conditions such as primary and secondary causes of dry eye, persistent epithelial defects **(Lopez-Garcia et al, ٢٠٠٨),** neurotrophic ulcers and following ocular surface reconstructive surgery **(Matsumoto et al, ٢٠٠٤)**

In the future, use of allogeneic serum preparations such as from family members, could also be advantageous for patients where autologous serum drops are not possible **(Chiang et al, ٢٠٠٩)** or patients are unsuitable for donation for various reasons such as anaemia (which is common in Sjőgren's syndrome, the elderly, or those receiving immunosuppressive therapy for ocular surface disease). Additionally, there are reports that serum drops prepared from umbilical cord blood, may be even more efficacious than peripheral blood serum possibly due to increased levels of nerve growth factor, EGF, TGF-b and substance P **(Yoon et al, ٢٠٠٧b)**.

Failure of Limbal Stem Cell Transplantation

and

Strategies to improve Outcomes

Failure of Limbal Stem Cell Transplantation and Strategies to improve outcomes

When evaluating whether a patient has had a successful result, it is imperative to consider the length of follow-up. Evaluation of short-term results (i.e., less than one year) reveals a high success rate with most techniques studied. It must be remembered that penetrating or lamellar keratoplasty in a patient with a total absence of stem cells will do well for the short term. When the inevitable sloughing of the donor epithelium occurs, it will be replaced by conjunctiva-like tissue. As a result, the ocular surface will fail **(Holland and Schwartz, ١٩٩٦)**.

We know from studies of epithelial rejection following keratoplasty that the donor epithelium can survive up to ١٣ months . **(Krachmer and Alldredge, ١٩٧٨)**.Therefore, when stem cell transplantation studies have follow-up of less than a year, it may very well be that the surface appears healthy because of the survival of donor corneal epithelium, rather than from repopulation by the transplanted stem cells **(Schwartz and Holland, ٢٠٠٢)**.

Other factors important in evaluating success or failure of a particular stem cell transplantation technique is the preoperative diagnosis and severity of disease of the patients enrolled in the study. Patients with total limbal stem cell deficiency will be more difficult to rehabilitate than those with partial limbal stem cell deficiency, and patients with active conjunctival inflammation will have a higher failure rate than those with normal conjunctiva. Also important are the various factors that one can evaluate in determining success versus failure. Stability of the ocular surface is the fundamental anatomic criterion with which to evaluate stem cell transplantation success. A stable ocular surface has the

clinical features of a healthy transparent epithelium and is devoid of neovascularization and inflammation. A stable ocular surface results not only in improvement in visual acuity, but also in resolution of the pain that typically occurs in these patients because of conjunctivalization or persistent epithelial defects **(Schwartz and Holland, ٢٠٠٢)**.

Another important factor in evaluating success of a limbal stem cell transplantation procedure is visual acuity. This factor is, of course, most important to the patient, and must not be forgotten by the clinician. Unfortunately, many patients with severe OSD have decreased visual acuity secondary to other ocular pathology, such as the aniridic patient with foveal hypoplasia. For this reason, visual acuity cannot be the only measure to evaluate the success of a particular technique. In experience of Schwartz and Holland, approximately ٥٠٪ of patients with OSD will require a subsequent penetrating or lamellar keratoplasty for visual rehabilitation. Many of these patients will develop with chronic endothelial rejection with a healthy ocular surface, and therefore graft clarity serves as another determinant of overall success **(Schwartz and Holland, ٢٠٠٢)**.

