Selective growth and expansion of human corneal epithelial basal stem cells in a three-dimensional-organ culture

Received November 16, 2004; accepted December 29, 2004

Abstract We report on a three dimensional (3D)-organotypic culture in vitro for selective growth and expansion of human corneal epithelial stem cells. Limbal corneal explants were cultured on porous collagen sponges submerged in Epilife medium containing 10% fetal bovine serum. The fragments were analyzed by immunohistochemistry for the expression and distribution of a spectrum of corneal epithelium markers: p63, CK-19, CK-3, Ki-67, pan-cytokeratins and vimentin. Early in culture the epithelium began to exfoliate losing its differentiated high-zone layers into the medium, maintaining only basal and few parabasal cells (mostly both p63 and CK-19 positive), which had remained attached to the specimen. After 14 days a new epithelium was formed displaying an increasing prominence of basal and suprabasal cells that, sliding onto the whole explant, showed the tendency to underlay stromal tissue and infiltrate into the underlaying sponge. After 21 days, sponge and fragments were incubated with trypsin-EDTA and dispersed epithelial cells were pipetted on a feeder monolayer of mitomycin-c-treated murine NIH.3T3 fibroblasts. Colonies of undifferentiated epithelial cells (p63, CK-19 and Ki-67 positive, CK-3 negative) were obtained: their cells, if seeded onto a collagen matrix containing embedded primary human corneal fibroblasts as feeder, provided the basic building blocks for reconstructing in vitro a 3D-multilayered corneal epithelium.

Key words corneal limbus · epithelial stem cell · human cornea reconstruction · epithelial engineering · 3D-corneal culture

Introduction

Corneal epithelium is a non-keratinized stratified squamous epithelium composed of five to six layers of tightly adherent cells that undergo a continuous process of self-renewal and regeneration, with the superficial epithelial cells being constantly released in the tear film and replaced, with a turnover of 4–6 days. Basal stem cells present in the corneal limbus self-replicate undifferentiated and also generate transient amplifying cells (both are the only cells which proliferate in the corneal epithelium) that eventually migrate from the inner limbal ring periphery toward the central part of the cornea and differentiate substituting the most superficial terminally differentiated cells (Daniels et al., 2001). The limbus has an optimal microenvironment, the microenvironmental niche first suggested by Schoefield (1983), including basement membrane contacts with surrounding cells, extracellular matrix interactions, local soluble milieus, and growth-differentiation factors, that altogether contribute to stem cell survival and
renewal, and to tissue homeostasis. This stem cell niche is characterized by abundance of unique attributes, such as specific enzymes, pigment, and presence of vimentin, cells with unique cytokeratin profiles. Despite the abundant literature, corneal epithelial stem cells are still elusive and their unique phenotypic profile is still undefined (Davanger et al., 1971; Cotsarelis et al., 1989; Gipson, 1989; Hall et al., 1989; Potten et al., 1990; Lindberg et al., 1993; Morrison et al., 1997; Pellegrini et al., 1997, 1999; Watt et al., 2000). Identification, maintenance, and ex vivo expansion of corneal stem/progenitor epithelial cells for autologous or allogeneic transplantation to patients who have lost corneal transparency has become a fundamental strategy.

The aims of these studies were the following: (1) to cultivate small limbal ring residues of human intact corneal explants, following penetrating keratoplasty, in a three-dimensional (3D)-organotypic culture immersed in liquid growth medium upon collagen sponges, at the liquid/air interface, in an attempt to favor the outgrowth of stem/progenitor cells from these explants; (2) to purify and expand ex vivo stem/reserve cells isolated from these primary 3D-culture and characterize their phenotypic characteristic profile; and (3) to test their stemness from their capacity to give rise to new stem cells and transient amplifying cells and to reconstruct in vitro a 3D-corneal epithelium, suitable for transplantation.

**Methods**

Human corneal tissue

Human corneal explants, which did not meet the criteria for clinical use, were obtained from the Corneal Tissue Bank of Tuscany, Center “P. Perelli”, Lucca, Italy. The tissue explants were maintained in EUSOL-C (Graft TEC, Alchimia Transplant srl, Padova, Italy Cat. No. REF-10040) for 2–4 days at 4°C to avoid contamination before being processed.

