Techniques for Culture and Assessment of Limbal Stem Cell Grafts

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ABSTRACT The therapeutic use of limbal cultures for the permanent regeneration of corneal epithelium in patients with limbal stem cell deficiency (LSCD) has been reported in many studies. According to the guidelines for good manufacturing practice (GMP), strictly regulated procedures and stringent quality control tests are now required to manipulate stem cells as "medicinal products" and make engraftment safer and eventually more successful. This paper describes techniques for optimal preparation of limbal stem cell grafts, including 1) a reliable impression cytology assay for the grading of LSCD, 2) culture methods that maintain high percentages of limbal stem cells, 3) the use of specific markers for the detection of corneal, conjunctival, and limbal stem cells, namely keratin 12, mucin 1, and ΔNp63α, and 4) assays to assess the presence of contaminants, such as murine fibroblasts, endotoxins, mycoplasmae, and viral particles, in the cultured graft. The use of some of these assays allowed us to obtain a regenerated normal corneal epithelium in approximately 80% of 166 LSCD patients who received transplants from 2004 to 2008.

KEY WORDS corneal regeneration, culture methods, Good Manufacturing Practice, impression cytology, keratin, limbal stem cell deficiency, limbal stem cell grafts, mucin, Q-FIHC assay, regenerative medicine, stem cell therapy

I. INTRODUCTION

Stem cell-based therapies have recently been classified as "medicinal products" by the European Union (**EU**), and manipulation is therefore regulated according to the manufacture of biological medicinal products for human use.[1] Groups providing cellular therapies within the EU must comply with EU laws (http://www.ema.europa.eu/htms/human/advanced_therapies/intro.htm), which require that grafts are produced only at accredited production sites authorized by the national regulatory agencies (in Italy, **AIFA**–Agenzia Italiana del Farmaco) and under the defined conditions of good manufacturing practice (**GMP**). In the USA, the Center for Biologics Evaluation and Research (**CBER**), which is within the Food and Drug Administration (**FDA**), regulates biological products for human use under applicable federal laws, including the Public Health Service Act and the Federal Food, Drug and Cosmetic Act. CBER protects the public health by ensuring that biological products are safe and effective and available to those who need them (http://www.fda.gov/BiologicsBloodVaccines/CellularGeneTherapyProducts/default.htm).

GMP conditions require accredited production sites to establish the following procedures:

1. Establish an adequately controlled cell storage system to allow proper maintenance and retrieval of cells without any alteration of their intended final characteristics; storage conditions must be optimized to ensure cell viability, density, purity, sterility and function.
2. Establish a specific microbiological screening program that is capable of detecting human infectious agents with appropriate sensitivity and that takes into consideration the medium components that might interfere with the assays (eg, antibiotics).
3. Establish an appropriately characterized Master Cell Bank (**MCB**) and Working Cell Bank (**WCB**).

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4. Document the quality of biologically active additives in culture media, such as growth factors, cytokines and antibodies, with respect to identity, purity, sterility and biological activity and absence of adventitious agents.
5. Provide a detailed description of the manufacture of the active substance and of the finished product, and the type of manipulation.
6. Control the manufacturing process by in-process controls at the level of critical steps or intermediate products.
7. Characterize the cellular components in terms of identity, purity, potency, viability, and suitability.
8. Validate the entire manufacturing process, including cell harvesting, cell manipulation processes, maximum number of cell passages, combination with other components of the product, filling, packaging, transport, storage.
9. Obtain complete traceability of the patient, as well as the product and its starting materials, in order to monitor the safety and efficacy of the cell-based medicinal product.
10. Use a safety database able to detect common adverse events.

In this paper, we will describe procedures and quality controls necessary for the optimal preservation and propagation of corneal stem cells.

