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Autologous transplantation of rabbit limbal epithelia cultured on fibrin gels for ocular surface reconstruction

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Purpose: Regeneration of the corneal epithelium could be severely impaired in patients suffering from limbal stem cell deficiency. The purpose of this study was to evaluate the restoration of the corneal epithelium by grafting onto denuded corneas autologous limbal cells cultured on fibrin gels. The rabbit model was chosen to allow the microscopic evaluation over time after grafting.
Methods: Rabbit limbal epithelial cells (RLECs) were isolated and cultured from small limbal biopsies (3 mm^2). The epithelium was separated from stroma after dispase digestion and put in culture on lethally irradiated fibroblasts used as a feeder layer. At the first passage, RLECs were cultured on a fibrin gel matrix. At confluence, the cultured epithelia were grafted in vivo on denuded autologous rabbit corneas. At different postoperative times, grafted and control (without graft or grafted with fibrin gels only) rabbit corneas were compared in vivo with a slit lamp microscope, and in situ by histological and immunohistological microscopy of harvested biopsies.
Results: A small limbal biopsy was sufficient to generate enough RLECs to prepare several grafts and to perform cell analysis. Only two weeks were required to produce a cultured epithelium suitable for autologous transplantation. One month after grafting, a normal corneal phenotype was observed on the ocular surface of grafted rabbits in contrast to the control rabbits (ungrafted or grafted with fibrin gel only) where histological signs of conjunctivalization were found. The absence of goblet cells and negative staining for keratin 4 confirmed that the cultured cells persisted and that the epithelium regenerated after grafting was not from conjunctival origin.
Conclusions: Our results demonstrate that an autologous epithelium cultured on a physiologically biodegradable matrix can be prepared from a small biopsy and grafted on denuded cornea. The autologous graft allows epithelial regeneration from cultured cells and promotes corneal healing of unilateral total stem cell deficiency.

Corneal stem cells, located in the limbus, account for epithelial regeneration of the cornea [1,2]. Limbal stem cell deficiency may result from chemical or thermal injuries, Stevens-Johnson syndrome, multiple surgery or cryotherapy to the limbal area, contact lens injury, aniridia, multiple endocrine deficiency, or idiopathic causes. Such a deficiency is accompanied by chronic surface problems characterized by corneal opacification and vascularization resulting in a marked decrease in vision. These patients are poor candidates for conventional corneal graft because grafting the central cornea does not restore limbal cells [3]. In addition, long-term healing of the grafted epithelial surface is plagued with recurrent and persistent epithelial defects with the risk of secondary infection or long-term inflammation leading to secondary reopacification and revascularization of the cornea.

The conjunctivalization observed on the cornea of individuals with total stem cell deficiency is the consequence of the inability of corneal epithelial cells to regenerate properly. Conjunctival epithelial cells take over and migrate over the corneal surface. Secondary neovascularization spreads from the limbus to cover the entire cornea. Conjunctivalization is often associated with epithelial defect, chronic inflammation, and abnormal scarring [4]. Markers that are associated with conjunctiva, but absent from the central area of normal cornea, such as the presence of goblet cells [3] and the expression of keratin 4 (K4) [5], have been used to confirm the conjunctival origin of cells and to diagnose conjunctivalization [3,6]. Goblet cells are visible by histological examination and may be identified by their specific mucin product, MUC5AC [7].

There are few treatment modalities available for patients with severe limbal stem cell deficiency. Limbal graft is still a prevailing option, although some potential disadvantages or complications may result from it. An autologous limbal graft is only considered as a potential alternative in cases of unilateral lesions [4], and limbal sampling bears the potential risk of causing limbal stem cell deficiency in the healthy contralateral eye [8]. Limbal allograft with systemic immunosuppression is another option. However, the risk of significant side effects from long-term immunosuppression is a major drawback of that technique. It has also been shown that rejection

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cannot always be controlled by cyclosporin A, the main immunosuppressive agent in use for organ transplantation for the last few years [9,10].

A new therapeutic approach for patients suffering from limbal stem cell deficiency was described by Pellegrini and coworkers [11,12]. They reduced the surface area required for an autologous limbal biopsy by expanding these human cells in culture and seeding them over a fibrin gel before grafting. The purpose of the present study is to document corneal restoration as a function of time following grafting of an autologous epithelium cultured on a fibrin gel. In contrast to the clinical context in which biopsies at various times post-transplantation are very hard to justify for patients and practically impossible to obtain, we characterized the fibrin gel degradation and corneal regeneration at five times after autologous grafting using a rabbit model. Herein, we report the successful isolation of epithelial cells from a 3 mm^2 limbal biopsy. These cells were first cultured on a lethally irradiated 3T3 fibroblast feeder layer before they were seeded on fibrin gels and used for autologous grafting on rabbit denuded corneas. Our results show that cultured cells survived after transplantation and could initiate corneal regeneration in vivo.

METHODS

All experiments described in this article were conducted according to the ARVO Statement for the Use of Animals and Humans in Ophthalmic and Vision Research and the Declaration of Helsinki. All procedures were approved by the Laval University Animal Care and Use Committee and by the institution's Committee for the Protection of Human Subjects.

Materials: Insulin, adenine, penicillin G, cholera toxin, bovine serum albumin (BSA), aprotinin, Hoechst reagent 33258, sodium azide, and dimethylsulfoxide were purchased from Sigma (Oakville, Ontario, Canada). L-glutamine, Ham's F12 medium, and Dulbecco's minimum essential medium (DMEM) were obtained from Invitrogen (Burlington, Ontario, Canada). Mouse anti-keratin 3 (AE5) and anti-keratin 4 (clone M6B10) antibodies were obtained from ICN (Aurora, OH); mouse anti-MUC5AC (clone CLH2) antibody from Chemicon (Temecula, CA); and Alexa 594-conjugated goat anti-mouse IgG H+L was from Molecular Probes (Eugene, OR). Ciprofloxacin hydrochloride 0.3% (Ciloxan), proparacaine chlorhydrate 0.5% (Alcaine), and atropine sulfate 1% (Isoptoatropine) were obtained from Alcon Canada (Mississauga, Ontario, Canada). Ketoprofen was purchased from Rhone Merieux (Athens, GA); acepromazine from Wyeth-Ayerst (St. Laurent, Québec, Canada); epidermal growth factor (EGF) from Austral Biologicals (San Ramon, CA); gentamicine from Schering Canada (Pointe-Claire, Québec, Canada); Tisseel (fibrin gel) from Baxter Hyland Immuno (Mississauga, Ontario, Canada); dispase II protease neutral from Roche (Laval, Québec, Canada); trypsin 1-500 from Intergen (Toronto, Ontario, Canada); optimal cutting temperature embedding medium (OCT) from Somagen (Edmonton, Alberta, Canada); FetalClone II serum from HyClone (Logan, UT); and Bouin's solution from Produits chimiques ACP (Saint-Léonard, Québec, Canada).

