Chapter 17: Cell therapy using extraocular cells: mesenchymal stem cells

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ABSTRACT

Extraocular mesenchymal stem cells (MSCs) represent an available, non-immunogenic source of stem cells that has proved to possess a potential therapeutic value in corneal epithelium regeneration based on results obtained in both preclinical and clinical studies. All published studies have revealed promising results in animal models and have shown significant corneal regeneration, improved corneal transparency and a rapid healing process associated with the restoration of vision. However, the studies performed to unravel the mechanisms underlying the beneficial effects of MSCs on the damaged ocular surface have shown that multiple mechanisms might contribute simultaneously to their therapeutic action. Although it remains uncertain if MSCs can transdifferentiate into corneal epithelial cells, these cells have shown a capacity of secreting trophic and growth factors capable of stimulating resident stem cells and reducing tissue injury, an ability to exert anti-inflammatory and immunomodulatory effects, and a capability to migrate into injured tissues. This book chapter is specifically focused on

the preclinical and clinical advancements on the use of extraocular MSCs for corneal epithelium regeneration.

Keywords: Mesenchymal stem cells; MSC; Corneal epithelium; Limbal stem cell deficiency; LSCD; Corneal failure; Ocular surface failure.

Abbreviations

ABCG2: ATP-binding cassette subfamily G member 2

ALDH3A1: Aldehyde dehydrogenase 3 family member A1

AM: Amniotic membrane

APCs: Antigen-presenting cells

AT-MSCs: Adipose tissue-derived mesenchymal stem cells

BM-MSCs: Bone marrow-derived mesenchymal stem cells

CAT: Catalase

C/EBPδ: Cytosine-cytosine-adenosine-adenosine-thymidine/enhancer-binding protein-δ

CCL: Chemokine (C-C motif) ligand

CCL2/MCP-1: Chemokine (C-C motif) ligand 2/monocyte chemoattractant protein-1

CD: Cluster of differentiation

CINC-1/CXCL1: Cytokine-induced neutrophil chemoattractant 1

CK: Cytokeratin

CLET: Cultivated limbal epithelial transplantation

Cox-2: Cyclooxygenase-2

CXCR4: C-X-C chemokine receptor type 4

Cx43: Connexin 43

DP-MSCs: Dental pulp-derived MSCs

EGF: Epidermal growth factor

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GMP: Good manufacturing practices

GPX: Glutathione peroxidase

GvHD: Graft *versus* host disease

HLA-DR: Human leukocyte antigen-DR

ICAM-1: Intercellular adhesion molecule 1

IDO: Indoleamine-2,3-dioxygenase

IFN-γ: Interferon gamma

Ig: Immunoglobulin

IGF-I: Insulin-like growth factor-I

IL: Interleukin

iNOS: Inducible nitric oxide synthase

iPSC: Induced pluripotent stem cells

iPSC-MSCs: Induced pluripotent stem cells-derived mesenchymal stem cells

IVCM: In vivo confocal microscopy

KGF-2: Keratinocyte growth factor-2

LESCs: Limbal epithelial stem cells

LSCD: Limbal stem cell deficiency

MCP-1: Monocyte chemotactic protein 1

MDA: Malondialdehyde

MHC: Major histocompatibility complex

MIP-1α: Macrophage inflammatory protein-1 alpha

MMP: Matrix metallopeptidase

MPO: Myeloperoxidase

MSCs: Mesenchymal stem cells

MSCT: Mesenchymal stem cell transplantation

M1: Macrophages type 1

M2: Macrophages type 2

NaOH: Sodium hydroxide

NF-kB: Nuclear factor-kappa beta

NK: Natural killer cells

NO: Nitric oxide

NT: Nitrotyrosine

PanCK: Pan-cytokeratin

Pax6: Paired box 6

PCNA: Proliferating cell nuclear antigen

PD-1: Programmed death-1

PDGF: Platelet-derived growth factor

PD-L1: Programmed death ligand-1

PEDF: Pigment epithelium-derived factor

PGE2: Prostaglandin E2

RT-PCR: Reverse transcription-polymerase chain reaction

SDF-1α/CXCL12: Stromal cell-derived factorα1/C-X-C motif chemokine 12

SGPT: Serum glutamic-pyruvic transaminase

SOD: Superoxide dismutase

SSEA4: Stage-specific embryonic antigen-4

TER: Transepithelial electrical resistance

TGF-β: Transforming growth factor beta

TLR: Toll-like receptors

TNF-α: Tumor necrosis factor alpha

Treg: Regulatory T cells

TSG-6: Tumor necrosis factor-a-stimulated gene/protein-6

TSP-1: Thrombospondin-1

UC-MSCs: Umbilical cord-derived mesenchymal stem cells

VCAM-1: Vascular cell adhesion protein 1

VEGF: Vascular endothelial growth factor

WJ-MSCs: Mesenchymal stem cells derived from the Wharton's jelly of the umbilical cord

XOX: Xanthine oxidase

ZO-1: Zonula occludens-1

1. INTRODUCTION

Maintenance of corneal epithelium integrity is essential for preserving corneal transparency and visual function. For that reason, a population of stem cells residing in the basal epithelial layer of the corneoscleral limbus, the so-called limbal epithelial stem cells (LESCs), is continuously renewing the corneal epithelium layers (1–4). Destruction or dysfunction of these limbal epithelial stem cells or their niche induces a syndrome known as limbal stem cell deficiency (LSCD), which can be caused by a wide variety of ocular surface injuries and disorders such as chemical or thermal burns, multiple previous eye surgeries, cicatrizing-autoimmune pathologies, severe dry eye syndrome, infections, congenital disorders, etc. LSCD is characterized by a deficient regeneration of the corneal epithelium that eventually leads to persistent epithelial defects, ingrowth of conjunctival tissue onto the cornea surface, neovascularization, and persistent ocular surface inflammation. All these events usually result in loss of vision and a chronic pain syndrome (5,6).

Transplantation of cultivated limbal epithelial cells (CLET) harvested from the contralateral healthy eye is the current treatment of choice for unilateral cases of LSCD, in which it is possible to harvest autologous limbal tissue from the healthy fellow eye and cultured it to expand the stem cell population before transplantation (7). In bilateral cases, which in fact are

more frequent, it is necessary to resort to allogeneic limbal tissue from deceased donors due to the scarcity of healthy tissue in patient's eyes, transforming an autologous transplant into an allogeneic one. Allogeneic transplantations require one year of systemic immunosuppression in order to avoid immune rejection, which consequently increases the risk of the patient's morbidity and the medical expenditures, although it avoids the costs related with the extraction of a biopsy (7,8). The use of alternative sources of extraocular cells could help to overcome the dependence on and the limitations of limbal epithelial cells, which are difficult to obtain and culture and that, in case of being allogeneic, can induce immune rejection (9–11).

Mesenchymal stem cells (MSCs) constitute a subset of stromal cells that can be isolated from a variety of tissues including bone marrow, adipose tissue, dental pulp, umbilical cord, and limbal stroma of the human eye, among others (9,12). They are partially defined by their adherence to plastic supports when cultured in standard conditions; their multipotent differentiation potential to bone, cartilage and adipose tissue *in vitro;* and the expression of a specific profile of surface antigens, including positivity for cluster of differentiation (CD) 73, CD90, and CD105 and negativity for CD34, CD45, CD11b or CD14, CD19 or CD79α, and HLA-DR markers (13).

The use of human MSCs in cell-based therapies has tremendously increased over the last decade in the field of regenerative medicine. There is a growing body of literature supporting that the therapeutic effects of MSCs not only rely on their differentiation ability to repair damaged tissue, but also on their immunomodulatory and anti-inflammatory properties, their capability to migrate to injured and inflamed tissues, and their capacity to modulate local environment, activate endogenous progenitor cell,s and secrete trophic factors. In addition, compared with other types of stem cells, MSCs have several advantages, such as availability

and easy harvesting from a variety of tissue types, few ethical concerns, elevated proliferative capacity *in vitro*, and low immunogenicity (12,14,15).

For all these reasons, MSCs have emerged as very attractive candidates for cell-based therapies in numerous clinical applications including autoimmune and inflammatory diseases (e.g., graft *versus* host disease (GvHD), Crohn's disease, and rheumatoid arthritis), skeletal disorders, heart diseases, diabetes, neurological disorders, etc. (12,15). In ophthalmology, the potential therapeutic applications are also many, and, according to the ClinicalTrial.gov research database, there are currently several ongoing clinical trials to test the efficacy of MSCs in the treatment of a variety ocular disorders such as retinitis pigmentosa, age-related macular degeneration, glaucoma, non-arteritic ischemic optic neuropathy, and different ocular surface disorders (16). This book chapter is specifically focused on the pre-clinical and clinical advancements on the use of extraocular MSCs for corneal epithelium regeneration.

2. EXTRAOCULAR MSCs FOR CORNEAL EPITHELIUM REGENERATION

2.1. Therapeutic potential of extraocular MSCs in corneal epithelium regeneration

There are abundant *in vitro* and *in vivo* studies on the use of extraocular MSCs for corneal epithelium regeneration. All published studies have revealed promising results in animal models and have shown significant corneal regeneration, improved corneal transparency, and a rapid healing process associated with the restoration of vision (10,17–19). However, the studies performed to unravel the mechanisms underlying the beneficial effects of MSCs on the damaged ocular surface have shown that multiple mechanisms might contribute simultaneously to their therapeutic action. Although it remains uncertain if MSCs can transdifferentiate into corneal epithelial cells (20), these cells have shown a capacity of secreting trophic and growth factors capable of stimulating resident stem cells, reducing tissue injury, an ability to exert anti-inflammatory and immunomodulatory effects, and a capability to migrate into injured tissues (11,17,19–21) (Figure 17. 1).

2.1.1. Transdifferentiation capacity into corneal epithelial cells

Corneal epithelium derives from the surface ectoderm during embryonic development (22). Although it seems pretty clear that MSCs can differentiate not only into mesodermal but also into non-mesodermal cell lineages including neuroectodermal and epithelial cells (23–25), the potential transdifferentiation capacity of MSCs into corneal epithelial cells is unclear and remains under investigation (20).

Transdifferentiation capacity of bone marrow-derived MSCs (BM-MSCs) into corneal epithelial cells

There are several studies published on the transdifferentiation capacity of human BM-MSCs into corneal epithelial cells. It has been shown that human BM-MSCs cultured on amniotic membrane (AM) and transplanted onto the surface of an alkali-burned rabbit cornea migrated to the cornea and expressed the corneal epithelial cell marker cytokeratin (CK) 12 when located in the corneal epithelium (26). Subsequently, the potential of human BM-MSCs to differentiate into corneal epithelial cells was confirmed when they were cultured first on pig Bowman's membrane and later under air-lifting conditions for creating a multilayer of cells. After 4 weeks of differentiation induction, Hou et al. saw that part of the BM-MSCs expressed the corneal and limbal epithelial markers CK12 and CK19, respectively, but not the limbal stem cell marker ATP-binding cassette subfamily G member 2 (ABCG2) (27). Afterward, Rohaina et al. also showed that while CK3 and p63 were not detected in human BM-MSCs, upon being cultured in limbal media for 10 days, they significantly increased their expression for the corneal and limbal epithelial markers CK3 and p63, respectively (28). They also demonstrated that transplantation of induced BM-MSCs stratified on AM onto a nude LSCD rat model remarkably improved corneal regeneration in terms of corneal transparency and vascularization. Although they did not track transplanted cells, they observed CK3 and p63 protein expression in the newly regenerated cornea (28). A recent study has also revealed

that human BM-MSCs positive for the early embryonic stem cell marker stage-specific embryonic antigen-4 (SSEA4) had higher potential to differentiate into corneal epithelial cells. Even though differentiated cells did not reach the typical transepithelial electrical resistance (TER) of corneal epithelium, they presented an epithelial-like morphology and were positive for the corneal and limbal epithelial markers CK3, CK12, CK8, CK14, CK15, β1-integrin, and E-cadherin (29). In addition, another *in vivo* study has demonstrated too that human BM-MSCs administered to mice by subconjunctival injection, migrated to the corneal tissues and displayed morphological characteristics of epithelial, stromal, and endothelial cells (30).