Abbreviations

CFE	colony forming efficiency
EU	European union
GMP	good manufacturing practice
IC	impression cytology
K3	keratin 3
K12	keratin 12
K19	keratin 19
LAL	limulus amebocyte lysate
LPS	lipopolysaccharide
LSCD	limbal stem cell deficiency
MUC1	mucin 1
PCR	polymerase chain reaction
Q-FIHC	quantitative fluorescence immunohistochemistry analysis

II. CORNEAL REGENERATION BY LIMBAL STEM CELLS

A. Impression Cytology: An Assay to Evaluate the Grading of Limbal Stem Cell Deficiency (LSCD)

We have employed a new method for the analysis of impression cytology (**IC**) specimens based on the use of a pair of markers, namely keratin (**K**)12 and mucin 1 (**MUC1**), which are able to distinguish corneal epithelia from conjunctival epithelia more accurately than previously reported markers (Figure 1). K12 is a specific marker for corneal cells, as reported by many studies. MUC1, although reported to be present in the cornea, is relatively more abundant in the conjunctiva and was therefore chosen as the marker for conjunctival cells. IC is a minimally invasive technique, which allows ophthalmologists to rapidly evaluate the health status of the ocular surface. Assessment of this feature requires specific markers of the ocular surface epithelia and the expression of these markers in the uppermost layers of the ocular surface. In fact, Z-stack analysis has shown that the thickness of the specimens obtained through IC corresponds to that of just one cell layer (the apical one) and only occasionally includes the deeper subapical flattened cell layers.[2] This suggests that only the most superficial cells of the ocular surface are normally collected onto the membranes used for IC and are available for analysis.

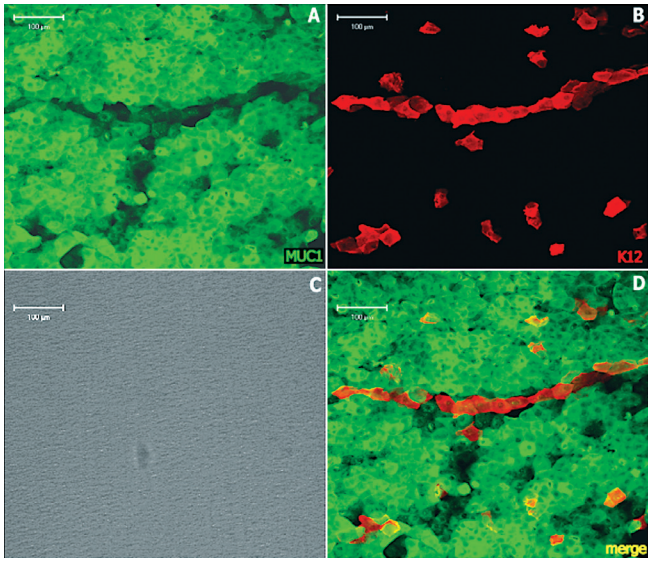

Figure 1. Analysis of IC specimens from LSCD patients using antibodies against MUC1 (A) and K12 (B). Transmitted light bright-field image of IC membranes (C). Note the presence of residual corneal epithelial cells (positive for K12) scattered within the conjunctival pannus (positive for MUC1) (D). Scale bar = 100 μm.