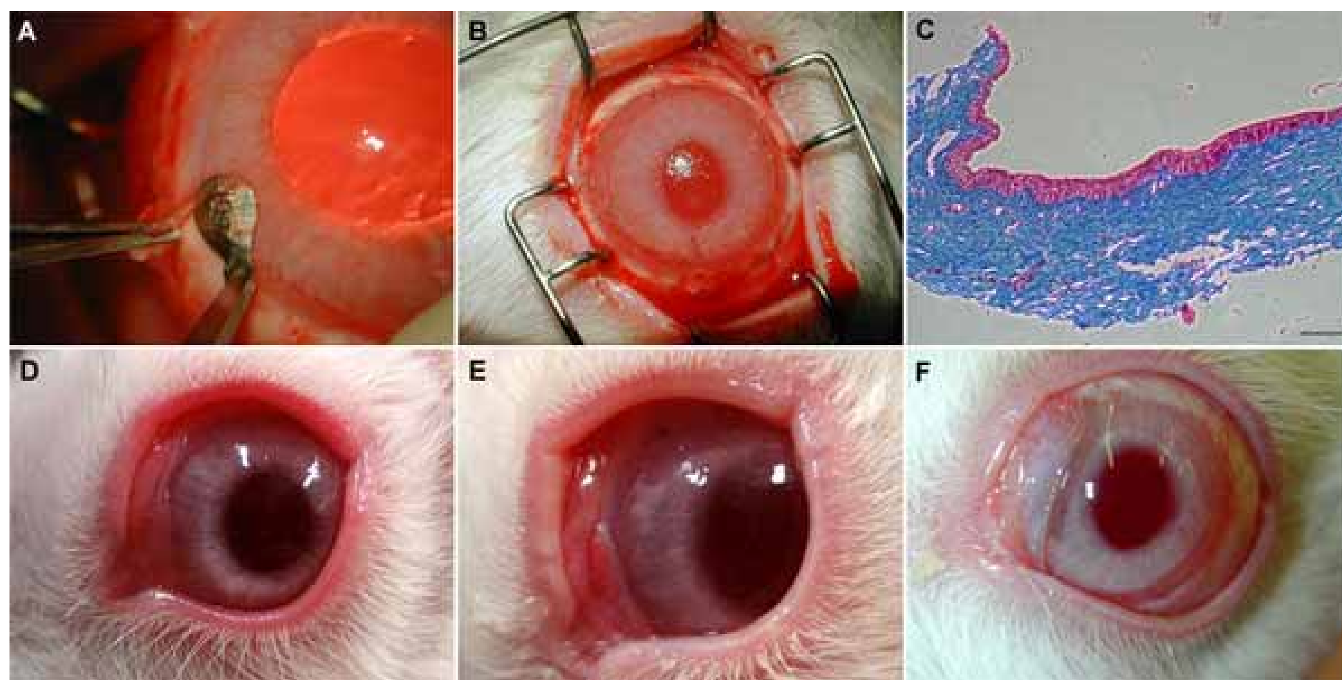

Figure 1. Surgical procedure and grafting. **A**: A limbectomy was performed over 360° with a round beveled corneal microblade. **B**: Macroscopic view of the fibrin gel, gently placed over rabbit cornea after complete epithelial removal including limbectomy, and secured with four interrupted sutures in the cardinal positions. **C**: Microscopic histological analysis of the resected tissue showing that all the epithelial cells were removed after limbectomy. **D**-**F**: Macroscopic aspect of the corneal surface after transplantation for ungrafted controls (**D**; after day 27), grafted with fibrin gel without RLECs (**E**; day 27) or with RLECs (**F**; day 23). The scale bar represents 100 μm.

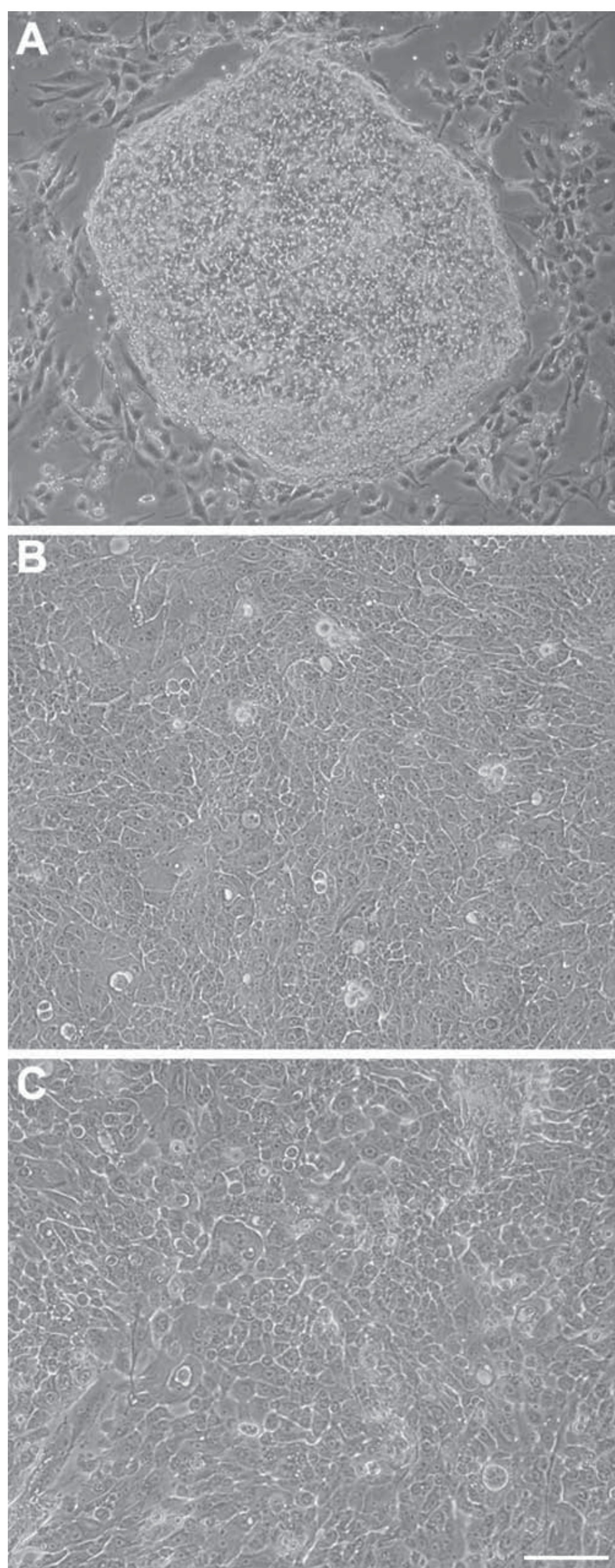

Figure 2. Morphological aspect of rabbit limbal epithelial cells in culture. **A**: Phase contrast micrographs of (**A**) a colony of rabbit limbal epithelial cells (RLECs) in primary culture surrounded by fibroblasts from the feeder layer. **B**: Phase contrast micrographs of confluent cultures (passage 1) of RLECs seeded on plastic. **C**: Phase contrast micrographs of confluent cultures (passage 1) of RLECs seeded on fibrin gel. The scale bar represents 50 μm.

