Propagation and Phenotypic Preservation of Rabbit Limbal Epithelial Cells on Amniotic Membrane

Der-Yuan Wang,1,2 Yi-Jen Hsueh,1 Vivian C. Yang,3 and Jan-Kan Chen1

PURPOSE. To describe the phenotypic characteristics of a limbal epithelial cell sheet outgrowth from a limbal explant cultured on amniotic membrane.

METHOD. Immunofluorescent staining and confocal microscopy were used to examine the expressions of p63, Ki-67, keratins 3 and 14, connexin 43, and the integrin αβ/β4 and α5/β1 subunits in corneal and limbal tissues in a limbal explant and epithelial outgrowth cultured for 2 weeks on amniotic membrane.

RESULTS. The expression patterns of p63, Ki-67, keratins, integrins, and connexin 43 in a limbal explant with an epithelial outgrowth cultured for 2 weeks on amniotic membrane resembled those in freshly prepared limbus. Moreover, the distribution of integrin subunits in positive cells of the limbal explant and its epithelial outgrowth was similar to that of the corneal epithelial cells during wound repair.

CONCLUSIONS. The epithelial cell sheet grown from a limbal explant on amniotic membrane exhibited a phenotype similar to that of the limbus, suggesting that amniotic membrane is a substrate capable of supporting the propagation and preservation of p63-positive limbal epithelial cells. (Invest Ophthalmol Vis Sci. 2003;44:4698–4704) DOI:10.1167/iovs.03-0272

The epithelium of the skin contains a subpopulation of basal cells that are slow cycling and self-renewing, have a high proliferative potential and long lifespan, and are able to maintain and repair the tissue in which they reside.1 These cells are termed keratinocyte stem cells. The expression status of several cellular proteins has been used to aid in the identification and/or isolation of the skin keratinocyte stem cells, including β-catenin,2 adhesion proteins α6 and β1,3 proliferating cell nuclear antigens PCNA and Ki-67, an antigen defined by a monoclonal antibody 10G7,4 keratin-145,6 and keratin-1.6,7 However, self-renewal and slow cycling are still the two most accepted characteristics of keratinocyte stem cells.8 Slow cycling is characterized by the long-term nuclear retention of tritiated thymidine (3H-TdR) or bromodeoxyuridine (BrdU) label in pulse-and-chase experiments.9 These undifferentiated, label-retaining stem cells have been shown to reside in the bulge area of the hair follicle10 and in the interfollicular basal layer of the epidermis.11 They are able to produce daughter transient amplifying (TA) cells that undergo a finite number of cell divisions before becoming terminally differentiated.12 Whether stem cells are all slow cycling is still uncertain, for there have been reports showing that not all epidermal stem cells are slow cycling13 and that putative stem cells and TA cells of the rodent epidermis can colocalize.14

The cornea is a transparent, avascular tissue. It is covered by stratified epithelium that is responsible for maintaining a smooth corneal surface and providing a barrier against environmental stress. Corneal epithelium is also renewable and, based on the ability to retain [3H-TdR] label and the expression of keratins (K)3 and K12, it has been suggested that corneal epithelial stem cells are located in the basal layer of the limbus.9,15 a transition zone between the cornea and conjunctiva.16 Recently, a p53 homologue essential for epithelial development,17 the p63 transcription factor, has been shown to be expressed in the basal cells of the human limbal epithelium, but not in TA cells of the corneal epithelium. Western blot analysis of the cultured keratinocytes has shown that p63 is abundantly expressed by holoclone cells of the limbal epithelium, but is undetectable in the paracorneal cells.19 p63 has thus been suggested to be a specific nuclear marker for limbal epithelial stem cells.

Clinical studies also appear to support the notion that corneal epithelial stem cells reside in the limbus. Limbus-deficient eyes manifest chronic inflammation, fibrous tissue ingrowth, and vascularized conjunctival invasion into the cornea, leading to functional blindness that cannot be treated by conventional corneal transplantation.19,20 Autologous and heterologous limbal transplantation have been used to treat such patients; however, donor site morbidity remains a major concern.21–25 Recently, Tasi et al.24 successfully reconstructed the corneal surface of six limbus-deficient eyes by transplantation of autologous limbal epithelial cells that had been ex vivo expanded from a limbal biopsy specimen on human amniotic membrane (AM).24 The success of this protocol in reconstructing a limbus-deficient corneal surface suggests that limbal epithelial stem cells may be preserved in the AM-based culture system. However, questions remain to be answered. First, it is not yet known whether AM can really preserve limbal epithelial stem cells, nor do we know the phenotypic characteristics of the limbal epithelial outgrowth on AM before transplantation. Second, and equally important, it is not clear how many stem cells are really transplanted, nor how many are needed to reconstruct a damaged cornea.