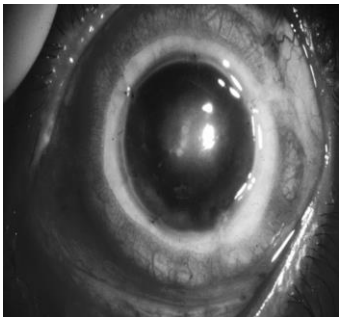
Early Stem Cell Transplant Failure

The causes of limbal stem cell transplantation failure can be categorized as early and late. Early failure is defined as occurring less than ١٢ months from the time of stem cell transplantation. The main causes of early failure include immunologic rejection, inflammation, eyelid abnormalities, and aqueous and mucin deficiency. Acute rejection typically occurs between ٢ and ١٢ months following transplantation. Review of the literature demonstrates several reports of acute stem cell transplant rejection. **In ١٩٩٣, Thoft and Sugar** described patients who had undergone a KLAL

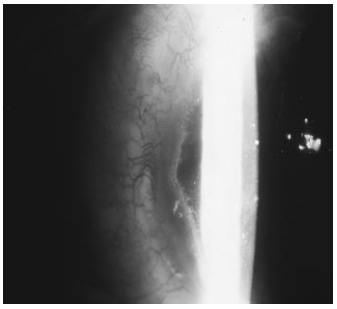
procedure that developed epithelial rejection. Clinical findings in these patients included injection, irregular epithelium, epithelial rejection lines, and subsequent epithelial defects. All three cases developed failed ocular surfaces despite aggressive topical anti inflammatory therapy.

In ٢٠٠٠, Daya described stem cell rejection as being either acute or low-grade. Common with KLAL. Patients with acute rejection had symptoms of pain, intense injection at the limbus, edema of the lenticules, punctate epithelial keratopathy, and epithelial defects. Typically, the defects were peripheral and located near the area corresponding to the area of rejection. Three patients had biopsy of the donor tissue that revealed T-lymphocyte infiltration (CD٤:CD٨, ٢:١) with strong HLA-DR (MHC class II) expression. Patients were treated with aggressive oral and topical immunosuppression, and required repeat KLAL for ocular surface rehabilitation.

Patients note symptoms of pain, redness and photophobia. Clinical examination demonstrates intense injection at the graft-host interface and an epithelial rejection line may be present (Figure ٢٧). With timely diagnosis and proper management, an acute rejection episode can almost always be resolved. Even though the graft will usually not immediately fail at the time of acute rejection, it has been our clinical experience that patients undergoing immunologic rejection of the stem cell graft do go on to have a higher rate of partial and total ocular surface failure **(Schwartz and Holland, ٢٠٠٢)**.



This patient had a keratolimbal allograft ٢ months before. Note the injection at the junction of recipient Conjunctiva and donor limbus.



Note the epithelial rejection line of keratolimbal donor tissue. .

Figure (٢٧): Photos of acute stem cell rejection. **(Schwartz and Holland, ٢٠٠٢)**

Nonimmunologic inflammation is another contributing factor toward transplant failure. Patients with Stevens–Johnson syndrome (SJS), ocular cicatricial pemphigoid (OCP), or severe alkali injuries may have nonspecific inflammation that persists for years. These patients have a higher failure rate with ocular surface transplantation probably due to inflammatory trauma to the delicate transplanted limbal stem cells. In addition, the increased level of inflammation leads to a higher rate of immunologic rejection of the transplanted cells **(Schwartz and Holland, ٢٠٠٢)**.

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Eyelid and adnexal abnormalities can also contribute to limbal stem cell transplantation failure. Entropion, trichiasis and distichiasis can cause failure from chronic, direct trauma to the transplanted stem cell population. Ectropion and lagophthalmos can lead to stem cell failure through exposure and abnormalities in tear film coverage of the ocular surface **(Schwartz and Holland, ٢٠٠٢)**.

Many patients with limbal stem cell deficiency will also suffer from severe aqueous and mucin deficiency, and keratinization of the ocular surface. **In ١٩٩٦, Holland**

described keratinization of the conjunctiva as a risk factor for limbal stem cell transplantation failure.١٠ Patients with preoperative conjunctival keratinization had a significantly poorer result when compared to those without keratinization. He also described aqueous tear production as another useful parameter to predict outcome. In his study, patients with a Schirmer test of ٢ mm or less at ٥ minutes without anesthesia had a significantly poorer prognosis.

Late Stem Cell Transplant Failure

Late failure is defined as failure of the ocular surface occurring greater than ١٢ months after limbal stem cell transplantation. Late causes include sectoral conjunctivalization, low-grade rejection, late acute immunologic rejection, and stem cell transplant exhaustion **(Schwartz and Holland, ٢٠٠٢)**.

