Progenitor Cells for Ocular Surface Regenerative Therapy

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Abstract
The integrity and normal function of the corneal epithelium are essential for maintaining the cornea’s transparency and vision. The existence of a cell population with progenitor characteristics in the limbus maintains a dynamic of constant epithelial repair and renewal. Currently, cell-based therapies for bio-replacement, such as cultured limbal epithelial transplantation and cultured oral mucosal epithelial transplantation, present very encouraging clinical results for treating limbal stem cell deficiencies. Another emerging therapeutic strategy consists of obtaining and implementing human progenitor cells of different origins using tissue engineering methods. The development of cell-based therapies using stem cells, such as human adult mesenchymal stromal cells, represents a significant breakthrough in the treatment of certain eye diseases and also offers a more rational, less invasive and more physiological approach to ocular surface regeneration.

The corneal epithelium has a key role in keeping the cornea transparent and free of blood vessels. Permanent repair is essential for the conservation of the cornea’s physiology. It has been observed that the stem cells responsible for the renewal of the corneal epithelium are located in the basal layers of the sclerocorneal limbus. The disappearance or deficiency of this cell population (limbal stem cell deficiency, LSCD), due to a variety of causes such as acid or alkali burns, immunological conditions, tumors, infections or even congenital conditions, can give rise to significant changes in the ocular surface, such as the occurrence of persistent corneal defects, epithelial keratinization, conjunctivalization phenomena with the development of newly formed vessels in the corneal tissue and scarring. All this compromises the corneal physiology, reducing transparency and decreasing vision [1–4].

The role of limbal stem cells (LSCs) in the maintenance of corneal epithelial integrity is widely accepted, due to their capacity for self-renewal and proliferation. These cells are characterized as an oligopotent progenitor cell population found in the basal layer of the limbal epithelium, presenting a high nucleus-to-cytoplasm ratio with a slow cell cycle, high proliferative potential and a great capacity for self-renewal with asymmetric division [4]. In the limbic region, there are a variety of cell types, such as subpopulations of different progenies (typical progenitors and immediate and transient amplifying cells), melanocytes, antigen-presenting cells (Langerhans cells), mesenchymal
cells, nerve endings and vascular elements, which form a unique characteristic microenvironment, or niche, responsible for the events of cell proliferation and self-renewal [2]. It is believed that the different progenies are intended for the production of cells which differentiate initially in the basal layer of the cornea, then in a postmitotic cell population in the suprabasal layer and, finally, in the surface layers. This continuous flow of cell differentiation, of centripetal type, from the deepest layers to the most superficial (the XYZ hypothesis), ensures the renewal of the corneal epithelium and the maintenance of its integrity [2, 3]. However, the exact roles of mesenchymal stromal cells, corneal nerve endings, perilimbal vascularization and cellular signaling pathways involved in controlling cellular activity in the niche remain to be defined [2, 4].

An alternative model of cell dynamics in the surface of the eye has recently been proposed. In this model, the sclerocorneal limbal region functions as a transition region in which continued cell-proliferative activity in the epithelia of both the conjunctiva and the cornea creates a balance through the bidirectional migration of cells with progenitor characteristics. The loss of this stability (e.g. in the case of a corneal wound) would result in the migration of LSCs into the regions of repair [5]. Thus, the model suggests the existence of an oligopotent progenitor cell subpopulation distributed continuously throughout the ocular surface. Therefore, corneal and conjunctival epithelia, structures with intense proliferative occupation, are in constant activity, but push in opposite directions; the point of convergence of the two is the limbic region. This model could explain why cells with progenitor characteristics accumulate in the limbic region in physiological conditions or why the limbic region acts as their main source of production [5]. However, there is evidence that limbal/corneal and conjunctival epithelia are not equipotent and that they represent two distinct cell populations managed by their own progenitor lineages, with their own proliferative characteristics [6]. The lack of human studies, the clinical data and the absence of specific markers for identifying progenitor cells of the ocular surface make it difficult to define the reparative dynamics and the cell behavior during corneal epithelial regeneration processes both in physiological conditions and in states of ocular surface aggression and corneal damage.

