
MINI REVIEW

Limbal Stem Cells in Health and Disease

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Stem cells are present in all self-renewing tissues and have unique properties. The ocular surface is made up of two distinct types of epithelial cells, constituting the conjunctival and the corneal epithelia. These epithelia are stratified, squamous and non-keratinized. Although anatomically continuous with each other at the corneoscleral limbus, the two cell phenotypes represent quite distinct subpopulations. The 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. In certain pathologic conditions, however, the limbal stem cells may be destroyed partially or completely resulting in varying degrees of stem cell deficiency with its characteristic clinical features. These include "conjunctivalization" of the cornea with vascularization, appearance of goblet cells, and an irregular and unstable epithelium. The stem cell deficiency can be managed with auto or allotransplantation of these cells. With the latter option, systemic immunosuppression is required. The stem cells can be expanded *ex vivo* on a processed human amniotic membrane and transplanted back to ocular surface with stem cell deficiency without the need of immunosuppression.

KEY WORDS: Ocular surface; corneal epithelium; conjunctival epithelium; corneoscleral limbus; stem cells; stem cell deficiency; limbal stem cells.

INTRODUCTION

Vision is probably the most important of the senses. We obtain more than 80% of our information from the external world by means of visual function. The phenomenon of good vision depends on the cornea and lens as effective refractive components; on the retina as a receptor system converting light into chemical and electrical energy; on transmission of the visual signal by the optic nerve to the brain; and on the final synthesizing function of the visual cortex.

The cornea is the gateway of the external images into the eye and accounts for more than two thirds of the total refractive power of the eye. Its principal physiological requirement is maintenance of its clarity. The avascular cornea is not an isolated tissue but is continuous with outer surface of the eye, and it forms the outer shell of the eyeball together with the sclera.

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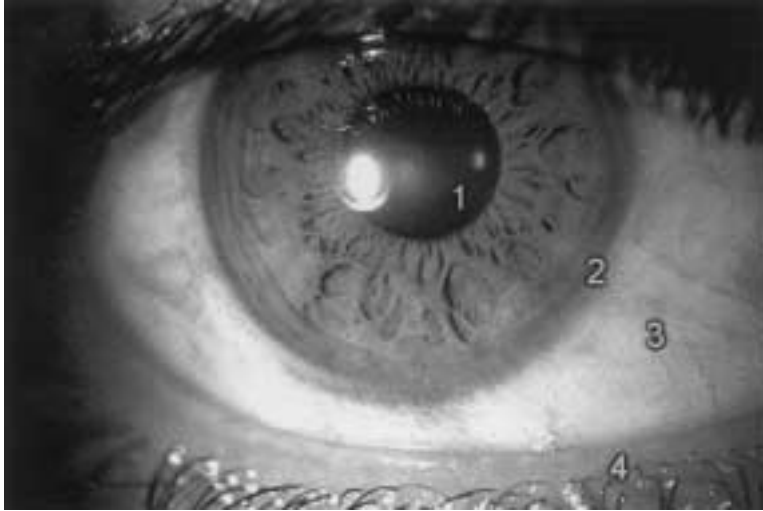


Fig. 1. Slit lamp photograph of the normal eye. 1. Central cornea; 2. Limbus; 3. Bulbar conjunctiva; 4. Lower lid.

The cornea, conjunctiva, and intervening transition area, known as limbus (Fig. 1) comprises the tissues at the ocular surface. All three are covered by a stratified, squamous, nonkeratinizing epithelium at the surface of the eye. These epithelia sit on a basement membrane and are connected through an identical adhesion complex to an underlying connective tissue stroma. Functionally, all three regions of the epithelium support the tear film and serve as barrier to fluid loss and pathogen entrance. The connective tissue of all the three regions serves not only as a structural support but also as the conduit of fluids and nutrients, and it houses support cells that provide for maintenance of the matrix and overlying epithelium. It is, however, the unique features of each region that indicate the special functional role of the tissue zones. The cornea, because of its critically important functions of light refraction and transmittance, has accordingly received the most attention in studies of its structure, function, and pathology. Recently, more attention has been given to surrounding limbal and conjunctival regions, which to some degree function as support tissues for cornea. Limbal stem cell research and its clinical application are the focus of this review.

PRINCIPLES OF CORNEAL EPITHELIAL REGENERATION

The corneal surface is, without question, the most specialized of the body's surface. Like the rest of the body surface, it is in a state of constant healing. Corneal epithelium is subject to a constant process of cell renewal and regeneration. Cells in the uppermost layer of the corneal epithelium are continuously desquamated from the surface and must be replaced by cell proliferation. The exclusive localization of dividing cells in the basal layer of the corneal epithelium suggests that proliferation is limited to basal cells [1–4]. Only cells, which are in contact with the basement membrane,

have the ability for mitotic cell division while cells that are displaced into the supra-basal layers become post-mitotic and lose their capability for cell division [5].

A vertical, as well as a horizontal, movement of cells characterizes the kinetics of the maintenance of the corneal epithelial mass. The vertical movement can be documented experimentally by the chase of previously labeled basal cells and might be due to the proliferative pressure in the basal cell layer [5]. The horizontal movement of corneal epithelial cells from the periphery to the center was observed after experimental corneal epithelial wounding [6, 7]. Similarly in rabbits, the centripetal replacement of corneal epithelium after corneal grafts, which originates in the donor epithelium has been shown to start in the periphery of the graft [8]. Tracing of peripherally located ink particles in the murine epithelium has proved that the centripetal movement of corneal epithelial cells also takes place under physiological circumstances in normal animal corneas [9]. Several observations indicate that the centripetal movement exists in human corneas as well. First, corneal erosions, which do not include the limbal epithelium, heal in a centripetal fashion [10]. Second, small subepithelial cysts, which develop between the sutures of corneal grafts, move towards the center when the sutures are removed [11]. Third, a centripetal movement of epithelial cells under physiological conditions was observed by specular microscopy [12].

The proliferation of basal cells, as well as horizontal and vertical cell movements, have been summarized in kinetic models which describe the maintenance of the corneal epithelial mass [13, 14]. While these mechanisms of the maintenance of corneal epithelium are generally accepted, the question of the origin of corneal epithelial cell proliferation has sparked a considerable controversy. Two opposing theories exist, one of which claims that the origin of corneal epithelium is derived from the adjacent conjunctiva by conjunctival transdifferentiation while the other claims that the origin of corneal epithelial proliferation depends on corneal stem cells in the limbal basal epithelium.

