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Ex vivo expansion of corneal limbal epithelial/stem cells for corneal surface reconstruction

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PURPOSE. *The management of severe ocular surface disease due to limbal stem cell deficiency has changed dramatically. The concept of limbal stem cells, as the source of corneal epithelium revolutionised the therapeutic approach of ocular surface reconstruction. Deficiency of limbal stem cells results in blinding ocular surface diseases. Grafting viable limbal tissue, from either fellow healthy eye or a donor eye, with the resident stem cell population may replenish limbal stem cells and can restore the corneal surface to normality. Transplanting the limbal tissue can be achieved through a variety of procedures that include cadaveric keratolimbal allograft (KLAL), live or living related conjunctival limbal allograft (lr-CLAL) and limbal autograft. Advances in tissue engineering techniques have offered a viable alternative to overcome the limitation of limbal tissue available for transplantation. Epithelial stem cells harvested from a small limbal biopsy can be expanded* in vitro *on a suitable carrier and then transplanted to the diseased cornea to successfully restore the corneal surface. This article is a chronological review of the important steps that brought* ex vivo *expanded stem cell transplantation in ocular, particularly corneal surface reconstruction.*
METHODS. *The MEDLINE data base was searched for the years 1966-2002, using key words cornea, cell culture, ex-vivo expansion, limbus, stem cell, ocular surface and transplantation. Several articles that were not found by MEDLINE search were taken from references from other articles. Inclusion or exclusion of article was based on the relevance to the subject.*
CONCLUSIONS. *Corneal epithelial reconstruction with ex vivo expanded limbal cells is a potential tool in ocular surface reconstruction, although the technique is currently investigational. Strategies to achieve conjunctival epithelial restoration and tear film replenishment will allow ophthalmic surgeons to truly reconstruct the ocular surface. (Eur J Ophthalmol 2003; 13: 515-24)*

KEY WORDS. *Culture, Limbal stem cells, Stem cell deficiency, Cornea*

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INTRODUCTION

The management of ocular surface disorders (OSD) has changed dramatically over the last two decades. Previously the patients with severe OSD had a very poor prognosis. The available techniques for ocular surface reconstruction at that time consisted of lamellar and penetrating keratoplasty, tarsorrhaphy and artificial tears. The outcome of keratoplasty was uniformly poor due to recurrence of ocular surface failure. The current approach to severe OSD is based on a scientific understanding of the role played by

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limbal stem cells in corneal surface maintenance (1-3). Deficiency of limbal stem cells results in blinding ocular surface disorders. Several clinical trails provided evidence to prove grafting viable limbal tissue, either from fellow healthy eye or a donor eye, with the resident stem cell population may replenish limbal stem cells (LCS) and may restore the corneal surface to normality (4-10). Transplanting the limbal tissue can be achieved through a variety of procedures that include cadaveric keratolimbal allograft (KLAL), live or living related conjunctival limbal allograft (lr-CLAL) and limbal autograft (11).

Advances in tissue engineering techniques have offered a viable alternative to overcome the limitation of limbal tissue available for transplantation. Tissue engineering is an interdisciplinary field that applies principles of engineering and the life sciences toward the development of biological substitute that restore, maintain, or improve tissue function (12). Bio-engineered tissue replacement is rapidly developing and therapeutic bio-artificial skin replacements are becoming routine in plastic surgical units (13). Much interest has been generated by the prospect of re-implanting *ex vivo* expanded limbal stem cells as a technique to replenish the corneal surface. This article is a chronological review of the important steps that brought *ex vivo* expanded stem cell transplantation in ocular, particularly corneal surface reconstruction.

The history of ocular surface reconstruction is a long record of challenges with very limited success. Many surgical approaches aimed at restoring the morphology and to some extent the physiological function of the ocular surface. The techniques employed included oral mucous membrane grafts, conjunctival flaps, conjunctival auto-grafts, lamellar keratoplasty, and keratoepithelioplasty (14-18).