Limbal biopsy and cell culture: Small biopsies (3 mm^2, 50 μm deep) were harvested from the right eye limbi of New Zealand White rabbits (Charles River Laboratories; Saint-Constant, Québec, Canada). The epithelium was separated from the stroma after a 45 min incubation in dispase (2.5 mg/ml). Rabbit limbal epithelial cells (RLECs) were seeded in culture with an irradiated murine 3T3 fibroblast feeder layer. RLECs were cultured in a modified supplemented hormonal epithelial medium (SHEM) [13] composed of a 1:1 mixture of DMEM and F12 with 10% FetalClone II and antibiotics (100 IU/ml penicillin and 25 μg/ml gentamicin). Cells were cultured in a 37 °C, 8% CO_2 incubator. In six to nine days, primary cultures reached 90% confluence. RLECs were trypsinized and seeded at 12,000 cells/cm^2 in the presence of a feeder layer on fibrin gel or on plastic culture dishes. Human cells were harvested and cultured in the same fashion from eyes provided by the eye bank less than 24 h after death. The fibrin gel was produced according to a method previously described [11]. Briefly, stock solutions were diluted to obtain a 3 IU/ml thrombin solution, 35-55 mg/ml fibrinogen solution, 1.1% NaCl, and 1 mM $CaCl_2$. The fibrin gel was produced by adding 330 μl of each solution using the Duploject-System (Baxter) in a 30 mm diameter plastic ring laid in a petri dish. Aprotinin (25 μg/ml) was added to each culture on fibrin gel to prevent fibrinolysis [14]. At this concentration, cell migration is not affected [15]. At confluence, fibrin gels were ready to graft. They were easily detached from the plastic with forceps.

Transplantation of cultured RLECs and experimental design: A limbal stem cell deficiency was created in each rabbit's left eye with a method adapted from Gipson et al. [16]. Each rabbit received a dose of tranquilizer (2 mg/kg ketoprofen and 1 mg/kg acepromazine, subcutaneous), and kept under adequate endotracheal general anesthesia with isoflurane 3%. Drops of proparacaine 0.5%, and atropine 1% were instilled onto the cornea at the beginning of the procedure. A 360° limbal peritomy was performed with Vanass scissors and Bonn forceps. The whole limbus was removed by first creating a superficial keratectomy from 2 to 3 mm inside the limbus at a depth of approximately 100 μm with a round beveled corneal microblade (Figure 1A). The dissection was then extended toward the sclera to include the stump of conjunctiva still attached to the limbus (Figure 1C). The remaining corneal epithelium was scraped off from the central cornea with a number 15 scalpel blade. This procedure was done on both treated (grafted with RLECs cultured on fibrin gels) and control (ungrafted or grafted with fibrin gel without cells) rabbits. For 25 rabbits, fibrin gel with autologous RLECs was cut at a diameter of 16 mm with a trephine and then grafted on the experimental eye as previously described by Rama et al. [11]. Briefly, the gel, placed on the bare cornea with cell side up, was secured in place by four interrupted cardinal sutures of 10-0 nylon (Figure 1B). The conjunctiva was stretched to cover the edges of the fibrin gel and attached with 9-0 vicryl interrupted sutures. A central complete tarsorraphy was achieved with a single 4-0 prolene mattress suture to protect the fresh graft. It was left in place for three days. For 10 control rabbits,

fibrin gels without cells were grafted. A sham surgical procedure was completed for 25 control animals without grafting the RLECs on the fibrin gel. An antibiotic ointment (ciprofloxacin hydrochloride) was applied on the eye immediately after the grafting and twice a day after lid opening for ten days.

Sixty rabbits with keratectomy were divided into two groups: grafted with RLEC on fibrin gels and control corneas. This latter group served for observation of the natural healing course in the absence of RLEC without or with a fibrin gel. On postoperative days 1, 3, 7, 14, and 28, rabbit corneas were observed with a slit lamp microscope in order to evaluate reepithelialization. Fluorescein was instilled at the end of the examination for monitoring whether or not epithelial defects were present. On each of these days, rabbits from each group were euthanized and both eyes were collected. Biopsies of cornea were processed for histological and immunofluorescence analysis.

Histological analysis: Biopsies were fixed with Bouin's solution and processed for paraffin embedding. Sections (5 μm) were stained by Masson's Trichrome and analyzed by light microscopy.

Indirect immunofluorescence analysis: Biopsies were embedded in OCT compound and kept at -70 °C until use. An indirect immunofluorescence assay was performed on acetone-fixed cryosections (5 μm) of rabbit corneas as previously described [17]. Primary antibodies used were mouse monoclonal anti-keratin 3, anti-keratin 4, and MUC5AC. Goat anti-mouse IgG H+L antibodies conjugated with Alexa 594 were used as secondary antibodies. Sections were incubated with antibodies, diluted in phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$·$2H_2O$), 0.48 mM $MgCl_2$·$6H_2O$ containing 1% BSA, at room temperature for 45 min (primary antibodies) or 30 min (conjugated antibodies). Cell nuclei were counterstained with Hoechst reagent 33258. Negligible background was observed for controls (primary antibodies omitted).