In recent years, several molecular markers have been proposed to identify LSCs, but none have achieved widespread acceptance. Reports in the literature [7–9] suggest that the basal epithelial cells of the limbal region in humans express several proteins, e.g. ATP-binding cassette subfamily G member 2 (ABCG2), cytokeratin (CK) 19, vimentin and α9 integrin, but were not especially positive for CK3/CK12, connexin 43, involucrin, some integrins (α2, α6, β4 subunits) and nestin compared with basal cells of differentiated corneal epithelium. High expression of transcription factor p63 and CK5/14 was also observed in basal limbal cells. Therefore, it seems that a combination of markers for differentiation (e.g. CK3/CK12, CK19, p63, ABCG2) may be required to identify human LSCs. At present, p63 (isoform ΔNp63α) and ABCG2 are considered the most useful cellular markers for the identification and isolation of progenitor cells in the limbal region. Recently, total RNA isolates of human limbal and central corneal epithelia were applied after transcription for hybridization on whole human genome expression microarrays [8] or quantitative real-time PCR [9]. A set of differentially expressed genes detected by both approaches was established, which may be useful for identifying putative corneal epithelial stem cells. Specific markers for stem cell progenies, their differentiation and signaling pathways for their maintenance (Notch and Wnt pathways) were identified [9]. Furthermore, several overexpressed genes related to development and differentiation (ITM2A, integral to membrane 2A), extracellular matrix components (SPON1, spondin-1), regulation of cell growth (IFITM1, interferon-induced transmembrane protein 1), melanin biosynthesis (DCT, dopachrome tautomerase) and Wnt pathway signaling transduction proteins (FZD7, frizzled 7; DKK4, dickkopf homolog 4) were also observed. Most were colocalized with other CCAAT enhancer binding protein-δ-positive putative resting LSCs [8].

The concept of ocular surface reconstruction was first introduced with the use of autologous conjunctiva in cases of unilateral chemical alkali burns. Since then, various surgical approaches have been developed with the goal of restoring the viability of the corneal epithelium on the diseased ocular surface, but they present technical difficulties and complications in both the short and the long term. In recent decades, limbal transplantation techniques using auto- or allografts have been introduced to improve and reconstruct the altered ocular surface, constituting a method of in vivo cell expansion applied to the bio-replacement of limbic tissues [10].

The first effective clinical results in regenerative medicine using stem cell therapy were applied to the treatment of patients with large surface areas of burned skin, specifically through the procedures developed to produce epithelial sheets from cultures of epidermal keratinocytes isolated from human skin and expanded ex vivo [11]. Building on this previous experience, epithelial cells of the ocular surface were obtained by cell culture tech-
niques for ex vivo expansion. Subsequently, the ocular surface was successfully reconstructed using LSCs in patients with severe unilateral ocular surface pathology, with good anatomic and functional results [12]. Since then, different translational approaches have been developed and optimized, with satisfactory long-term clinical results [13–17].

The use of stem cells for the regenerative purposes of organ and tissue repair is a subject of great current scientific interest. As there are ethical and practical obstacles to the use of human embryonic stem cells, an excellent alternative is the use of adult or somatic stem cells, which present significant advantages due to their immediate clinical applicability [18, 19].

**Epithelial Bio-Replacement by ex vivo Expansion of LSCs in Culture**

Ex vivo expansion of LSCs is the most innovative approach for ocular surface bio-replacement (cultured limbal epithelial transplantation; CLET). From a minimally invasive biopsy (1–2 mm²) of the healthy limbal region (the same or contralateral eye), an explant culture technique can be applied on a suitable substrate [such as amniotic membrane (AM)] or by separating the epithelial layer from the fragment obtained by an enzymatic treatment in 1 or 2 stages [17, 20–23]. In the latter approach, the cells obtained need to be cocultured in vitro using cell culture techniques on feeder layers (3T3 murine fibroblasts arrested by irradiation or mitomycin C). Once cell growth is achieved, the cell suspensions can be transferred to suitable substrates such as fibrin, collagen or biocompatible polymers. The bio-replacement is carried out after removal of most of the diseased tissue from the ocular surface [12–15]. This methodology has many advantages over the limbal transplantation techniques used to date; essentially, it requires a substantially smaller limbic biopsy, which mitigates the risk of induction of limbal deficiency in healthy donor tissue. Its other advantages include a final high cell population that is more efficiently selected, homogeneous and, theoretically, more enriched with progenitor characteristic cells [20, 22]. However, enzymatic techniques are characterized by a more complex approach, with additional manipulation of the tissue and the need for xenoproducts at different stages of the primary cell culture production. For its part, the explant technique has certain advantages, among them its technical simplicity, the lack of xenoproducts and its cost-effectiveness, despite the heterogeneity of the cell population cultured (sclera fibroblasts, antigen-presenting cells, melanocytes, conjunctival epithelium cells and others) [17, 20–23].