Among the variety of procedures, conjunctival auto-graft and keratoepithelioplasty described by Thoft, were remarkable (14, 19). His work laid the foundations for future developments. Conjunctival autograft was based on the theory of trans-differentiation of conjunctival epithelium into cornea like epithelium (14) but this procedure did not truly result in normal corneal epithelium (20, 21). In keratoepithelioplasty, peripheral cornea lenticules consisting of epithelium and a thin layer of stroma which served as a carrier source of epithelium was grafted (19). Four of these Lenticules were sutured to the corneoscleral limbus. The epithelium from the lenticules spread and covered the recipient cornea. Although these two procedures probably did transfer limbal stem cells the results were not satisfactory and many of these patients had immune mediated rejection of transplanted cells. Although Thoft did not have the benefit of the limbal stem cell concept, he did appreciate the potential of the limbus as a source for epithelial growth (19). With the understanding of the stem cell concept, Thoft's work opened a new era in ocular reconstruction.

1. Stem cell theory

The single most important breakthrough in managing OSD was the understanding of the location and function of limbal stem cells. The first hint of the stem cell concept was provided by Ida Mann in the 1940s, when she observed melanin pigments from the limbus slide toward a healing corneal abrasion (22). In one late 1940s evidence for centripetal movements of corneal epithelial cells were provided by several investigators, including Maumenee, Scholz and Buschke (23, 24). Eventually Davanger and Evensen raised the possibility that the limbus was the source of these migrating cells (25). Further experimental and clinical observations provided additional evidence that the source of proliferating cells is located at the basal layers of the corneal limbus (26-28). There is a substantive body of evidence to suggest the sub-population of cells at the basal layer of the corneal limbus exist in an "undifferentiated " state, and are termed "stem cells" (6, 26, 29-31). These cells are capable of (a) proliferation, (b)self-renewal, (c) producing large number of terminally differentiated functional progeny and (d) regenerate the tissue after injury (2, 32, 33).

2. Limbal transplantation

Kenyon and Tseng applied stem cell theory and adopted a direct approach to replenish the limbal stem cells population through grafting healthy limbal tissue with the resident stem cells either from the fellow healthy eye or from a donor (8, 34). Limbal transplantation is a collective term to describe

various procedures that include Cadaveric Keratolimbal Allograft (KLAL), live or living related Conjunctival Limbal allograft (lr-CLAL), Conjunctival Limbal Autograft (CLAU). This classification considers the location as well as origin of tissue and the common goal is to restore the limbal stem cell population. Conditions such as Steven-Johnson's syndrome, cicatricial pemphigoid and chemical burns, among others, can severely compromise the ocular surface due corneal vascularisation, chronic inflammation, in-growth of fibrous tissue and corneal opacification (6, 28, 29). The common pathological feature of this diverse group of disorders is the depletion of limbal stem cell population responsible for maintaining corneal epithelial equilibrium (6, 28, 29). In these conditions, limbal transplantation allows restoration of the corneal limbal stem cells and significant long term corneal epithelial healing may be achieved (7, 35-41). Limbal transplantation requires more than a span of two to three clock hours of limbal tissue excision and may seriously compromise the living donor eye (5). Moreover, limbal allograft, requires prolonged immunosuppression to prevent graft rejection and the systemic side effects of immunosuppressive agents remain a therapeutic concern.

3. Amniotic membrane transplantation

Amniotic membrane transplantation (AMT) offers a simple and effective approach to restored ocular surface abnormalities resulting from partial stem cell deficiency. In 1910 Davis reported the usefulness of AMT in skin reconstructive surgery (42). Although limited ocular application was reported in the 1940s the initial enthusiasm waned. Kim and Tseng successfully reintroduced the technique to reconstruct ocular surface irregularities and currently the indication of AMT is ever increasing (43-48). Amniotic membrane serves as “transplanted basement membrane” facilitating migration of epithelial cells and reinforces epithelial cell adhesion. Amniotic membrane provides a potential substrate and various growth factors, that promote epithelialisation and enhance wound healing (36). AMT alone is not effective in treating total stem cell deficiency (35, 36). AMT may be combined with limbal transplantation and is a useful adjunct in ocular surface reconstruction (6, 35, 36).