In the present study, we compared the expression patterns of corneal keratinocyte differentiation markers, including p63, K3 and K14, integrin subunits, connexin 43, and Ki-67 in freshly prepared rabbit corneal and limbal tissues and in a limbal explant with an epithelial outgrowth cultured for 14 days on AM. Our results indicate that the epithelial outgrowth of the limbal explant on AM assumes a phenotype similar to that of the limbus, suggesting that AM is a substrate capable of supporting the propagation and preservation of p63-positive limbal epithelial cells.
MATERIALS AND METHODS

Dulbecco’s modified Eagle’s medium (DMEM), Ham’s F-12 nutrient, trypsin-EDTA, fetal bovine serum (FBS), and dispase II were purchased from Invitrogen-Gibco (Gaithersburg, MD); Dimethyl sulfoxide (DMSO) and bovine insulin were from Sigma-Aldrich (St. Louis, MO); Cholera toxin A subunit (type Inaba 569B, azide free) was purchased from Calbiochem (La Jolla, CA). Mouse receptor grade epidermal growth factor (EGF) was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Recombinant human basic fibroblast growth factor (bFGF) was from PeproTech EC, Ltd. (London, UK). The primary antibodies against p63 nuclear protein (4A4 clone); K3 (AE5 clone) and K14 (LL002 clone); integrin-α3 and -α6 (MK1-Gol5 clone); β1 (MB1.2 clone), and β4 subunits; connexin 43 (4B6.2 clone); and all fluorescent dye–conjugated secondary antibodies were purchased from Chemicon International, Inc. (Temecula, CA). The anti-human Ki-67 antibody was purchased from Serotec, Ltd. (Oxford, UK). All plastic cell culture wares were from Corning Costar Co. (Corning, NY).

Preparation of Human AM

Human AMs were obtained from Chang Gung Memorial Hospital (Linko, Taiwan) with proper informed consent, according to the Declaration of Helsinki, and were processed as described.23,24 Briefly, the AM was aseptically washed three times in 200 mL of 1× phosphate-buffered saline (PBS) containing 50 μg/mL penicillin, 50 μg/mL streptomycin, 2.5 μg/mL amphotericin B, and 25 ng/mL gentamicin. The membrane was then serially washed once each with 0.5, 1.0, and 1.5 M DMSO in PBS. The membrane was frozen and stored at −80°C in DMEM containing 50% glycerol. Before use, the AM was thawed and incubated with 2.4 U of dispase II at 37°C for 25 minutes to loosen the epithelial layer. The epithelial cells were then removed by gentle scraping with a cell scraper (Nunc Inc., Naperville, IL). The denuded AM was placed on a culture plate with the basement membrane side up and incubated at 37°C in a humidified incubator under 95% air and 5% CO2 overnight before use.

Treatment of the Limbal Biopsy Specimen and In Vitro Culture of Limbal Epithelial Cells

A limbal biopsy was performed on healthy eyes of a New Zealand White rabbit, housed and treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and according to an experimental procedure approved by the committee for Animal Research at Chang Gung University. The eyelid was sterilized with providone iodine, and a 1 × 2-mm limbal tissue specimen containing epithelial cells and part of the corneal stroma was separated from the limbal margin and excised from the superficial corneal stroma by lamellar keratectomy. The limbal epithelial cells were cultured as previously described.24 Briefly, the limbal biopsy specimen was inoculated onto the basement membrane side of the denuded human AM and cultured in DMEM/Ham’s F-12 (1:1, 10 mM HEPES buffered) supplemented with 5% FBS, 0.5% DMSO, 2 ng/mL EGF, 25 μg/mL bovine pituitary extract, 1 μg/mL bovine insulin, and 0.1 μg/mL cholera toxin. Cultures were incubated in a humidified incubator at 95% air and 5% CO2. The culture was maintained for 2 to 3 weeks, and the medium was replaced with the same medium every 2 days.