Regarding rabbit BM-MSCs, Gu et al. showed that when they were co-cultured with limbal epithelial stem cells, or their conditioned medium, they changed their fibroblastic morphology to a more flattened epithelial-like shape and transiently increased their expression of the corneal epithelial-specific marker CK3 (31). They also saw that when they were suspended in fibrin gels and further transplanted onto alkali-burned rabbit corneas, rabbit BM-MSCs induced a successful ocular surface reconstruction, and some of those that located in the corneal epithelium expressed the corneal epithelial-specific marker CK3, implying BM-MSC differentiation into corneal epithelial cells (31). Interestingly, another study evidenced that, under standard culture conditions, rabbit BM-MSCs were negative for the limbal and corneal epithelial markers ABCG2, p63, and connexin 43 (Cx43), but positive for CK3/12 and α-enolase, suggesting, for the first time, that CK3 and CK12 markers were not highly specific for differentiated corneal epithelium (32).

Concerning BM-MSCs isolated from rats, Jiang et al demonstrated that co-cultivation of rat BM-MSCs with corneal stromal cells induced the expression of the corneal epithelial specific marker CK12 and the acquisition of morphology and cell structures (tight junctions) typical of epithelial cells. Transplantation of the induced rat BM-MSCs cultured on AM for 7 days

demonstrated that those cells significantly improved the reconstruction of the cornea surface in an alkali-burned LSCD rat model (33). Zhang et al. also supported that rat BM-MSCs had the ability to transdifferentiate into corneal epithelial cells when they cultured them on a xenogeneic acellular corneal matrix *in vitro* for 7 days and showed that cells acquired an epithelium-like shape and expressed the corneal epithelial marker CK3 (34).

In regard to mouse BM-MSCs, a recent *in vitro* study demonstrated that when they were cultured with corneal extract in the presence of insulin-like growth factor-I (IGF-I), mouse BM-MSCs differentiated to cells with features of corneal epithelial cells and keratocytes and maintained MSC properties. They observed that the expression of the corneal-specific markers CK12, keratocan, and lumican was upregulated after culture with corneal extract and that adding IGF-I to the culture medium significantly increased the expression of those genes (35).

In contrast to the evidence supporting the differentiation potential of BM-MSCs into corneal epithelial cells, a study conducted by Ma et al. revealed that human BM-MSCs transplanted over AM onto a chemically burned rat cornea survived and successfully reconstructed the damaged rat corneal surface but that the therapeutic effect did not come from epithelial differentiation of BM-MSCs but as result of inhibition of neovascularization and inflammation (36). Likewise, another study showed that rabbit BM-MSCs systemically administered in a rabbit corneal alkali-burned model, engrafted to injured cornea, promoted wound healing by stimulating and synergizing with native cells and by differentiating not into corneal or limbal epithelia cells but into myofibroblast (37).

Although the outcome of many *in vitro* experiments supports the idea that BM-MSCs are able to transdifferentiate into corneal epithelial cells under certain conditions, *in vivo* data published up to date has shown contradictory and inconclusive results pointing out that

further investigations are required to confirm whether BM-MSCs can or cannot acquire a corneal epithelial cell phenotype.

Transdifferentiation capacity of subcutaneous adipose tissue-derived MSCs (AT-MSCs) into corneal epithelial cells

Up to now, results from the few studies specifically performed on AT-MSCs differentiation into corneal epithelial cells do not bring enough evidence to support the hypothesis of their real transdifferentiation into functional corneal epithelial cells since all of them are only based on *in vitro* experiments. While a first study showed that orbital fat-derived stem cells had more potential to differentiate into corneal epithelial cells that MSCs derived from subcutaneous AT due to their same developmental origin during embryonic development (38), a subsequent study found that human AT-MSCs expressed the progenitor markers p63 and ABCG2 and the cytokeratins for differentiated epithelial cells CK12, and CK76. Furthermore, the study showed that CK12 expression spontaneously and progressively increased by cell adhesion over time in culture, suggesting that AT-MSCs had the potential to acquire corneal epithelial-like characteristics under the appropriate conditions (39). Those results were further confirmed by Nieto-Miguel et al. when they demonstrated that human AT-MSC grown under basal culture conditions expressed the corneal epithelial markers CK3 and CK12, and that AT-MSCs acquired a more epithelial-like morphology and an up-regulated expression of CK12 when they were cultured in corneal epithelial cell-conditioned medium for 15 days (40). Later, the transdifferentiation capacity of AT-MSCs was also advocated when another *in vitro* study showed that paired box 6 (Pax6) stable transfection stimulated differentiation of murine AT-MSCs into corneal epithelial-like cells by induction of the expression of the corneal epithelium-specific marker CK12 (41).

Transdifferentiation capacity of MSCs derived from the Wharton's jelly of the umbilical cord (WJ-MSCs) into corneal epithelial cells

Base on the accessibility, abundance, lack of ethical issues, and capability of transdifferentiating into epithelial cells (42), Garzón et al. chose human Wharton's jelly MSCs to generate a three-dimensional heterotypic human cornea with fibrin-agarose scaffolds. They showed that WJ-MSCs were able to differentiate into corneal epithelial-like cells with results similar to the native corneas in the expression of corneal epithelial key markers such as CK3/12, plakoglobin, zonula occludens 1 (ZO-1) and Cx43 (43). On the other hand, Sidney et al. studied the expression of specific epithelial cell markers at different passages and activity stages of corneal stromal cell and compared it to the one in WJ-MSCs. Results demonstrated that *in vitro* culture of stromal cells not only caused phenotypical changes but also induced expression of specific cytokeratins. Despite not being from epithelial origin, WJ-MSCs were positively stained for CK3/12, CK19, panCK ,and E-caherin, while corneal stromal cells only were stained for CK3/12 and CK19. Reverse transcription-polymerase chain reaction (RT-PCR) analyses revealed significantly decreased gene expression of CK3, CK12, and CK19 in WJ-MSCs compared to corneal stromal cells, and showed detectable levels of both CK5 and E-cadherin genes, suggesting that there might be some specificity to the cytokeratins expressed in stromal cells isolated from different tissues (44).

Transdifferentiation capacity of dental pulp-derived MSCs (DP-MSCs) into corneal epithelial cells

There are only few studies in which it has been explored the potential capacity of DP-MSCs to differentiate into corneal epithelial cells (45–48). In the first published study, Monteiro et al. demonstrated that human immature dental pulp stem cells expressed *in vitro* limbal epithelial stem cell markers such as ABGC2, Cx43, integrin-β1, p63, and vimentin, while they did not express or weakly expressed the corneal epithelial-specific markers CK3 and CK12, respectively (45). The subsequent transplantation of DP-MSC sheets onto the ocular

surface of a mild and severe LSCD rabbit model also confirmed that DP-MSCs successfully induced corneal epithelium reconstruction and had the capacity to transdifferentiate into corneal epithelial cells *in vivo* (45,46). Later, it was demonstrated that stem cells from human exfoliated deciduous teeth also had the potential to be induced to differentiate into corneal epithelial-like cells *in vitro*. Tsai et al. showed that both direct and indirect co-culturing with corneal epithelial cells upregulated the expression of the corneal epithelial marker CK3 and the corneal epithelial progenitor marker CK19 (48). Furthermore, when Kushnerev et al. used soft contact lenses to deliver MSCs derived from pulp tissue explants to an *ex vivo* model of human epithelial-debrided corneas in organ culture, they saw that, once the cells had transferred, they began to express the corneal epithelial-specific markers CK3 and CK12, supporting that DP-MSCs differentiated into corneal epithelial-like cells once they attached to Bowman's membrane (47).

Although the results derived from several *in vitro* experiments support the idea that MSCs are able to differentiate into a corneal epithelial-like cell phenotype under certain conditions, it remains uncertain whether the expression of certain epithelial markers like cytokeratins represents a real functional differentiation or just the result of MSC culturing (20,44). Moreover, the fact that results from several *in vivo* studies do not support the data shown by *in vitro* experiments suggests the need of performing additional *in vivo* studies to address whether MSCs can or cannot transdifferentiate into functional corneal epithelial cells.

2.1.2. Trophic activity

The Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy proposed the minimal criteria to define human MSCs in 2006 (13). Nevertheless, even before an official definition about MSCs was established, it was known that mesenchymal progenitor cells obtained from bone marrow could secrete different cytokines which had specific functions inside tissues under physiological conditions and whose

expression was modified under inflammation situations (49). This fact, together with results obtained from different studies in which BM-MSCs enhanced corneal wound healing in spite of the absence of MSC migration to damaged tissue (50,51), contributed to establish the concept currently known as “trophic effect of MSCs.”

At the moment, it is known that MSCs secrete a large panel of soluble bioactive molecules which can cause direct intracellular signaling (autocrine effect), direct extracellular effects, or indirect effects by causing another cell in the vicinity to secrete the functionally active agent (52,53). The main functions of these bioactive molecules are (1) to inhibit apoptosis and limit the field of damage or injury (anti-apoptosis mechanism), (2) anti-inflammatory action, (3) to regulate angiogenesis (pro- and anti-angiogenic), (4) to stimulate and support the growth and differentiation of local stem cell, and (5) oxidative stress protection (52,54,55)**.** The trophic effects of MSCs have been also detected in corneal epithelium regeneration, showing that different factors secreted by MSCs can be implicated in a set of processes, such as corneal reepithelialization, and anti-inflammatory and anti-angiogenic actions that finally end in total or partial corneal tissue regeneration.

Corneal wound healing, reepithelialization, and anti-apoptosis mechanism

Corneal wound healing is a complex multistage process. For corneal wounds to heal optimally, a cascade of multicellular interactions and tissue remodeling must proceed in an orderly fashion. This process can be enhanced by several tissue repair growth factors secreted by cells and those recruited to the wound immediately after tissue injury (34,56). Currently, it is known that BM-MSCs secrete factors as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), pigment epithelium-derived factor (PEDF), and transforming growth factor beta (TGF-β) 1 (34), with the EGF and TGF-β1 playing an important role in the proliferation and migration of corneal epithelial cells (57–59) and, therefore, in the stimulation of corneal epithelium regeneration. When the effect of rat MSCs *versus* rat LESCs

was compared for *in vitro* healing corneal epithelium wounds, it was observed that the secretion of EGF and TGF-β1 was higher by MSCs than by LESCs. This difference would indicate that MSCs could be better than LESCs for corneal wound healing (34). However, different studies showed that rabbit BM-MSCs, AT-MSCs, and LESCs secreted, spontaneously or after stimulation with lipopolysaccharides, levels of TGF-β without significant differences among them (60,61). In addition, when these three types of cells were cultured on nanofiber scaffold and transplanted onto a de-epithelialized rabbit corneal surface, the transfer of stem cell-seeded nanofiber scaffolds onto the damaged ocular surface significantly improved corneal healing, in part because the number of apoptotic caspase 3+ cells decreased in the groups treated with stem cell-seeded nanofibers with respect to untreated injured corneas or with respect to corneas treated with cell-free nanofiber scaffolds (60). This fact support the important implication of numerous trophic and growth factors secreted by stem cells, such as MSCs or LESCs, on wound healing. In addition, the best re-epithelialization was observed in corneas treated with nanofiber scaffolds cultured with BM-MSCs or LESCs, reporting that MSCs from different sources can have different trophic effects on wounded corneal epithelium (60). On the other hand, it is known that MSCs promote the survival and proliferation of LESCs in the tissue, and that these effects may be mediated in a paracrine manner, due to the fact that cell proliferation rate and expression of EGFs were significantly higher in LESCs co-cultured with MSCs than in LESCs cultured alone (62). In addition, the expression of matrix metallopeptidase (MMP)-9 by damaged human corneal epithelial cells is strongly inhibited by human MSCs (63). Taking into account that MMP-9 interferes with normal re-epithelialization of the wounded cornea (64), this fact supports the hypothesis that MSCs transplanted after corneal and limbal epithelium debridement could promote the proliferation of LESCs and decrease the induced apoptosis in corneal epithelial cells. Recently, it has been also shown that tumor necrosis factor-a-

stimulated gene/protein-6 (TSG-6), secreted by murine BM-MSCs, promoted corneal epithelial wound healing in damaged corneal epithelium of diabetic mice through activation of LESCs (65).