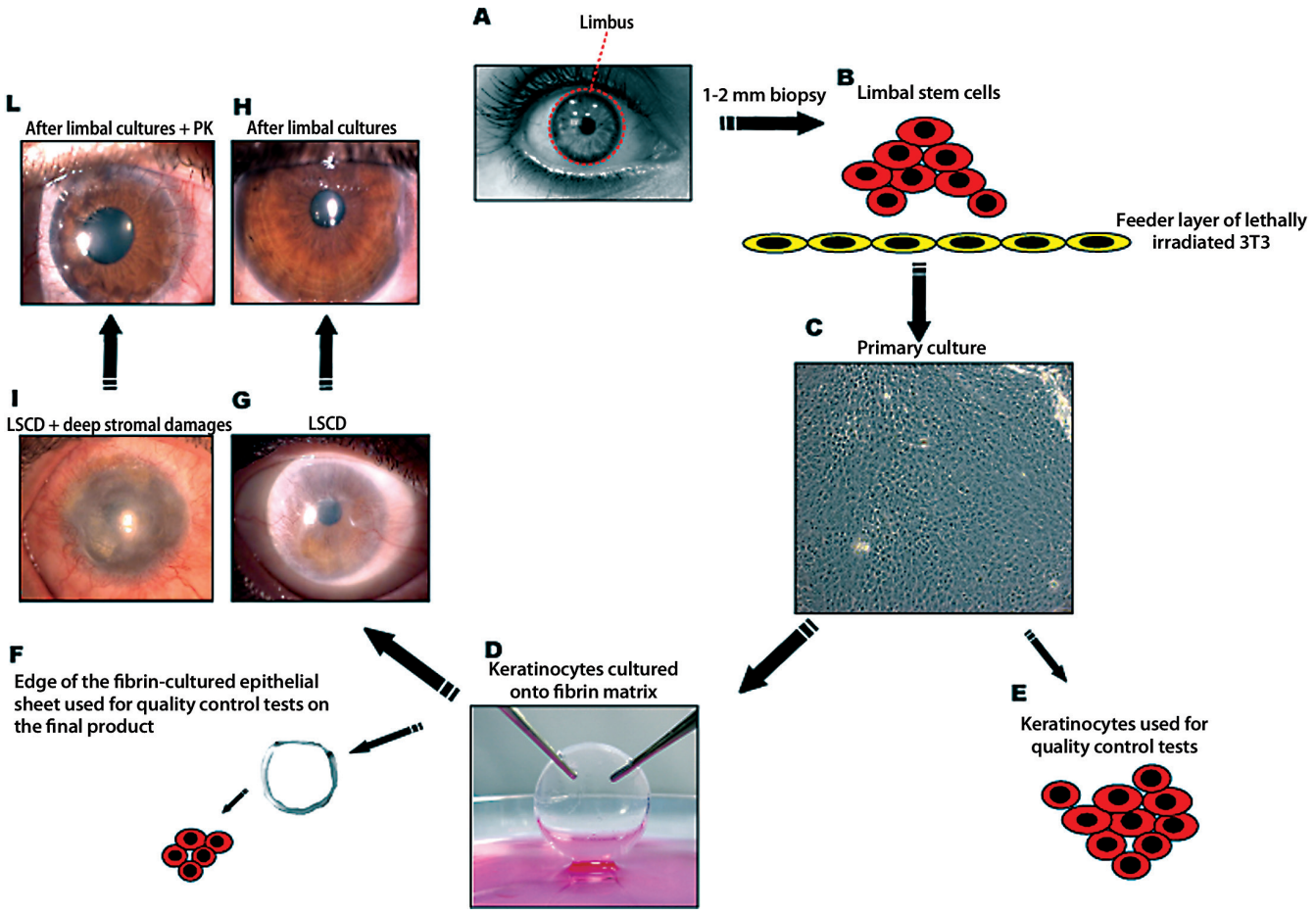

Figure 2. Phases of corneal stem cell therapy. Limbal keratinocytes are isolated from a 1-2 mm^2 limbal biopsy taken from the contralateral healthy eye of a LSCD patient (A) and cultured onto a feeder-layer of lethally irradiated 3T3-J2 cells (B). After 7-10 days, subconfluent primary cultures (C) are trypsinized and transferred onto a fibrin matrix (D). A small number of keratinocytes from the primary culture are used for quality control tests (E). At confluence, the edges of the fibrin-cultured epithelial sheet are removed, keratinocytes are isolated through enzymatic treatments, and quality control tests are repeated (F). If the latter are satisfactory, the corneal stem cell graft is transplanted onto the eye of the patient (G). After grafting, the corneal surface is covered by a transparent epithelium (H). To restore visual acuity (L), patients having deep stromal damages (I) need a penetrating keratoplasty after limbal stem cell grafting.

We selected K12 and MUC1 because they are able to distinguish clearly between corneal and conjunctival epithelia and are expressed by suprabasal epithelial cells. The conventional cytology techniques used to distinguish conjunctival epithelia from corneal epithelia are represented by hematoxylin & eosin, periodic acid-Schiff, or mucin 5AC staining to identify goblet cells, or by IC staining with the markers K3 and K19 to distinguish between corneal and conjunctival epithelia, respectively. Compared to traditional assays based on IC, our test 1) uses more reliable markers that can discriminate between corneal and conjunctival epithelium with no overlapping signals, 2) is carried out through double immunofluorescence, which allows all the information to be obtained from just one cytological specimen per eye, thus reducing the invasiveness of the technique, and 3) allows marker signals to be carefully quantified through Quantitative Fluorescence ImmunoHistoChemistry (**Q-FIHC**) analysis.[2,3]

The use of specific diagnostic tests able to characterize the status of the ocular surface is fundamental for both pretreatment and follow-up analyses of LSCD patients undergoing cell therapy-based treatments.