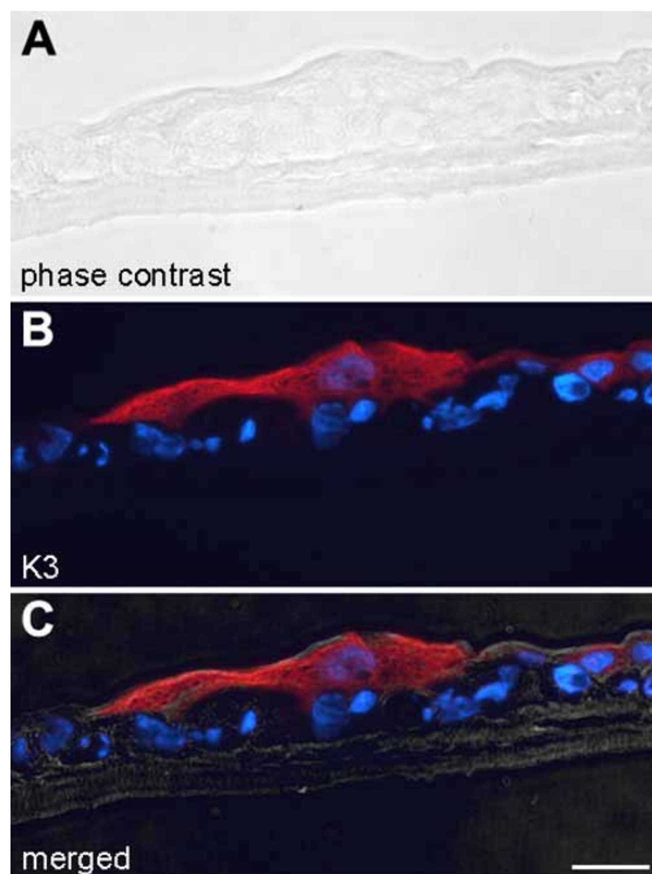

Figure 3. Keratin 3 staining of rabbit limbal epithelial cells cultured on a fibrin gel in vitro. **A**: Phase contrast of confluent rabbit limbal epithelial cells (RLECs) cultured on fibrin gel. **B**: Keratin 3 (K3) immunostaining (red) of the same confluent RLECs cultured on fibrin gel. **C**: A merger of the phase contrast (**A**) and K3 immunostaining (**B**). At this step, the RLECs cultured on fibrin gels were ready for grafting. Nuclei were counterstained with Hoechst (blue). The scale bar represents 50 μm.

RESULTS

Cell isolation and culture: We initially investigated whether a small biopsy provided enough epithelial cells to prepare grafts, to perform analyses, and to freeze cells for a subsequent use. A small limbal biopsy (3 mm^2) proved sufficient to isolate epithelial cells following digestion with dispase and separation of the epithelium from the stroma. When seeded on an irradiated fibroblast feeder layer, epithelial cells adhered, quickly started to proliferate after a few days in culture, and established a confluent culture within six to nine days. RLECs in primary culture (P0) produced regular, round-shaped colonies of small cells (Figure 2A). At the first passage (P1), cells were seeded with a feeder layer either directly on plastic or on fibrin gels. An identical growth was obtained with both substrates when cells were seeded at the same density. RLECs at P1 were small but slightly larger than cells in primary cultures. Whether cultured on plastic (Figure 2B) or on a fibrin gel (Figure 2C), cells exhibited a similar morphology. Within two weeks, cells covered approximately 15,000 mm^2, a surface area that represents a 5,000 fold expansion. These RLECs could be subcultured for several passages and were still proliferating well after three passages at which point we stopped the cultures. Thus, in the subsequent experiments, a 3 mm^2 limbal biopsy was harvested for the initiation of autologous cultures for grafting.

Graft procedure and ocular surface observation: Limbal stem cell deficiency was surgically induced in rabbits. Biopsies of freshly keratectomized corneas processed for histological staining provided evidence that the epithelium was completely removed from the limbal and corneal regions (data not shown). Thus, this procedure resulted in a complete ablation of limbal stem cells before the beginning of the transplantation experiment. One eye was keratectomized while the other was left intact (used for in situ corneal biopsies at the end of the experiment). Twenty-five rabbits were grafted with RLECs cultured on fibrin gels whereas 35 rabbits were keratectomized and grafted with fibrin gel only (10) or left ungrafted (25) as controls.

At time of grafting, RLECs cultured on fibrin gels that were identical to those grafted, were processed for analysis. A corneal epithelial marker, keratin 3 (K3), and a conjunctival epithelial marker, K4, were used in order to confirm cell source. One or two cell layers were present in confluent cultures, indicating that stratification occurred in vitro. Immunostaining revealed that the superficial cells expressed K3 (Figure 3). In contrast, basal cells were not stained with this marker of corneal differentiation. K4 was not expressed in RLECs cultured on fibrin gels (data not shown). We therefore conclude that before grafting, basal cells from the in vitro cultured RLECs were K3-negative (Figure 3) and K4-negative, a distinctive feature of limbal basal cells in situ (Figure 4B,I).

Macroscopic evaluation of grafted and ungrafted rabbit corneas: Fibrin gels, with or without autologous cultured RLECs, were successfully sutured to bare corneas. The examination of the external appearance of rabbit eyes with a slit lamp at various times after surgery did not reveal large differences between grafted and control rabbits (Figure 1D-F). At the time of lid opening, the lids were swollen. The appearance of the lids, cornea, and conjunctiva improved with time after surgery. At seven postoperative days, most of the corneal regions from controls, either ungrafted or grafted with an acellular fibrin gel, had not epithelialized completely, forming a central epithelial defect that was responsible for diffuse fluorescein stromal staining (data not shown). This was rarely observed in treated animals. Afterwards, fewer differences were observed between the eyes of treated or control animals in the macroscopic appearance of the cornea. Neovascularization and corneal opacification were absent in both groups of animals within the four week period studied after surgery (Figure 1D-F). Therefore, a faster reepithelialization was obtained in the group of rabbits grafted with epithelial cells cultured on fibrin gels.

Epithelial regeneration on cornea grafted with RLECs cultured on fibrin gels: To better assess the nature of the epithelium eventually present on the ocular surface, biopsies were harvested at various times after grafting to evaluate fibrin degradation and corneal regeneration. Histological staining revealed that the fibrin gel was visible under the RLECs 2 and 24 h after grafting (Figure 5H). However, three days after grafting, the RLECs were in contact with the corneal stroma (Figure 5H right insert), indicating that the fibrin gel was already completely degraded. In contrast, no epithelium was present in keratectomized corneas that were left ungrafted (Figure 5B). Remnants of the gel were also visible three days after grafting