Cell cultures

The murine embryonal fibroblast cell line NIH.3T3 was a gift from Dr. Eva Matouskova (Czech Academy of Sciences, Praha, Czech Republic). Primary cultured human corneal stromal fibroblasts (HPC.33 cells) were isolated and expanded in monolayer culture, as described elsewhere (Chen et al., 2003; Papini et al., 2003). All cells were maintained in Epilife medium (Cascade Biologics, Portland, OR, Cat. No. M-EPIcf-500) supplemented with 1.0×10^7 U/ml penicillin, 100 μg/ml streptomycin sulfate, and 0.29 mg/ml glutamine all from Euroclone (West York, UK), and Epilife-defined growth factors (EDGF) (Cascade Biologics, Cat. No. M-EPIcf-500), a combination of soluble factors with epithelial growth promoting activity, including purified bovine serum albumin, purified bovine transferrin, hydrocortisone, recombinant human insulin-like growth factor, type-I prostaglandin-E2, recombinant human epidermal growth factor. Several lots of fetal bovine serum (FBS) (Euroclone) were pre-screened for optimal epithelial cell growth and only one lot was selectively used in all these experiments. Epilife supplemented with EDGF and 10% FBS was routinely used for organ and single cell culture (Epilife10). Cells were grown in a humidified incubator in a 5% CO₂ in air atmosphere at 37°C, with changes of cell-conditioned medium with fresh medium every 2 or 3 days and split (1:5) at about 70% confluence.

3D-organotypic cultures on gelatin sponges

Small corneal explants close to limbus were cut with blades and scissors into (6–9-mm²) fragments under sterile conditions, removing the conjunctival tissue as much as possible. The fragments were washed once with sterile physiologic solution and then with Epilife10. Sterile Gelfoam® sponges from purified pork skin gelatin (Pharmacia & Upjohn, Kalamazoo, MI; Cat. No. NDC 009-031503) (20×60×7 mm size) were moisturized with Epilife10 and pressed to release air bubbles just before use, as described (Hanto et al., 1982; Chishima et al., 1997; Chen et al., 2003; Papini et al., 2003, 2004). Each sponge was cut into three pieces (20×20×7 mm size), and each piece was transferred to a well of a six-well tissue culture plate (Falcon Plastics Inc.; London Ontario, Canada; Cat. No. 353046). The fragments were then transferred (two to three/well) and placed on top of the sponge. Epilife10 was added up to the level of the upper part of the sponge (3.5–4 ml/well). The plates were maintained in a humidified incubator at 37°C and 5% CO₂ in air atmosphere. The culture medium was changed every 2 days. Fragments and sponges were collected prior to culture (T0d), after 7 days (T7d), 14 days (T14d), and 21 days (T21d). The samples were fixed in Gliofix (Italscientifica S.p.A, Genova, Italy, Cat. No. 9186764262) for 4 hr at room temperature and then maintained in a solution of 75% Ethanol at 4°C until they were processed for histology and immunohistochemistry.

Isolation and clonal expansion of epithelial basal stem cells in a two-dimensional monolayer in co-culture with NIH.3T3 fibroblasts

Corneal specimens were first cultured on top of sponges for 21 days in Epilife10, as described above. Fragments and sponges were removed from the plate, washed with physiologic solution and digested separately with a trypsin–EDTA solution 1× (Sigma, St. Louis, MO, Cat. No. T3924) at 37°C for 80 min. Cells released in suspension were collected every 20 min and the enzymatic reaction was neutralized with Epilife10. Cells were plated in six-well-tissue culture plates, containing a post-mitotic mitomycin-C (mit-c)-treated monolayer of NIH.3T3 fibroblasts seeded in culture with Epilife10 24 hr before as a feeder cell layer, and maintained in a humidified incubator at 37°C and 5% CO₂ in air atmosphere. The cell-conditioned medium was changed every 2 days. At sub-confluence (about 70% saturation of the well) the cells of a single well were detached, transferred to a glass culture chamber slide (Falcon Plastics Inc., London Ontario, Canada, Cat. No. 354104), and processed for histological and immunohistochemical analysis. Individual epithelial clones were selected, based on their active growth capacity and phenotypic traits as revealed by immunocytochemical staining (see below), their cells pooled together and seeded in co-culture with the mit-c-treated NIH.3T3 fibroblast feeder for further expansion.