B. Culture Method and Preservation of Limbal Stem Cells

The clinical success of keratinocyte-mediated cell therapy for LSCD patients[4,5] depends primarily on the quality of the cultures used to prepare the grafts. These must contain a sufficient number of stem cells (holoclones) to guarantee longterm epithelial renewal. Keratinocytes isolated from a 1-2 mm^2 limbal biopsy taken from the contralateral eye of an LCSD patient are processed within 24 hours from harvest, cultivated in the presence of selected fetal calf serum and plated onto a feeder layer of lethally irradiated 3T3-J2 fibroblasts.[6] Subconfluent primary cultures are then plated onto a modified fibrin sealant in the presence of a feeder layer (Figure 2). The fibrin matrix represents an ideal support, as it does not alter the characteristics of cultured cells, is highly manageable, is able to preserve the proliferative compartment of the epithelium during transportation and surgery,[6]

and has adhesive properties, so that no sutures are needed to affix the cultured epithelium to the corneal stroma.

Keratinocytes cultured onto a fibrin matrix have the same growth capacity and stem cell content as those cultured directly on plastic surfaces. This would suggest that fibrin is able to preserve longterm proliferation of limbal stem cells and to maintain high percentages of holoclone-forming cells[5] (Figure 3). After application of the culture to the wounded corneal surface, the underlying fibrin is degraded rapidly, thus allowing the overlying epithelium to interact with the wound surface, where it readily engrafts.

A small number of keratinocytes from the primary culture (approximately 30,000 cells) are not plated onto the fibrin glue, but instead are used for quality control tests to assess percentage of corneal epithelial stem cells, clonogenic potential (through Colony Forming Efficiency [**CFE**]), and presence of contaminant cells (conjunctival cells, 3T3-J2 fibroblasts, etc). Quality control tests are repeated on the final product, ie, before the corneal stem cell graft is transplanted onto the patient. At confluence, the edges of the fibrin-cultured epithelial sheet are removed and keratinocytes are isolated from the fibrin matrix through enzymatic treatments with Dispase II and trypsin-EDTA. These cells are also used to evaluate the levels of endotoxin and potential bacterial/viral contaminants within the graft.

C. Evaluation of the Percentage of Stem Cells in Cultured Grafts

The accurate determination of stem cell content within cultured epithelial grafts is considered the best quality control to ensure optimal clinical performance.[5,7] Antibodies raised against the epithelial stem cell marker p63 are currently used to determine the percentages of stem cells contained in the graft.[7] However, corneal epithelial stem cells mainly express the ΔNp63α isoform,[8] which was shown to be essential to maintain the proliferative potential of limbal stem cells and the regenerative capacity of ocular surfaces. Antibodies recognizing all p63 isoforms might therefore not be ideal for quantification of limbal stem cell percentages in corneal grafts, as they could erroneously indicate higher levels of stem cell content.[3]

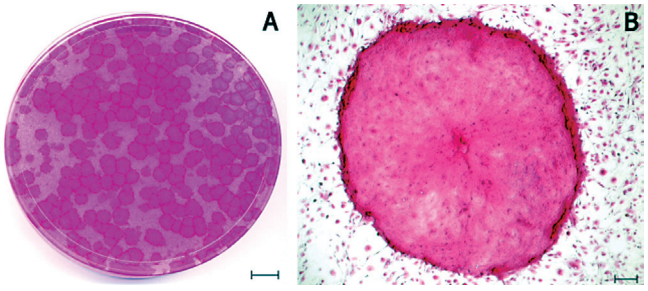

Figure 3. To evaluate the clonogenic potential of primary cultures, CFE assays are performed. Cells from primary cultures are trypsinized and approximately 1,000 cells are plated into 100-mm dishes for 12 days. Colonies are stained with Rhodamine B and scored to calculate the number of proliferative and aborted colonies (A). CFE values are expressed as the ratio between the number of colonies found and the cells seeded. Scale bar = 1 cm. Colonies arising from a single clonogenic cell can be of three types (holoclone, meroclone or paraclone) depending on the growth potential, morphology and phenotype of the cells forming the colony.[17] Holoclone type colony (B). Scale bar = 50 μm.