Sectoral conjunctivalization is a process in which a wedge of conjunctiva-like epithelium invades a sector of the cornea (Figure ٢٨). Patients present with a slow progression of abnormal, irregular epithelium moving centrally from the limbus. This abnormal epithelium is often accompanied by superficial neovascularization. Impression cytology of this tissue demonstrates goblet cells, and this finding confirms the conjunctival nature of this abnormal tissue. Chronic conjunctivalization will lead to subepithelial scarring; if the central cornea becomes involved, the patient may experience significant visual loss **(Schwartz and Holland, ٢٠٠٢)**.

Sectoral conjunctivalization is typically seen in gap areas between transplanted areas of stem cell tissue. In the KLAL procedure, it can be seen in areas where a gap has been left at the time of surgery, or areas where there has been contraction during the postoperative healing process. It

is not uncommon for conjunctival invasion to occur at the site of prior immunologic rejection **(Tan et al, ١٩٩٦)**. In lr-CLAL procedures, the donor tissue is most often placed in the superior and inferior quadrants, and the nasal and temporal quadrants are left without transplanted stem cell tissue. In these patients, sectoral conjunctivalization will often occur at the nasal and temporal quadrants. This situation is one in which the stem-cell-derived epithelium seems to compete with the abnormal host conjunctiva-like epithelium for repopulation of the corneal surface. Fortunately, rapid epithelization by the transplanted tissue usually impedes growth of conjunctiva-like tissue onto the corneal surface **(Schwartz and Holland, ٢٠٠٢)**.

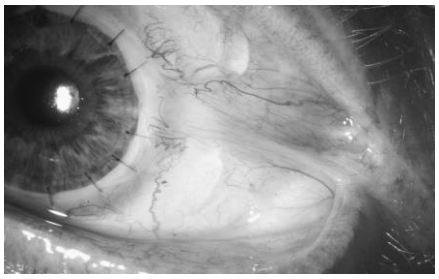

Figure (٢٨): Conjunctivalization invasion between gaps in keratolimbal allograft donor tissue **(Schwartz and Holland, ٢٠٠٢)**.

The treatment of sectoral conjunctivalization is dependent on the size of abnormal epithelium and the amount of inflammation. In cases of quiet eyes with small areas of conjunctival invasion, patients may benefit from sequential conjunctival epitheliectomy **(Dua et al, ٢٠٠٠)**. Patients with several clock hours or more involvement of the conjunctivalization process may benefit most from a partial repeat stem cell transplant to the involved area. If there is an inflammatory reaction associated with the conjunctivalization process, the clinician must entertain the possibility of chronic immunologic rejection. In these cases, topical and possibly even system

immunosuppression may stabilize the ocular surface **(Schwartz and Holland, ٢٠٠٢)**.

Late failure can also occur because of an episode of acute rejection occurring more than a year after limbal stem cell transplantation. These patients present similarly to those experiencing early failure acute rejection. Patients complain of pain, redness and photophobia. On examination they may have injection at the graft-host interface and may exhibit an epithelial rejection line. The ocular surface of these patients can usually be saved with intense topical and systemic immunosuppressive therapy **(Schwartz and Holland, ٢٠٠٢)**.

There are also patients who fail late because of chronic, unresolved, low-grade inflammation. This inflammation most likely represents a chronic low-grade form of rejection. These patients will demonstrate mild limbal injection, diffusely or in a sector and they develop conjunctivalization of the cornea in areas corresponding to the conjunctival injection **(Daya et al, ٢٠٠٠)**.