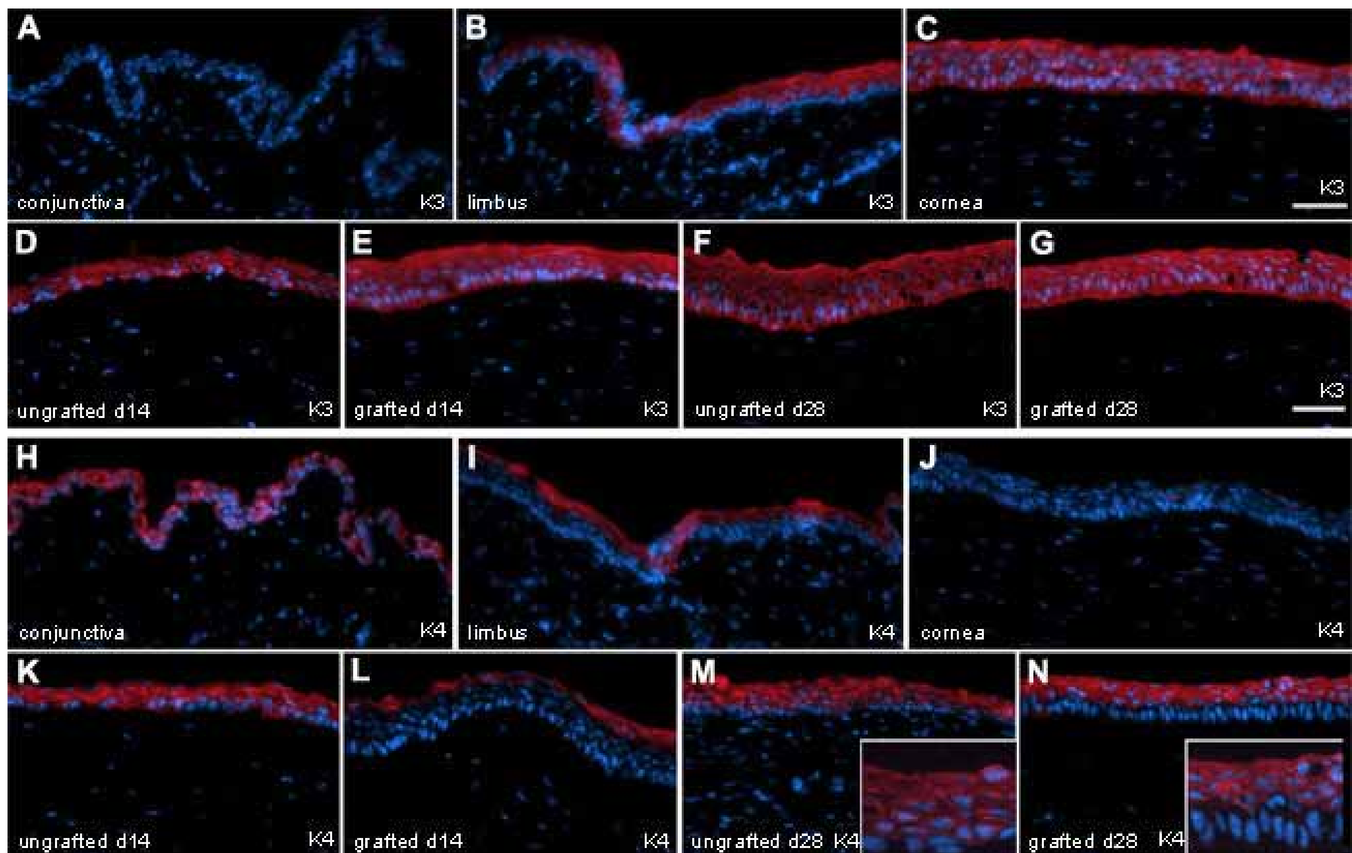

Figure 4. Keratin 3 and 4 analysis of native or grafted rabbit cornea. Keratin 3 (K3) and keratin 4 (K4) staining of native rabbit ocular surfaces in situ and following grafting of rabbit limbal epithelial cells (RLECs) cultured on fibrin gels or controls (ungrafted). **A**-**G**: These show immunostaining with K3 (red). **H**-**N**: These show immunostaining with K4 (red). **A**,**H**: Native conjunctiva. **B**,**I**: Native limbus. **C**,**J**: Native central cornea. Biopsies harvested from denuded cornea left ungrafted (**D**,**F**,**K**,**M**) or grafted (**E**,**G**,**L**,**N**) with autologous limbal epithelium cultured on fibrin for 14 days (**D**,**E**,**K**,**L**) or 28 days (**F**,**G**,**M**,**N**). Indirect immunofluorescence staining was performed on frozen sections. Nuclei were counterstained with Hoechst (blue). The scale bars represent 50 μm.

for control rabbits that received a graft of fibrin gel without RLECs (Figure 5E). The fibrin gel was completely degraded seven days after grafting (Figure 5F). Thus, in vivo degradation of the fibrin gel was rapid as it occurred within three days, upon which time RLECs attached directly to the stroma.

The epithelium thickened with time after grafting of cultured RLECs. Two to four epithelial cell layers were present in corneas seven days after grafting. The number of epithelial layers increased until five to six layers were present by 14 to 28 days after grafting cultured RLECs (Figure 5I,J). The basal cells presented the typical cuboidal morphology. Cells were flattened in the suprabasal layers, indicating a normal corneal histology. In the control ungrafted animals, the epithelium was absent on the seventh postoperative day (data not shown). It was however present but thinner than the grafted corneas by day 14 and reached the expected full thickness by day 28. In contrast to the grafted corneas, the epithelium of the controls, ungrafted or grafted with fibrin gels only, did not appear completely normal with basal cells more flattened (Figure 5C,F) and a variable number of cell layers (Figure 5D,G).

Presence of goblet cells in rabbit epithelium: The presence of goblet cells in the central region of the cornea is a strong indicator of conjunctivalization [3]. In order to better characterize the type of epithelium regenerated in the healed corneas, goblet cells were sought on histological sections and by staining for MUC5AC, a mucin specific to goblet cells. As expected, on the normal ocular surface, goblet cells were numerous in the conjunctiva but absent from the central cornea (Figure 5A, Figure 6A). In the intermediate region of the limbus, rare goblet cells could be observed in the higher suprabasal layers but never in the basal layer (Figure 5A, Figure 6A).