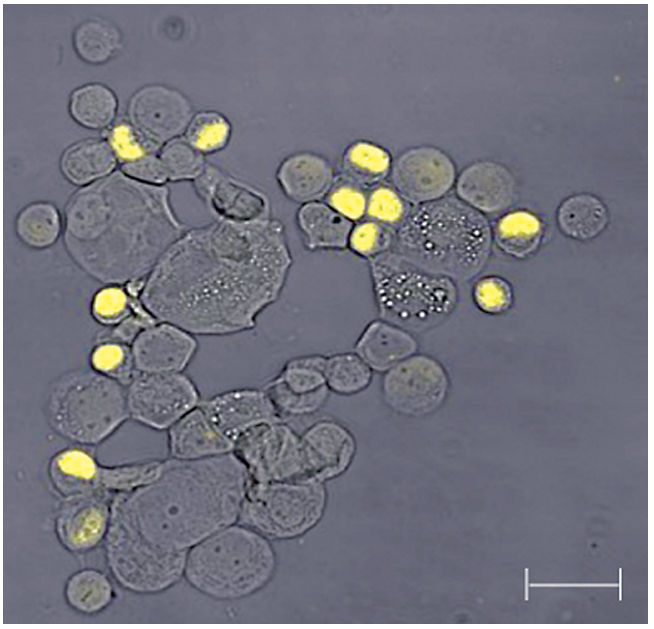

Figure 4. Evaluation of ΔNp63α (+) cells and expression levels allows the number of stem cells within the graft to be determined before it is transplanted onto the patient.[10] Scale bar = 20 μm.

To more accurately evaluate the stem cell content within corneal cell grafts, we developed a rabbit polyclonal antibody against the ΔNp63α isoform. A FITC-conjugated form was also developed to allow quantitative evaluations through Q-FIHC based assays.[2] ΔNp63α is expressed in a small sample of undifferentiated and small cells (holoclones = stem cells [Figure 4]), and its level of expression can be assessed by automated quantitative immunocytochemistry. In primary cell cultures, the percentage of these cells

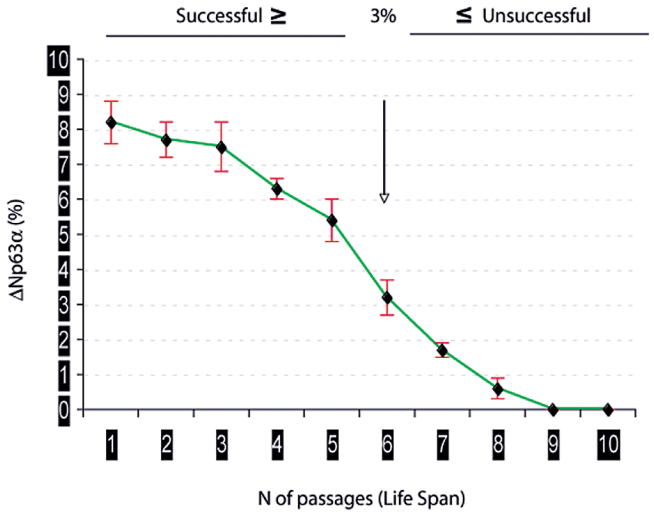

Figure 5. Example of ΔNp63α decrease during stem cell primary culture life-span. ΔNp63α values ≤ 3% result in unsuccessful clinical outcomes.

that are ΔNp63α-positive ranges between 3% and 8% and decreases progressively both during clonal conversion (the transition from holoclones to meroclones and paraclones) and serial in vitro propagation (life span [Figure 5]). High levels of ΔNp63α and longterm proliferative capacity of cells are highly correlated.[7] Based on 1) in vitro evaluations by analyzing ΔNp63α content and comparing it to colony forming efficiency during serial cultivation of corneal cultures, and 2) successful clinical outcomes of LSCD patients transplanted with cultured limbal stem cells, primary cultures with less than 3% of cells containing high levels of ΔNp63α are currently considered to be unsuitable for graft preparation.[9]