The last form of late failure occurs secondary to limbal stem cell exhaustion. In these patients, a slow, quiet conjunctivalization of the corneal surface occurs years after otherwise successful limbal stem cell transplantation (Figure ٢٩). This type of failure occurs more commonly following penetrating keratoplasty, or in patients with prior history of acute stem cell rejection. The etiology of stem cell transplant exhaustion is not well understood, and they may, in fact, represent a mild, quiet form of stem cell rejection. We, however, believe it to be secondary to the loss of mitotic activity of the transplanted stem cells over time. Mere transplantation of these delicate cells from one individual may limit their useful life span to ٢ to ٣ years in many cases. Recipient eyes are often inflamed, and exhibit

aqueous tear and mucin deficiency, and these factors may also diminish the lifespan of the transplanted tissue. Subsequent penetrating or lamellar keratoplasty, bringing the added mitotic stress to repopulate the new corneal surface, may transform a marginally functioning transplanted limbal stem population into a case of limbal stem cell exhaustion **(Holland and Schwartz, ١٩٩٩)**.

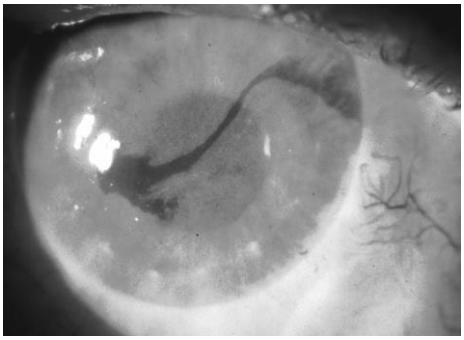

Figure (٢٩): Late stem cell failure. Note hazy epithelium and late staining with fluorescein for most of the cornea. A wedge of normal epithelium persists **(Holland and Schwartz, ١٩٩٩)**.

Strategies to Improve Outcomes

Patients with severe OSD disease secondary to limbal SC deficiency provide a significant challenge to the clinician. Long-term success following limbal SC transplantation can be as low as ٠٪ and as high as ٧٥٪, depending on the surgical procedure, the preoperative diagnoses, and the staging of the patients. Unfortunately, many patients' ocular surfaces may go on to fail despite not only an excellent short-term surgical result, but also diligent postoperative care. Strategies have been developed to improve long-term results following limbal stem cell transplantation. These strategies can be categorized based upon whether they are undertaken preoperatively, intraoperatively, or postoperatively **(Schwartz and Holland, ٢٠٠٢)**.

Preoperative Strategies

Long-term success following limbal SC transplantation can be maximized by recognizing and correcting certain preoperative risk factors for success. Many patients with limbal SC deficiency with conjunctival inflammation, such as patients with alkali injuries, SJS, or OCP will also have abnormal eyelid architecture. Cicatricial entropion, distichiasis, and trichiasis will often lead to SC transplantation failure from direct trauma. Ectropion and lagophthalmos can lead to failure of the ocular surface from exposure. To maximize outcomes, it is imperative that all eyelid abnormalities be repaired prior to, or concurrently with, the limbal stem cell transplantation procedure **(Schwartz and Holland, ٢٠٠٢)**.

Patients with severe mucin and aqueous tear deficiencies must also be recognized prior to stem cell transplantation. Patients with severe tear abnormalities should be treated aggressively. We advocate tarsorrhaphy for the majority of patients undergoing SC transplantation. Patients with dry eyes will also benefit from punctal occlusion prior, and nonpreserved artificial tears after, SC transplantation. If a patient has total keratinization of the ocular surface, the surgeon should consider not performing SC transplantation, since, in our clinical experience, nearly ١٠٠٪ of these patients will go on to eventually fail **(Schwartz and Holland, ٢٠٠٢)**.

Many patients with severe OSD from limbal SC deficiency will also have significant ocular inflammation. Clinical impression indicates that nonspecific inflammation is a significant risk for limbal SC transplantation failure from both direct trauma to the transplanted cells and an increased likelihood of developing subsequent immunologic

rejection. For these reasons, it is imperative that any inflammation be treated aggressively prior to performing limbal SC transplantation. For patients with inflammation from exogenous sources (i.e., alkali, acid or thermal injuries), the eye will often quiet considerably over time. Therefore, it is best to wait months to years after the injury before performing transplantation on these patients. For patients with inflammation from autoimmune sources (i.e., SJS, OCP), the surgeon usually does not have the luxury of the disease quieting down over time. These patients must be treated aggressively with topical and systemic immunosuppression in order to increase the likelihood of SC transplantation success **(Schwartz and Holland, ٢٠٠٢)**.