4. Cultured corneal epithelial grafts

The feasibility of cultured corneal epithelial transplants has been considered in the treatment of ocular surface disorders since 1982. Friend et al demonstrated, that *in vitro*, suspensions of cultured rabbit epithelial cells could repopulate denuded corneal stroma and was capable of producing hemidesmosomes and basement membrane (49). Gipson et al enzymatically dissected rabbit corneal epithelium and then transplanted the epithelial sheets to denuded corneas of living rabbits. Their results indicated that it was technically feasible to transplant corneal epithelial sheets to wound models *in vivo* (50). However the epithelial sheets failed to remain adherent to the cornea after 24 hours (50). The technical difficulties of handling and transferring the fragile epithelial sheets led to a search for a carrier to transfer cultured epithelium. The eye tolerates various collagen preparations without provoking an inflammatory reaction. Based on this observation, Geggel and co workers bio-fabricated a collagen gel substrate which was non toxic to epithelial cells. This collagen gel substrate allowed transfer of epithelial sheets obtained by enzymatic digestion to wounded corneas *in vitro* (51). He and Macully documented that limbal epithelial stem cells could be grown *in vitro* and would become stratified on type IV collagen shields (52). These collagen shields with epithelial cells were then transferred to denude *ex vivo* human corneal stroma in organic cultures. Histological examination revealed that the epithelial cells attached tightly to the recipient stromal surface even after removal of collagen shields (52).

4.1 Epithelial-fibroblasts co-culture systems

Although epithelial culture techniques are well established, dis-aggregated epithelial cells have very limited proliferative capacity *in vitro*, and sub-culturing is extremely difficult. *In vitro* they quickly differentiate to loose their proliferative capacity (53-55). Reinwald and Green introduced a co-culture system which maintained the dermal epithelial cells in a relatively undifferentiated state and preserved their proliferative capacity (56). They cultured the dermal epithelial cells on a growth arrested mouse embryonic fibroblast known as 3T3-J2 cells, as a feeder layer in medium containing foetal calf serum. This system re-

ferred to as "3T3 system " supported proliferation of, presumably the stem cells. It is possible to culture large sheets of epithelial cells from a small number of dis-aggregated cells (56, 57). This system also showed that the epithelial cells depend on the presence of fibroblasts or their products for their maintenance and growth (56, 57). The growth promoting potential of the 3T3 system has been extensively applied to *in vitro* cultures of dermal epithelial cells in bio-fabricating dermal substitutes (58-60).

4.2 Limbal stem cell culture

The 3T3 system also allow corneal limbal epithelial proliferation, and maintains the cells in a relatively undifferentiated state and permitted clonal growth (31, 61, 62). LSC cultured *in vitro* by the 3T3 system adhere firmly to *in vitro* corneal graft bed, and deposits basement membrane. The cells also retain substantial proliferative capacity (62). The ability to expand large numbers of limbal epithelial cells *in vitro* from small number of cells obtained from a small limbal biopsy, enabled investigators to develop a novel therapeutic approach to ocular surface reconstruction.

4.3 Transferring the *ex vivo* expanded LSC

Although the 3T3 system enabled expansion of epithelial cells from a limited number of cells, a suitable carrier to transfer the cultured cells without inducing toxicity and loss of proliferative capacity remained a challenge. Pellegrini et al transplanted a sheet of in-vitro cultured autologous limbal corneal epithelial cell layer to stem cell deficient cornea (37). They reported successful long-term restoration of the corneal epithelial surface in two cases (37). They performed a 1 x 2 mm limbal corneal biopsy from the contra-lateral eye. The limbal tissue was digested enzymaticlly and the dis-aggregate epithelium was expanded in medium containing, foetal calf serum on a lethally irradiated embryonic murine 3T3 fibroblast feeder layer. Three weeks later the confluent epithelial sheet was grafted to the diseased cornea. Handling of the fragile sheets of epithelial layer was fraught with difficulties and additional clinical application of this technique had not been reported. Technical difficulties associated in transferring the *ex vivo* expanded cells led the search for a suitable carrier