It is always desirable to use autologous cells for ex vivo expansion to avoid the risk of immune response. However, in the presence of severe bilateral ocular pathology, the use of heterologous epithelial cells is acceptable [13, 14, 17]. Heterologous epithelial cells can be obtained from cadaveric or related living donor corneas [15]. Sheets of oral mucosal epithelial cells expanded ex vivo have also been used successfully as an alternative source of autologous epithelial cells (cultured oral mucosal epithelial transplantation; COMET) [24]. One of the reasons for the use of epithelial cells from the oral mucosa is their marked expression of CK3.

Clearly, the ideal cells for use in corneal reconstruction are autologous corneal LSCs. However, if both eyes have serious surface damage, the source of healthy LSCs will be lost, and the only current therapeutic options include cultured oral mucosal epithelial transplantation or limbal allografts. The latter approach is associated with potential adverse effects, including the risks of immunosuppressive therapy after heterologous graft application. As a consequence, other strategies, including the use of mesenchymal stem cells (MSCs) from adult tissue, e.g., bone marrow mesenchymal stromal cells (BM-MSCs) or adipose-derived stromal (ADS) cells, for cell-based therapy in corneal injuries are gaining prominence.

**Can MSCs from Adult Tissue Develop into Corneal Epithelial Cells?**

Currently, MSCs from adult tissue represent an attractive source for cell-based therapy using autologous cells. Recent studies have highlighted their much broader plasticity and applicability than those described above [19]. These cells are characterized by a low grade of differentiation and by high capacities for self-renewal and proliferation in vivo and in vitro. Furthermore, they have properties of pluripotent or multipotent cells and have a great potential for differentiating into mesodermal and nonmesodermal cell lineages. Adult MSCs can be isolated from different tissues, e.g., bone marrow (BM-MSCs), white adipose tissue (ADS cells), umbilical cord, peripheral blood, liver and fetal tissues [25]. Once seeded, MSCs can be selected by plastic surface adhesion and characterized to demonstrate their mesenchymal origin. Thus, they should express (≥95%) CD105, CD73, CD90, CD44 and CD29 markers and lack (≤2%) expression of HLADR-II, CD34, CD45, CD14 and CD11c markers [26]. More-
over, their ability to differentiate into three different mesenchymal lineages, adipocyte, chondrocyte and osteoblast, should be tested. MSCs have both endothelial and epithelial tissue coding genes [27] and can be induced to differentiate into epithelial or epithelial-like cells both in vitro and in vivo [28–30].

Due to their potential for multilineage differentiation and their immunomodulatory properties, in addition to the ease with which they can be isolated from lipospi-rates, ADS cells represent a promising tool for cell therapy and are currently being tested in clinical trials for myocardial ischemia, chronic graft versus host disease, limb ischemia, fistulous Crohn’s disease, rectovaginal fistula, multiple sclerosis and spinal cord injury. Clinical trials for degenerative arthritis, spinal cord injury and Romberg’s disease have already been completed (http://clinicaltrial.gov).

ADS cells can also be used as a source for regenerating the corneal stroma. In one study, diseased corneas were repopulated and repaired in an animal model, and transparency was preserved for several weeks after adipose-derived progenitor cell therapy [31]. As described in our earlier report [30], the expression of stem cell progeny markers and CK12 in ADS cell cultures indicates the capacity to acquire epithelial-like characteristics in appropriate conditions. In addition, topical application of autologous ADS cells seems to promote corneal epithelium healing in patients with a persistent sterile corneal epithelial defect refractory to other treatments [32]. Interestingly, there is also evidence for the targeted differentiation capacity of human ADS cells into neuroepithelial retinal pigment epithelium analogs [33].

The potential of BM-MSCs to transdifferentiate and repair epithelial cells in the skin, the laryngeal epithelium, the renal tubular epithelium and elsewhere has been explored by systemic administration [39, 40]. It is also known that MSCs can express a variety of neurotrophic factors, such as brain-derived neurotrophic factor, ciliary neurotrophic factor, insulin-like growth factor-1, nerve growth factor and basic fibroblast growth factor, which can protect injured retina [39, 40]. The pleiotropic action of MSCs has also been reported in wound healing and fibrosis inhibition, linked to upregulation of matrix metalloproteinase-9, tumor necrosis factor-α and interleukin (IL)-10 [41]. MSCs possess a ‘bystander immune modulation’ capacity [25] that can be used to control the proliferation and function of naïve and memory T cells, B cells and natural killer cells, in addition to reducing the secretion of inflammatory cytokines [42, 43]. Another remarkable ability of MSCs is their ‘homing mechanism’, which allows cells to migrate into other areas of the body undergoing pathological processes [19]. The molecular mechanism underlying MSC migration and homing is not fully understood, although the chemokine receptor CXCR4 is expressed on BM-MSCs [44, 45], and its ligand, stromal cell-derived factor-1, is known to be upregulated in tissues that have been injured or are suffering from ischemia [44].