D. Identification of Corneal and Conjunctival Cell Markers to Determine the Characteristics of Cultured Grafts

The characteristics of 166 cell cultures set up for transplantation in LSCD patients were evaluated through analysis of the number of corneal and conjunctival cells, using antibodies against K3 and K19, respectively, as quality control determinants. All the corneal epithelial cultures used for grafting showed expression levels of the corneal marker K3 ranging between 60% and 90% and the conjunctival marker K19 between 5% and 30%. Analysis of all the grafts prepared indicated a higher prevalence of corneal cells in primary cultures and a low contamination of conjunctival cells. Cultures with K3 and K19 in the ranges noted above were considered suitable for clinical application, as the "contamination" (conjunctival cells) was low. However, recent reports have demonstrated that these two markers are not as specific as previously shown.[2,10,11] Their use could therefore lead to incorrect evaluation of the percentages of the two cell lineages. As mentioned above, we have recently shown that K12 and MUC1 are more specific and reliable markers of corneal and conjunctival cells, respectively. In the future, they might therefore replace K3 and K19 for quality control tests of cells, thus leading to better selection of the cell cultures suitable for transplantation.

E. Evaluation of the Percentage of Murine Fibroblast Contaminants in Cultured Grafts

Murine 3T3-J2 fibroblast-based feeder layers are essential for growing epithelial stem cells,[6] maintaining stem cell characteristics, and delaying activation of senescence/differentiation pathways.[12] 3T3-J2 feeder layers have been used for the treatment of massive full-thickness burns and to regenerate the urethral epithelium in patients with posterior hypospadias,[13] as well as to restore the ocular surface in patients with LSCD.[14,15] Although the murine fibroblasts were lethally irradiated and therefore unable to replicate, we found their percentage in the transplanted graft to be around 5% (Figure 6). A potential downstream effect is that the application of cultured epithelial grafts could stimulate inflammatory responses against the murine antigens, thus potentially reducing the chances of clinical success. Therefore, as a further quality control, specific antibodies against murine fibroblast markers are used to monitor the percentage of murine fibroblast contaminant/impurity in the final product (Figure 6). If it was above 5% (our threshold level), the corneal epithelial graft was considered unsuitable for clinical use and therefore was not transplanted. This value has been established following the absence of inflammatory/immune responses in 166 LSCD patients transplanted with corneal epithelial grafts carrying approximately 5% of murine fibroblasts. This would suggest that this percentage of murine cells is not associated with dramatic adverse effects.

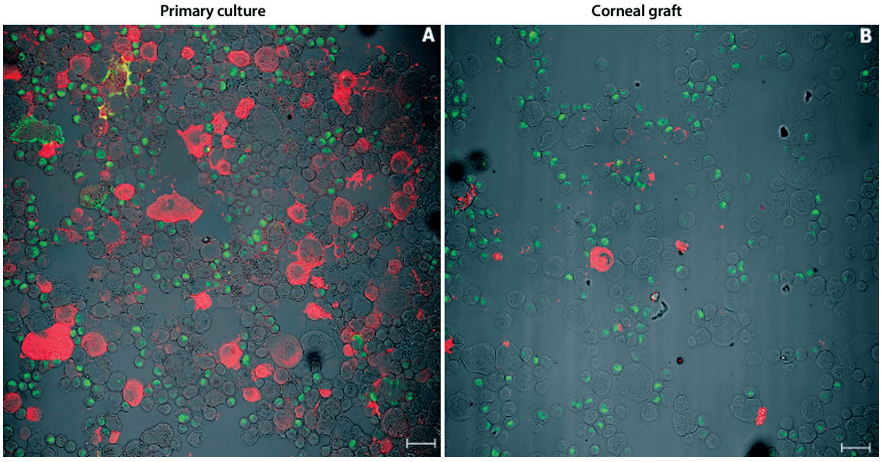

Figure 6. Evaluation of the stem cell content (through ΔNp63α – green) and impurity due to the presence of feeder layer cells (through an antibody against murine fibroblasts – red) are carried out both on primary cultures (A) and on fibrin-cultured epithelial grafts (B). Note that ΔNp63α expression levels were not altered during the manufacturing process. A decrease in murine fibroblasts is observed in the final product (corneal graft) (B). Scale bar = 20 μm.