4.4 Amniotic membrane as a carrier

In 1995 Noguchu et al first showed tracheal epithelium could be cultured on amniotic membrane (63). Tsai et al chose human amniotic membranes as a carrier to expand the limbal stem cells *in vitro* and transfer the expanded cells to ocular surface. AM is being extensively used in ocular surface disorders and this was a logical choice. Amniotic membrane serves as "transplanted basement membrane" facilitating migration of cells and reinforces epithelial cell adhesion. Amniotic membrane alone is not effective in treating total stem cell deficiency (35, 36). Cultivation of limbal epithelial cells on amniotic membrane for transplantation offers the additional advantage of replenishing the stem cells and providing the potential growth factors present in amniotic membrane. This method gave an advantage of ensuring a compatible substrate for the graft. Technically, transferring the amniotic membrane-epithelial sheet composite, to the cornea was easy and was an advance over the previous methods. Tsai et al report transplantation of autologous limbal epithelial cells cultured on amniotic membrane in successful ocular reconstruction. They achieve visual improvement in 6 cases of OSD secondary to chemical burns and pseudopterygium.

4.5 Preparation of amniotic membrane -limbal stem cell composite graft

In this technique a 1 X 2 mm limbal tissue was biopsied aseptically from the healthy eye and transferred to the laboratory in culture medium. Then limbal epithelial/stem cells were dis-aggregated into single cells by Trypsin digestion. These single cells were then cultured on a growth arrested murine fibroblast (3T3-cells) feeder layer in Green's medium (contains Dulbecco's Modified Eagle's medium, foetal calf serum, glutamine, epidermal growth factor, hydrocortisone and cholera toxin). When expanded epithelial cells reach 40-50% confluence, the cells were passed on to human amniotic membrane and allowed to attach to the AM for 10-14 days. This formed the composite graft ready for transplantation (Fig. 1).

Surgical technique: The abnormal tissue over the cornea was removed and the conjunctiva was resected and recessed. The composite graft was placed atop