Immunofluorescent Staining and Confocal Microscopic Examination

The immunofluorescent staining was performed conventionally. Briefly, rabbit corneal and limbal tissues, limbal explant, and epithelial outgrowth on human AM were fixed with 4% freshly buffered paraformaldehyde (pH 7.4) at room temperature for 10 minutes and were dehydrated and paraffin embedded. For dual-color immunostaining, 3-μm sections were rehydrated three times with 1× PBS containing 5% normal donkey serum for 45 minutes to minimize nonspecific signals. Sections were incubated with the following primary antibodies in respective dilutions: anti-p63 (1:100), anti-K3 and anti-K14 (1:100), anti-α3/α6/β4/β1 integrin subunits (1:25), anti-connexin 43 (1:50), or anti-Ki-67 (1:25). After samples were washed with 1× PBS, they were incubated with cyanine-3 (Cy3)-conjugated (donkey anti-mouse IgG for p63, K3, and connexin 43) or FITC-conjugated (donkey anti-rabbit IgG for the α3 and β4 integrin subunits, donkey anti-rat IgG for the β1 integrin subunits) secondary antibodies. Sections were mounted with anti-fade mounting medium (Gel Mount; Biomedea Co., Foster City, CA), and examined with a confocal laser scanning microscope (model LSM 510; Carl Zeiss Meditec, Oberkochen, Germany) and a fluorescence microscope (Carl Zeiss Meditec). All images were acquired with a digital photograph system and processed with image-analysis computer software (Photoshop; Adobe Systems, Mountain View, CA).

RESULTS

The epithelial sheet grown from the limbal explant on AM was usually more or less circular, and in 2 weeks, it assumed a diameter of 0.9 to 1.2 cm. The central region of the epithelial cell sheet was 2 to 3 cells thick (Fig. 1C), whereas the marginal advancing edge was 9 to 12 cells thick (Fig. 1D). No goblet cells were observed in the limbal explant or the outgrowth, indicating that the ex vivo expansion of limbal epithelium on AM does not follow the differentiation lineage of the conjunctival epithelium and that the original limbal explant was not contaminated with conjunctival tissue. To characterize the phenotypic characteristics of the limbal epithelial outgrowth on AM, we examine the expression patterns of several differentiation markers of corneal epithelial cells with immunofluorescent staining and confocal microscopy in freshly prepared corneal and limbal tissue (Fig. 1A) and in a limbal explant with an epithelial outgrowth on AM. Sections (5 μm) of paraffin-embedded rabbit corneal tissue (A) and limbal tissue expanded on AM (B) were stained with hematoxylin. (B) Direction of epithelial outgrowth from limbal explant on AM is from left to right. (C, D) Magnification images of boxes (B, left and right, respectively).

FIGURE 1. Histologic examination of rabbit cornea, limbus, and a limbal explant with an epithelial outgrowth on AM. Sections (5 μm) of paraffin-embedded rabbit corneal tissue (A) and limbal tissue expanded on AM (B) were stained with hematoxylin. (B) Direction of epithelial outgrowth from limbal explant on AM is from left to right. (C, D) Magnification images of boxes (B, left and right, respectively).

Corneal epithelium; Lu, limbal epithelium; Bars: (A, B) 100 μm; (C, D) 40 μm.
Several subunit combinations of integrin have been identified in the corneal epithelium.\textsuperscript{53} The α6/β4 integrin localizes to hemidesmosomes and is defined as an adhesion receptor for laminins. It is expressed primarily on the basal layer of epithelium and has been shown to be involved in corneal epithelial wound healing.\textsuperscript{32} The α3/β1 integrin functions as a basement membrane receptor. It is involved in modulating adhesion, migration, and cytoskeletal organization of epithelial cells.\textsuperscript{33} In the present study, expression of the integrin β4 subunit was weakly positive in both suprabasal and basal cells of the corneal epithelium. In the basal cells, it was mostly localized to the lateral membranes (Figs. 4E, 4I). In the limbus, the integrin β4 subunit was confined to the basal cells and was localized mostly at the cell periphery (Figs. 4F, 4J). In the limbal explant with an epithelial outgrowth on AM, the integrin β4 subunit was evenly distributed throughout the epithelium (Figs. 4G, 4H, 4K, and 4L). In the cornea, limbus, and limbal explant with outgrowth on AM, the expression pattern of the integrin α3 subunit was very similar to that of the integrin β4 subunit (Figs. 5F–H, 5J–L). However, the expression level of α3 subunit appeared to be lower than that of the β4 subunit in the cells of an ex vivo cultured limbal explant with an epithelial outgrowth. In the cornea, expression of the integrin β1 subunit was confined to the suprabasal and basal cells (Figs. 4I, 5A). In fresh limbus and in a limbal explant with an epithelial outgrowth on AM, the expression pattern of integrin β1 subunit was also similar to that of the integrin α6 subunit (Figs. 5B–D, 5J–L). In summary, cornea and limbus expressed lower levels of integrin-β4 and -α3 than the limbal explant with an outgrowth on AM.