CONJUNCTIVAL TRANSDIFFERENTIATION

The first theory is based on early studies of corneal wound healing in humans which observed an ingrowth of conjunctival epithelium onto the denuded cornea following large epithelial wounds extending beyond the limbus [15, 16]. Further observations and experimental studies showed that in the absence of vascular ingrowth conjunctival epithelium on the corneal surface loses its conjunctival phenotype and becomes cornea-like [16–18]. This transformation of conjunctival epithelium to a corneal epithelium was described by the term “Conjunctival transdifferentiation”. This phenomenon was investigated in numerous animal studies [19–22] and led to the assumption that normal corneal epithelium is maintained by the surrounding conjunctival epithelium [13].

However, there are several pieces of experimental evidence against the above assumption and favor the notion that “the corneal and conjunctival epithelia are not equipotent”. Some of them are as under:

1. Wei *et al.*, demonstrated in culture media that corneal and limbal cells synthesize identical keratins, including large amounts of the K3 and K12

- markers of corneal-type differentiation. By contrast, the conjunctival epithelium produced another keratin pattern with large amounts of simple epithelial keratins but only minimal amounts of K3/K12 keratin [23].
2. The glycogen content and several other biochemical properties of conjunctiva-derived “corneal epithelium” remain abnormal long after the completion of the transdifferentiation [24].
 3. The conjunctiva-derived corneal epithelium can respond to corneal vascularization by forming goblet cells [25, 26] and by expressing immunoglobulin A secretory component [26]—two markers of normal conjunctival epithelium.
 4. Although conjunctiva-derived corneal epithelium appears normal on light microscopic studies, electron microscopic studies showed that the epithelium has much wider intercellular spaces than the corneal epithelium [27].
 5. It was found that, in humans, the conjunctiva-derived corneal epithelium is frequently associated with persistent epithelial defects, recurrent erosion, stromal vascularization, necrosis, and retarded healing rate [28].

Taken together, these data strongly suggest that limbal-corneal epithelium and conjunctival epithelium represent two separate cell lineage that are intrinsically divergent. These data also indicate the conjunctival transdifferentiation does not represent the true conversion of a differentiated corneal phenotype but rather describes an environmental modulation of the conjunctival epithelium. The transdifferentiation events are usually complete and not readily reversible and should be distinguished from environmental modulation of cellular phenotypes [23]. Although the modulation process can be accompanied by significant changes in cellular morphology and gene expression, it usually does not result in the complete conversion of one cell type to another and the process is readily reversible. Such a distinction is important because transdifferentiation represents an unusual (and rare) process in which “differentiated adult cells reverse their commitment and engage in a new pathway of differentiation thus violating the general rule of stability of determination in differentiated animal somatic cells.” The evidence presented above confirms the notion that corneal and conjunctival epithelia are not equipotent [23]. This leads to the second theory regarding the origin of the corneal epithelium, which claims that the origin of corneal epithelium lies in corneal stem cells located in the limbal basal epithelium [29].

STEM CELLS AND THE CORNEAL EPITHELIUM

In the adult organism, many tissues undergo rapid continuous cell turnover. These tissues, which include simple and stratified epithelium as well as the hematopoietic system, must repopulate and continuously maintain the integrity of the tissue. Similarly the corneal epithelium exists in a state of dynamic equilibrium, with the superficial cells being constantly shed into the tear pool [29]. Terminal differentiation of cells, coupled with cell death by apoptosis, prompts the cell loss via desquamation [29]. The cells ultimately responsible for repopulation are termed “stem cells” and can be defined as any cell with high capacity for self-renewal extending through

adult life of the organism [30]. These cells are a small sub-population of the total tissue and have been estimated to make up from 0.5% or less to 10% of the total cell population [31]. Stem cells thought to share a common set of characteristics (discussed below) including high proliferative potential and a long cell cycle.

Basic Concept of Stem Cells

Stem cells (SC) are by definition present in all self-renewing tissue [32]. These cells are long-lived, have great potential for clonogenic cell division and are ultimately responsible for cell replacement and tissue regeneration. Most of our knowledge about stem cells comes from studies on blood cells and some epithelial tissue e.g., intestinal epithelia, seminiferous epithelia, and skin epidermis [32]. Based on cell kinetic studies [34–35], all cells in a tissue consisting of a clonogenic cell lineage can be placed into either one of the following two tissue compartments: proliferative or non-proliferative [32]. Cells in the proliferative compartment are capable of preceding cell mitosis with DNA synthesis. This compartment includes stem cells (SC) and transient amplifying cells (TAC) that are derived from each SC mitosis and amplify their number by undergoing a few rounds of cell division. Cells in the non-proliferative, differentiative compartment are in theory all post-mitotic cells (PMC) that are committed to cellular differentiation. In the latter compartment, cells at

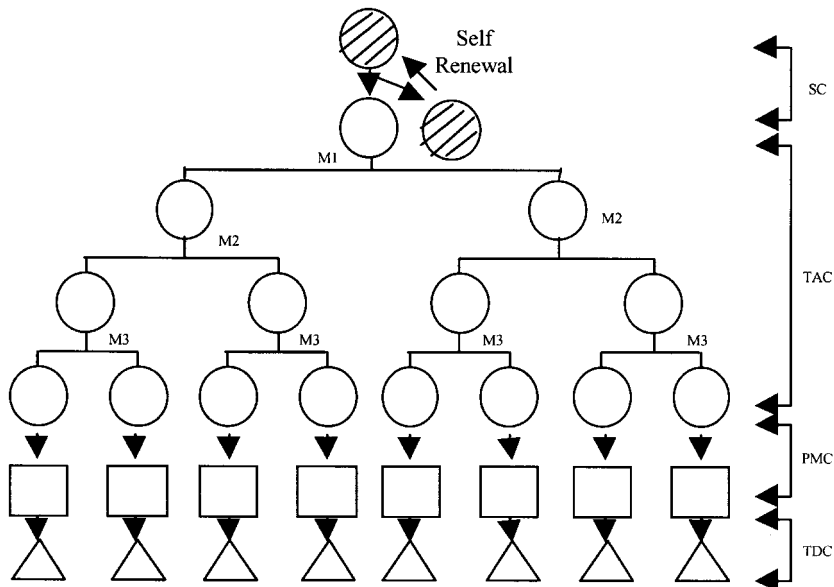


Fig. 2. Schematic drawing of the hierarchy of a defined tissue consisting of stem cell (SC), transient amplifying cell (TAC), post-mitotic cell (PMC), and terminally differentiated cells (TDC). SC is depicted by shaded circle and its population can be recovered via the self-renewal process during an asymmetrical cell division. Three mitotic cycles: M1, M2 and M3 are assigned to TAC. This allows amplification of cell numbers of eight. Modified from Tseng S. C. G. (1989) *Eye* 3:141–157.