Anti-inflammatory action

In 2006, it was reported the first evidence that human BM-MSCs could be used for the treatment of damaged corneal epithelium through an anti-inflammatory mechanism, since pro-inflammatory molecules which were detected in injured corneas treated with AM alone, such as CD45, interleukin (IL) 2, and MMP-2, were not detected in the damaged corneal surfaces treated with AM plus MSCs (36). Interestingly, the therapeutic effect of the transplantation could be associated with the anti-inflammatory trophic effect of MSCs, although this hypothesis must be contrasted. Some years after, it was observed that topical application of MSC-derived conditioned media contributed to restoration anti-inflammatory property of the rat corneal epithelium in damaged animals (66). These authors suggested that the exogenous high concentration of IL-6 secreted by MSCs to extracellular medium, and subsequently administrated to damaged corneas, could suppress dendritic cell activation in injured ocular surface (66–68). In accordance with this, several research groups have supported that soluble factors secreted by the MSCs played an important role as anti-inflammatory therapy in wounded corneas, since decreased corneal inflammation can be observed in spite of the absence of MSC migration to damaged tissue (51,69), establishing that the anti-inflammatory mechanism of MSCs could be mediated by paracrine pathways. Several works have supported that trophic factors secreted by MSCs could suppress the infiltration of inflammatory cells and $CD68^+$ macrophages by suppressing the expression of the macrophage inflammatory protein-1 alpha (MIP-1α) (51,70,71) and that factors secreted by MSCs are capable of inhibiting production of pro-inflammatory tumor necrosis factor alpha (TNF-α) by macrophages in locally burned corneas (51,72,73). In addition, it is known

that MSCs, activated by inflammatory signals, secreted the anti-inflammatory protein TSG-6 (69,74). Specifically, it is known that chemical injury to corneal epithelial cells activated human MSCs to secrete anti-inflammatory protein TNF-α that stimulated TSG-6 expression, molecule which interacts through the CD44 receptor on resident macrophages, attenuating the inflammatory cascade that is initiated by them (74) and reducing inflammatory damage to the cornea (69). A meticulous study showed that intraocular injection of TSG-6 into the anterior chamber reduced markedly the expression of different pro-inflammatory cytokines and chemokines (IL-6, IL-1β, cytokine-induced neutrophil chemoattractant 1 (CINC-1/CXCL1), and chemokine (C-C motif) ligand 2/ monocyte chemoattractant protein-1 (CCL2/MCP-1)), and levels of MMP-9, as well as the infiltration of neutrophils and proteases into the cornea after corneal and limbal epithelial debridement, being the effects of TSG-6 dose-dependent (75). These results strongly support the hypothesis that TSG-6 produced by human BM-MSCs in response to injury signals can protect the corneal surface from the excessive (75).

Anti-angiogenic function

In 2008, it was proposed that MSCs could have anti-angiogenic actions that could be mediated by paracrine pathways, in accordance with a work in which it was shown that thrombospondin 1 (TSP-1), a powerful anti-angiogenic factor, was upregulated at high levels in response to corneal epithelium injury treated with topical MSC-derived conditioned media (66). Currently, it is known that human BM-MSCs secreted different molecules in basal conditions, such as VEGF, MMP-2, and TSP-1 (75). In addition, MSCs secrete high amounts of VEGF when these cells are stimulated under inflammatory environment (66,68,72,75). VEGF and MMP-2 play a role as pro-angiogenic molecules in the cornea, while TSP-1 is a powerful anti-angiogenic factor that acts inhibiting VEGF-induced angiogenesis (76–78), maintaining the characteristic avascular corneal situation. Another molecule secreted by MSCs that has been proposed as anti-angiogenic factor after corneal damaged is the TSG-6,

since it can decrease the neovascularization in corneas with their epithelium debrided (75). On the other hand, it is known that MSCs and LESCs secrete, spontaneously or after stimulation with lipopolysaccharides, levels of VEGF without significant differences among them, suggesting that there is not additional risk of inducing corneal neovascularization by MSCs (34,60). Therefore, when cultured rabbit BM-MSCs, AT-MSCs, or LESCs on nanofiber scaffold were transplanted on a de-epithelized rabbit corneal surface, the neovascularization decreased with all three types of stem cells. Surprisingly, however, the greatest decrease was found in injured corneas treated with nanofiber scaffolds seeded with BM-MSCs or LESCs (60).

Stimulation and support of the growth and differentiation of local stem cell

Several studies have reported that MSCs can induce re-epithelialization on wounded corneas. This fact can be explained by the transdifferentiation of MSCs transplanted or by other mechanism. A group of authors observed that when de-epithelialized rat corneas were treated with rabbit BM-MSCs, AT-MSCs or LESCs cultured on nanofiber scaffold, the wound healing and the epithelial expression of CK3 and CK12 significantly enhanced on the cornea (60). They thought that MSC transdifferentiation is not the main mechanism of the re-epithelialization effect, suggesting that important effects represented by the production of numerous trophic and growth factors that can support the growth of residual corneal epithelial cells and LESCs (60).

Oxidative stress protection

Currently, it is known that reactive oxygen species increased in corneal epithelium after alkali corneal injury (79,80). However, rabbit BM-MSCs cultured on nanofiber scaffold can reduce alkali-induced oxidative and nitrosative stress in de-epithelized corneas. It is believed that MSCs cultured on nanofiber scaffolds protect the formation of toxic peroxynitrite, lower apoptotic cell death, and decrease matrix metalloproteinase and pro-inflammatory cytokine

production, resulting in reduced corneal inflammation as well as neovascularization and significantly accelerated corneal healing (80). In addition, it is known that the expression of antioxidant enzymes superoxide dismutase (SOD) and xanthine oxidase (XOX) is restored in the regenerated corneal epithelium similar to healthy corneas after BM-MSCs or LESCs nanofiber treatment (61). In accordance with these results, some authors postulated that MSCs can exert direct antioxidant activities through the secretion of antioxidant molecules, due MSCs secrete the extracellular antioxidant molecule SOD3 (81).

In summary, paracrine mechanisms of MSCs may exert a significant impact in promoting corneal wound repair, which involves the joint participation of different soluble factors that modulate inflammation and angiogenesis as well as improve tissue regeneration (Table 17. 1). However, the involved biophysiological factors and the underlying mechanism in corneal wound healing remain unclear (82).

Recently, important reviews reporting the trophic effects of MSCs, obtained from different sources, had been published (53,83). Concretely, in corneal regeneration, the potential less therapeutic effects of AT-MSCs in comparison with BM-MSCs or LSCs could be due to the lower differentiation potential of AT-MSCs and to the different spectrum of growth and immunoregulatory factors produced by these cells (53,83), although this fact is currently in controversy. On the other hand, several authors have reviewed that commonly referred to as the MSC secretome, describing exhaustibly soluble factors, and reporting new mechanisms related with factors released in extracellular vesicles, for example, exosomes and microvesicles (83–85). Exosomes are one of several groups of secreted vesicles, which have a size of 40–100 nm, and can be distinguished from microvesicles and apoptotic bodies by size and morphology (86). They can fuse with cellular membranes and it has been suggested that exosomes are secreted by a variety of cell types; between them we can find the MSCs, transporting genetic material and proteins (87,88). They are thought to mimic the roles played

by MSCs from which they originate (88,89). Currently, the effect of MSC exosomes on tissue wound healing had been reported (90). Although currently there are no studies in which this action was checked in corneal epithelium, it is possible via for the trophic action of MSCs (83).

3.1.3. Immunomodulatory ability

Over time, several studies have confirmed that MSCs have important immunomodulatory properties (82). This question was proposed some years ago thought studies in which MSCs directly inhibited proliferation of T cells (91,92). The immunomodulatory action of MSCs is often attributed at the action of different components that this type of cells can secrete. To effectively influence immunoregulation, MSCs require to be activated by an inflammatory microenvironment and stimulation by pro-inflammatory cytokines, such as interferon gamma (IFN-γ) and TNF-α (82). This immunomodulatory action can be characterized by a final immunosuppression action or by an final immunostimulatory effect (82,93). In addition, MSCs can suppress mechanisms regulated by innate and adaptive immune cells (82,93). Regarding innate immune reaction regulation, MSCs secrete molecules such as granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-6, and prostaglandin E2 (PGE2). GM-CSF elicits the anti-inflammatory state of macrophages type 2 (M2) which have decreased the capacity of antigen presentation function and do not secrete proinflamatory molecules (73,94). IL-6 secreted by MSCs inhibit neutrophil action, preventing respiratory bursts (95,96), while PGE2 secreted by these cells inhibit mast cell degranulation (97). In addition, PGE2 together with TGF-β or indoleamine-2,3-dioxygenase (IDO) can decrease natural killer cell proliferation and the pro-inflammatory cytokine production carried out by these cells, while PGE2 with IL-6 inhibit dendritic cell maturation, cytokine expression, and migration to lymph nodes (98,99). In adaptive immune response, the programmed death-1 (PD-1) and programmed death ligand-1 (PD-L1) factors, both secreted by MSCs, can inhibit B cell

proliferation and reduce production of immunoglobulin (Ig) G, IgM, and IgA (100–103). In addition, MSCs inhibit T cell proliferation and regulate cytokine expression, being both mechanisms regulated through molecules secreted by MSCs, such as nitric oxide (NO), IL-10, PD-1, PD-L1, TGF-β, etc (72,91,104,105). For example, MSCs can decrease the expression level of IFN-γ (proinflamatory molecule) from Th1 cells and increase the expression levels of IL-4 and IL-10 from Th2 cells, both with anti-inflammatory effects (72,106,107). Moreover, MSCs promote expansion and differentiation of regulatory T cells (Treg), which inhibit T cell response (82). On the other hand, MSCs have pattern-recognition receptors which can regulate specific immunostimulatory properties of these cells, such as toll-like receptors (TLR). For example, in response to TLR3 signaling, MSCs maintain an anti-inflammatory phenotype, while in response to signaling through TLR4, MSCs adopt a pro-inflammatory phenotype, upregulating production of IL-8, IL-1, and CCL-5 (93,108–110).