Demonstration of “stemness” activity of epithelial cells outgrown from the explants

Epithelial cells, expanded in vitro at sub-confluence in a monolayer with the murine NIH.3T3 fibroblast feeder, were recovered after light enzymatic treatment, as described above, and subsequently incubated on top of primary cultured human corneal stromal fibroblasts (HPC.33 cells) as feeder cells, embedded in a collagen layer (Papini et al., 2003). Cultures were maintained immersed in Epilife10 with changes of medium every 2 days, in a humidified incubator at 37°C in a 5% CO₂ in air atmosphere. After 18–21 days of culture, the conditioned medium was removed, the cultures were fixed with Gliofix, and processed for paraffin embedding and staining.
Histology and immunohistochemistry

At pre-determined time intervals prior to culture (T0d), after 7 days (T7d), 14 days (T14d) and 18 days (T18d) or 21 days (T21d) of culture tissue fragments, sponges and reconstructed epithelium in 3D-collagen layers were embedded in paraffin and sections of <5 μm of thickness from each block were stained with Gill's hematoxylin (Bio-optica, Milan, Italy, No. 05-060132) and eosin (Eosin Y Sigma Cat. No. E4009) (H&E). Immunohistochemistry was performed on sections using Dako Envision+™ System HRP (DakoCytomation, Carpinteria, CA; Cat. No. K4007). Table 1 reports the antigens detected by the antibodies used. The positive immunoreaction of the primary antibody was detected by a secondary antibody conjugated with peroxidase-labeled polymer with diaminobenzidine (DAB) as chromogen. Immune-staining was done in order to identify epithelial cell types and, additionally, to evaluate some specific characteristics, such as the proliferative rate with mouse monoclonal antibodies (mAb) anti-Ki-67, that detects all cycling cells. Cells grown in monolayer on glass culture-chamber slides were first fixed for 6 min in absolute ethanol and then manually stained with antibodies. Monoclonal antibodies mAb 4A4 were used to detect p63, a transcriptional nuclear factor homologous with p53 whose production is essential for epithelial cell survival, growth, and differentiation (Signoretti et al., 2000). p63 is currently considered as a marker for epithelial progenitor cells (Koster et al., 2004). Monoclonal antibodies anti-pan cytokeratins (mAb AE1/AE3 which detect both high and low molecular weight cytokeratins) were used for staining epithelial cells at different differentiation stages; mAb anti-cytoplasmic CK-3 detects corneal cytokeratins (Koster et al., 2004). Monoclonal antibodies AE1/AE3 (not shown) stained the parabasal cells of the peripheral cornea, but was not detected toward the central part of the cornea. Monoclonal antibodies AE1/AE3 (not shown) stained the whole surface epithelium, whereas mAb anti-cytoplasmic CK-3 detected corneal epithelial suprabasal and epibasal differentiated cells (Joseph et al., 2004); anti-vimentin mAb were used to detect corneal endothelial and stromal cells; Envision+™ kit was used to reveal bound antibodies, following the manufacturer’s procedure.

Results

3D-organotypic cultures on sponges

Histologic examination of corneal fragments, cultivated in native state on gelatin-supported histocultures, revealed good maintenance of cellular heterogeneity and 3D-tissue-architecture throughout the period of the experiment. As shown in Fig. 1, prior to culture (T0d) p63 expression (A) was exclusively confined to the nucleus of cells of the limbal basal layer and in occasional parabasal cells of the peripheral cornea, but was not detected toward the central part of the cornea. Monoclonal antibodies AE1/AE3 (not shown) stained the whole surface epithelium, whereas mAb anti-CK-19 (B) detected this antigen only in the cytoplasm of the basal and parabasal cells of the limbus and the peripheral corneal ring, but not in most differentiated corneal epithelial cells, and mAb anti-CK-3 (C) detected this antigen only in the cytoplasm of more differentiated cells in the epibasal layers from the peripheral toward the central cornea. Vimentin-positive cells (D) were detected in endothelial cells within the stroma and also in rare cells infiltrating the epithelium. Compared to prior culture at T0d, at T7d-intervals some modifications were evident. The more external corneal superficial epibasal epithelial layers, formed almost exclusively by CK-3 positive cells, had rapidly detached, and the remaining epithelium was reduced to a monolayer, sometimes a bilayer, of progenitor cells most of which expressed selectively the p63 (E) and CK-19 (F) antigens; this epithelium had covered the entire surface of the corneal specimen including the original area toward the central cornea. These basal/parabasal epithelial cells tended to divide forming a progeny of undifferentiated and transient amplifying cells that at the T14d interval lined the outer surface of the whole fragment forming a cell bilayer and even a multiple layer of epithelial cells, both p63 (G) and CK-19 (H) positive cells, that subsequently infiltrated into the underlaying sponge. CK-3 positive cells (I) were seen predominantly in the cultured fragments forming multiple, tightly adherent layers showing the tendency of being exfoliated and released into the medium, with an apparent turnover of a week. Vimentin-positive cells were present in small vascular structures and within the corneal stroma and a small number was also seen infiltrating into the surface epithelium (J). At this time interval, we found that the whole external surface of each fragment was lined by a non-keratinized multilayered squamous epithelium (three to five layers of AE1/AE3 positive cells) containing a prominence of positive p63 cells in the basal layer and CK-19 cells in the basal and suprabasal layers, in fragments of both limbal and paracentral cornea, suggesting a rather homogeneous, limited differentiated population that was becoming predominant in our 3D-histotypic long-term culture, as a function of time. Almost every cell infiltrating into the sponge, was both p63 (K) and CK-19 (L) positive. Immune-staining was weak or absent for the most differentiated CK-3 positive cells (not shown). Many cells were cycling, as detected by positive immune-staining with anti-Ki-67 mAb (M). The