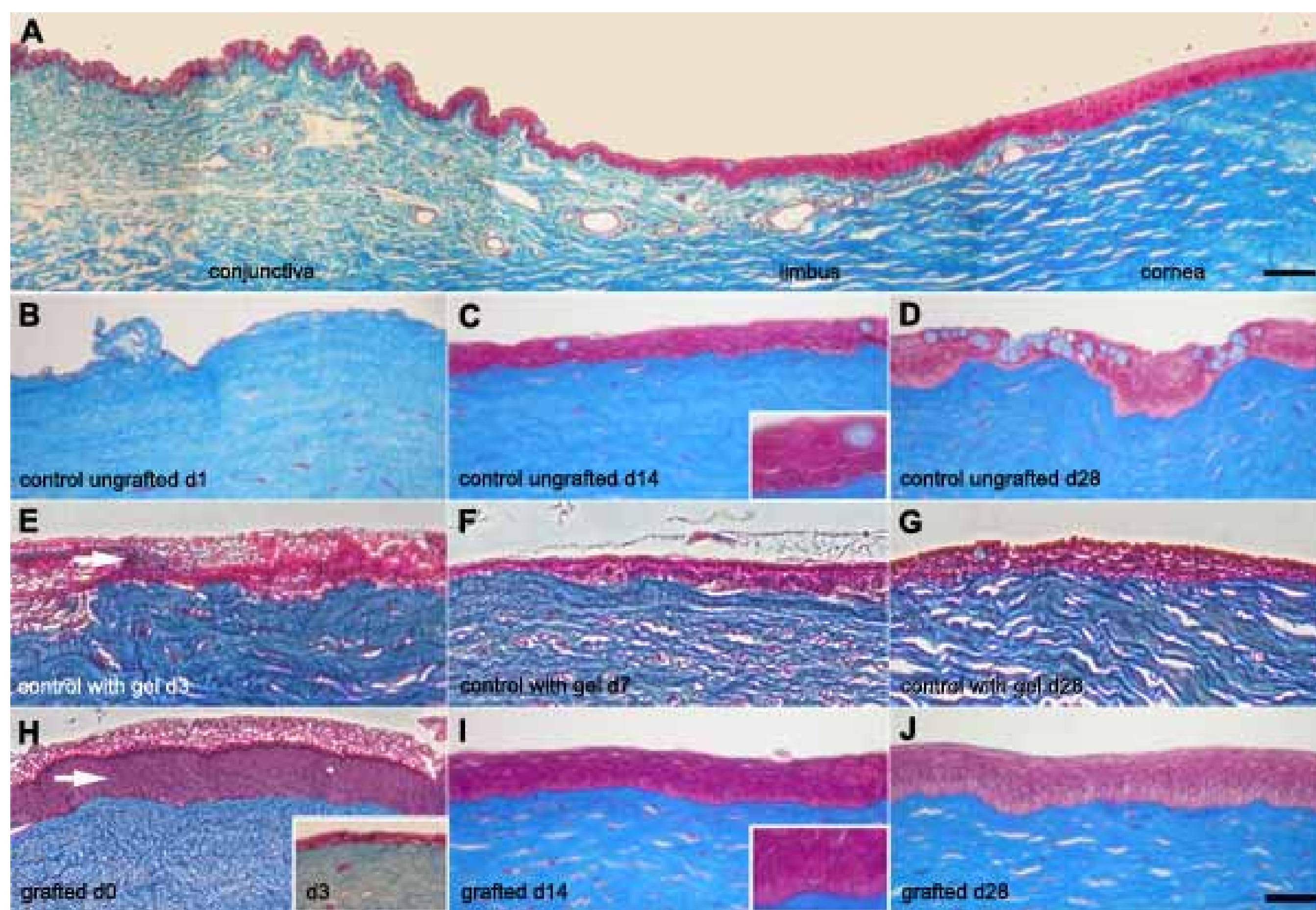

Figure 5. Histological analysis of native and grafted rabbit cornea. Masson's Trichrome staining of native rabbit ocular surface in situ (**A**) and following grafting of rabbit limbal epithelial cells (RLECs) cultured on fibrin gels (**H**-**J**) or controls (ungrafted [**B**-**D**] or grafted with fibrin gels without RLECs [**E**-**G**]). **A**: Normal rabbit cornea in situ showing the conjunctival, limbal, and corneal areas of the ocular epithelium. Control corneas (ungrafted [**B**-**D**] or grafted with fibrin gels without RLECs [**E**-**G**]) or corneas grafted with RLEC cultured on a fibrin gel (**H**-**J**) at postoperative day 1 (**B**), day 3 (**E**, insert in **H**), day 7 (**F**), day 14 (**C**,**I**), day 28 (**D**,**G**,**J**) and 2 h after grafting (**H**). Note that the characteristic staining of goblet cells (a pale blue coloration of round cells) is increasing with time after surgery in control corneas (ungrafted or grafted with fibrin gels without RLECs) as seen at day 1 (**B**), day 3 (**E**), day 14 (**C**), and day 28 (**D**,**G**). In contrast, the staining was absent or rare in the corneal epithelium 2 h (**H**), 14 days (**I**), and 28 days (**J**) after grafting RLECs cultured on fibrin gels. Note also that the fibrin gel was present 2 h after grafting (**H**) but was absent under the epithelium three days post-grafting (insert in **H**). In contrast, when gels without RLECs were grafted, the fibrin gel was still present three days after grafting (**E**). The scale bars represent 50 μm.

In the epithelium regenerated after grafting cultured RLECs following keratectomy, no goblet cells were observed in the corneal region before 28 days post-surgery (Figure 5H,I). Rare goblet cells were observed 28 days after grafting in the peripheral region but were totally absent from the central region (Figure 5J). In contrast, goblet cells were detected at 14 days in all control corneas (both ungrafted or grafted with fibrin gel only; Figure 5C). Their number largely increased as they formed clusters by day 28 (Figure 5D). The goblet cell's identity was confirmed using specific mucin immunostaining. Indeed, staining for MUC5AC was present in cells dispersed through the entire epithelium of ungrafted corneas 14 days after surgery. Positive cells were more numerous by day 28 (Figure 6B,C). In contrast, when RLECs cultured on fibrin gels were grafted, the epithelium was generally negative for MUC5AC (Figure 6D,E). The restriction of the rare positive cells observed close to the limbal region and their absence from the central area of the grafted corneas strongly suggest that RLECs cultured on fibrin gels persisted after grafting.

Characterization of rabbit epithelium with keratins: In normal cornea, K3 is expressed in all cell layers of the central cornea and the superficial cells of the limbus but absent from the conjunctiva (Figure 4A-C). K4 is present in all conjunctival epithelial cells and in superficial limbal cells but absent from the central cornea (Figure 4H-J). Basal cells of the limbus did not stain for these markers. On day 14 and 28 postoperative, a positive K3 staining was observed in both control and grafted corneas (Figure 4D-G). In contrast, K4 staining was not detected in the basal cells of the grafted corneal epithelium but was present in all layers of control corneas (both ungrafted or grafted with fibrin gels without RLECs; Figure 4K-N). The presence of cells negative for this conjunctival-specific marker (K4) indicates that the grafted RLECs persisted for at least one month.

Xenogeneic grafting of cultured human limbal cells in rabbits: In order to evaluate whether human epithelial cells behaved similarly to RLECs, limbal epithelial cells from human cornea were cultured on fibrin gels and grafted on denuded