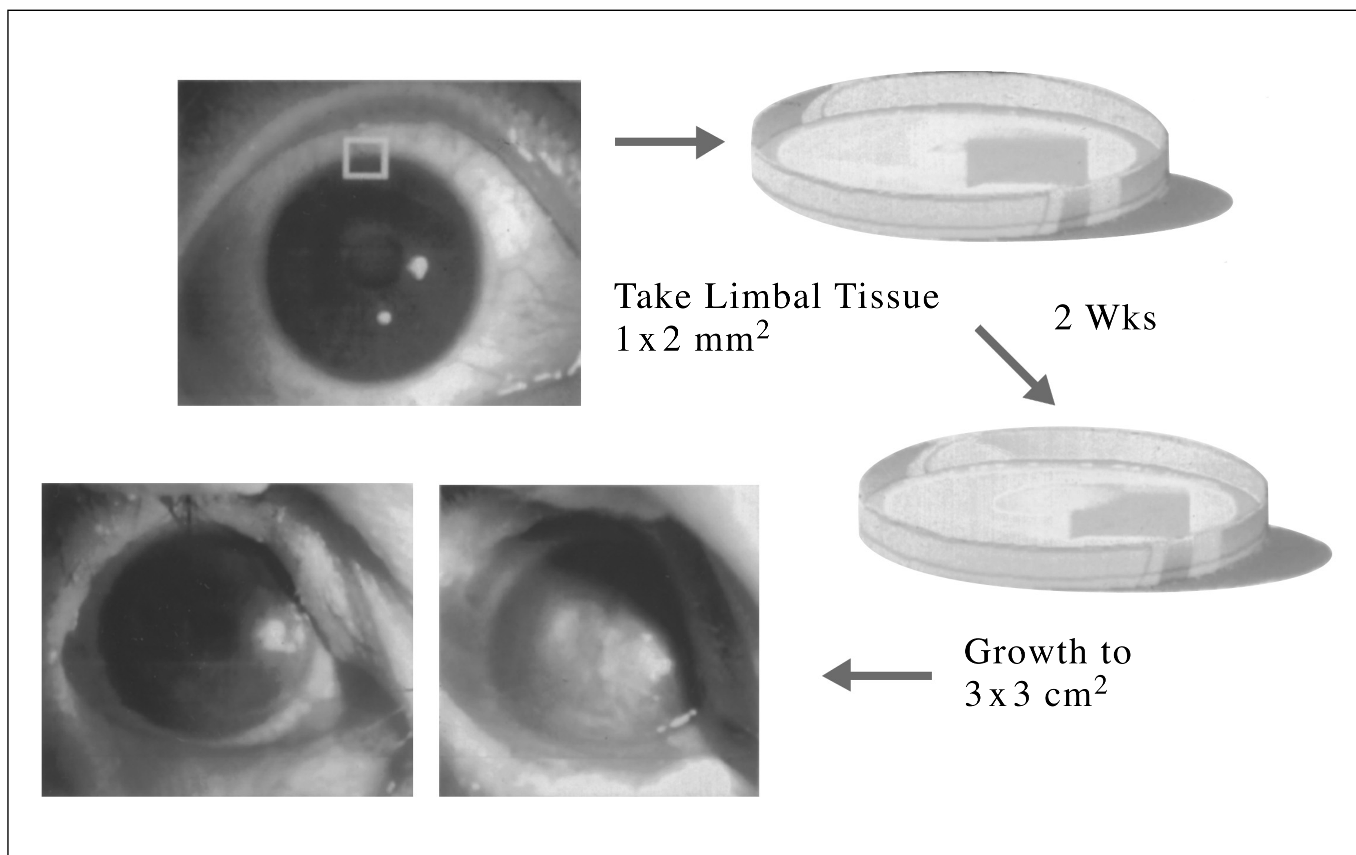

Fig. 1 - *A 1 x 2 mm limbal tissue was biopsied aseptically from the healthy eye and transferred to the laboratory in culture medium. Then limbal /stem cells were isolated by Trypsin digestion. These single cells were then cultured on a growth arrested murine fibroblast (3T3-cells) feeder layer in Green's medium. When expanded epithelial cells reach 40-50% confluence, the cells were passed on to human amniotic membrane and allowed to attach to the AM for 10-14 days. This composite was grafted to a stem cell deficiency eye. Visual improvement was achieved with the improvement of the clarity of cornea (courtesy of professor R. J. Tsai, Chang Gung Memorial Hospital, Taipei, Taiwan).*

the defect, and the corneal edge was sewn to the peripheral cornea with 10-0 nylon. The posterior peripheral edge of the amniotic membrane is sewn to the resected and recessed conjunctiva, and a bandage contact lens was placed. The amniotic membrane gradually dissolves. The bandage contact lens is placed to help to protect the cells, as AM dissolved within weeks and the cells take longer to attach to the cornea. Systemic immune suppression should be continued in case of allografts.

Encouraging results have been reproduced by other investigators applying similar techniques and the current indications for *ex vivo* expanded limbal stem cell translation includes Steven-Johnson's syndrome, ocular cicatricial pemphigoid, recurrent pterygium and chemical burns (38-41, 64). These investigators have also used allograft cells from a living donor and cadeveric cornea (40, 64). For a successful allograft transplantation immune rejection, needs to be countered with immune suppressive agents (40, 64).