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Specificity</th>
<th>Species</th>
<th>Clone</th>
<th>Code</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>p63</td>
<td>Epithelial progenitor cells</td>
<td>Mouse</td>
<td>4A4</td>
<td>SC-8431</td>
<td>Santa Cruz Biotechnology</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Proliferating cells</td>
<td>Mouse</td>
<td>MIB-1</td>
<td>M7240</td>
<td>DakoCytomation</td>
</tr>
<tr>
<td>CK-3</td>
<td>Central corneal epithelial cells</td>
<td>Mouse</td>
<td>AE5</td>
<td>CBL-218</td>
<td>Cymbus</td>
</tr>
<tr>
<td>CK-19</td>
<td>Limbal corneal epithelial cells</td>
<td>Mouse</td>
<td>RCK108</td>
<td>M-0888</td>
<td>DakoCytomation</td>
</tr>
<tr>
<td>Vimentin</td>
<td>Mesenchymal cells</td>
<td>Mouse</td>
<td>VIM384</td>
<td>M-7020</td>
<td>DakoCytomation</td>
</tr>
<tr>
<td>High and low molecular weight cytokeratins</td>
<td>All epithelial cells</td>
<td>Mouse</td>
<td>AE1/AE3</td>
<td>M3515</td>
<td>DakoCytomation</td>
</tr>
</tbody>
</table>
phenotype and behavior of the cells in long-term cultured corneal fragments was therefore different from that seen in the normal adult cornea.

After 21 days of culture, at Td21, the same trend observed at the 14-day interval was commonly detected, with a significantly increased proportion of p63 and CK-19 positive cells infiltrating into the sponge. At Td21, the proportion of Ki-67 positive cells was still well maintained. In contrast, the cells did not express CK-3 (not shown).

Prior to culture, and at each given subsequent time in culture, the stromal compartment appeared well maintained, with few vessels in the limbal area, which was apparently intact and unchanged throughout the experiment. The endothelium monolayer under Descemet’s membrane was similarly well maintained in long-term culture, as confirmed by positive vimentin expression (not shown).

Expansion of basal epithelial stem cells in monolayer in the presence of primary cultures of mit-c-treated murine NIH.3T3 fibroblasts as a feeder layer

Enzymatic digestion of small corneal explants prior to cultures (T0d) or previously maintained on gelatin sponges for 21 days (T21d) in a 3D-organotypic culture was performed in an attempt to isolate and then expand ex vivo corneal basal stem/progenitor cells using mit-c-treated NIH.3T3 fibroblasts as a source of feeder cells providing a suitable microenvironmental niche. The culture medium currently used was Epilife10.