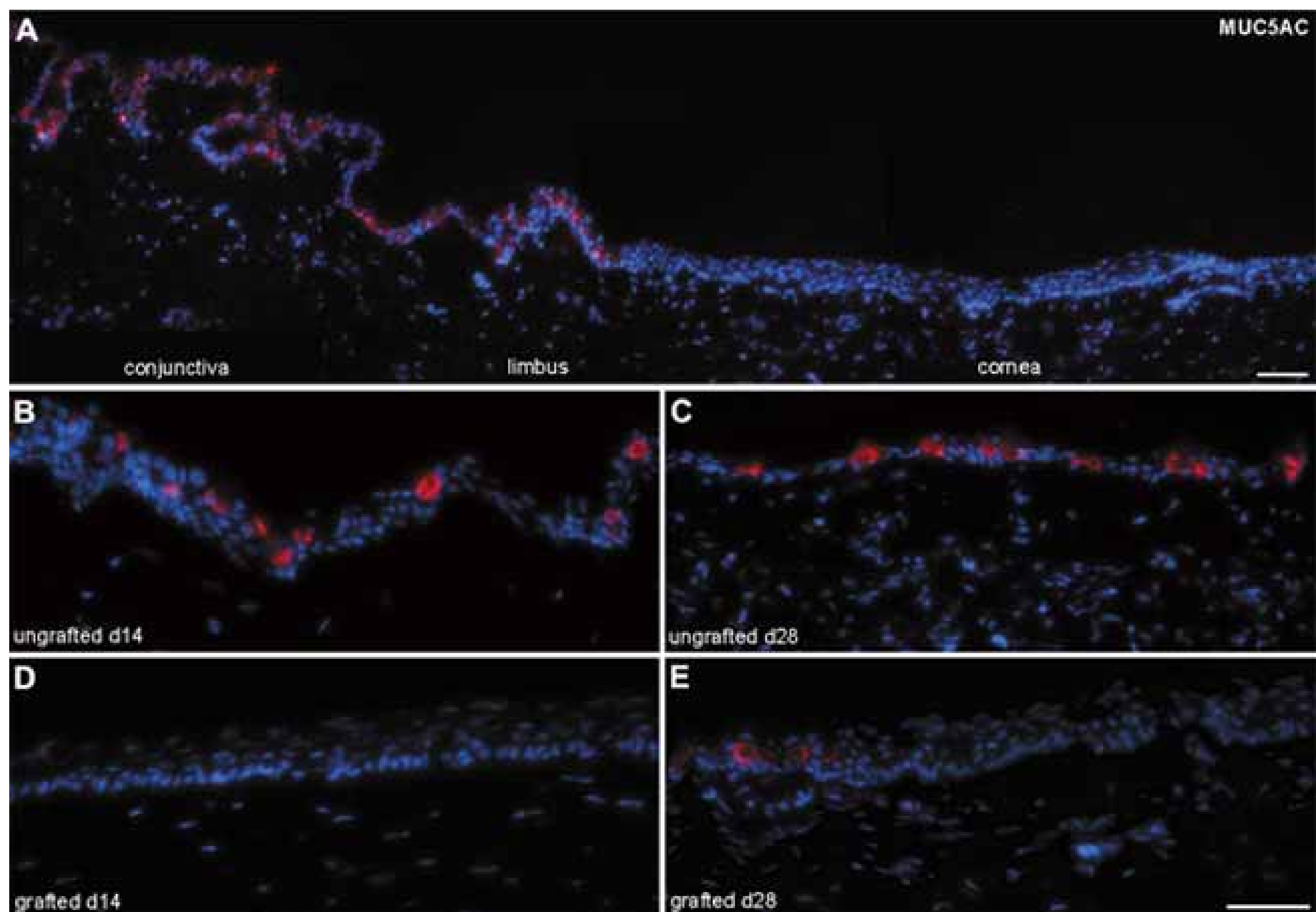

Figure 6. Goblet cell detection in native and grafted rabbit cornea. Goblet cell detection in native rabbit ocular surface in situ and following grafting of rabbit limbal epithelial cells (RLECs) cultured on fibrin gels or controls (ungrafted). Sections were stained with MUC5AC (red), and nuclei were counterstained with Hoechst (blue). Normal rabbit cornea in situ (**A**) showing the conjunctival, limbal, and corneal areas of the ocular epithelium. Note that the characteristic goblet cell marker, MUC5AC, is increasing with time after surgery in ungrafted corneas from 14 days (**B**) to 28 days (**C**). In contrast, the staining was absent or rare in the corneal epithelium 14 days (**D**) and 28 days (**E**) after grafting RLECs cultured on the fibrin gel. The scale bars represent 50 μm.

rabbit corneas using the same procedure as that selected for RLECs. Rabbits were kept for only ten days after grafting to avoid rejection of the xenogeneic epithelium. A thin epithelium containing flattened epithelial cells and goblet cells was present on the keratectomized corneas of the ungrafted control rabbits (Figure 7A). In contrast, a good reepithelialization was obtained with the grafted corneas. Ten days after grafting, the fibrin gel was absent and human corneal epithelial cells gave rise to a layer of epithelium 3 to 5 cells thick (Figure 7B). MUC5AC staining was greater in ungrafted corneas than in grafted corneas (data not shown). We therefore conclude that human cells isolated from a small limbal biopsy and cultured on fibrin gels can be successfully grafted to regenerate the corneal epithelium.

DISCUSSION

The culture and grafting of autologous limbal epithelial cells is an attractive approach for the treatment of limbal stem cell deficiency. The rapid progress of tissue engineering in the production of skin, and more recently also corneal substitutes [18,19], has led to a few clinical applications in the corneal domain [11,20-25]. The purpose of this study was to follow the regeneration of the corneal epithelium in a rabbit model that allows biopsy harvesting at various time postgrafting. Our results demonstrate that RLECs cultured on fibrin gels persist after grafting on denuded rabbit cornea and can regenerate a normal corneal epithelium within one month after grafting.

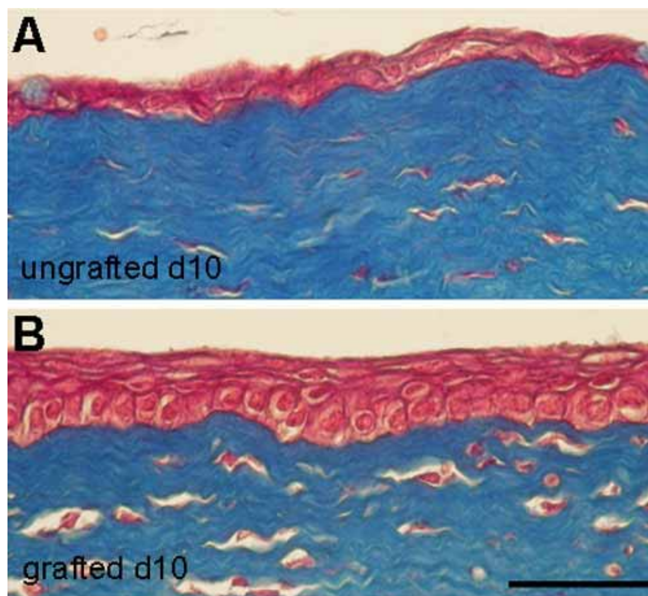

Figure 7. Histological analysis following xenogeneic grafting of human limbal epithelial cells cultured on fibrin gels. Histological corneal staining following xenogeneic grafting, on rabbit, of human limbal epithelial cells cultured on fibrin gels. Masson's Trichrome staining of control rabbits (**A**; ungrafted) and xenogeneic grafting of cultured human epithelium on rabbit (**B**). Note that 10 days after grafting, the epithelium regenerated by human epithelial cells is a 5 cell thick layer whereas the ungrafted cornea presents only one or two cell layers containing some goblet cells. The scale bar represents 50 μm.