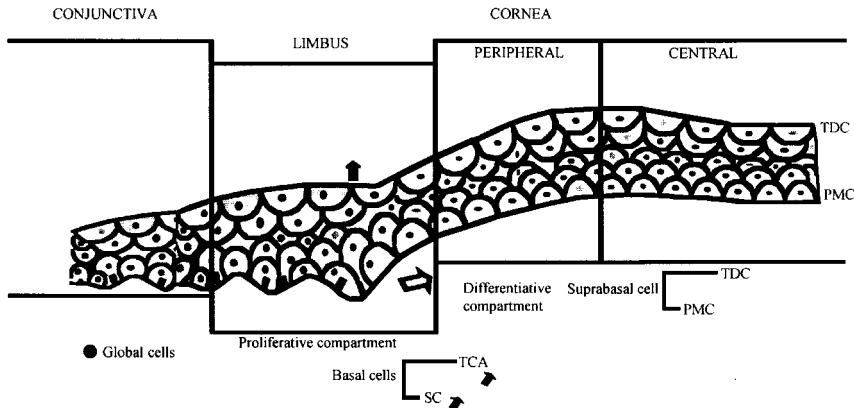


Fig. 3. Ocular surface epithelia covering the cornea, limbus, and conjunctiva. The conjunctival epithelium differs from the limbal/corneal epithelium in that it has mucin-expressing goblet cells. Some of the limbal basal epithelial cells are considered to be stem cells (SCs) for the corneal epithelium. By means of centripetal movement (open arrow), SCs generate corneal transient amplifying cells (TACs) located in the corneal epithelial basal layer. Both SCs and TACs are regarded as progenitor cells in the proliferative compartment, and they give rise to postmitotic cells of the suprabasal layers, as well as terminally differentiated cells of the superficial layers. The latter two cell types belong to the differentiative compartment. Suprabasal cell movement at the limbus creates a barrier to separate the conjunctival epithelium from the corneal epithelium (solid arrow). Adapted from Tseng, S. C. G. and Sun, T. T. (2000) In: *Corneal Surgery: Theory, Technique, and Tissue*. (F. S. Brightbill, ed.), Mosby-Year Book, Inc., St. Louis, Missouri, pp. 12.

different stages of differentiation can be identified during the process of tissue maturation. The terminally differentiated cells (TDC) achieve the ultimate expression of the functional aspect of the tissue. All cells except SC have a limited life span and are destined to die. These serial steps of clonogenic cell lineage in a defined tissue are summarized in Figs. 2 and 3 in which one can see an organized cellular hierarchy containing heterogeneous populations of cells that are arranged in the order of SC-TAC-PMC-TDC. From this schematic diagram (Fig. 2), one thus appreciates the fact that the loss of TDC is compensated by the gradual terminal differentiation of the preceding higher hierarchy, PMC and, eventually by the source of cellular proliferation, SC, at the highest rank. Furthermore, to ensure the normal health of the tissue, cellular proliferation and differentiation in a coordinated manner at different levels of this hierarchy is important, even indispensable.

CHARACTERISTICS OF STEM CELLS

Stem cells are responsible for ultimate cellular replacements and tissue regeneration. Several unique, inherent properties, as discussed below, enable stem cells to accomplish this important task.

1. Error-free Proliferation: Ideally, error-free mitosis is absolutely essential since any genetic error at the level of the stem cell (SC) will continuously

and permanently pass on to the whole clone of cells, resulting in abnormal differentiation and cellular dysfunction. To minimize any error made in SC mitosis, several protective mechanisms have been developed. First, SC are relatively quiescent during the state of steady growth. They leave the job of active DNA synthesis and cell number amplification to transient amplifying cells (TAC). So that even if an error is made at the TAC level it will be self-limited because all cells except SC have a limited life span. In cell kinetic terms, SC have a longer cell cycle time, 180 vs. 90 hr, and a shorter *S*-phase duration, 2–3 hr vs. 9–21 hr as compared to those of TAC in the case of skin epidermis [32]. These data explain (if [³H] thymidine is used to label the mitotic cells) why several studies of skin epidermis have shown that SC have a low labeling index or mitotic activity; i.e., 1–4% for SC as compared to 10–15% for TAC in skin epidermis [33]. This slow-cycling property of SC indicates a long *G*₀ phase or resting state. Secondly, Potten *et al.* [34] demonstrated that there is asymmetrical DNA segregation during the SC mitosis, suggesting that SC retains its original genetic message during the mitosis, and allows the new copy to be passed on to TAC. This result also explains why SC utilizes a long-lived thymidine pool in DNA synthesis [35].

2. Poor differentiation: It has been emphasized that the concept of “stemness” does not include further differentiation as a necessary property. Therefore, it has long been recognized that the cytoplasm of SC appears “primitive” and contains few, if any, differentiation products. If differentiation is envisioned as a “reprogramming” of the genome, then the process of differentiation also means “removal” of cells from the SC population. Therefore, a SC having responded to a differentiation stimulus is “out” of the SC population. Two possible mechanisms can explain how a differentiation event is induced. First, a full mitotic cell cycle is needed to produce an asymmetrical cell division into two different daughter cells. One will remain a SC (self-renewal), and the other is destined for cellular differentiation (Fig. 2). Secondly, a full cell cycle may not be needed: instead, differentiation stimuli affect SC at the *G*₀ state, where most stem cells are in steady state growth [36, 37]. The affected cells will be “removed” from the SC population by virtue of the change induced by the differentiation process, and will not enter the SC mitotic cell cycle.
3. Stem cells have a long life span, which might be equivalent to the life of the organism in which they reside [38].
4. Stem cells have a long cell cycle time or slow cycling (which indicates low mitotic activity). Although stem cells are endowed with high proliferative potential, under steady-state conditions, they exhibit extremely low rates of proliferation [38].
5. Cell division within stem cells can be intrinsically asymmetric, asymmetric only with regard to daughter cell fate, or symmetric [39–41]. When cell division is obligatorily asymmetric [41], one of the daughter cells remains as its parent and serves to replenish the stem cell pool (Fig. 2), whereas the other daughter cell is destined to divide and differentiate with the acquisition of

features that characterize the specific tissue. On the other hand, the asymmetry in division perhaps induces otherwise similar daughter cells to behave differently. Finally, all divisions of the stem cell may be symmetric, but are “self-renewing” only half the time [41].