Intraoperative Strategies

The most important intraoperative strategies are precise surgical technique, meticulous preparation of the transplanted tissue, and careful selection of the proper surgical procedure **(Schwartz and Holland, ٢٠٠٢)**.

Postoperative Strategies

Postoperative strategies exist that are crucial for successful ocular surface rehabilitation following limbal stem cell transplantation. The first of these is the appropriate use of systemic immunosuppression. Systemic and topical immunosuppression are mandatory during the postoperative period to ensure successful transplantation. Many reasons for stem cell transplant failure are related to inflammation. Early failure can be secondary to nonspecific inflammation and may be secondary to acute rejection. Late failure may be secondary to long-term rejection, late acute rejection, chronic low grade nonspecific inflammation, or stem cell exhaustion (which may have inflammatory causes). Because inflammation is at the root of so many causes of early and

late stem cell transplant failure, and because inflammation can normally be treated with aggressive topical and systemic medications, it is imperative to treat patients with failing ocular surface after transplantation with immunosuppression. The overwhelming number of cases of a failing ocular surface can be abated or even reversed with high-dose systemic and topical immunosuppression, and therefore almost all patients with failing surface should be treated with a trial of aggressive immunosuppression **(Schwartz and Holland, ٢٠٠٢)**.

One other strategy in treating these patients after transplantation is to follow them postoperatively at short time intervals, even while asymptomatic. Early stages of failure such as mild inflammation or conjunctivalization may go unnoticed by the patient. Yet, if the signs and symptoms of these harbingers of surface failure are observed by the clinician, aggressive treatment may be initiated that may prevent subsequent failure of the ocular surface. In practice we typically examine limbal SC transplant patients at postoperative days ١, ٢, and ٣, weeks ١, ٢, and ٤, then monthly for the next ٦ months. Obviously, patients may be seen more often as needed **(Schwartz and Holland, ٢٠٠٢)**.

Summary

Summary

The ocular surface consisted of three main parts; cornea, conjunctiva and intervening zone called limbus. The cornea is the clear window at the front of the eye that allows accurate focussing of light to produce a sharp image on the retina for subsequent visual perception.

The limbal epithelium that separates the corneal epithelium and conjunctival epithelium is made up of a nonkeratinizing stratified squamous epithelium but is much thicker than the corneal epithelium (up to ten cell layers. It is thought to contain the source of the stem cells (SCs) that provide the source of corneal epithelial renewal and also provide a barrier preventing the conjunctival epithelium from encroaching onto the corneal surface. These cells are known as corneal epithelial SCs, or limbal stem cells (LSCs) which settle in stromal folds called palisades of Vogt (PV).

During homeostasis, cells are constantly lost from the surface of the corneal epithelium. The ultimate source of cell renewal is the LSC population. LSCs are defined as unipotent, or ‘progenitor’ cells because they only give rise to corneal epithelial cells. An SC exists in an optimal microenvironment or ‘niche’ that promotes its maintenance in an undifferentiated state. When SCs undergo asymmetric division, only one of the daughter cells can re-enter the niche to replenish the SC population. The other cell loses the protection of the niche and is destined to differentiate and become a transient amplifying cell (TAC). The role of the TAC is to divide at a regular rate to provide increased cell numbers. The ability of the TAC to multiply is limited and will eventually differentiate into a post-mitotic cell (PMC) that can no longer multiply. The PMCs are

committed to cellular differentiation and mature to form terminally differentiated cells that represent the final phenotypic expression of the tissue type.

Precise LSC identification is difficult because of a lack of specific and reliable markers. However, there are several pieces of evidence that indicate the presence of an LSC population in the basal layer of the limbal epithelium such as migration of pigmented epithelium lines from the limbus centrally, after central corneal epithelia wounding.

Failure of ocular surface may be due to limbal stem cell deficiency (LSCD), the major type of ocular surface disorders (OSD) or due to squamous metaplasia (the hallmark of dry eye disorders).