### Expression Pattern of p63 and Ki-67

In the present study, we first examined the distribution pattern of p63-positive cells in rabbit cornea and limbus by immunohistochemistry and confocal microscopy. Consistent with ob-

### Expression Pattern of Keratins

K3 is a specific differentiation marker of corneal epithelium.\textsuperscript{9,20} K14 is regarded as a marker of keratinocyte proliferation in skin\textsuperscript{27}, however, it is also expressed in epithelial cells of the ocular surface.\textsuperscript{28} In our results, corneal epithelium was strongly positive for K3 and negative for K14 (Figs. 2A, 2B). In limbus, K3-positive cells were confined to the upper stratified epithelium, and K14-positive cells were confined to the suprabasal and basal layers (Figs. 2C, 2D). In limbal explant cultured for 2 weeks on AM, the distribution of K3- and K14-positive cells remained similar to that of the freshly prepared limbal tissue (Figs. 2E, 2F). In the limbal epithelial outgrowth on AM, K3- and K14-positive cells were confined to the upper suprabasal and lower basal layers, respectively (Figs. 2G, 2H). Thus, the expression pattern of K3 and K14 in the limbal outgrowth on AM was also similar to that observed in the freshly prepared rabbit limbal epithelium.

### Expression Pattern of Connexin 43

Connexins are a group of transmembrane proteins in gap junctions and are involved in direct cell-cell communication.\textsuperscript{29} Connexin 43 has been implicated in corneal functions and repair.\textsuperscript{30} In the current study, connexin 43 was strongly expressed throughout the corneal epithelium (Fig. 3A), whereas in the limbus, connexin 43-positive cells were confined to the superficial layer and the expression level was low (Fig. 3B). In a limbal explant cultured for 2 weeks on AM, connexin 43-positive cells were also confined to the upper stratified epithelial cells, and the expression level was as weak as in the freshly prepared limbus (Fig. 3C). In the limbal epithelial outgrowth on AM, connexin 43 signal was absent throughout the entire epithelium (Fig. 3D). The basal and suprabasal layers of limbal epithelium and the limbal explant with epithelial outgrowth on AM were all negative for connexin 43.

### Expression Pattern of Integrin Subunits

Integrins are heterodimers of α and β subunits and connect the cytoskeletal components of cells to extracellular matrix (ECM).
observations made in human limbus, we found that p63-positive signal was specifically confined to the nuclei of the basal cells in the limbal-peripheral corneal junction and the basal and suprabasal cells of the limbus (Fig. 6). To see whether the p63-positive cells proliferate, the presence of Ki-67, a proliferating cell nuclear marker, was also examined. Most of the basal and suprabasal cells of the limbus were positive for p63 and Ki-67 (Figs. 7C, 7H, 7M). In the limbus–peripheral corneal junction, there are a few suprabasal cells that were Ki-67 positive but p63 negative (Figs. 7B, 7L, arrows), however, the basal cells were positive for both p63 and Ki-67 (Figs. 7G, 7L). In contrast, corneal epithelium was negative for both markers (Figs. 7A, 7F, 7K). In the limbal explant and its outgrowth on AM, p63- and Ki-67-positive cells were present only in the basal layer (Figs. 7D, 7E, 7I, 7J, 7N, 7O). Similar to observations in the limbal-peripheral corneal junction, there were a few suprabasal cells expressing only Ki-67 in the limbal explant (Figs. 7D, 7N, arrows). In the epithelial outgrowth on AM, the p63 and Ki-67 signals were coexpressed in the basal and suprabasal cells (Figs. 7E, 7J, 7O). There were some suprabasal cells that were p63 positive alone when viewed in a single x-y-axis scanning field (Figs. 7G, 7H, 7L, 7M, arrowheads); however, when viewed through the serial z-axis scanning field of the same section (data not shown), all cells that were positive for p63 appeared to be positive for Ki-67 as well.