Regardless of which mechanism is operative during or soon after cell division, one of the daughter cells may follow the path of differentiation. Such a cell is called a “transient amplifying cell” and is less primitive than its parent stem cell. Transient amplifying cells divide more frequently than stem cells, but have a limited proliferative potential and are considered the initial step of a pathway that results in terminal differentiation [42]. They differentiate into “post-mitotic cells” and, finally, to “terminally differentiated cells” (Figs. 2 and 3). Both post-mitotic and terminally differentiated cells are incapable of cell division [43].

IDENTIFICATION AND LOCATION OF LIMBAL STEM CELLS

To date, methods for identifying stem cells in self-renewing tissues such as the epithelium of the skin or the ocular surface are indirect except in the case of hematopoietic stem cells, which have been positively identified by an antibody [44]. Direct markers for stem cells have not been established, but there is clinical and experimental evidence supporting the location of corneal epithelial stem cells at the limbal region. Davanger and Evensen first proposed the concept that epithelial cells in the limbal region are involved in the renewal of corneal epithelium in 1971 [45]. In healed eccentric [38] corneal epithelial defects in heavily pigmented eyes, they observed pigmented epithelial migration lines (cells) that migrated from the limbal region toward the central cornea [45]. They suggested that the limbal papillary structure (palisades of Vogt) serve as a generative organ for corneal epithelial cells. Later, experimental studies by Schermer *et al.* and Cotsarelis *et al.* [46, 47] confirmed that the source of cell proliferation and migration after a corneal epithelial defect is the sclero-corneal limbus. The current evidence of the limbal location of stem cells is summarized below.

1. The analysis of epithelial keratins has generated useful information about, not only the differentiation, but also the lineage of epithelial cells. Keratins are a group of water-insoluble cytoskeletal proteins that form the desmosome-associated 10-nm intermediate filament in almost all epithelia [48]. There are a total of approximately 30 keratins that can be divided into an acidic (Type I) and neutral-to-basic (Type II) subfamily [23]. Detailed analysis of keratins in a number of epithelial cell types helped to establish that the expression of these keratins follows a set of rules [49]. Most notable is that each basic keratin tends to co-express with a particular acidic keratin, forming a so-called keratin “pair” [23]. In addition, each keratin pair tends to be expressed in a tissue-restricted and differentiation-dependent fashion. Perhaps most important for ocular surface epithelia is the K3/K12 keratin pair, which is synthesized by corneal and some oral mucosal epithelium but only in small amounts in conjunctival epithelia [23]. The immunofluorescence studies have shown the expression of these keratins in the suprabasal cell

layers of cultured rabbit corneal epithelium indicating they represent markers for an advanced stage of corneal epithelial differentiation [47, 50]. Using a monoclonal antibody (AE5) it was demonstrated K3 is expressed suprabasally in limbal epithelium but uniformly in the central corneal epithelium [47]. This finding implies that the (K3-positive) basal cells of central corneal epithelium may have attained a more advanced state of differentiation than the (K3-negative) basal cells of limbal epithelium. Based on this and several other considerations, Wei *et al.*, proposed that corneal epithelial stem cells are not uniformly distributed in the basal layer of the entire corneal epithelium but are restricted to the basal layer of limbal epithelium [4, 46]. Other studies that evaluated the expression of different proteins and indicators of a relatively undifferentiated phenotype provided further evidence for the low level of differentiation of limbal basal epithelium [51–54].

2. The limbal basal epithelium contains cells that exhibit the proliferative characteristics of stem cells. Limbal basal epithelial cells have a higher proliferative potential in culture than central and peripheral corneal epithelial cells [55–56]. Limbal basal cells respond to central corneal wounds and to tumor-promoting agents by undergoing higher proliferation than central corneal epithelial cells, which terminate proliferation-initiating differentiation [46, 57]. Labeling studies have demonstrated that the mitotic index of the corneal epithelium tends to be higher toward the periphery, suggesting that the peripheral corneal basal cells are more active in DNA synthesis [58]. Cotsarelis *et al.* [47] found that titrated thymidine was incorporated for long time intervals only into limbal cells. This labeling indicated that these cells exhibited a long cell cycle [47, 59]. These cells are also resistant to the induction of differentiation [29, 47, 57, 60]. Growth factors, retinoic acid, and calcium have been shown to affect the limbal and central corneal epithelial cell types differently [60–62].
3. Further support for the limbal location of corneal epithelial stem cells is derived from experimental studies and clinical observations of abnormal corneal epithelial wound healing when the limbal epithelium is partially [63–64] or completely [65–66] removed. These studies produced a spectrum of corneal surface abnormalities characterized by conjunctival epithelial ingrowth (conjunctivalization), vascularization, and chronic inflammation, which indicated limbal stem cell deficiency. The conjunctival source of the epithelial ingrowth was proved by immunofluorescent staining with monoclonal antibodies [63–66] and by the detection of goblet cells with impression cytology [67]. Kenyon and Tseng suggested that the original corneal phenotype could be reconstituted by transplantation of healthy corneal stem cells in patients [31]. The effectiveness of limbal transplantation for the treatment of experimentally induced stem-cell deficiency, confirmed in rabbits by Tsai and coworkers [68], further supports the limbal location of corneal epithelial stem cells.
4. It has been shown that surgical removal of limbal epithelium results in defective corneal epithelial regeneration [68–69], and that human limbal epithelium provides a much better tissue source than the conjunctival epithelium for corneal epithelial regeneration [31].