Limbal stem cell deficiency (LSCD) results from the loss or dysfunction of LSC, most often because of injury or inflammation. LSCD or dysfunction can result from severe chemical and thermal burns to the surface of the eye, inflammatory diseases (such as Stevens–Johnson Syndrome and ocular cicatricial pemphigoid), long-term contact lens wear. There are also various iatrogenic causes of LSCD, which include extensive limbal surgery or cryotherapy and therapeutic radiation. Exposure of the limbus to cytotoxic agents such as mitomycin C has also been known to cause LSCD. Hereditary causes of LSCD include aniridia and ectodermal dysplasia. It is probably in these cases that the niche for LSCs is altered and this results in subsequent LSC dysfunction and loss.

The hallmarks of LSCD are conjunctivalization of the cornea. chronic epithelial defects on the corneal surface and Both of these result in a chronically painful and visually impaired eye.

Diseases that affect the ocular surface are multifactorial and present different stages of severity. Choice of treatment and visual prognosis are dependent upon a wide variety of factors. The most important features to consider in evaluating these patients include the degree of limbal stem cell (SC) loss, partial or total LSCD; the extent of conjunctival disease; and presence and etiology of conjunctival inflammation; Laterality of the disease, unilateral or bilateral.

Treatment of OSD varied from symptomatic treatment in mild cases to surgical intervention in severe cases. The goal of treatment for severe LSCD is to re-establish the anatomic and physiologic environment of the ocular surface by optimizing lids and the tear film, controlling inflammation, and the management of glaucoma preoperatively then reconstruction of the corneal and conjunctival epithelium.

Numerous techniques to replace limbal stem cells have been described based on the source of the donor tissue, the carrier tissue employed, whether conjunctival or limbal tissue is transplanted. Currently, the main clinical procedures that are performed include a conjunctival limbal autograft (CLAU) using tissue from the fellow eye; a living related conjunctival limbal allograft (lr-CLAL), where a living relative donates conjunctiva and limbal tissue; and keratolimbal allograft (KLAL), utilizing a cadaveric donor where the peripheral cornea is used to transfer the limbal stem cells. More recently, ex vivo expanded limbal stem cells have also been used successfully to reconstruct the ocular surface with or without amniotic membrane transplantation (AMT) and penetrating keratoplasty (pk).

New modalities tried recently in limbal stem cell transplantation to facilitate its procedures and improve its results such as modification of amniotic membrane membrane; alternative substrates for epithelial cell transfer; and serum eye drops as epitheliotrophic agent.

Results of limbal stem cell transplantation are almost promising unless failure occurs which may be early (that occurs less than ١٢ months from the time of stem cell transplantation) or late (that occurs greater than ١٢ months after limbal stem cell transplantation). Each type has its causes and methods to overcome it.

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الملخص العربى

تعتبر القرنيه الموجوده علي السطح الامامي للعين نافذتنا الي العالم الخارجي، و تستوجب قدرتها علي الابصار هذه وجود تكامل بنائي ووظيفي لخلايا النسيج الطلائي بالطبقه الخارجية لسطح القرنيه، و تتجدد هذه الخلايا من خلال مجموعه من الخلايا الجذعية الموجوده بحافة القرنيه عن طريق الامداد الدائم للخلايا الابنه التي تعوض باستمرار الخلايا التي تفقد من سطح مقلة العين أثناء عملية التآكل و التفتت التي تحدث طبيعياً وكذلك التي تفقد بعد أي إصابة للعين.

ومن الجدير بالذكر أن فقد شفافية القرنيه يسبب فقد البصر بالعينين لخمسة واربعين مليون شخص في العالم ويسبب أيضاً ضعف البصر الشديد بالعينين لمائه وخمسة وثلاثون مليون شخص في العالم، و يتراوح العلاج من عقاقير موضوعية الي زرع قرنية العين و حديثاً العلاج بالخلايا الجذعية.