Figure 2 shows the results of a representative experiment. A large fraction of epithelial cells (AE1/AE3 positive) isolated from sponges or fragments, adhered to the plate. However, with cells isolated directly from the corneal explant prior to culture, at T0d, only a small fraction of the initial primary cultured mass (<0.01% of the cells) could initiate colonies, forming in many cases mosaics of slowly enlarging clones of tightly adherent polygonal cells that frequently rapidly differentiated, and the colonies aborted expressing a typical differentiated epithelial phenotype (AE1/AE3 and CK-3 positive, CK-19, p63 and Ki-67 negative); only a very limited proportion of adherent cells (<0.1%) showed high cloning efficiency and continuous proliferative capacity. On the contrary, using epithelial cells isolated from sponges and fragments maintained 18–21 days in 3D-organotypic culture, the frequency of adherent cells forming enlarging colonies, based both on the size and the phenotype of the growing colonies, in addition to aborted colonies, was markedly higher (over 15% of the seeded population). At T18d or T21d, we selected homogeneous colonies that appeared to contain cells with a prolonged proliferation capacity; the cells of these enlarging colonies were pooled and when the cultured mass reached subconfluence again, the cells were divided. The proportion of self-maintaining cells increased significantly within two successive cell passages, accounting for almost the entire population of the mass culture. These cells could be sub-cultivated by division at sub-confluence for at least four to five passages.

Fig. 1 Marker profiles. Prior to culture, at T0d, corneal limbal basal epithelial progenitors expressed nuclear p63 (A); CK-19 antigen positive expression was limited to basal epithelial cells in the limbus (B); in contrast, CK-3 was expressed in suprabasal and epibasal cells toward the central part of the cornea (C); vimentin was produced by endothelial cells and sub-epithelial stromal cells (D). At 7 days, only basal and parabasal cells, still attached to the limbal corneal fragment, expressed p63 (E) and CK-19 (F). After 14 days, at T14d, the newly generated multilayered corneal epithelium revealed that the pattern of p63 expression was similar to that observed prior to culture: cells expressing nuclear p63 were detected in one row of basal cells, sometimes also in two rows of parabasal cells, lining the inner layer of the epithelium (G); CK-19 was expressed selectively in the basal and parabasal layers (H); in contrast, CK-3 was expressed exclusively in the most differentiated epibasal layers (I); only endothelial and scattered stromal cells showed vimentin (J). A largely predominant population of p63 positive and CK-19 positive cells was constantly detected lining the entire surface of the fragment: these cells also migrated to the underlying sponge (p63 positive, K; CK-19 positive, L; Ki-67 positive, M). (Original magnification: A, B, C, D, G and H, × 200; E, F, J, K, L and M, × 400; I, × 100.)
Immune-staining showed that a great number of them expressed constitutively both p63 (>85% positive) (A) and CK-19 (over 90% positive) (B) markers and all cells were constantly CK-3 negative (C), clearly representing an enriched and rather homogeneous source of epithelial stem/progenitor corneal basal epithelial cells (basal cell metaplasia), consistent with their limbal origin (Joseph et al., 2004). These results are in line with a similar behavior that we have previously reported when trying to isolate selectively basal epithelial cells from human skin (Papini et al., 2003) or prostatic epithelium (Papini et al., 2004) in a similar type of 3D-organ culture.

The presence of post-mitotic murine embryonic fibroblasts as a source of feeder cell layer markedly enhanced the proliferative potential of the primary epithelial explants. In the absence of the feeder layer, only a few epithelial cells were able to self-replicate, provided the cells were initially seeded at a sufficiently high number (>10^4 cells/cm^2). However, when these cells reached a saturation density and were divided for further expansion, very few of them were able to reattach to the plate and those that became adherent generated rapidly abortive colonies (data not shown). These findings demonstrate the requirement of a suitable 3D-microenvironmental *niche* and of specific growth factors for corneal epithelial stem cell survival and extensive growth *ex vivo*.

Demonstration of “stemness” activity of corneal epithelial basal cells outgrown from corneal explants and expanded *ex vivo* in the presence of human primary HPC.33 corneal fibroblasts as feeder