5. Recently Schwab *et al.* [70] reported a novel technique of harvesting the presumed corneal epithelial stem cells from limbus (in human subjects with ocular surface disease), expanded successfully *in vitro* and grown on denuded amniotic membrane. The resultant composite cultured tissue was transplanted which appeared to successfully manage eyes with difficult ocular surface disease, including those with stem cell deficiency.
6. The limbal location of corneal epithelial stem cells could account for the relative preponderance of limbal neoplasm and the scarcity of corneal epithelial tumors, assuming that neoplasm arise mainly from relatively “undifferentiated” cells [38].
7. A mathematical analysis of the kinetics of maintenance of the corneal epithelial mass confirms that the corneal epithelium can be maintained by cellular proliferation originating from limbal stem cells without contribution of the adjacent conjunctiva [14].

All the above investigations can be summarized as follows. Stem cells are located exclusively in the limbal basal epithelium. The specific location of corneal epithelial stem cells in the limbus provides several functional advantages. Because central corneal epithelium has to be transparent, its basal cells are devoid of pigment and, consequently, are highly susceptible to solar damage. Basal cells in the limbal region do not have this constraint; they are heavily pigmented (Fig. 1) and, thus, are well protected [46, 71]. The transparency of the cornea also dictates a smooth epithelial-stromal junction. This minimal anchorage renders corneal epithelium susceptible to physiological shearing. In contrast, limbal epithelium is very resistant to shearing forces and displays a highly undulating epithelial-stromal junction [38]. This would give a natural advantage for retention of stem cells in the face of environmental onslaught [38].

DIFFERENCES BETWEEN LIMBAL STEM CELLS AND CORNEAL TRANSIENT AMPLIFYING CELLS

It is most likely that the limbal basal epithelium consists not only of stem cells but also of transient amplifying cells. In fact, limbal and peripheral corneal epithelium seem to contain a population of very early transient amplifying cells, which display some of the characteristics of stem cells such as long life, slow cycling with low mitotic activity, and less differentiated in the normal steady state. In contrast, corneal transient amplifying cells have a short life span, are rapid cycling, and can amplify cell mass effectively through limited rounds of mitosis. At a critical point the transient amplifying cells stop mitosis and differentiate into corneal suprabasal postmitotic and terminally differentiated cells. The above concept is supported by several studies that show proliferative rate of cultures [72, 73] in the expression of differentiation markers [50, 54, 74, 75] and in cell cycle length between limbal and corneal epithelia [76]. The limbal and corneal epithelia respond differently to treatment by phorbol ester tumor promoter [76, 77]. These results indicate that the control of mitotic kinetics for limbal stem cells is different from that for corneal transient amplifying cells, although the exact mechanism remains unknown. This

partitioning of all progenitor cells into stem cells and transient amplifying cells can be found in the hematopoietic system and the epidermal and the intestinal epithelia [39, 78]. The former can be activated by demand for tissue regeneration to increase their own population in the process of stem cell renewal and/or to differentiate into corneal transient amplifying cells. During each mitotic cycle, stem cells must decide whether they should be differentiated into transient amplifying cells that will naturally deplete the stem cell population, or be kept undifferentiated as stem cells so that stem cell population may be maintained. How stem cells balance their act between these two conflicting demands is an ultimate secret dealing with how the normal corneal epithelium is maintained. Transient amplifying cells have an important role in corneal epithelial wound healing. Corneal epithelial repair is a multistep process involving the flattening and sliding of pre-existing cells to cover the wound area followed by cell mitosis to allow repopulating and restratification of the corneal epithelium. Aside from stem cell replication, the efficiency of transient amplifying cell replication is increased by shortening the cycling time and increasing the number of times the transient amplifying cells can divide [43]. Increasing the proliferative capacity of transient amplifying cells has the advantage of; (1) amplifying each stem cell division and minimizing the need for stem cell proliferation, (2) minimizing the chance of introducing replicative DNA errors into the stem cell population, and (3) providing new cells that are much closer to the terminally differentiated, functional cellular compartment, for example, the epithelium that covers the central cornea [38].

REGULATION OF STEM CELLS

What maintains the “stemness” of a stem cell is not well understood. In addition to characteristics inherent to the stem cells, extrinsic influences from the microenvironment surrounding the stem cells may also play a role [41, 62, 78]. Tissue cultures studies can be used to support or argue against the hypothesis that stem cells are maintained by intrinsic properties alone in that even cultures of limbal epithelium senesce, indicating the loss of “stemness.” Cultured corneal epithelial cells can be transplanted, resulting in a normal-appearing tissue, suggesting that stem cells are retained in culture [56, 79–80]. However, this might well represent the persistence of “transient amplifying cells,” rather than stem cells. A recent study [81] demonstrated that the donor derived corneal epithelium survived for up to 30 months after limbal allograft transplantation (LAT), which is significantly longer than the conventional keratoplasty (donor epithelial cells of the graft are replaced with recipient epithelial cells by end of one year). LAT is likely to function as stem cell transplantation of the corneal epithelium.

Schofield suggested that stem cells exist in an optimal microenvironment that promotes the maintenance of the stem cell in an undifferentiated condition [82]. An important difference between the limbus and central cornea is the presence of blood vessels at the limbus. These vessels derive from the pallasades of Vogt and provide the limbal epithelium with nutrition and greater interaction with blood-borne cytokines [38, 83]. Several proteins have been identified and found in higher concentrations in basal cells of limbal epithelium than in basal cells of central corneal epithelium, such

as cytochrome oxidase [84], $\text{Na}^+\text{-K}^+\text{-ATPase}$ [38], and carbonic anhydrase [85]. It is not clear whether any of these proteins is involved in the maintenance and regulation of the stem cells. There seem to be regional differences in the distribution and concentration of various regulatory factors, such as retinoic acid in human limbal and corneal epithelium, as well as underlying stroma and fibroblasts [80, 86]. The resulting concentration gradients could have regulatory functions.

The undifferentiated cells in the limbus contain the highest levels of epithelial growth factor receptor (EGFR) [87]. The cells express lower levels of EGFR with maturation. It has been suggested that high levels of EGFR may inhibit differentiation by signaling the cells to maintain the proliferation potential [83, 87]. Zieske *et al.* [54] also demonstrated an increased concentration of alpha Enolase in limbal epithelial cells and proposed this as a potential marker for corneal epithelial stem cells. In addition, two components of intermediate filaments, vimentin and keratin [88] have been identified in the limbus [52, 74]. Intermediate filaments are involved in the maintenance of cell architecture. It is not known whether these proteins may somehow be involved in the anchorage of stem cells into a certain microenvironment.