The process of culturing the cells takes three to four weeks. Potentially the cells may be cryo-preserved for any length of time in liquid nitrogen. This offers plenty of time to plan surgery and achieve the best possible immune histo-compatibility between the recipient and donor, thus may reduce the dependency on immune suppression.

4.6 Fibrin as a carrier

Investigators from Italy, have shown that limbal stem cells can be preserved when cultivated on fibrin and

transplantation of autologous fibrin-cultured limbal cells permanently restores corneal integrity of patients with total limbal deficiency unresponsive to conventional therapy (65). In this novel technique, stem cells obtained by biopsy was first cultured on a feeder-layer of lethally irradiated 3T4-J2 cells. Sub-confluent primary cultures were trypsinised and plated on fibrin sealant, in the presence of feeder layer. At confluence the fibrin culture epithelial sheets were washed and placed in sterile holders for contact lenses. A 360 degree conjunctival peritomy was performed to remove abnormal epithelium and fibro-vascular tissue. Fibrin cultures epithelial sheets were placed on the prepared corneal scleral surface and fitted under the dissected conjunctiva. This technique offers the advantage of long distance transportation of fibrin cultured epithelial sheets and the technology can be widely applied. Further fibrin sealant is freely available commercially and handling is less demanding than amniotic membrane.

4.7 Graft rejection and role of immunosuppression

Patients receiving allograft transplantation of *ex vivo* expanded limbal stem cells run the potential risk of immune mediated rejection and should be countered with immunosuppressive agents. Topical treatment with steroids and antibiotics are indicated for the first 4-6 weeks. Systemic immunosuppression with cyclosporin or similar agent is indicated for at least one year. Immunosuppression will compromise the natural anti microbial protection of the ocular surface and vigilant observation is essential.

4.8 Limitations

Several factors such as corneal anaesthesia, tear film abnormalities, dry eyes, conjunctival scarring, entropion, corneal epithelial keratinisation, mucous depletion contribute to ocular surface abnormalities beside limbal stem cell deficiency. For a successful *ex vivo* expanded graft to be effective the ocular surface micro environment needed to be corrected to near normal states. Further these techniques require a great deal of preparation and laboratory facilities.

5. *Potentials of* ex vivo *expanded limbal cells in gene therapy to cornea*

The external position of the corneal and limbal epithelium offers the option of gene therapy not available to deeper tissues. It is possible to inactivate a gene producing an abnormal substance or insert a gene lost due to mutation (66). In theory, transfer of genes expressing anti inflammatory proteins or growth factors could be a valuable way to prevent an inflammatory cascade or to supply components for healthy epithelial turn over (66).

The genetic and biochemical basis of many inherited corneal epithelial dystrophies have been characterised and target genes identified. To manipulate the tissue or cells affected by mutation, the gene delivery technique should be efficient and non-toxic and should be able to express the transfected gene for a prolonged period of time. The potential for the genetically manipulated cells to become resident cells and continue to proliferate depends on the transgene vector employed in gene manipulation. Expression of the transgene following non viral plasmid DNA and adeno-viral vectors gene transfer is short lived (67). Retroviral vectors integrate into the host genome, providing the potential for long term passenger DNA expression (68). Proof of concept for this approach has been provided in a rabbit model. In this model limbal autografts were transduced with retrovirus carrying a marker gene *in vitro*. The cornea *in vivo* continued to express the marker gene for long period of time (68). Similarly *ex vivo* expanded limbal stem cells could be transduced with a target gene with a suitable vector before transplantation. Given the special external access afforded by the corneal limbus gene delivery through *ex vivo* expanded limbal cells have clear potential for therapeutic application in the cornea.

6. *Concerns*

Ophthalmologists are in an advantageous position to gain the experience of plastic surgeons who widely applied cultured autologous dermal epithelial cells to treat massive skin burns and chronic ulcers (60, 69, 70). The initial optimism declined among these surgeons due to inconsistency in the clinical results and several surgeons have questioned the usefulness of cultured epithelial cells (58, 71-73). Recently it has been pointed out that

the poor performance and the un-explained loss of a graft after an initial good performance may arise due to depletion of stem cells in culture (60). This may arise due to sub optimal culture conditions, environmental damage inappropriate transfer techniques damaging the stem cells function and stem cells differentiation (31, 59). These issues are equally applicable to bio-engineered reconstruction of the ocular surface.