Finally, MSCs show reduced expression of major histocompatibility complex (MHC) class I and do not express MHC II neither the classic co-stimulatory molecules (72). In addition, MSCs reduce the expression of MHC class II and co-stimulatory molecules such as CD80, CD86, and CD40 on the surface of other cells (82), facts that wake it possible for MSCs to likely have a non-immunogenic phenotype, showing immunoprivileged features. Therefore, MSCs can potentially be used allogenically or xenogenically in a variety of tissue disease states (54).

Immunosuppression and immunostimulation induced by MSCs on corneal epithelium

All these immune modulatory characteristics of MSCs play a determinant role in corneal epithelium regeneration. It is known that when human corneal epithelial cells are cultured under a pro-inflammatory environment (IFN-γ and TNF-α stimulation), they increased their expression of intercellular adhesion molecule 1(ICAM-1), MHC-I, and HLA-DR (MHC-II) (111). However, MSCs effectively decreased IFN-γ/TNF-α-induced ICAM-1, MHC-I, and

MHC-II expression on this type of cells (111). Since these adhesion molecules play an important role in the infiltration of activated leucocytes in the injured corneal epithelium (112,113), this fact could be one of the mechanisms through MSCs decrease the infiltration of leukocytes in damaged corneas. Currently, it is suggested that MSCs reduce the expression of adhesion molecules in corneal epithelial cells though nuclear factor-kappa beta (NF-kB) signaling pathways (111), due to the specific inhibition of NF-kB nuclear translocation which decreases the expression of molecules such as ICAM-1 and vascular cell adhesion protein 1(VCAM-1) on epithelial cells (66,114–116). On the other hand, contrary to the expectations, MSCs significantly attenuated cytokine-induced IDO expression on human corneal epithelial cells (111), molecule which is strongly linked to prolong corneal allograft survival (117). Since some years, the immunomodulatory effect of MSCs on corneal epithelium also was studied by *in vivo* experiments. Currently, it is known mechanical injury on mice corneal-limbus epithelium induced an inflammatory reaction characterized by production of IFN-γ and inducible nitric oxide synthase (iNOS) in the ocular surface (118). In addition, IL-6 is also expressed when damaged ocular surfaced is treated with allogeneic limbal transplantation. However, both inflammatory situations are decreased when nanofiber scaffolds with BM-MSCs are transplanted in a mice model of damaged corneal/limbal epithelium, suggesting that inflammation inhibition was partially regulated by MSC immunomodulatory effects, since MSCs inhibited IFN-γ secretion and T cell proliferation *in vitro* (118). On the other hand, it has been reported the immunoregulatory effect of rabbit BM-MSCs and AT-MSCs cultured on nanofiber scaffold in a rabbit model of corneal de-epithelialization, which showed a strong tissue infiltration with executor cells of adaptive (T lymphocytes) and innate (iNOS-expressing cells) immunity (60). The MSC transplantation significantly decreased all of the harmful manifestations of the injury; although less pronounced therapeutic effects of AT-MSCs were observed in comparison with BM-MSCs.

The effects of MSC transplantation were related with the expression of genes for the immunoregulatory molecules IDO-2, cyclooxygenase-2 (Cox-2), and iNOS by these cells, since these molecules secreted by this type of cells like to inhibit proliferation of activated T cells, inhibit natural killer (NK) cells, and suppress mast cells in the presence of low concentration of IFN-α/IL-1, respectively (93,97). On the other hand, the effect of BM-MSC intravenous injection in a mouse model of autoimmune ocular inflammation (autoimmune uveitis) has been tested, observing an increase of Treg cells and M2 in peripheral blood and lung, respectively (119). However, since high levels of M2 lasted longer than Treg, it was reported that M2 were effectively protected against experimental autoimmune uveitis independently of Treg, indicating a direct role of M2 in the MSC-induced tolerance (119). This fact was confirmed by other authors, who co-cultured MSCs with macrophage isolates from mouse peritoneum. When macrophages where cultured alone, they differentiated into classical macrophages type 1 (M1) (pro-inflammatory); however, when these polarized M1 were exposed to human umbilical cord-derived MSCs (UC-MSCs), these macrophages began to express markers typical of M2 (anti-inflammatory) (120), although the effect of UC-MSCs on M1 was inhibited when UC-MSCs were modified, removing from their surface extracellular matrix, dermatan sulfate, and hyaluronan (120). In addition, currently, it is known that the presence of extracellular matrix and hyaluronan in the surface of UC- MSCs is vital for these MSCs to suppress inflammation and escape host rejection when they were transplanted in a model of epithelial corneal alkali burn (120). Another action that the hyaluronan of MSCs can regulate is the induction of regulatory T cells (120,121). In conclusion, currently it is suggested that UC-MSC glycocalyx play an important role in host immune modulation.

Effect of immunomodulatory properties of MSCs in corneal transplantation

Currently, MSCs have been widely used as immunotherapy in solid organ transplantation. Over time, several authors have reported that MSCs show potential therapeutic effect for corneal allograft transplantation, decreasing the immune rejection and promoting corneal allograft survival (82). The first study developed in this field was carried out in 2009, supporting that the survival of corneal xenografts was not significantly prolonged by rat commercial MSC application (122). However, some years later, a research group found that intravenous injection of rat BM-MSCs prolonged corneal allograft survival when cells were administered immediately after transplantation (123), while preoperative infusion was ineffective, in accordance with the results previously reported (122,123). In this work, when mononuclear cells were isolated from the spleen and lymph nodes of both MSC-treated and vehicle-treated rats, previously transplanted with allogenic cornea, the proliferation of T cells from MSC-treated rats was significantly reduced compared with T cells from vehicle-treated postoperative rats, suggesting that MSCs may prevent rejection at least in part by reducing effector T cell proliferation *in vivo*. It was also observed that media obtained from T lymphocytes isolated from rats treated postoperatively with MSCs showed a decrease quantity of Th1 pro-inflammatory cytokines and elevated IL-4 secretion. In addition, Treg were upregulated in mononuclear cultures isolated from rats treated with MSCs. Consequently, it was suggested that MSCs can decrease corneal allograft rejection, postulating that this effect could be mediated by immunomodulatory reactions, as inhibition of pathogenic T cell responses, an anti-inflammatory shift in the Th1/Th2 balance, and activation of Treg cells (123). In addition, currently, it is known that intravenous injected mice BM-MSCs to allografted mice recipients, 3 h after surgery, increase allograft corneal survival by inhibiting antigen-presenting cell (APC) maturation and induction of alloreactive T cells (119,124). On the other hand, the ability of rat BM-MSCs from three distinct sources to prolong rat corneal allograft survival has been tested. MSCs obtained from same strain of recipient (syngeneic

transplant, syn-MSCs), from same strain of donor (Allo-MSCs) or from a third-type strain (third-MSCs) were injected intravenously in rat recipient. A high percentage of untreated and syn-MSC-treated allografts were rejected (80% and 100%, respectively). Conversely, corneal allograft survival was significantly prolonged in allo-MSC (90%)- and third-MSC (80%)-treated allograft recipients (125). Fact that could be due to inferior number of corneal natural killer T cells coupled with a higher proportion of splenic regulatory T cells showed in animals treated with allo- or third strain- MSCs compared to animal corneas treated with syn-MSCs. It was also shown a decrease of CD4 T and B cell activated in corneas from animals receiving either of the MSC types compared with untreated animals (125). All these results induce to conclude that the effect of MSCs on inhibiting the immune rejection response can depend of MSC source.

However, in spite of the data previously reported, the *in vivo* effect of MSCs on inhibiting the immune rejection response is controversial (82), and it is known that other parameters, as the moment of MSC application with respect to tissue transplant or the total cell doses applied, can play an important role about the effect of MSCs on corneal transplantation (82,93). When the effect of local and systemic injection of human AT-MSCs into two rabbit models of corneal allograft rejection (normal- and high-risk model) was studied, in contrast to the expectation, it was observed that local injection of AT-MSCs, at the time of surgery, induced an increase of inflammation, corneal edema, and higher levels of infiltration of lymphocytes in both models, inducing a decrease in graft survival (126). This results could not be explained by to the use of human MSCs could create a xenogeneic environment, because the results were similar when transplanted MSCs were isolated from human or rabbit. It has been speculated that MSCs could be recognized by the adaptive immune system (127). In the current scenario in which an inflammatory environment is already established, the secretion of IL-6 and IL-8 by AT-MSCs can exacerbate the chemotaxis of leukocytes to the injected

zone, thus increasing inflammation. In this case, the action of pro-inflammatory cytokines IL-6 and IL-8 seems to be higher than the immunosuppressive effects of IDO and NO also secreted by AT-MSCs (126).

In conclusion, although the immunomodulatory effect of MSCs has been shown in repeated occasions and some action mechanisms are known at the moment (Table 17. 2), more research is necessary in order to clarify all immunomodulatory actions that MSCs can perform in damaged corneal epithelia.

3.1.4. Tissue homing capacity

Currently, it is known that MSCs have capacity to migrate specifically to inflamed tissues, in accordance with general the premise that injury and inflammation induce stem cell mobilization, migration, and colonization (82,128). Although the tissue homing capacity of MSCs has been robustly demonstrated by several authors along the time (55,82,83,129–133), at the moment, the mechanisms involved in the regulation of MSC homing capacity still remain unclear, while it is thought that this property is mainly regulated by growth factors and/or chemokines expressed by damaged tissues and by extracellular matrix receptors expressed on the surface of MSCs (55,82,134). For example, the interactions of stromal cell-derived factor 1/C-X-C motif chemokine 12 (SDF-1α/CXCL12) and C-X-C chemokine receptor type 4 (CXCR4) directly mediate the trafficking of transplanted MSCs, while the presence of inflammatory cytokines as platelet-derived growth factor (PDGF), TGF-β1, TNF-α, and SDF-1α/CXCL12 increases MSC motility (83,135–137). One of the several tissues where MSCs can migrate is to the inflamed corneal epithelium; due to the fact that damaged corneal epithelium expresses specific chemoattractants that can mobilize MSCs to this region. BM-MSC migration into peripheral blood, bone marrow, and cornea was monitored after intravenous administration in a rabbit model of alkali burn, observing that successful engraftment of MSCs occurred in all treated animals, although different cell migration

patterns were observed. When MSCs were administrated to no immunosuppressed animals, MSCs rapidly migrated to bone marrow and corneo-limbal wound (epithelium, stroma, and corneal endothelium), although, subsequently, the presence of MSCs decreased over time. However, when MSCs were transplanted in immunosuppressed animals, MSCs slowly migrated to bone marrow. In this case, MSCs remained long time in peripheral blood, and presence of MSCs in cornea, mainly in stomal area, slowly increased over time, observing high infiltration of MSCs after one month. Consequently, currently it is known that bone marrow state plays an important role in BM-MSC migration capacity when these cells are systematically injected. Other experiments showed that intravenously injected mice BM-MSCs, in a mouse model of corneal epithelial thermal cauterization, could migrate specifically to the injured corneas, persisting MSC infiltration at least for 50 days, while MSCs did not migrate into healthy contralateral corneas (138). In addition, it was shown that when exogenous MSCs were not applied in damaged animals, increased levels of endogenous MSCs were found in the peripheral blood 48 h after the stimulus of corneal injury, a fact that correlated with the presence in blood circulation and corneal damaged tissues of high levels of SDF-1/CXCL12 factor and substance P molecules which reported to possess stem cell chemoattractant properties (139–141). It was speculated that the injured corneal tissue was the source of these MSC chemoattractants (138). Recently, a new study has supportted the important role of SDF-1/CXCL12 in corneal epithelium healing when MSCs are implicated in this repair function. In an *in vitro* model of wound healing, in which a scratch was made through rat limbal MSC monolayer, the presence of SDF-1/CXCL12 stimulated MSC migration from wound edge to damaged culture area in order to repair cell monolayer (89). In another study in which systemically MSC administration was used as therapy for the treatment of corneal damage, specifically in corneal transplantation, it was shown that BM-

MSCs, intravenously administrated, preferentially home to the inflamed ocular surface and draining lymph nodes, while these cells did not migrate to the healthy corneas (124).