Two gap junction proteins have been identified in the corneal epithelium [89–90]. Gap junction intercellular communication has been linked to the regulation of cellular growth, development, and differentiation. The limbal stem cells are devoid of these connections, suggesting incompetence of intercellular communication of stem cells in site [91]. This property may reflect the need of these unique cells to maintain a distinct intercellular environment.

The basement membrane of the limbus is different from that of the central cornea. The basement membrane of the limbus is modified to enhance epithelial cell adhesion. It also possesses an abundance of type IV collagen (which is absent from the central cornea) [92] and anchoring fibrils and a rough undulating surface that enhance epithelial cell adhesion. Conversely, a protein identified by an antibody AE27, and associated with cells expressing keratin 3, is present in the basement membrane of the central cornea in large concentrations, with low levels in the limbal area [38]. This evidence has led to the hypothesis that basement membrane can also influence stem cell differentiation in areas (stem cell niche) containing high levels of type IV collagen and low levels of AE27 binding antigen [92].

STROMAL CELLS IN STEM CELL REGULATION

For different hematopoietic progenitor cells, long-term cell survival and growth can be enhanced when co-cultured with stromal cells derived from bone marrow [93], fetal liver [94] and umbilical cord [95]. For malignant T lymphoma cells, apoptosis can be inhibited when co-cultured with lymph node stromal cells [96]. Survival of several neurons can also be enhanced by co-culture with different glial cells [97]. Since the pioneering work by Rheinwald and Green in 1975 [98], many studies have shown that long-term survival and serial propagation of many types of epithelial cells become possible only if cocultured with fibroblast feeder layers [98–101]. This system, abbreviated herein as the 3T3 system (3T3 fibroblasts), has been successfully applied to grow rabbit [23, 102] and human [73] ocular surface epithelial

cells. In this system, a sub-population of epidermal progenitor cells is maintained which is resistant to treatment by a phorbol ester tumor promoter [103], a feature considered unique for stem cells [104, 105]. Based on clonal analysis, epidermal [106], hair follicle [100] and limbal [73] epithelial stem cells are found to be preserved in the 3T3 system. Furthermore, epidermal keratinocytes expanded in the 3T3 system can be transplanted back into patients with burned skin [101]. These results taken together, strongly support the notion that the 3T3 system can maintain epithelial stem cells while promoting their clonal growth. It has been noted that human limbal but not corneal fibroblasts also reproduce the above 3T3 fibroblast-derived growth promoting activity and such an activity is characterized as anti-apoptotic [62]. These findings together with the above observed fibroblast-dependency for stem cell maintenance support the idea that functions of limbal stem cells might be modulated by their own stromal fibroblasts.

Epithelial–Mesenchymal Cytokine Interactions in Stem Cell Regulation

To reconstitute the stem cell stromal microenvironment, Tseng *et al.* [62] have successfully adapted an organotypic culture by incorporating 3T3 fibroblasts into native collagen gel onto which ocular surface epithelial cells are seeded, and then by culturing at the air–fluid interface [107]. In this organotypic culture epithelial proliferation, exhibited by increased stratification [107] and areas of epithelial intra-gel invasion [108] is promoted together with the expression of the normal corneal epithelial phenotype, mimicking the *in vivo* situation. Importantly, all these phenomena are also fibroblast-dependent and can still be produced by 5-fluorouracil-resistant, slow-cycling progenitor cells [108]. Furthermore, 3T3 or human limbal fibroblasts in collagen gel remain active in secreting the above-described epithelial growth-promoting activity. These results strongly indicate that there are active epithelial–mesenchymal interactions in the limbal stem cell microenvironment.

Many studies have shown that epithelial mesenchymal interactions play a vital role in embryonic development, postnatal morphogenesis, wound healing, and tumor metastasis of several epithelial tissues [62]. In theory, these epithelial–mesenchymal interactions can be mediated by signals transmitted from mesenchymes to epithelia or *vice versa* in reciprocal manner [62]. Such signals can be extracellular matrix components, cell membrane-associated molecules and soluble factors, i.e., cytokines. These three types of signals are not mutually exclusive because the action of one may be dependent on or mediated by the expression of others [109]. The limbal location of corneal stem cells makes it an ideal model to investigate the role of mesenchymal–epithelial interactions for the regulation of corneal transient amplifying cells and limbal stem cells [62].

Tseng *et al.* investigated the role of cytokines in the regulation of limbal stem cells [110]. They have identified four patterns of cytokine dialog of which three constitute the basic network of potential epithelial–mesenchymal interaction using cytokines [62]. Type I cytokines include transforming growth factor- α (TGF- α), interleukine-1 β (IL-1 β) and platelet-derived growth factor (PDGF-BB), which can be expressed by epithelial cells to converse with fibroblasts [62]. Type II cytokines include insulin-like growth factor type 1 (IGF-1), TGF- β 1, TGF- β 2, leukemia

inhibitory factor (LIF) and basic fibroblast growth factor (bFGF), which are cytokines crosstalking between the epithelium and fibroblasts [62]. Type III cytokines include keratinocyte growth factor (KGF) and hepatocyte growth factor (HGF), which are expressed by fibroblasts to stimulate epithelial proliferation and migration. Consistent with others [111, 112], these results indicate that ocular surface epithelial cells and fibroblasts express a myriad of cytokines. Intriguingly, KGF transcript and protein are expressed most by limbal fibroblasts, whereas HGF transcript and protein are expressed most by corneal fibroblasts. This differential expression suggests that KGF and HGF might be important in modulating limbal cells and corneal transient amplifying cells, respectively [62].

Clinical Application of the Concept of Limbal Stem Cell

The understanding that the limbus contains a self-renewing stem cell population of the corneal epithelium explains why ocular surface dysplasia and neoplasia are known to have a limbal predilection [113]. It also clarifies why it had not been possible for delayed epithelialization or persistent corneal epithelial defects created in animals through repetitive debridement of the corneal epithelium sparing the limbus [114]. It has been shown that rate of re-epithelialization after successive debridements is often faster than that single debridement [114], a finding that supports the concept that limbal epithelial stem cells have been activated in response to previous injury.