Corneal epithelial basal cells (AE1/AE3, CK-19, p63 and Ki-67 positive, CK-3 negative cells) isolated after
one to two passages in co-culture with mit-c-treated NIH.3T3 fibroblasts, were detached by enzymatic treatment, dispersed by gentle pipetting and then transferred onto a collagen-matrix layer containing embedded human HPC.33 corneal stromal fibroblasts seeded 2–3 hr before as a source of feeders on a six-well plate (Fig. 2). The medium was then added in an amount sufficient to keep the corneal epithelial cells fully immersed. The conditioned medium was changed at 1:1 dilution with fresh medium every 2 days thereafter. When the cultures were analyzed 18 or 21 days later by histology and immunohistochemical staining on paraffin-embedded preparations, it became evident that a 3D-multilayered corneal epithelium had developed on top of the collagen layer with epithelial cells expressing the same basal, parabasal, and epibasal phenotypic traits and immunological staining mimicking that of a multilayered corneal epithelium, although containing less differentiated cells than those that are normally seen in a native normal adult human cornea. Figure 2 shows the results of a representative experiment of corneal reconstruction. The epithelial progenitor cells (AE1/AE3, p63, CK-19, and Ki-67 positive, and CK-3 all negative) isolated from epithelial cells recovered after ex vivo expansion in a 3D-organotypic culture and then from clones which had developed in co-culture with NIH.3T3 fibroblasts, were pipetted onto the collagen matrix containing embedded feeder HPC.33 cells; the cells began to divide forming first a monolayer of homogeneous undifferentiated epithelial cells tightly adherent on top of the collagen matrix layer. These basal cells eventually differentiated and after division migrated upwards, forming new layers of tightly adherent more differentiated cells. Figure 2D shows the morphology of an epithelium reconstructed in 3D-culture after 21 days. The basal and parabasal layers of the reconstructed epithelium contained p63 (E), CK-19 (F), and Ki-67 positive cells (not shown) in the basal/parabasal layers, in the absence of cells expressing CK-3; in contrast, epibasal layers included AE1/AE3 (not shown) and CK-3 positive cells (G), in the absence of cells expressing p63 and CK-19 markers. After another week in culture, there was a marked decline of cycling cells in the new epithelium, implying that the proliferative status of these cells declined as a function of more prolonged time in culture (not shown).

The phenotype of the reconstructed corneal epithelium, ranging from basal progenitor epithelial cells to superficial differentiated epithelial cells, was remarkably well maintained in 8 separately performed experiments run with corneal explants from different donors. The differentiation capacity of basal cells depended on tissue freshness (the best interval of time was the same day from the operation) and conditions of preservation (the best were those above reported), but not on donor age. Moreover, differentiation required specific growth factors, since in the presence of murine fibroblasts instead of human corneal stromal fibroblasts as feeder, a 3D corneal reconstruction was not obtained (data not shown).

Discussion

Our study reports the development of an original multi-step procedure for the selective growth, ex vivo expansion, and purification of corneal epithelial basal stem cells that can be successfully used for ex novo surface corneal reconstruction.

First, we established a 3D-organ culture that maintained the most appropriate microenvironmental niche for the survival and expansion in vitro of human corneal limbal stem/progenitor cells. The method is simple and feasible. Small fragments of limbal corneal explants were seeded in native state on gelatine sponges immersed in Epilife. For 3 weeks, at weekly intervals, the morphology of the corneal fragments was evaluated by conventional histology and immunohistochemistry. Compared with prior to culture, during the first few days there was a rapid initial loss of the two to three more superficial and differentiated layers of the original corneal epithelium, which were released into the culture medium. Subsequently, these cells were substituted by new cells generated from the basal layer of the epithelium that had remained tightly attached to the basal membrane and this occurred again, with a turnover of approximately one week. In this ex vivo 3D-organotypic culture, the surface of the cultured corneal epithelium revealed an overall less differentiated phenotype than that in a normal adult corneal epithelium seen in vivo, due to the prevalence of basal p63 and CK-19 positive cells over the whole explant of the cornea, including its more central area, in long term culture. Their phenotypic traits resemble those of the primitive epithelial basal cells that can be seen in corneas of at term anencephalus fetuses (Revoltella R.P., unpublished observations), confirming the presence of a more undifferentiated phenotype of the fetal cornea, in contrast with the immune-detected pattern in the normal adult cornea.