Several studies [37, 46–49] have described the function of BM-MSCs in corneal wound healing, in which their immunosuppressive and anti-inflammatory properties act on inflammatory and angiogenic processes. In BM-MSC-transplanted corneas, decreases in inflammatory cell infiltrates and corneal vascularization were observed, associated with repression of inflammatory cytokine expression levels (IL-2, interferon-γ and IL-6) and a higher production of anti-inflammatory cytokines such as IL-10 and transforming growth factor-β [46]. In addition, rat BM-MSCs injected subconjunctivally into chemically damaged corneas showed lower levels of monocyte chemoattractant protein-1 and tumor necrosis factor-α associated with lower degrees of inflammatory infiltrating cells. Reductions in vascular endothelial growth factor and matrix metalloproteinase-2 levels were also observed with less vascularized corneas [47, 49]. This help during corneal wound healing due to the immunosuppressive, anti-inflammatory and angiogenic effects of MSCs has been explored by systemic administration [37, 38].

An additional role for MSCs has been linked to their paracrine abilities, that is, their capacity to locally secrete molecules able to modify the behavior of surrounding cells. Neurotrophic and immunomodulatory effects have been suggested as mechanisms by which MSCs can create a regenerative microenvironment to promote local tissue healing [37]. In this way, neurotrophic factors have been reported to play an important role in maintaining stem cells in the human limbal region [38]. It is also known that MSCs can express a variety of neurotrophic factors, such as brain-derived neurotrophic factor, ciliary neurotrophic factor, insulin-like growth factor-1, nerve growth factor and basic fibroblast growth factor, which can protect injured retina [39, 40]. The pleiotropic action of MSCs has also been reported in wound healing and fibrosis inhibition, linked to upregulation of matrix metalloproteinase-9, tumor necrosis factor-α and interleukin (IL)-10 [41]. MSCs possess a ‘bystander immune modulation’ capacity [25] that can be used to control the proliferation and function of naïve and memory T cells, B cells and natural killer cells, in addition to reducing the secretion of inflammatory cytokines [42, 43]. Another remarkable ability of MSCs is their ‘homing mechanism’, which allows cells to migrate into other areas of the body undergoing pathological processes [19]. The molecular mechanism underlying MSC migration and homing is not fully understood, although the chemokine receptor CXCR4 is expressed on BM-MSCs [44, 45], and its ligand, stromal cell-derived factor-1, is known to be upregulated in tissues that have been injured or are suffering from ischemia [44].

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When the impact of MSC homeostasis on corneal injury and the potential of MSCs to home to injured tissue and promote corneal repair are analyzed, the mobilization of endogenous MSCs and elevation of stem cell chemotactic factors in the peripheral blood are observed. Thus, systemically administered MSCs are able to home specifically to the injured cornea and promote epithelial regeneration, highlighting the therapeutic implications of MSC-mediated tissue repair in corneal injury [37]. Future studies are needed to determine whether BM-MSCs also exert their beneficial effects through a transdifferentiation cellular mechanism.

However, clinical roles for MSC therapy in neuroprotection and immune suppression are likely to emerge. These therapeutic effects will be particularly advantageous in neurological tissues such as the retina and the optic nerve, because MSC-based molecular therapy may overcome some of the difficulties of long-term drug delivery to tissue such as the eye, which is relatively inaccessible to systemic delivery due to the blood-retinal barriers [19].

BM-MSCs may also be used as alternative feeder layer cells for LSC cultures, thus avoiding the xenogenic risk presented by murine 3T3 fibroblasts. Similar patterns of expression of CKs (CK3 and CK15), ΔNp63α and ABCG2 were found in rabbit LSCs cocultured with both mitomycin-arrested human BM-MSCs and 3T3 fibroblasts. When epithelial sheets produced by both cocultures were transplanted onto chemically damaged rabbit corneas, a similarly robust expression of CK3 was observed without corneal neovascularization or conjunctivalization [50]. More recently, the ability of BM-MSCs to promote LSC proliferation and influence improvements in cell viability, growth factor expression and was reported in cocultures [51]. These data also confirmed the suitability of BM-MSCs for supporting ex vivo expansion of LSCs.