There is a well-defined group of human corneal diseases with stem cell deficiency [115]. The stem cell deficiency could focal or diffuse (Figs. 4 and 5) depending upon the extent of limbal involvement with underlying disease process. Patients with this

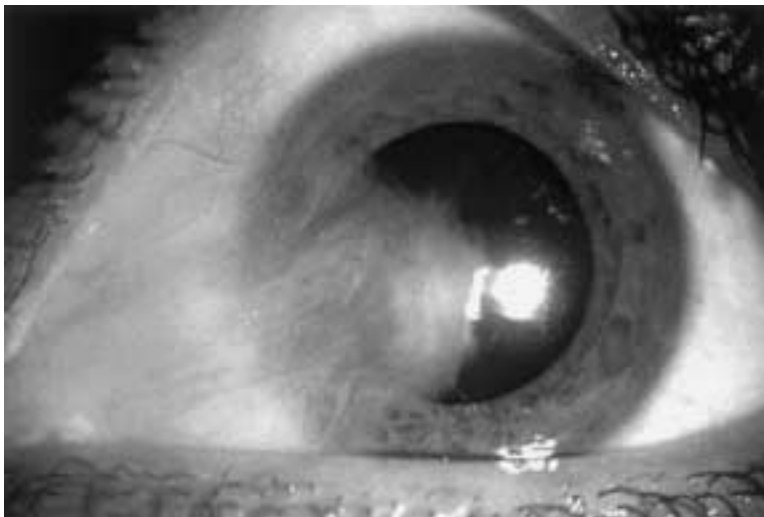


Fig. 4. Slit lamp photograph of left eye of patient with nasal pterygium representing presumed focal stem cell deficiency. There is growth of conjunctiva onto the clear cornea nasally.

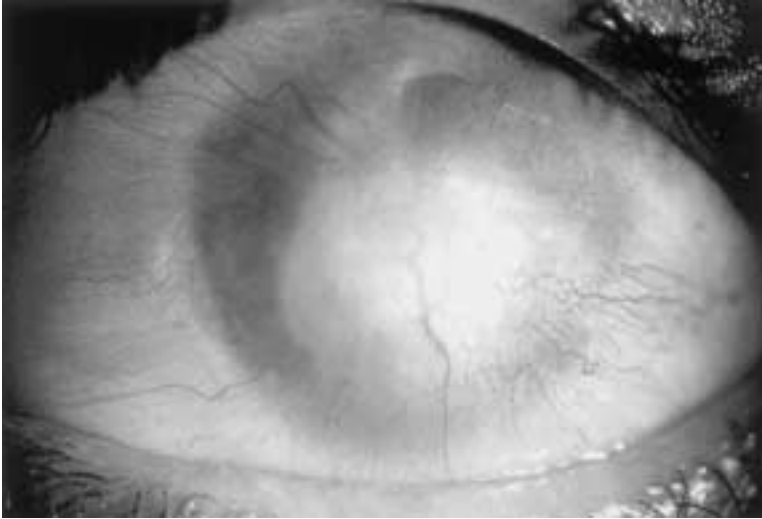


Fig. 5. Slit lamp photograph of the left eye with status post acid burn with diffuse damage to the limbal stem cells resulting in the conjunctivalization of the corneal surface with fibrovascular pannus.

group of diseases often suffer from severe photophobia, decreased vision, and other complications. They are generally poor candidates for conventional corneal transplantation (where limbal stem cells are not part of the graft) for several reasons:

- (1) Only corneal transient amplifying cells are transplanted.
- (2) Pre-existing corneal vascularization and inflammation increases the risk of allograft rejection.
- (3) They tend to undergo recurrent conjunctivalization owing to the loss of stem cell function [116].

Ocular surface diseases such as Stevens–Johnson syndrome, chemical and thermal burns, ocular surface tumors, immunologic conditions, radiation injury, inherited syndromes (such as aniridia), and ocular pemphigoid can severely compromise the ocular surface and cause catastrophic visual loss in otherwise healthy eyes. The common pathogenic feature of this seemingly diverse group of diseases is the depletion of the stem cell population from corneal limbus [67]. Damage or depletion of the corneal stem cells results in “conjunctivalization” or ingrowth of conjunctival elements on the surface of the cornea with associated profound visual loss (Fig. 4).

Based on the pathogenic nature of limbal involvement, corneal surface diseases can be divided in two categories (Table 1). Category I diseases are characterized by a clear pathogenic cause that is identifiable from history, which has destroyed the limbal epithelial stem cell population. The destruction can come from chemical/thermal injuries, Stevens–Johnson syndrome, and multiple surgeries or cryotherapies applied on the limbal regions. In the rare situation of contact lens-induced keratopathy, lens-wearing injuries or toxic effects from lens-cleansing solutions can also cause limbal stem cell (SC) damage. The category II diseases include diverse causes

Table 1. Human Corneal Diseases Involving Limbal Deficiency.

Category I. Destruction of limbal epithelial stem cell population
<ul style="list-style-type: none"> a. Chemical or thermal injuries. b. Multiple surgeries or cryotherapies of the limbus. c. Stevens–Johnson syndrome. d. Contact-lens-induced keratopathy. e. Severe microbial infection.
Category II. Dysfunction of stromal microenvironment of limbal epithelial stem cells.
<ul style="list-style-type: none"> a. Aniridia (hereditary). b. Keratitis associated with multiple endocrine deficiencies (hereditary). c. Neurotrophic keratopathy (neuronal or ischemic). d. Chronic limbitis or peripheral corneal inflammation and ulceration. e. Pterygium and Pseudopterygium. f. Idiopathic keratopathy.

Adapted from Puangsricharern, V. and Tseng, S. C. G. (1995) Cytologic evidence of corneal diseases with limbal stem cell deficiency. *Ophthalmology* **20**:192.

such as aniridia, keratitis associated with multiple endocrinal deficiencies, neurotrophic keratopathy, and pterygium/pseudopterygium (Table 1). This represent milder form of corneal diseases (category II diseases) where limbal SC dysfunction is not due to the total loss of limbal SC, but rather are associated with either a gradual loss of SC population or poor TAC generation and amplification. Because it has not resulted from traumatic loss, the underlying pathogenesis might come from poor microenvironment support of limbal SC or TAC, or a poor regulatory mechanism. In the case of aniridia, a hereditary example of limbal SC dysfunction, such poor regulation is probably associated with microenvironment alteration due to the anomalous development of the adjacent angle-iris structures. Poor nutritional supply of endocrine factors and of neuronal trophic cytokines might be the basis for the development of limbal SC deficiency in keratitis associated with multiple endocrine deficiency and neurotrophic keratopathy derived from a primary neuronal or ischemic component, respectively. The introduction of adverse undesirable cytokines secreted by chronic inflammation of various natures might inhibit or antagonize normal regulators and create a state of limbal SC dysfunction. These mechanisms might explain the poor support of SC function in clinical examples of chronic limbitis and pterygium (Fig. 5) or pseudopterygium.