Some authors have suggested that after systemic MSC administration, most of the transplanted cells migrate to the main organs, such as the lung, liver, kidney, and spleen, and, therefore, MSCs have lower capacity to migrate to areas with tissue damage (82,142), as the damaged corneal epithelium. Consecutively, some researchers began to perform topical MSC transplantations. Some years ago, rat BM-MSC migration to damaged corneal epithelium after topical application was evaluated, finding MSCs in corneal epithelium three weeks after the injury. This fact support that MSCs were successfully engrafted underneath the epithelial cell layers up to 3 weeks after topical application (66). The migration capacity of topically administrated MSCs to injured corneal epithelium was also shown by another research group, who administrated human AT-MSCs in suspension (topical drops) in a rat model of corneal burn (143). On the other hand, when human MSCs obtained from amniotic membrane were administrated subconjunctivally on damaged corneal epithelia of rabbits, it was observed that MSCs migrated to limbal-corneal tissues at day 28 after administration (144). In addition, human BM-MSCs in damaged corneal tissue of mice after its subconjunctival administration could migrate to corneal epithelium, in which MSCs appeared at different epithelial layers. In addition, these cells showed different morphologies from cubic to flattened, depending on their position within the tissue (30). Also, when MSCs were cultured on amniotic membrane, with or without keratinocyte growth factor-2 (KGF-2) and/or with human serum, and this cell-substratum complex was transplanted on rat with damaged corneal epithelium, MSCs generally could migrate to injured cornea (145). In a recently developed study, in which human AT-MSCs cultured on amniotic membrane were transplanted on rabbits in which a partial or a total limbal stem cell deficiency had been previously induced, AT-MSCs could migrate from amniotic membrane to ocular surface, since MSCs were found in the inflamed

areas of the superior and inferior limbal stroma and corneal 8 weeks after transplantation (18). This cell migration could be regulated by the signaling pathway mediated by SDF-1/CXCL12 and its receptor CXCR4 expressed by MSCs (146). However, MSC migration to inflamed tissues has not been supported by all studies performed in this area (50,51).

At the moment, cell mechanisms involved in MSC migration to damaged tissues remain unclear, as well as the different physiological and pathological situations that regulate MSC migration (82); although in general, results reported by several authors suggested that MSCs have the capacity to migrate to injured corneal epithelium, enhancing epithelial wound healing and reducing corneal opacification and neovascularization, independently of the administration route. In conclusion, scientific community accepts that homing capacity of MSCs, together with their tropic and immunomodulatory action, plays an important role in corneal epithelium regeneration.

2.2. Extraocular MSCs in experimental models of corneal epithelial damage

2.2.1. Experimental models of corneal epithelial damage

In order to study different potential therapies to treat diseases, it is necessary to deepen in the knowledge and the understanding of diseases. Experimental models represent one of the best tools that help to accomplish this goal.

There are a lot of different kinds of experimental models of corneal epithelial damage with diverse characteristics, such as the size of the affected area in the cornea and/or the limbus, the severity of the model, and the methodology used to develop it, among others.

Experimental models with central corneal damage but without limbal injury are considered corneal burn or corneal injury models. Corneal burn models are induced by a chemical agent, whereas corneal injury is induced by mechanical damage. Experimental models that include limbal damage are designated as LSCD models. The most used agent during the induction of

both corneal burn and LSCD models is sodium hydroxide (NaOH). However, ethanol, n-heptanol, and mechanical scraping have been also used to induce corneal epithelial damage. There are several studies that describe the use of MSCs to treat corneal epithelial injuries using different experimental models and methodology. One of the principal factors that could condition the results of these works is the administration route: systemically (intravenous or intraperitoneal), using different carriers (amniotic membrane, fibrin, biopolymers, etc.), topically, or even by subconjunctival injection. In addition, some researchers have studied the therapeutic effect of endogenous MSCs in the ocular surface after induced corneal epithelial damage.

2.2.2. Mobilization of endogenous MSCs

Different researchers have demonstrated that endogenous MSCs can carry out a therapeutic effect in the cornea when a corneal epithelial damage has been induced. In a partial LSCD model, endogenous MSCs could reduce the epithelial corneal defects and opacity in rabbits with normal bone marrow function (147), while this did not happen in bone marrow-suppressed rabbits. Furthermore, MSCs and hematopoietic stem cells were able to migrate from bone marrow to the injured cornea in rabbits with competent bone marrow, but it did not occur in the bone marrow-suppressed animals (147). Additionally, a significant increment in the number of circulating endogenous MSCs was observed in animals with a partial LSCD (138). This increment in circulating MSCs was accompanied by an increase of SDF-1/CXCL12 and substance P levels both in peripheral blood and in corneal tissue. These factors then contribute to the mobilization of the endogenous cells (138). Therefore, these data demonstrate that endogenous MSCs can migrate from the bone marrow to the cornea in order to trigger their therapeutic effect in corneal epithelial damage.

3.2.3. Intravenous administration of MSCs

There is evidence that MSCs administered by intravenous injection can help during the wound healing process and reduce the clinical signs of the corneal epithelial damage in different experimental models. BM-MSCs intravenously administered reduced significantly the epithelial defects in corneal injury and partial LSCD models (19,138). Furthermore, the neovascularization developed in a partial LSCD model was also reduced by the intravenous administration of BM-MSCs (37). In addition, corneal opacity has been widely studied in experimental models of corneal epithelial damage, showing that intravenous MSCs were able to diminish it in both corneal injury and LSCD models (19,37,69,148). Some of these studies showed that MSCs effectively reduce corneal opacity in corneal epithelial damage, but their effect was variable depending on the cell donor; MSCs from some donors were more efficient at reducing corneal opacity than cells from other donors (148). Additionally, intravenous induced pluripotent stem cells (iPSC)-derived MSCs (iPSC-MSCs) could also reduce corneal opacity in a LSCD model (149). Therefore, all these data together indicate that the intravenous administration of MSCs in experimental models of corneal epithelial damage can contribute to the reduction of clinical signs. However, MSCs administration in those studies was performed in the acute phase of the corneal epithelial damage. This fact could contribute to restrain the progression of the damage in an early phase. In contrast, there are a lot of patients suffering LSCD or corneal epithelial damage in a chronic phase; hence it would be necessary to study the effect of the MSCs intravenously administered in corneal epithelial damage or LSCD models well established in a chronic phase.

To trigger the therapeutic effect of MSCs in the corneal epithelium when they are intravenously administered, it would be necessary that the cells migrate from the peripheral blood to the injured cornea or that they carry out their actions from the distance. Some authors observed the presence of intravenously administered MSCs in the injured cornea (37,138,150). Furthermore, they also described that the systemic administered cells were

found in the peripheral blood and migrated to the bone marrow (37). Although some authors advocate the migration of the MSCs intravenously administered to the injured cornea, others have located fewer than 10 iPSC-MSCs in the cornea (149) or have not found data that supports the migration of MSCs (69). In this case, the authors postulated that the therapeutic effect of the MSCs was produced from the distance (69).

The anti-inflammatory properties of MSCs have been widely described in many tissues, such as the cornea. This anti-inflammatory effect could be beneficial for the regeneration of the corneal epithelium after damage. In this regard, some authors have reported the anti-inflammatory role of the MSCs after their intravenous administration in LSCD models. Less infiltration of inflammatory cells in the corneal stroma and less disorganized cornea were observed when BM-MSCs were intravenously administered in LSCD models (150). Furthermore, the level of myeloperoxidase (MPO), a molecule released by activated neutrophils, was lower in the injured corneas treated with intravenous BM-MSCs than in the untreated corneas (69,148), indicating less infiltration of neutrophils in the cornea of the treated animals (149). Nevertheless, a variable effect on the BM-MSC capacity to reduce the MPO levels has been observed depending on the donor of the cells (148). Moreover, a direct relation between the level of TSG-6 expression in the BM-MSCs and the capacity of these cells to reduce MPO has been described (148). In addition, the expression of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, CCL2, CCL3, and CCL4 was reduced in the corneas with LSCD and treated with MSCs (69,149). Also the expression of tenascin C, a proangiogenic and profibrotic molecule, was reduced in the corneas administered with MSCs (69). Additionally, the anti-inflammatory factors TGF-β and IL-1Ra grew up in the corneal tissues after the intravenous administration of BM-MSCs in LSCD models (138). Thus, MSCs seem to have the capacity to release these anti-inflammatory factors in the injury site. Furthermore, in order to demonstrate if the therapeutic effect of MSCs is mediated by TSG-6,

some authors knocked down the expression of TSG-6 in MSCs, and these cells were intravenously administered without showing therapeutic effect in the cornea (69,149). These data could indicate that the TSG-6 is responsible for the beneficial effect of the MSCs in the cornea. With all these data, we can conclude that the administration of intravenous MSCs promotes an anti-inflammatory effect in the corneal epithelium when it has been damaged. Furthermore, this effect seems to be induced to a large degree by the factor TSG-6, secreted by MSCs.

Regarding corneal epithelium phenotype, there are data to support that MSCs can help to the progenitor residence cells to recover their phenotype and function through the release of trophic and paracrine factors. The expression of the stem cell markers ABCG2, p63, Hes1, and C/EBPδ, as well as the epithelial cell markers CK12 and CK19, increased in the corneas of partial LSCD models treated with intravenous BM-MSCs in comparison with the non-administered animals (37,138). In addition, the cell proliferative activity marker proliferating cell nuclear antigen (PCNA) showed its expression not only in the basal limbus but also in the peripheral corneal epithelium in the MSC-treated group (37). Therefore, MSCs promote a positive effect in the recovery of the phenotype of the corneal and limbal cells after a corneal epithelial damage.

2.2.4. Intraperitoneal administration of MSCs

The knowledge about the effect of MSCs intraperitoneally administered in experimental models of corneal epithelial damage is limited. As in the case of the intravenous administration, a decrease in corneal opacity and MPO levels was observed after intraperitoneal administration of MSCs in a rat LSCD model (69). The expression of pro-inflammatory cytokines and chemokines IL-1β, CXCL1, CCL2, CCL3, and CCL4 as well as tenascin C was also decreased in the group with intraperitoneal administration of MSCs. As in the intravenous administration, the MSCs administered intraperitoneally were not detected in

the cornea (69). Therefore, the therapeutic effect of the MSCs was produced from the distance. However, IL-17 levels in corneal tissues were lower in a mice model of LSCD treated with intraperitoneal BM-MSCs in comparison with untreated animals (151). Thus, the authors concluded that BM-MSCs have an anti-inflammatory effect on the IL-17 secreting cells by blocking both non-Th17 cells and Th17 cells (151). These data demonstrate that the MSCs intraperitoneally administered have an anti-inflammatory effect in corneal epithelial damage. However, there is no data to support the migration from the peritoneal area to the damage cornea, so their effect could be produced from the distance. It is important to note that, as in the case of the intravenous administration, all the studies with MSCs intraperitoneally administered were performed in an acute phase of the epithelial damage, and this fact could condition the results. Therefore, further studies applying the cells in a chronic phase of the corneal epithelial damage or in chronic LSCD models are necessary to deepen on the knowledge of the beneficial effects of the MSCs systemically administered.