F. Q-FIHC Assay for Quality Control of Limbal Cultures

To obtain qualitative and quantitative information on both the manufacturing process and the final medicinal product (ie, the limbal stem cell graft), we have developed a new assay, Q-FIHC, which uses laser scanning confocal microscopy and advanced image analysis to detect and quantify fluorescent intensity.[3] The results obtained with Q-FIHC assay are similar to those obtained with other more traditional techniques, such as quantitative Western blotting (Qdot Western) for protein evaluation or flow cytometry for cell cycle analysis. The advantages of Q-FIHC are that it requires fewer cells and the staining of any individual cell can be detected and quantified. To obtain similar results with other assays, at least 100 times as many cells would be required. The ability of this assay to correlate size and levels of expression has allowed us to use a ΔNp63α clinical marker to predict the percentage of stem cells in

keratinocyte cultures. Thus, we have introduced Q-FIHC as the main quality control for our limbal cultures before they are grafted back onto patients.

G. Evaluation of Endotoxin Content, Mycoplasma, and Viral Contamination to Reduce Post-Grafting Inflammatory Responses

The presence of gram-negative bacterial lipopolysaccharide (**LPS**) molecules (also known as endotoxins), mycoplasma, and viruses in grafts could produce immune/inflammatory responses, potentially reducing the rates of successful stem cell grafting. Evaluation of endotoxin content, mycoplasma, and viral contamination are recognized as crucial information by the guidelines of the Italian Minister of Health for optimal cell therapy procedures. Endotoxin levels are assessed with an FDA-approved Limulus Amebocyte Lysate (**LAL**)-based assay, which is able to recognize endotoxin concentrations ranging from 10 EU/ml to 0.01 EU/ml. Potential mycoplasma contamination is assessed with use of a chemioluminescence-based assay. Positive cultures are re-assessed through real-time polymerase chain reaction (**PCR**) using mycoplasma-specific kits able to detect at least four different mycoplasma strains (genitalium, hominis, oral, and pneumoniae).

The presence of viruses is evaluated through real-time PCR assays using virus-specific kits. Only viruses known to affect corneal epithelial cells, such as herpesvirus I, cytomegalovirus, and varicella zoster virus, are tested for.

An additional control that regulatory agencies are likely to require is testing for the presence of potential murine viruses in the 3T3 cells used for limbal stem cell propagation. The batch of murine 3T3 fibroblasts that we currently use has been tested for the presence of murine viral contaminants by inoculation of adult mice, suckling mice, guinea pigs, rabbits, and embryonated chicken eggs with 3T3 murine fibroblasts, after which the effects are observed for 28 days. Adult mice, suckling mice, guinea pigs, and rabbits are some of the preferred animals in virology studies, according to the British Institute for Biological Standardization and European Pharmacopeia. No viral contaminants were detected within the sensitivity limits of the assay. In vitro assays have also been performed to detect the presence of viral contaminants by using monolayers of indicator cell types, such as Vero and MRC-5 cell lines. No cytopathic effects were observed in any of the test article-inoculated indicator cells or the indicator cells inoculated with the negative control.

III. OUTCOME OF AUTOLOGOUS CULTURED LIMBAL STEM CELL GRAFTS

From 2004 to 2008, 166 patients affected by LSCD and unresponsive to previous standard medical and surgical treatments received autologous cultured limbal stem cell grafts (Table 1). Cell cultures were obtained in compliance with GMP standards and requirements set by the Italian Ministry of Health at the time of the study (Decree of the Italian Ministry of Health, dated December 5, 2006). Few centers worldwide are known to produce grafts of limbal stem cells prepared under GMP conditions. Only recently, the results of the first study describing the use of ex vivo limbal stem cells cultured in accordance with GMP standards and the EU Tissues and Cell Directive have been reported.[16]

Table 1. Patient demography and baseline clinical characteristics (N=166)