With the advent of the limbal stem cell concept, new surgical procedure, termed “limbal transplantation” was developed by Kenyon and Tseng [31] to transplant limbal stem cells. In experimental rabbits Tsai and coworkers [117] demonstrated that limbal transplantation could restore effectively the corneal epithelial phenotype on severely damaged corneal surface; in contrast conjunctival transplantation results in conjunctival epithelial phenotype. These results further confirmed limbal location of corneal epithelial stem cells; they also established limbal autograft as an ideal

method of corneal surface reconstruction for patients suffering from the diseases listed in Table 1, especially for focal or unilateral limbal deficiency [31, 118–120]. For patients with diffuse and bilateral limbal deficiency, corneal surface reconstruction requires an allograft from either HLA-matched living [119–121] or nonmatched cadaver [115]. Unlike autografts, allografts can be rejected, especially when they are transplanted to such vascular tissues as the limbus and conjunctiva. The clinical features and treatment of limbal allografts has been recently reported [122].

Schwab and coworkers [70] reported *ex vivo* expansion of the harvested (2-mm² limbal biopsy) cell population and cultivation directly upon a modified amniotic membrane surface. The resultant composite graft tissue was transplanted on to severely damaged corneal surface. They reported [70] a successful outcome, defined as restoration or improvement of vision, along with maintenance of corneal re-epithelialization and absence or recurrence of surface disease, in 6 of the 10 patients with autologous procedures and in all allogenic transplants. Another study reported the use of “bioengineered cornea” for reconstruction of severely damaged corneal surface with stem cell deficiency [123].

FUTURE DIRECTIONS

The concept of limbal stem cells has resulted in greatly improved understanding of corneal epithelial proliferation, migration, and regeneration. This concept has also contributed directly to improved and more rationally based clinical and surgical management of a wide range of ocular surface disorders. There are, of course, many remaining questions. Clinically most important is the issue of rejection of limbal allografts and long term survival of limbal transplant by improving the regimen for immunosuppressive therapies. In terms of stem cell biology there are many questions unanswered such as; how the “stemness” of stem cells is maintained? What factor(s) regulate asymmetric division of stem cells? What are external and internal modulators influencing stem cells? What is the role of microenvironment in stem cell function and regulation?

Tsai and Tseng [124] found that chronic inflammation in the perilimbal region precedes progressive failure of limbal autografts in rabbit. This raises the possibility of gradual dysfunction of the limbal stromal microenvironment as a result of inflammation. This explains possible pathogenesis of the diseases that have been classified as category II (Table 1). This also emphasizes the importance of considering that limbal stem cells can be regulated by their underlying stroma. The notion that the limbal stem cell deficiency can be caused by a dysfunctional stromal microenvironment mimics murine hematopoietic stem cell deficiency [115]. In the latter disorder, an identical phenotype [125] has long been found to be caused by mutation of the gene at the W locus encoding c-kit tyrosine kinase receptor [126] that has been expressed by hematopoietic stem cells, as well as the gene at the S1 locus encoding stem cell growth factor (i.e., ligand for c-kit that has been expressed by stromal fibroblast) [115]. Future studies are needed to understand the role of stromal microenvironment in the regulation of stem cell function.

Another emerging approach is the use of amniotic membrane transplantation for ocular surface reconstruction. This procedure has been found useful for a variety

of ocular surface disorders with and without stem cell deficiency [115]. Tseng and coworkers reported that amniotic membrane transplantation is efficacious as the first-stage procedure before the use of limbal allografts for patients with total limbal deficiency [115]. Schwab *et al.* [70] reported the use of modified amniotic membrane for *ex vivo* expansion of presumed corneal stem cells. The resultant composite tissue termed as “bioengineered cornea” was successfully transplanted in-patient with severe ocular surface disease, including those with stem cell deficiency. Bioengineered tissue replacement may represent the future for the repair of many tissues and organs. Using autologous cultures corneal stem cells allows for banking of the patient’s cells so that repeat grafts can be constructed from the patient’s own cells. This procedure (primary or repeat) would address two major problems associated with allograft stem cell transplantation, namely, allograft rejection and the need for immunosuppression with its associated cost and potential side effects. If the autologous limbal tissue is not available for harvest (as in severe bilateral disease), a very small tissue (2 mm²) can be harvested from a related donor, minimizing introgenic danger to the donor eye [70]. Combining the *ex vivo* expanded cells with cultivation on a modified amniotic membrane confers the advantage of supplying a compatible extracellular matrix (ECM) to the graft, enhancing its durability and manipulability [70]. Although much progress has been achieved, there is still much to be learned before a totally “off-the shelf” tissue-engineered cornea replacement can be easily produced and transplanted [70].

The conjunctival epithelial stem cells have been localized in the forniceal region [115]; additional studies are required to determine if ocular surface reconstruction can be expanded more effectively to the conjunctival surface using transplantation of conjunctival epithelial stem cells as well.

It has been reported that hematopoietic stem cells can be converted into hepatocytes in animal model of tyrosinemia type I [127]. The authors reported that within bone marrow, only rigorously purified hematopoietic stem cells gave rise to donor-derived hematopoietic and hepatic regeneration [127]. This result seems to contradict the conventional assumptions of the germ layer origins of tissues such as the liver, and raises the question of whether the cells of hematopoietic stem cell phenotype are pluripotent hematopoietic cells that retain the ability to transdifferentiate, or whether they are more primitive multipotent cells [127]. This raises an interesting possibility of harvesting hematopoietic stem cells in patients with severe bilateral ocular surface disease and converting these cells into limbal stem cells in laboratory and transplant back (autologous) on to diseased ocular surface. Only future studies will answer this question. Our laboratory, as are many other laboratories around the world, is actively engaged in this area of research.

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