2.2.5. Ocular topical administration of MSCs

Topical administration is the easiest way to apply a drug on the ocular surface; however, it has some drawbacks, such as low retention time, high washing rate, and low permeability of the corneal epithelium, among others. The administration of topical AT-MSCs in corneal burn models reduced the epithelial defects similarly to the effect of the treatment with topical serum (143). The lack of differences in the therapeutic effect between the serum group and the AT-MSC-treated group could represent a drawback. However, the mildness of the corneal burn model, along with the small number of animals and the short follow-up time of the study, could have contributed to the lack of differences between the treatment groups. In addition, the corneas of a LSCD model topically treated with BM-MSCs did not show positive healing results (80). Thus, the rapid washing away of the topically administered BM-MSCs to the ocular surface could have avoided their therapeutic action. In contrast,

neovascularization and corneal opacity decreased in the corneas treated with BM-MSCs or with BM-MSCs conditioned medium in a rat LSCD model, mainly in the BM-MSC-treated group (66). Additionally, whereas the anti-angiogenic factor TSP-1 increased, the pro-angiogenic factor MMP-2 decreased only in the BM-MSC-treated corneas (66). All these data indicate a poor beneficial effect of the MSCs topically administered on the clinical signs of a corneal epithelial damage.

AT-MSCs topically administered were found in the corneal epithelium and stroma of a corneal burn model developed in rat (143). Therefore, cells were able to migrate from the corneal surface to deeper layers of the corneal epithelium and stroma. However, this migration experiment was only performed in both eyes of one rat; more data should be obtained from more animals to draw any valid conclusions.

The inflammation of the corneal tissue was lower in corneal burn and LSCD models treated with AT-MSCs or BM-MSCs than in other experimental groups (66,143). In addition, the expression of the pro-inflammatory factors IL-2 and IFN-γ decreased in the corneas treated with BM-MSCs. However, the expression of the anti-inflammatory factors IL-10 and TGF-β increased in the BM-MSC-treated corneas. Although, the level of IL-6 was mild, it was higher in the BM-MSC-treated group than in the vehicle-treated group (66). This data point out that IL-6 could mediate the immunomodulatory action of MSCs. In general, BM-MSCs applied topically to the damaged corneas for 2 h showed better results than conditioned medium administration. It could indicate that cell-to-cell contact has additive effects to the action of soluble factors or that the continuous secretion of factors induces better outcomes in the administration of these factors by the conditioned medium. These data along with the poor therapeutic effect of the topical MSCs observed in the clinical signs could indicate that the low retention time, the high washing rate, and the low permeability of the corneal epithelium are responsible for these results. Furthermore, there are not enough data about the migration

capacity of the MSCs topically administered to the ocular surface; thus, further investigations in this field are necessary.

2.2.6. MSCs seeded on amniotic membrane

The AM is the innermost layer of the placenta, and it is increasingly used for ocular surface reconstruction in a variety of ocular pathologies, even as carrier for the *ex vivo* expansion of stem cells in order to treat corneal epithelial damage or LSCD. AM have several beneficial properties, such as low immunogenicity, stimulation of the epithelialization, and anti-fibrotic, anti-inflammatory, anti-angiogenic, and antimicrobial effects (152). In this field, the AM has widely used as carrier to transplant MSCs to the ocular surface of experimental models with corneal epithelial damage. The improvement of epithelial defects, neovascularization, and corneal opacity was observed in LSCD models after the transplantation of MSCs seeded on AM to the ocular surface (18,28,33,36,145). Additionally, the expression of the pro-angiogenic factor MMP-2 was reduced in the corneas treated with BM-MSCs (36). Furthermore, in order to check visual function of the rats, a head tracking method showed that the animals transplanted with BM-MSCs had better visual capacity than the untreated groups (36). Nevertheless, transplantation of BM-MSCs on AM did not show significant improvement of the clinical signs in a corneal burn model, whereas BM-MSCs subconjunctivally injected showed therapeutic action (50). However, it is important to note that the cell dose administered by subconjunctival injection was higher than the cell dose seeded on the AM, thus it could have influenced the results. In general, the transplantation of MSCs seeded on AM to the ocular surface of experimental models with corneal epithelial damage not only improves the wound healing process but also reduces the neovascularization and corneal opacity.

Some authors have demonstrated that MSCs migrate from AM to corneal tissues in experimental models of corneal epithelial damage (18,36,145). Therefore, MSCs transplanted

to the ocular surface using AM as carrier are able to migrate to the corneal tissues and exert its beneficial effect there.

The anti-inflammatory properties of the MSCs have been also confirmed when they are transplanted on AM to the ocular surface of experimental models with corneal epithelial damage. Less inflammatory cells and less disorganization of the cornea were observed in LSCD models after the MSC transplantation using AM as carrier (18,28,33). In addition, the expression of the leukocyte marker CD45 and the pro-inflammatory cytokine IL-2 was significantly lower in eyes transplanted with BM-MSCs (36). All these results indicate that the MSCs seeded on AM and transplanted to the ocular surface can reduce the infiltration of inflammatory cells in the experimental models of corneal epithelial damage. However, the mechanism that triggers the anti-inflammatory action of the MSCs transplanted on AM to the ocular surface has not been deeply studied.

Regarding corneal markers, the recovery of the corneal epithelial markers CK3 and E-cadherin in damaged corneas has been demonstrated after the transplantation of AT-MSCs or BM-MSCs seeded on AM (18,28). Nevertheless, other authors did not find the expression of the corneal epithelial markers CK3, CK12, and panCK in the epithelium of the BM-MSCs treated eyes (33,36). Therefore, they postulated that the therapeutic effect of BM-MSCs may not come from differentiation of these cells into corneal epithelial cells. Moreover, the expression of the limbal stem cell markers CK15 and p63 was partially restored in the limbal epithelium of the AT-MSC-treated eyes (18,28). Additionally, the rats transplanted with BM-MSCs showed positive expression of CK19 in the limbal epithelium, while the untreated rats did not express this marker (145). However, the location of the CK19 marker is controversial because its expression has been described not only in the limbal epithelium but also in the conjunctival epithelium (153). These works altogether show poor evidence of MSCs having

effect in the recovery of the corneal and limbal phenotypes. Therefore, further works are necessary to elucidate this issue.

In the case of MSC transplantation using AM as carrier, the cell dose is lower than in the administration by other routes. Furthermore, the dose of the transplanted MSCs is not totally controlled in several of the studies performed using AM as carrier, because some authors seed a number of cells on the AM, but then they keep them in culture for some days. Additionally, MSCs seeded on AM are administered in a less acute phase of the disease than with other administration ways; this fact could help to know the effect of the MSCs in a chronic phase of the corneal epithelial damage.

2.2.7. Amniotic membrane pocket

AM has also been used in order to create a pocket in which BM-MSCs were administered in a LSCD model developed in rabbits (32). However, there were no differences in clinical signs among the different experimental groups (32). The corneal epithelial markers CK3/12 were more strongly expressed in the corneas of the BM-MSC-treated group than in those of the untreated group. Moreover, the expression level of the epithelial marker Cx43 and the limbal stem cell markers ABCG2 and β1-integrin was higher in the cornea of the BM-MSCs-treated group than in the untreated group (32).

2.2.8. MSCs seeded on fibrin matrix

Fibrin gel is prepared from fibrinogen and thrombin, obtaining a matrix able to house cells and suitable for transplantation. The transplantation of fibrin matrix could reconstruct the corneal epithelium in a LSCD model not only when it contained BM-MSCs but also without cells (31). Furthermore, BM-MSCs were located in the corneal epithelium 4 weeks after transplantation. In addition, the expression of the corneal epithelial marker CK3 was observed in both experimental groups; however the expression was more regular and continuous in the cornea treated with BM-MSCs. In addition, some of the BM-MSCs transplanted to the ocular

surface expressed CK3 (31). However, other authors have described that MSCs cultured in normal conditions express CK3 (32,40); thus the expression of CK3 in the transplanted BM-MSCs might not be indicating a real differentiation of MSCs into corneal epithelium.

2.2.9. Contact lens carriers

Other carriers used to transplant MSCs to the ocular surface are contact lenses. The effect of human AT-MSCs seeded on scleral contact lenses applied over the cornea of a LSCD model has been studied (154). Epithelial defects, neovascularization, and corneal opacity were reduced in the animals treated with AT-MSCs in contrast to the group with contact lenses without cells. The authors also observed less disorganization and fewer inflammatory cells in the stroma of the AT-MSC-treated corneas (154). Thus, contact lenses could be an adequate substrate to seed stem cells and to transplant them to the ocular surface in order to treat corneal epithelial damage.

2.2.10. Poly-L-lactic acid carriers

Biopolymers are one of the alternative carriers that have been investigated in order to find reproducible substrates that allow cell adhesion and proliferation, as well as the re-epithelialization of the ocular surface when they are transplanted to the ocular surface of experimental models with corneal epithelial damage. The most studied biopolymer in this kind of experimental model is based on poly-L-lactic acid. Epithelial defects, neovascularization, corneal opacity, and corneal thickness showed a reduction in corneal burn and LSCD models transplanted with poly-L-lactic acid carriers containing MSCs both BM-MSCs and AT-MSCs (60,61,80,155). However, AT-MSCs showed less pronounced therapeutic effect in LSCD models than BM-MSCs (60,61). In addition, the expression of the pro-angiogenic factor VEGF was also reduced in the BM-MSC-treated corneas (60,61,80,155). All these data demonstrate that MSCs seeded on poly-L-lactic acid nanofibers and transplanted to the ocular surface can mitigate the clinical signs in experimental models

of corneal epithelial damage. Furthermore, BM-MSCs show higher therapeutic effect than AT-MSCs when they are transplanted using poly-L-lactic acid carriers.

There are no data about the migration capacity of the MSCs from the poly-L-lactic acid carriers to the ocular surface. It would be very interesting to perform further experiments to elucidate the migration of the cells transplanted in this kind of substrates.

Regarding corneal inflammation, MSCs transplanted to the ocular surface on poly-L-lactic acid carriers were able to decrease the presence of CD3+ cells and macrophages in the corneal tissues of LSCD models (60,80). Furthermore, the expression of the pro-inflammatory cytokines TGF-β, IL-6, IL-8, IL-1β, IL-2, and IFN-γ also decreased in the corneas with epithelial damage and treated with BM-MSCs seeded on poly-L-lactic acid carriers (61,80,155). On the other hand, the increment of the oxidative stress in corneal epithelial damage has been demonstrated (80). In this regard, the BM-MSCs seeded on nanofibers reduced the expression of the oxidative stress markers iNOS, caspase-3, nitrotyrosine (NT), and malondialdehyde (MDA), and on the other hand promoted the increment of the protective enzyme aldehyde dehydrogenase 3 family member A1 (ALDH3A1) (60,61,80,155). Furthermore, the expression of XOX and the levels of the antioxidants SOD, glutathione peroxidase (GPX), and catalase (CAT) were similar between the BM-MSC-treated corneas and the healthy control corneas, while SOD, CAT, and GPX showed lower levels in AT-MSC-treated corneas than healthy corneas (61). Moreover, in the injured corneas treated with BM-MSCs the expression of MMP-9 was reduced or suppressed (61,80,155). Therefore, all these results demonstrate the anti-inflammatory properties of MSCs, as well as their beneficial role against the oxidative stress produced in a corneal epithelial damage. Nevertheless, the treatment was applied always in an acute phase of the damage. In this regard, it has been described that the sooner the treatment is applied the better the healing is, mainly in an inflammatory and oxidative environment. However, in the clinical setting there

are a lot of patients in a chronic phase of the disease, and then it is necessary to know the behavior of these cells in those patients. Therefore, further experiments in a chronic phase of corneal damage are necessary to overcome this issue. In addition, as in the AM experiments, the number of stem cells that can be transplanted using this kind of substrates is very low in contrast to the cell dose that can be administered by injection. It is important to note that the cell dose transplanted in these carriers to the ocular surface was not totally controlled because the cells were kept in culture for some days before transplantation.