**DISCUSSION**

Since the initial successful reconstruction of the corneal surface by transplantation of limbal epithelial cells expanded on human AM in limbus-deficient patients, several slightly modified methods for reconstruction of the corneal surface have been reported. The clinical success of these protocols in reconstructing the corneal surface suggests that limbal epithelial stem cells may be preserved in the AM-based culture system. However, there has been no direct proof of the presence of stem cells in the transplant, nor do we know the phenotypic characteristics of the limbal outgrowth on AM before transplantation. In this study, we compared the expression patterns of specific markers of squamous epithelial differentiation in freshly prepared corneal and limbal tissues, and in a limbal explant with an epithelial outgrowth cultured for 14 days on AM. We showed that the basal cells of the epithelium ex-
Figure 6. The expression of p63 in epithelium of rabbit cornea and limbus. Sections (5 μm) were reacted with p63 (4A4 clone)-specific antibody, visualized with a chemical DAB-HRP system, and counterstained with hematoxylin. (A, D) The corneal epithelium was negative for p63. In the limbus, p63-positive cells were located in the basal and suprabasal layers (B). The p63-positive cells were gradually confined to the basal layer toward the limbal-peripheral corneal junction together with a decreasing staining intensity (C). (B, arrows) Cells in the superficial layer of the limbal epithelium expressed relatively weak p63 signal in the limbal superficial layer. Bars: (A) 100 μm; (B, C, D) 40 μm.

Figure 7. Immunostaining of p63 and Ki-67 in rabbit cornea, limbus, and a limbal explant with epithelial outgrowth on AM. Sections were double stained with p63- and Ki67-specific antibodies and Cy3/FITC-conjugated secondary antibodies. Confocal microscopic examination showed that most of the basal and suprabasal cells of the limbus were positive with both p63 and Ki-67 (C, H, M). In the limbal-peripheral corneal junction, the basal cells were positive with both p63 and Ki-67 (B, G, L). However, the corneal epithelium was negative for both markers (A, F, K). In the limbal explant and its outgrowth on AM, both p63- and Ki-67-positive cells were present only in the basal layer (D, E, I, J, N, O). In the limbal-peripheral corneal junction, there were some suprabasal cells that were p63 positive alone when viewed through a serial z-axis scan of the same section (data not shown), all cells that were positive for p63 appeared to be positive for Ki-67 as well. Dashed lines: boundaries of the epithelia. Bars, 6 μm.

The expression of p63 in epithelium of rabbit cornea and limbus. Sections (5 μm) were reacted with p63 (4A4 clone)-specific antibody, visualized with a chemical DAB-HRP system, and counterstained with hematoxylin. (A, D) The corneal epithelium was negative for p63. In the limbus, p63-positive cells were located in the basal and suprabasal layers (B). The p63-positive cells were gradually confined to the basal layer toward the limbal-peripheral corneal junction together with a decreasing staining intensity (C). (B, arrows) Cells in the superficial layer of the limbal epithelium expressed relatively weak p63 signal in the limbal superficial layer. Bars: (A) 100 μm; (B, C, D) 40 μm.

The absence of connexin 43-containing gap junctions has been suggested as being involved in maintaining the stemness of limbal epithelial stem cells in their microenvironment. In the present studies, the basal cells in the limbus and limbal explant and outgrowth cultured for 2 weeks on AM were all connexin 43 negative. This indicates again that the AM-based culture system has the capability of propagating and preserving the limbal epithelial basal cells. In addition, it has been reported that connexin 43 is absent in the entire human limbal epithelium. However, we found connexin 43-positive cells that were confined to the superficial layer of the rabbit limbus. This specialized expression pattern is in accordance with the observation in New Zealand and Dutch belted rabbits reported by Matic et al. They found that connexin 43 is absent in the human and chicken limbus and is expressed at very low levels in the rabbit limbus.

The rate of corneal epithelial renewal is dependent on a highly integrated balance between the processes of proliferation, migration, differentiation, and even apoptosis. The processes of cellular proliferation, migration, and differentiation involve the interactions between cells and the underlying ECM. The basement membrane consists mainly of type IV collagen and laminins. These two components mediate epithelial cell attachment, migration, proliferation, and differentiation. Fukuda et al. have shown that the basement membrane of the AM and the region ranging from cornea to conjunctiva share the same composition of laminins-1 and -5, fibronectin, and type VII collagen. The α6/β1, α6/β4, and α3/β1 integrin heterodimers serve as laminin receptors in a variety of cell types. In the present study, the expression patterns of the integrin-α6 and -β1 subunits were similar in the cornea, limbus,

growth on AM, both p63- and Ki-67-positive cells were present only in the basal layer (D, E, I, J, N, O). In the limbal-peripheral corneal junction, there were some suprabasal cells that were p63 positive alone when viewed through a serial z-axis scanning field (arrowheads). However, when viewed through a serial z-axis scan of the same section (data not shown), all cells that were positive for p63 appeared to be positive for Ki-67 as well. Dashed lines: boundaries of the epithelia. Bars, 6 μm.