In conclusion, do MSCs have the potential to differentiate into corneal epithelial cells? Can they be used to treat LSCD? The development of tissue engineering technology opens up new possibilities. Research has shown that adult MSCs are pleiotropic, multipotent and easy to isolate and can differentiate into corneal epithelial-like cells in vitro. Meanwhile, the ability of these cells to fully regenerate the corneal epithelium by acquisition of a corneal phenotype needs to be further examined. There is also evidence that BM-MSCs and ADS cells exert their therapeutic effects in wound healing via inhibition of inflammation and angiogenesis, leading to an improvement of the regeneration of damaged corneas. Future studies will need to determine the molecular mechanisms underlying these cellular events. Nevertheless, although the mechanisms of ocular tissue reconstruction remain elusive, MSCs may provide a feasible approach to corneal regeneration.

Challenges and Future Perspectives

To date, the search for innovative strategies and approaches in the field of ocular surface reconstruction has produced some encouraging results. One emerging alternative source of stem cells for therapeutic purposes is the umbilical cord. Umbilical cord stem cells have a high proliferative potential, are less immunogenic and nontumorigenic and can be obtained by minimally invasive methodologies [52]. Recently, the expression of several putative markers found in human LSCs, such as ABCG2, CK15 and several integrins (α6, α9, β1 subunits), in progenitor cells derived from umbilical cord has been confirmed [53]. In addition, growth and ex vivo expansion on AM showed a stratified cellular arrangement with expression of specific corneal CKs (CK3, CK12), suggesting a therapeutic potential for corneal epithelial regeneration.

Furthermore, there is evidence of human embryonic stem cell differentiation into corneal epithelial cells [54] and of transdifferentiation of progenitor cells obtained from the skin epidermis into cells with characteristics of the corneal epithelium [55]. However, these methods are experimental and have yet to be applied in clinical practice, due to technical difficulties regarding cell collection.

More recently, the capacity of human progenitor cells of immature dental pulp for corneal reconstruction was described in a rabbit LSCD model [56]. Transplanted corneas showed better transparency and less vascularization in LSCD animals than in controls after 3 months. The capability of human progenitor cells of immature dental pulp to regenerate the ocular surface is due to the fact that they share similar characteristics with human LSCs, such as the acquisition of an epithelial phenotype and the expression of ABCG2, CK12 and connexin 43 [57].

Interestingly, the potential for transdifferentiation of hair follicle bulge-derived stem cells (HFSCs) into corneal epithelium through modulation of specific microenvironmental factors related to the limbal region has also been assessed [58]. Stratified sheets of epithelial-like cells from murine HFSCs cultured on specific substrates (laminin-5) and in the presence of conditioned medium derived from stromal fibroblasts from the region of the limbus were obtained. This condition increased the expression levels of Pax-6 and CK12, evidencing the HFSCs'
ability to differentiate in vitro into corneal epithelial cells when exposed to specific conditions [59]. Moreover, HFSCs transplanted in a murine model of LSCD showed differentiation into corneal epithelial phenotypes with expression of CK12. In most of the transplanted animals, a re-epithelialization of the cornea was observed with ocular surface repair, associated with the suppression of conjunctivization and a significant decrease in corneal vascularization [58]. These data highlight the therapeutic properties of HFSCs as an alternative, readily available source of autologous adult stem cells for application in the regeneration of corneal epithelium.

At present, one of the major problems associated with cell-based therapy on the ocular surface is the absence of a suitable scaffold for LSC growth and transfer. To treat LSCD, various carriers for the culturing cells and for their transplantation onto the recipient eye have been tested. The ones used in human clinical treatment include human AM [13, 60], fibrin gel [13, 14] and thermoresponsive dishes [24, 61]. In recent years, promising scaffolds for ex vivo expansion and transfer of different types of stem cells have been developed by tissue engineering [62] and nanotechnology. Soon, additional suitable substrates are likely to be found such as nanofiber scaffolds [63] or different bioengineered polymers [64] which will be acceptable for LSC growth and biocompatible for human use.

These experimental innovations should help to develop therapeutic approaches in the near future for treating diseases of the ocular surface, in which stem cell-based therapies represent a more physiological, more rational and less invasive treatment.

References


8. Casaroli-Marano/Nieto-Nicolau/Martínez-Conesa

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