Regarding corneal phenotype, the expression of the corneal epithelial markers CK3 and CK12 increased in the MSC-treated groups, mainly in the BM-MSC-treated group (60).

2.2.11. Polyamide 6/12 carriers

Polyamide nanofiber scaffolds containing LESCs + BM-MSCs have been transplanted to the ocular surface of a LSCD model (118). A decrease of the iNOS, IL-2, and IFN-γ levels was observed in the treated corneas in comparison with the untreated eyes (118). This anti-inflammatory effect could be originated by both cell types, or just by one of them, but the design of the study does not allow to know.

2.2.12. Subconjunctival injection of MSCs

Subconjunctival injection is a technique widely used in ophthalmology. Although this way of administration allows to apply a local treatment with a high cell dose, the volume administered have to be low. A reduction in epithelial defects, neovascularization and corneal opacity has been observed after subconjunctival administration of MSCs in corneal burn experimental models (50,51,156,157). Furthermore, BM-MSCs administered by subconjunctival injection immediately after injury produced a decrease of the epithelial defects in the diabetic mice, showing similar reepithelialization rate of the non-diabetic mice (65). It has also been demonstrated that the expression of the pro-angiogenic factors VEGF and MMP-9 is lower in the BM-MSC-treated corneas, whereas the anti-angiogenic TSP-1

factor increase its expression in the corneas of the BM-MSC-treated groups (50,51,156). Therefore, MSCs, both BM-MSCs and AT-MSCs, subconjunctivally administered have a positive effect in the reduction of the clinical signs in experimental models with corneal epithelial damage.

In regard to the migration of the MSCs subconjunctivally administered, although some authors have located the BM-MSCs in the wound edge of the cornea (65), others located the BM-MSCs in the injection site but not in the cornea (50,51). These data seem to indicate that the MSCs subconjunctivally administered tent to keep in the injection site and do not migrate to the central cornea. Nonetheless, the therapeutic effect of the cells has been demonstrated, thus the MSCs trigger their beneficial role from the injection site without migrating to the damaged area.

The MSCs subconjunctivally administered also showed an anti-inflammatory role in the ocular surface. Less infiltration of inflammatory cells, macrophages (CD68+ cells), and leukocytes (CD45+ cells) was observed in the MSC-treated eyes of experimental models with corneal epithelial damage (50,51,65). In addition, the expression of MPO was lower in the BM-MSC-treated corneas than in the PBS-treated corneas (65). Furthermore, the expression of the chemotactic factor MIP-1α, the monocyte chemotactic protein-1 (MCP-1), the pro-inflammatory cytokines TNF-α and IL-1β, and the protein MMP-2 was lower in the corneas of the BM-MSC-treated groups in contrast to the untreated groups (51,65,156). Additionally, the anti-inflammatory factor TGF-β showed an increment in the BM-MSC-treated corneas (156). Moreover, the expression of TSG-6 was elevated in the corneas treated with BM-MSCs, whereas no changes were observed in other factors that could be also related with the immunomodulatory effect of MSCs (65). In addition, BM-MSCs knocked down for TSG-6 did not show therapeutic effect in the diabetic corneas with epithelial damage (65). Therefore,

TSG-6 seems to be one of the mediators of the therapeutic effect of the MSCs when they are subconjunctivally injected into a damaged cornea.

An increase in the expression of the epithelial markers Cx43 and β-catenin in the AT-MSC-treated corneas was described, whereas differences were not seen in the epithelial marker E-cadherin and in the limbal stem cell marker p63 (157). Therefore, the role of the MSCs subconjunctivally administered in the recovery of the corneal and limbal phenotypes is not yet clear.

2.2.13. Combined administration of MSCs

AT-MSCs have also been administered to the cornea of a partial LSCD model using combined routes: topically, subconjunctivally and in a stromal pocket (159). The AT-MSC-treated group showed attenuated epithelial defects, neovascularization, and cornea opacity in contrast with the untreated corneas. The expression of the proliferation marker Ki67 increased in the AT-MSC-treated corneas, whereas a reduction of the secreted VEGF factor was observed in these corneas. Twenty-eight days after damage and treatment, the AT-MSCs administered were found in the cornea. In regard to inflammation, the authors noted inflammatory cells in the untreated corneas, while the animals treated with AT-MSCs presented a normal corneal stroma without inflammation. Furthermore, the indicator of damage serum_glutamic-pyruvic transaminase (SGPT) was reduced in the corneas treated with AT-MSCs. Therefore, the combined administration of MSCs, using three local applications, is effective in the treatment of corneal epithelial damage in experimental models.

In conclusion, both BM-MSCs and AT-MSCs have shown therapeutic effects at reducing clinical signs in corneal epithelial damage or LSCD experimental models using different administration routes. Nevertheless, all of these ways of administration have limitations; the intravenous or intraperitoneal administrations inject the MSCs very far from the cornea, the transplantation of MSCs on carriers do not allow high cell dose, in the topical administration

the cells are washed away very quickly, and the volume administered by subconjunctival injection has to be low. Despite these drawbacks, all these administration routes of MSCs are effective in the treatment of experimental corneal epithelial damage. Additionally, migration of MSCs from the application site to the cornea has been confirmed using intravenous and intraperitoneal injection or transplantation of the cells on a carrier. However, subconjunctival injection does not allow the migration of the cells to the central cornea, and further experiments are needed to know the migration of topically administered MSCs. Furthermore, the anti-inflammatory properties of these cells, as well as their beneficial role against the oxidative stress triggered by the epithelial corneal damage, have been demonstrated. In addition, one of the principal factors implicated in the anti-inflammatory effect of MSCs is TSG-6. Although some authors advocate the transdifferentiation of extraocular MSCs to corneal epithelial cells, only one of them have demonstrated that MSCs expressed CK3 in the corneal epithelium when they were transplanted to the ocular surface of an experimental corneal epithelial damage (31). But since some authors have shown that MSCs express CK3 in basal conditions (18,32), the CK3 expression might not indicate a real transdifferentiation. Therefore, transdifferentiation of MSCs into corneal epithelial cells has not be confirmed *in vivo*, but all the results seem to indicate that MSCs contribute to the recovery of the corneal epithelium by secreting factors that act at a paracrine level promoting proliferation and differentiation of the resident stem cells in the tissues.

2.3. Extraocular MSCs for corneal epithelial damage in humans

MSCs have been used successfully to repair and regenerate tissues (160,161). In the 1980s, it was concluded that MSCs could differentiate into osteoblasts, chondrocytes, adipocytes, and myoblasts, and, consequently, they have been used to repair infracted myocardium (162), in hepatic cirrhosis (163), in gastrointestinal cancer (164), in periodontal therapy (165), or for bone deformities and fractures (166). It was also demonstrated that these cells could

contribute to the regeneration of ectodermal tissues, such as bronchial and alveolar epithelial cells (167), glomerular and tubular cells, or gastrointestinal cells (168). This process of differentiation will depend on induced and control factors that cohabit in the environment where they are located (169).

In the field of ophthalmology and vision-related therapies, some approaches have been made using MSCs, mainly at the ocular surface. But even though there are publications using these cells in animal models (10,17–19,60,82), there is practically no studies in the clinical area.

MSCs have been used to treat the deleterious consequences of chronic GvHD, one of the most dreaded complications of otherwise successful allogeneic hematopoietic stem cell transplantation to treat hematological malignancies (170,171). Some years ago, a study published the beneficial effect of intravenous injection of MSCs to treat the severe ocular surface involvement that often occurs in this disease. This study described improvement in clinical dry eye scores in 54.55% of the 22 patients enrolled, also accompanied by in increased levels of regulatory $CD8^{+}CD28^{-}$lumphocytes but not $CD4^{+}CD25^{+}$, which led authors to conclude that the immunomodulatory effect by which MSC transfusion improved the refractory dry eye disease could be due to regulating the Th1/Th2 balance by triggering the generation of $CD8^{+}CD28^{-}$T lymphocytes (171).

There is also a single case report in which a patient suffering from keratoconus and a persistent corneal epithelial defect unresponsive to conventional therapy was treated by topical administration of adipose tissue-derived MSCs, achieving corneal epithelial healing after 1 month (172).

MSCs have been experimentally used to treat not only the acute phase of ocular chemical burns (173) but also the chronic devastating consequences of severe ocular chemical injuries. To this later aim, there exists now enough experimental evidence showing that MSCs are beneficial in dealing with the chronic sequelae of chemical injuries, helping to provide

corneal epithelium with the lost regenerative capacity due to the destruction of the limbal stem cells and usually their niche entirely (10,17–19). But, although MSCs are shown useful in animal models of LSCD, they have not been yet fully approved to clinical use, as there is only one clinical trial on the use of MSCs for LSCD in humans. This exploratory proof-of-concept clinical trial, performed in our institution (Calonge M et al. Invest. Ophthalmol. Vis. Sci. 2017;58(8):3372. ARVO 2017 Annual Meeting), explored the safety and efficacy of bone marrow-derived MSCs for bilateral LSCD of any etiology in which the more widely accepted autologous cultivated limbal epithelial transplantation (CLET) was not feasible due to the complete absence of healthy limbus where to remove a biopsy from (7–9). In this randomized controlled clinical trial, we compared patients subjected to CLET, as we had enough previous experience in this procedure (8,174), with patients subjected to MSC transplantation (MSCT), enrolling though a small but significant number of patients due to its exploratory nature. Using an extensive evaluation endpoint analysis, there was enough evidence showing that MSCT was as safe and as effective as CLET, with a 73-86% success rate after 12 months (Calonge M et al. Invest. Ophthalmol. Vis. Sci. 2017;58(8):3372. ARVO 2017 Annual Meeting) (Figure 17. 2). These results, although must be replicated in a larger clinical trial, provide hope to those patients that are not eligible to have autologous limbal transplantation procedures due to extensive bilateral disease.

3. FUTURE PERSPECTIVES AND CONCLUSIONS

Extraocular MSCs represent an available, non-immunogenic source of stem cells that has proved to possess a potential therapeutic value in corneal epithelium regeneration based on results obtained in both preclinical and clinical studies, although only a few clinical studies have been performed yet, as this approach is quite novel. However, MSCs isolated from different tissues by different techniques may possess different properties and levels of activity, which may directly influence their efficacy. In addition, most of the MSC isolation,

propagation and characterization protocols vary between laboratories, and clinical translation of MSC-based therapies implies that their manufacturing process should comply with good manufacturing practices (GMP) in order to preserve the quality and safety standards of the final cell product. Therefore, the development of standardized protocols for the preparation, characterization, and evaluation of the biological potential activity (potency assays) of MSC, is essential to better understand MSCs function and clinical utility and to improve comparison of results between research and clinical centers. To achieve this goal, cooperation between basic researchers, physicians, industry, and regulatory authorities is fundamental.

Although several scientific groups, including ours, have reported promising results on the use of MSCs for corneal epithelial regeneration, the precise mechanism of how these cells exert their therapeutic effects on the ocular surface remains unclear and deserves further investigation. Additional research in LSCD animal models is needed to address whether MSCs can or cannot transdifferentiate into corneal epithelial cells, to determine how long MSCs can survive in the cornea secreting factors that reduce tissue injury, and to answer questions such as which is the most appropriate cell dose and best route of administration for applying MSCs onto the ocular surface.