Number of individuals (%)	
Male	126 (75.8)
Female	40 (24.2)
Years of age	
Mean (SD)	46.3 (14.8)
Range	9–78
Median (25th, 75th)	46.0 (35.5; 57.5)
Cause of stem cell deficiency (%)	
Alkali injury	107 (64.5)
Acid injury	20 (12.1)
Thermal injury	10 (6.0)
Keratitis or keratopathy*	7 (4.2)
Multiple corneal surgeries	6 (3.6)
Unspecified chemicals	5 (3.0)
Unknown	11 (6.6)
Years from the event	
Mean (SD)	18.4 (16.6)
Range	1–60
Median (25th, 75th)	13.0 (4.0; 32.0)
Previous ocular surgery (%)	
None	23 (14.0)
≥ 1 surgery†	102 (61.8)
AMT	46 (29.3)
Keratoplasty	43 (27.4)
Eyelid surgery#	43 (27.4)
Conjunctival flaps	11 (7.0)
CLAU, KLAL, cell cultured	11 (7.0)
PTK	3 (1.9)
Unknown	34 (20.6)

* Acanthamoeba infection; pseudomonas infection; contact lens use

† Some patients had ≥ 1 surgery or different surgeries repeated several time before limbal stem cell transplantation.

Lysis of symblephara, fornix reconstruction with conjunctiva or oral mucosa

AMT: amniotic membrane trasplantation; CLAU: conjunctival limbal autograft; KLAL: keratolimbal allograft; PTK: phototherapeutic keratectomy.

To undergo treatment, patients were required to have no exposure of the ocular surface and mild or absent signs of symblepharon; Shirmer test I with >5 mm/5 min; and no ongoing topical or systemic anti-inflammatory treatments. LSCD was clinically assessed in all patients on the basis on the loss of Vogt palisades, corneal conjunctivalization revealed by fluorescein staining, irregularity of the corneal epithelium, IC findings, and presence of fibrovascular tissue (pannus). About 60% of patients showed complete corneal conjunctivalization and the remaining 40% showed a mixed corneal/conjunctival epithelial pattern. The corneal surface

was evaluated at 3, 10, 15, 25, and 40 days and at 3, 6, 9, and 12 months post-surgery by slit lamp examination. Fluorescein staining was done starting from the third month.

The recovery of a stable and clear epithelium, with no superficial vessels growing onto the 8-mm central cornea was graded as a complete success. Success was considered partial if conjunctival in-growth reappeared in some sector of the 8-mm central cornea and if the corneal surface was unstable. The treatment was considered to have failed when the postoperative clinical conditions were the same as the preoperative ones. Grafting of cultured limbal cells was successful in approximately 80% of the patients, including both complete and partial successes. Twelve months (or longer) after surgery, 33 patients (19.9%) underwent either penetrating or lamellar keratoplasty for optical or tectonic rehabilitation, and 10 other patients were scheduled for keratoplasty. After keratoplasty, a clear cornea with a stable and transparent epithelium was maintained in about 90% of patients after a mean follow-up time of 29.9 months (range 6–50).

IV. SUMMARY AND CONCLUSIONS

In the 10 years following the first report of restoration of damaged corneal surfaces with autologous cultivated corneal epithelium,[15] many other studies describing the clinical application of in vitro cultured limbal stem cells have been reported.6,[16-29] The protocols used to cultivate cells vary widely. Similarly, several scaffolds have been used to transfer the cells to the eye. The method we employed allowed us to obtain a regenerated normal corneal epithelium in approximately 80% of our patients. Evaluation at 12 months post-intervention is consistent with the establishment of a physiological self-renewal likely due to transplanted limbal holoclones.

In cell therapy, it is crucial to define the suitability and quality of cultured epithelial grafts, similar to the evaluation of chemical identity and purity in the use of pharmaceuticals. To meet the requirements for the manufacture of biological medicinal products for human use, increasing numbers of quality control tests have been introduced in compliance with the pharmaceutical quality requirements set by the regulatory authorities (Decree of the Italian Ministry of Health, dated 5th December 2006). In the next few years, it is likely that regulations will become more stringent and will limit the use of murine feeder layers and growth media containing serum of bovine origin. Cell factories will have to deal with these issues.

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