With this autologous graft technique, it will become possible to bypass some of the major problems often occurring in conventional limbal grafting such as long term systemic immunosuppression or wide limbal sampling from the normal contralateral eye.

The grafting of autologous cells is highly suitable as there is no risk of rejection for the host. The small size of the limbal biopsy required to initiate the culture is a major advantage of this method. In contrast to a conventional limbal graft, where there is a risk of depleting all stem cells from the contralateral donor's eye [8], removal of a small biopsy of limbal tissue (3 mm^2) allowed the rabbit donor's eye to heal completely within two weeks while producing enough cells to populate a fibrin gel large enough to cover the corneal and limbal regions. Moreover, the fibrin gel disappeared after grafting, and an epithelium of normal thickness was regenerated from the cultured cells within a month. For humans, cultures are also possible from small biopsies since enough epithelium can be expanded from a biopsy as small as 1 mm^2 and can be transplanted on the patients [11,26,27]. We documented the postgrafting fate of cultured cells on 60 rabbits using histological and immunohistological analyses with results consistent with the clinical observations obtained by the group of Pellegrini after grafting 18 patients [11,27]. However, we presented results at short time periods impossible to achieve in humans. Our results are consistent with those obtained with the amniotic membrane, a different substratum shown to be adequate for culturing the epithelium before grafting in a rabbit model [28,29]. However, in contrast to the amniotic membrane that lasted 10 days after grafting, the fibrin gel was degraded very rapidly within three days without any deleterious effect on the survival of the cultured epithelial cells [30].

The use of dispase for separating the epithelium from deeper tissues yielded pure cultures without any contamination by stromal fibroblasts [31]. With this technique, epithelial cells may be detached from the basement membrane in order to isolate a viable sheet of epithelium [32] from which cultures are established [18]. Obtaining cultures of epithelial cells from every biopsy constituted an important step in our study as a single small biopsy was harvested for each rabbit. Many cell colonies grew after seeding epithelial cells on irradiated fibroblasts. The addition of a feeder layer is advantageous since large colonies can be grown from a single cell under these conditions [18,20,33]. The morphology of cultured cells, small cells with regular shapes and a low cytoplasm to nuclei ratio, was consistent with the preservation of stem cells in culture as previously demonstrated for epidermal cells cultured with a feeder layer or on fibrin gels that can permanently regenerate the epidermis in massive full-thickness burns [34-39]. Moreover, the phenotype of RLECs attached on fibrin gels and the negative immunostaining for both keratins K3 and K4 further suggest a low differentiation status as the only cells with this phenotype in situ are the basal cells from the limbus where stem cells are located.

The absence of macroscopic differences between grafted and control animals can be easily explained by the fact that macroscopic signs characteristic of conjunctivalization, such

as the emergence of blood vessels and opacification of cornea, happen between the fourth and sixth week following stem cell deficiency [6,23,28]. Thus, a microscopic study was needed to assess the changes occurring on the ocular surface over time after grafting. Such a controlled study was only possible in an experimental setting using animals since numerous biopsies are required.

The fibrin gel over which RLECs were cultured was still visible 24 h but not three days after grafting. The rate of degradation was even more rapid than the one obtained following the graft of cultured epidermal cells with a fibrin tissue glue (seven days) [40-42]. The good adhesion of the cultured epithelium to the stroma was consistent with these previous results in which a complete basement membrane was shown to be regenerated under the grafted epidermis 21 days after grafting. Twenty-eight days after grafting RLECs, the epithelium of the central corneas presented a normal morphology with cuboidal basal cells and flattened superficial cells. The number of cell layers was consistent with that observed in normal cornea in situ. The phenotype of basal cells, expressing K3 but not K4, indicates their corneal origin. Goblet cells and MUC5AC staining were rarely observed in the superficial layers of the epithelium in the periphery 28 days after RLECs grafting. It is likely that these rare cells migrated over the grafted RLECs as they were absent from the central cornea and that basal cells were K4 negative. These results strongly suggest that the grafted epithelium adhered to bare cornea, proliferated and began regenerating the corneal epithelium. It is likely that a proportion of cells in the superficial layers probably derive from the conjunctiva but are doomed to be replaced by the underlying corneal cells with time. The difference was striking when control corneas were analyzed by microscopy. Many characteristics of conjunctivalization [3,5,6] could then be observed, as expected. The presence of goblet cells is a major indicator of conjunctivalization since they originate from conjunctival keratinocytes [20]. In normal eyes in situ, these cells and their specific marker, MUC5AC, are only associated with conjunctiva [43]. In control corneas, goblet cells and MUC5AC were uniformly present over the whole cornea even in deep epithelial layers and their number increased until day 28 after surgery. The irregular morphology of the epithelial cells, the thinner epithelium compared to the normal cornea in situ, and the presence of a K4-positive staining in all layers of the epithelium all constitute clear signs of conjunctivalization that also indicate that the keratectomy induced a stem cell deficiency.

Keratin (K3) is also often used as a marker of corneal epithelial cells, K3 being normally expressed by differentiated corneal cells [5,44-46]. As expected, corneas grafted with RLECs indeed stained positively for this keratin. However, K3 was also present in the epithelium regenerated over the control corneas. These results are consistent with the works of Kurpakus et al. [47], who demonstrated that K3 expression is inactivated in the conjunctiva but not when conjunctival cells are maintained in a corneal environment. Indeed, at the early stages of conjunctivalization, K3 is present in the conjunctival cells migrating over the cornea but is undetectable at later stages when the macroscopic signs of conjunctivalization occur [2,48]. Moreover, a weak K3 staining was detected in normal bovine, rabbit, and human conjunctiva [49-51].

In summary, rabbit or human limbal epithelial cells cultured on fibrin gels restored the ocular surface with a corneal epithelium that differs from the conjunctival epithelium observed on controls left ungrafted or grafted with fibrin gel without RLECs. Our results therefore support the clinical findings reported by Pellegrini et al. [11,27] who used human cultured limbal cells to treat several ocular surface disorders characterized by the destruction of both the corneal and limbal epithelia. Long-term survival of skin epithelia cultured in the same manner [12] allows to anticipate that grafted cultured cells persist and could regenerate a corneal epithelium. Thus, the benefits of this technique include the rapid healing of the donor’s eye and the preservation of the stem cells, which are essential to repopulate the corneal epithelium during the culture process. The use of autologous cultured cells will be particularly relevant in clinical settings characterized by the total destruction of the limbus in one eye but necessitate the presence of a spared limbal area to harvest the biopsy in the other eye.

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