Altogether, these findings demonstrate that our method extends the lifespan of basal stem/progenitor cells beyond that reported with most other traditional methods (Koizumi et al., 2001; Du et al., 2003; Grueter-rich et al., 2003; Mealett et al., 2003). In our present study, we found that the phenotype and behavior of the cells in long-term cultured corneal fragments is different from that seen in the normal adult cornea. Similar to our findings, Kim et al. (2003) recently reported a method which allows corneal epithelial cell expansion ex vivo from corneal limbal explant (14–21 days) and single cell culture (10–14 days), demonstrating the maintenance of cells with a broad phenotype, ranging
from basal cells to superficial most highly differentiated cells, including a small fraction of slow-cycling BrdU label-retaining cells, characteristic of stem cells. However, these authors did not demonstrate that these cells were able to reconstruct a 3D-corneal structure. Previous experiments have shown that the epithelial cells recovered directly from fresh corneal explants could be successfully expanded ex vivo when seeded on an amniotic membrane used as a source of niche (21–24). However, in contrast to our method, these cultures do not selectively separate corneal from contaminant conjunctival epithelial cells, and could be therefore dangerous for transplantation.

The second step of our procedure was to expand ex vivo and purify basal epithelial cells. The corneal epithelium in our 3D-limbal explants showed early in culture an increasing prominence for the basal cells, detected by strong positive staining for p63 and CK-19 markers and negative staining for CK-3. Subsequently, a rapid epithelial basal cell metaplasia was seen, with basal progenitor cells showing the tendency to migrate and outgrow from the native epithelium, sliding over the outer surface of the whole corneal explant, with a tendency to infiltrate the underlaying sponge. These findings therefore demonstrate that our culture conditions favor the occurrence of a homeostasis that mimics what normally happens in vivo (Daniels et al., 2001). Within the sponge, the epithelial progenitor cells (CK-19 and p63 positive) found an appropriate structural microenvironment and actively expanded, forming colonies of tightly adherent cells expressing the typical phenotype of immature basal epithelial cells. The proliferative activity of the basal cells, expressing both p63 and CK-19 markers, was apparently more intense inside the sponge, than on the surface of the fragment, suggesting that they had a prolonged cell renewal capacity once they had migrated from the original explanted specimen and attached to the artificial collagen sponge, a response which was probably mediated by soluble growth factors present in the culture medium. The massive expansion of epithelial cells of the basal cell compartment inside the sponge provided us with the possibility of isolating and trying to purify a highly enriched population of undifferentiated corneal epithelial progenitors. These cells were detached from the sponge by enzyme digestion and cultured on a mit-c-treatment NIH.3T3 fibroblast feeder layer, using a method which was originally designed for selectively enhancing the survival, growth, and expansion of embryonal stem cells in vitro (Chen et al., 2003; Papini et al., 2003). Following this method, primary cultured corneal basal epithelial cells attached to the feeder layer and to the plastic plate, forming new colonies of enlarging epithelial cells that kept stable the same immature phenotype typical of basal stem cells.

The third step of our procedure aimed to demonstrate the “stemness” of these ex vivo expanded epithelial progenitor cells. The clones were detached, washed with fresh medium and the cells were then pipetted onto a collagen matrix layer containing freshly prepared human corneal fibroblasts (HPC.33 cells). The dispersed epithelial cells adhered to the gelatin matrix and generated cell progeny that eventually differentiated forming a 3D-multilayered epithelium, demonstrating a phenotypically successful reconstruction of the corneal tissue, mimicking a corneal epithelium in vivo. The growth rate of these epithelial stem cells depended on the tissue freshness and the presence of species-specific growth factors released by fresh human corneal fibroblasts as a source of feeder, since in the presence of feeder murine fibroblasts, a 3D-corneal reconstruction was not obtained.

In conclusion, here we have shown that corneal epithelial basal stem cells, normally present in the corneal limbal ring in vivo, can be successfully expanded ex vivo in a microenvironmental niche represented by native corneal fragments, and subsequently selectively purified and further expanded to produce an amplifying epithelial basal cell population, in co-culture with mit-c-treated NIH.3T3 fibroblasts as a feeder layer. These cells contained basal stem cells, characterized for their phenotype, the capacity of prolonged self-renewal and the capacity to form ex novo a 3D-multilayered epithelium, a successful reconstruction of the corneal surface epithelium present in vivo, if seeded on a collagen matrix containing embedded human primary corneal fibroblasts as a feeder layer. This demonstrates unequivocally that they were the basic building blocks required for corneal epithelial engineering.

The accessibility of corneal stem cells, contained in eye-banked corneal tissues, indicates the opportunity to investigate whether the corneal stem cells expanded in culture may be multipotential, representing a convenient source of adult stem cells capable of conversion along different tissue lineages.


References
can be preferentially stimulated to proliferate: implications on epithelial stem cells. Cell 57:201–209.