For a successful bio-engineered reconstruction of the ocular surface, ensuring sufficient stem cells are being transplanted is necessary and the *ex vivo* environment should maintain the replicative function of the stem cells. On transplantation the cells should differentiate into the corneal phenotype. Currently there are no reliable markers form limbal stem cells (6, 27). To ensure sufficient numbers of progenitor cells are present in the *ex vivo* expanded pool, one has to rely on the isolated single cell's ability to proliferate to from large colony in culture (31, 49, 59, 60). Recently p63, a variant of p53 was suggested as a reliable limbal stem cells marker but this claim needs further validation (74).

6.1 Long term outcome

For a long term bio-engineered repair of ocular surface disorder the transplanted stem cells should integrate into the limbus of the recipient eye. Positive proof of surviving transplanted stem cells remain difficult and DNA finger printing results have given mixed results on the persistence of transplanted stem cells. Shimazaki et al were able to detect persistence of donor specific DNA sequences 30 months after limbal allograft (75). However similar studies on successful allograft failed to detect donor cells as evidenced by DNA (76-80). DNA finger printing techniques applied to analyse the fate of clinically successful bio-engineered grafts failed to demonstrate donor cells in the cornea. In successful bio-engineered skin replacement, although the donor cells persist for a long time, they are slowly replaced by the host cells. This observation suggests transplantation of cultured LSC may trigger activation of resident inactive stem cells by altering the micro-environment and the graft acting like a "biological dressing" by providing cytokoines or growth factors. To answer the question, what will happen to the cells transplanted after many years, will require many years of careful observation and follow-up.

6.2 Potential risk of slow viral infections

The potential risk of transmitting infection during transplantation of *ex vivo* expanded stem cell is a concern. In the current techniques limbal epithelial cells are cultured in a system containing 3T3-J2 fibroblasts as a feeder layer and a medium containing foetal calf serum (56, 57, 61). 3T3-J2 cells are immortalised cell lines and were derived from mouse embryos (56, 57). This cell line is extensively used in dermal epithelial culture for human autologous transplantation without any unwanted adverse effects (81, 82). There are concerns over transmission of unknown agents from the mouse cell line to humans. Furthermore the culture media contains foetal calf serum. Currently, the serum is obtained from regions free of CJD and VCJD. Although the cells are washed well before transferred to recipient sites, there are no studies validating the claim that there is no animal protein being transferred along with the cells. Currently used serum-free culture media favour the proliferation of transient amplifying cells and therefore not a preferred system for *ex vivo* expansion of limbal stem/epithelial cells (83-85). Similarly allograft limbal tissue and AM may potentially transmit infections. Strict guidelines have to be observed in harvesting AM for human therapeutic application and infections such as exclude HIV-I, HIV-2, and hepatitis must be excluded. Human transmission of variant CJD is a concern and the current methods are not effective in detecting prion proteins present in the AM (35, 36).

CONCLUSIONS

A normally functioning conjunctiva with its goblet cells are essential for a properly functioning limbal and corneal surface. Conjunctival disease in combination with limbal stem cells deficiency results in the most severe form of OSD. *Ex vivo* expanded limbal stem cell transplantation does not correct conjunctival disease or replace conjunctival stem cells. When OSD is due to a combination of conjunctiva and limbal disorders the value of *ex vivo* expanded limbal stem cell transplantation is questionable.

Corneal epithelial reconstruction with *ex vivo* expanded limbal cells is still in its infancy. Strategies to achieve conjunctival epithelial restoration and tear

film replenishment will allow ophthalmic surgeons to truly reconstruct the ocular surface. Only then will the therapeutic potential of this technology be fully realised.

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