Our proof-of concept clinical trial have shown that MSCs transplantation onto the ocular surface of patients suffering from LSCD can safely and effectively restore the corneal epithelium. Nevertheless, to keep moving forward it is needed further research and to decipher the mechanism by which transplanted MSCs improve corneal epithelial cell phenotype. To accomplish this, it is first essential to agree on LSCD diagnostic criteria and clinical evaluation endpoints so that the data collected from different clinical centers applying cell-based therapies for LSCD treatment can be effectively compared.

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5. COMPLIANCE WITH ETHICAL REQUIREMENTS

T Nieto-Miguel, S Galindo, M López-Paniagua, I Pérez, JM Herreras and M Calonge declare that they have no conflict of interest.

All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients for being included in the study.

All institutional and national guidelines for the care and use of laboratory animals were followed.

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FIGURES

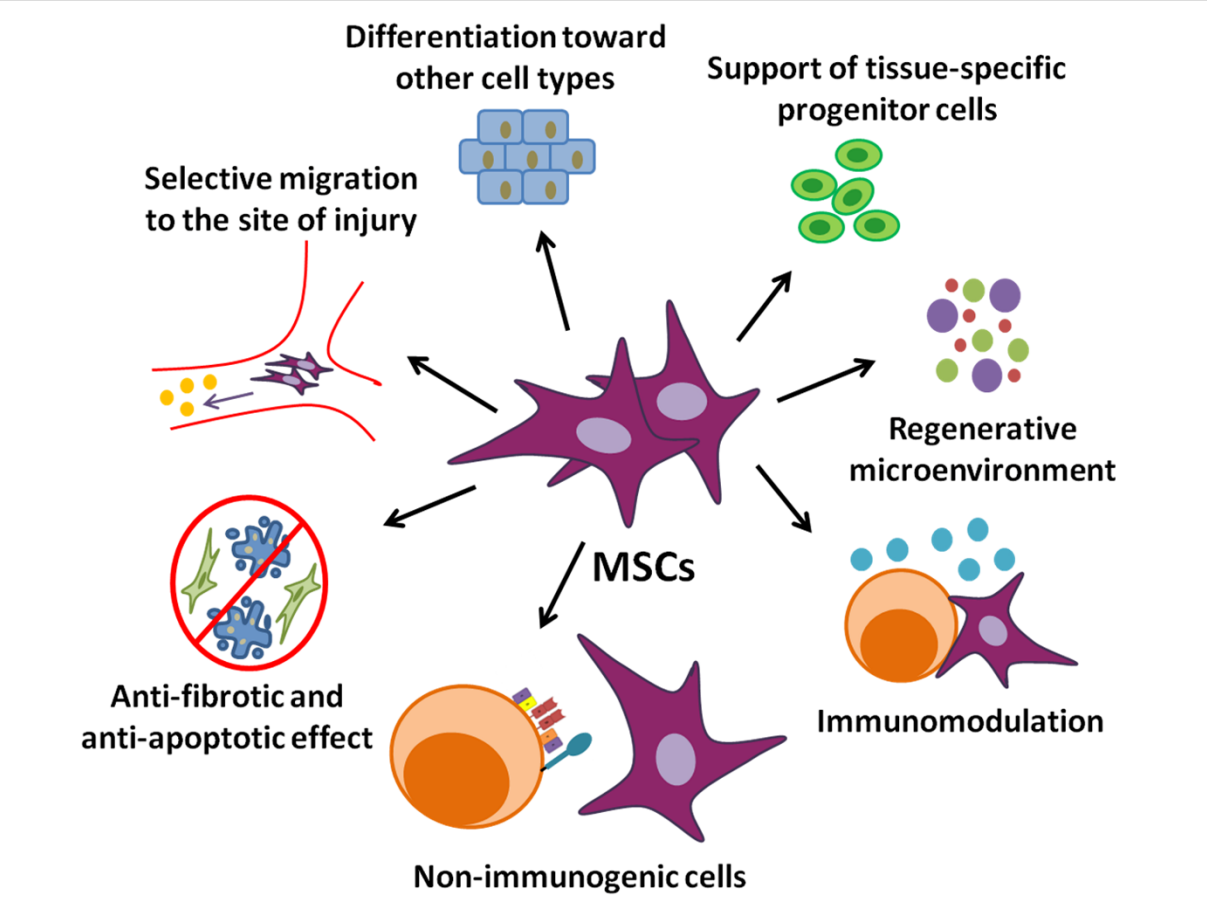

Figure 17. 1. Properties of mesenchymal stem cells (MSCs). MSCs have the capacity to differentiate into other cell types, to support of tissue-specific progenitor cells, to provide a regenerative microenvironment, to be non-immunogenic, to have immunomodulatory, anti-fibrotic, and anti-apoptotic properties, and to migrate to the site of injury.

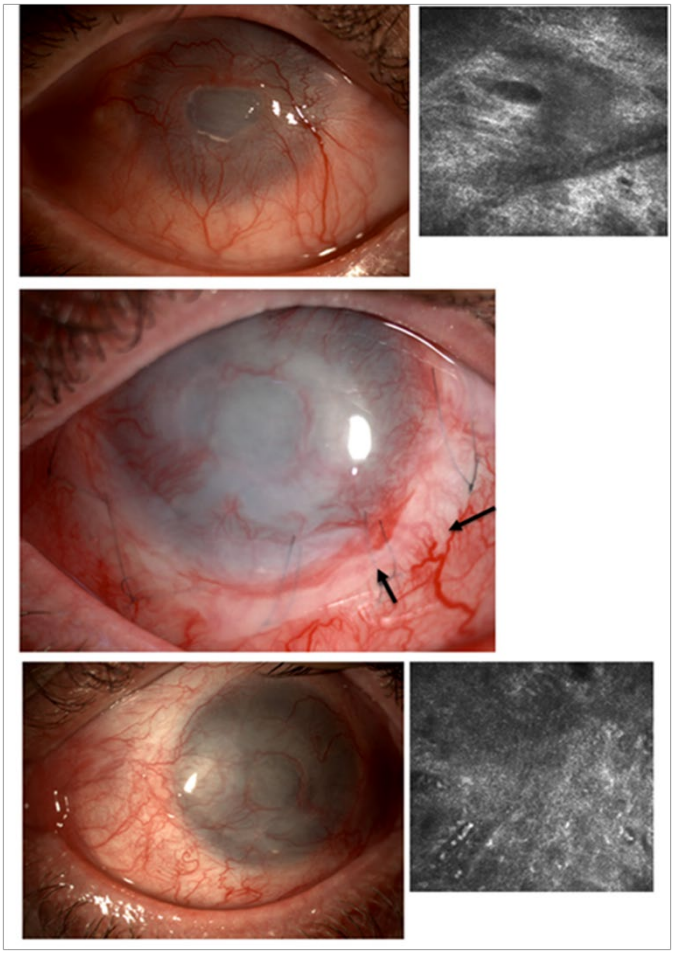

Figure 17. 2. This 48 year-old man had a bilateral chemical injury 4 years before, for which multiple amniotic membrane transplantations were performed, as per standard of care. Both eyes show the typical end-stage picture of a severe chemical burn: residual corneal vascularized opacification that is due to a full-thickness stromal scarring. Not seen in these pictures are bilateral cataracts, many areas of synechiae between the iris and posterior cornea and glaucoma. Thus, three surgical procedures, i.e., stem cell transplantation, further penetrating keratoplasty, and another non-corneal surgery (cataract removal), would be required to recover vision. He underwent MSC transplantation in his left eye. The upper row displays left eye 4 weeks before cell transplantation. The middle row shows transplant in place with the cells facing down and the amniotic membrane (arrows) facing the scleral contact lens two days after surgery. Lower row displays successful transplant after 12 months. *In vivo* confocal microscopy showed a conjunctival-like phenotype in the central cornea before transplantation (upper row). Twelve months after surgery, a corneal-like phenotype was present in his left central cornea (lower row). The continued improvement of the central corneal epithelial phenotype 12 month after transplantation indicates that this patient would have a better chance for a successful penetrating corneal transplant to improve his vision.

Table 17. 1. Trophic activity of mesenchymal stem cells (MSCs)

Trophic factors secreted by MSCs	**Effect on corneal regeneration**
EGF	Induction of proliferation and migration of corneal epithelial cells
IL-6	Anti-inflammatory effect, suppressing dendritic cells
SOD3	Antioxidant effect
TGF-β	Induction of proliferation and migration of corneal epithelial cells
TNF-α	Stimulation of TSG-6 expression
TSG-6	Activation of LESCs. Inhibition of local activated macrophages, decreased expression of pro-inflammatory cytokines, chemokines, and, MMP-9. Decreased neutrophil infiltration on the cornea and decreased corneal neovascularization
TSP-1	Anti-angiogenic effect
Trophic factors inhibited by MSCs	**Effect of inhibition action on corneal regeneration**
IL-2	Anti-inflammatory effect
MMP-2	Anti-inflammatory effect
MMP-9	Increase of proliferation of LESCs and decrease apoptosis of corneal epithelial cells
VEGF	Anti-angiogenic effect

Abbreviations: EGF, epidermal growth factor; IL, interleukin; LESCs, limbal epithelial stem cells; MMP, matrix metallopeptidase; MSCs: mesenchymal stem cells; SOD, superoxide dismutase; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha; TSG-6, tumor necrosis factor-a-stimulated gene/protein-6; TSP-1, thrombospondin 1; VEGF, *v*ascular endothelial growth factor.

Table 17. 2. Immunomodulatory ability of mesenchymal stem cells (MSCs).

Immunomodulatory factors secreted by MSCs	**General effects on corneal regeneration**
Cox-2	Inhibition of natural killer
GM-CSF	Induction of anti-inflammatory state of macrophages
IL-6	Inhibition of neutrophil action or exacerbation of the chemotaxis of leucocytes to damaged cornea
IL-10	Inhibition of T cell proliferation
iNOS	Inhibition of mast cells
NO	Inhibition of T cell proliferation
PD-1	Decrease of production of IgG, IgM, and IgA Inhibition of B and T cell proliferation
PD-L1	Decrease of production of IgG, IgM, and IgA Inhibition of B and T cell proliferation
PGE2	Inhibition of mast cells degranulation. PGE2 + TGFβ or IDO: decrease of natural killer cell proliferation. PGE2 + IL-6: inhibition of dendritic cells
TGF-β	Inhibition of T cell proliferation
Immunomodulatory factors inhibited or downregulated by MSCs	**Effect of inhibition action on general immune response detected on corneal regeneration**
IDO	Increase of T cell proliferation
IFN-γ	Decrease of ICAM1 and MHCI and II expression on corneal epithelial cells. Consequently, decrease of leucocyte infiltration on corneal inflamed epithelium
TNF-α	Decrease of ICAM1 and MHCI and II expression on corneal epithelial cells. Consequently, decrease of leucocyte infiltration on corneal inflamed epithelium

Abbreviations: Cox-2: cyclooxygenase-2; GM-CSF, granulocyte-macrophage colony-stimulating factor; IDO, indoleamine-2,3-dioxygenase; IFN-γ, interferon gamma; Ig, immunoglobulin; IL, interleukin; iNOS, inducible nitric oxide synthase; MSCs: mesenchymal stem cells; NO, nitric oxide; PGE2, prostaglandin E2; PD-1, programmed death-1; PD-L1, programmed death ligand-1; TGF-β, transforming growth factor beta; TNF-α, tumor necrosis factor alpha.