يشكل كلاً من القرنية ، الملتحمة وحافة القرنية النسيج المكون للسطح الخارجي لمقلة العين، ويغطي كلاً منهم بطبقات قشرية غير كراتينية للخلايا الطلائية وكذلك بطبقة مستقرة من الدموع، تتحقق صحة سطح العين بالعلاقة المتوازنة والسليمة بين هذين المكونين السابقين و هما طبقات الخلايا الطلائية لسطح العين و طبقة الدموع امام سطح العين.

يوجد نوعان من اضطرابات سطح مقلة العين:
النوع الاول يتميز بتحول في نوعية وطبيعة الخلايا القشرية وفقد الخلايا التي تشبه الكأس و المسئولة عن اخراج المخاط مما يؤدي الي عدم استقرار طبقة الدموع وهو ما يعرف بأمراض جفاف العين.
النوع الثاني يتميز بإحلال خلايا النسيج الطلائي بالقرنية فيما يسمي بنقص الخلايا الجذعية بحافة القرنية.

تضم أمراض سطح العين التي تتميز بفقد الخلايا الجذعية بحافة القرنية و الذي يؤدي الي اضطراب في التئام سطح العين كثيراً من الامراض منها: ظفرة العين، اورام حافة العين، مرض عدم تكون القزحية في الجنين، الندبة الشديدة الناتجة بعد حرق بسطح العين، متلازمة ستيفن جونسون، اصابة الخلايا بفيروس الهيربس، اعتلال قرنية العين بعد التعرض للاشعاع، اعتلال قرنية العين الناتج عن العدسات اللاصقة، التهاب قرنية العين الناتج عن شلل الاعصاب والتسمم الدوائي.

توجد الخلايا الجذعية في كل الانسجة المتجددة تلقائياً و لها خواص فريدة، يتكون سطح العين من نوعين مختلفين من الخلايا الطلائيه وهما خلايا القرنية و خلايا ملتحمة العين، وبالرغم من اتصالهم ببعض تشريحياً عن حافة القرنية و الملتحمة الا أن كل نوع له صفاته وسماته التي تختلف عن الآخر، و يعتبر الوسط الداخلي لحافة القرنية عامل هام في استمرار نمو الخلايا الجذعية و هو أيضاً يعمل كعائق لخلايا النسيج الطلائي للملتحمة من الانتقال الي خلال القرنية إلا أنه في بعض الظروف المرضية يحدث تدمير كلي أو جزئي لخلايا الساق الموجوده بحافة القرنية مما يؤدي الي درجات متفاوته من مظاهر نقص الخلايا الجذعية و منها أن تغطي القرنية بنسيج يشبه نسيج الملتحمة مع ظهور الاوعية الدموية علي سطح القرنية وظهور الخلايا الشبيهه بالكأس و التي تفرز المخاط و كذلك عدم انتظام واستقرار النسيج الطلائي علي سطح القرنية.

يعالج النقص الجزئي للخلايا الجذعية بإزالة النسيج الطلائي المريض و السماح لباقي نسيج القرنية المكشوط و خاصةً الموجود في محور الابصار بإعادة تكوين سطح القرنية من الخلايا الموجوده في النسيج الطلائي السليم المتبقي،أما النقص الكلي للخلايا الجذعية يعالج بزرع حافة القرنية في العين المصابة و هذه الحافة قد يكون مصدرها ذاتي من نفس الشخص من العين السليمة أو خارجي من شخص آخر قريب له أو من شخص متوفي وفي هذه الحاله يلزم إعطاء مثبطات عامة للجهاز المناعي، و في بعض الحالات نحتاج الي نقل الغشاء الامنيوسي المحيط بالجنين كدعم مفيد للاجراءات السابقة.

العلاج بالخلايا الجذعية في إضطرابات سطح مقلة العين

رسالة

توطئة للحصول علي درجة الماجستير في طب وجراحة العيون

مقدمة من

الطبيبة / دينـــا سيـــد ســـعد عبـد ربـــــه

بكالوريوس طب وجراحة العيون

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جـــامعـــة عيـن شـمـس
مصر- القاهره
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