The expression of p63 in epithelium of rabbit cornea and limbus. Sections (5 μm) were reacted with p63 (4A4 clone)-specific antibody, visualized with a chemical DAB-HRP system, and counterstained with hematoxylin. (A, D) The corneal epithelium was negative for p63. In the limbus, p63-positive cells were located in the basal and suprabasal layers (B). The p63-positive cells were gradually confined to the basal layer toward the limbal-peripheral corneal junction together with a decreasing staining intensity (C). (B, arrows) Cells in the superficial layer of the limbal epithelium expressed relatively weak p63 signal in the limbal superficial layer. Bars: (A) 100 μm; (B, C, D) 40 μm.

The absence of connexin 43-containing gap junctions has been suggested as being involved in maintaining the stemness of limbal epithelial stem cells in their microenvironment. In the present studies, the basal cells in the limbus and limbal explant and outgrowth cultured for 2 weeks on AM were all connexin 43 negative. This indicates again that the AM-based culture system has the capability of propagating and preserving the limbal epithelial basal cells. In addition, it has been reported that connexin 43 is absent in the entire human limbal epithelium. However, we found connexin 43-positive cells that were confined to the superficial layer of the rabbit limbus. This specialized expression pattern is in accordance with the observation in New Zealand and Dutch belted rabbits reported by Matic et al. They found that connexin 43 is absent in the human and chicken limbus and is expressed at very low levels in the rabbit limbus.

The rate of corneal epithelial renewal is dependent on a highly integrated balance between the processes of proliferation, migration, differentiation, and even apoptosis. The processes of cellular proliferation, migration, and differentiation involve the interactions between cells and the underlying ECM. The basement membrane consists mainly of type IV collagen and laminins. These two components mediate epithelial cell attachment, migration, proliferation, and differentiation. Fukuda et al. have shown that the basement membrane of the AM and the region ranging from cornea to conjunctiva share the same composition of laminins-1 and -5, fibronectin, and type VII collagen. The α6/β1, α6/β4, and α3/β1 integrin heterodimers serve as laminin receptors in a variety of cell types. In the present study, the expression patterns of the integrin-α6 and -β1 subunits were similar in the cornea, limbus,

growth on AM, both p63- and Ki-67-positive cells were present only in the basal layer (D, E, I, J, N, O). In the limbal-peripheral corneal junction, there were some suprabasal cells that were p63 positive alone when viewed through a serial z-axis scanning field (arrowheads). However, when viewed through a serial z-axis scan of the same section (data not shown), all cells that were positive for p63 appeared to be positive for Ki-67 as well. Dashed lines: boundaries of the epithelia. Bars, 6 μm.
and the limbal explant and outgrowth cultured for 2 weeks on AM, suggesting that in these tissues, integrin-α6/β1 may play important roles in cell binding to laminin-1 and/or laminin-5 of the basement membrane. As for the expression of α6/β4 integrin in normal human corneal epithelium, Espana et al. showed that a α6/β4 integrin signal is limited to the bottom membrane of the basal layer and is in contact with the underlying basement membrane. In the present study in rabbit cornea, the integrin β4 subunit was present in the bottom and lateral membranes of the basal cells, and in the limbus, it was present around the periphery of the same cell layer. However, the integrin-α6 subunit is expressed throughout the rabbit corneal epithelium and is confined to the basal cells in the limbus. Thus, there may be cross-species variations in the expression of α6/β4 in the epithelia of cornea and limbus between human and rabbit. The α6/β4 integrin also interacts with several signaling molecules that regulate cell migration and invasion. Studies of an in vivo rabbit corneal epithelial injury model by Song et al. showed that integrin α6 subunit is expressed constitutively at relatively higher levels than is β4 subunit over time. They also showed that an increase in the expression ratio of β4 to α6 correlates with cell migration. In the present study, the expression levels of the β4 subunit in a limbal explant and outgrowth cultured for 2 weeks on AM were usually higher than in freshly prepared limbus and cornea. Therefore, our finding is in accordance with a role for the β4 subunit in mediating cell mobility. In human epidermis, the putative stem cells express higher levels of the α3/β1 integrin than that of the TA cells, and α3/β1 integrin has been used to localize the epidermal stem cells. In the present study, limbal explant and its outgrowth on AM expressed a higher level of β4 and α3 than freshly prepared cornea and limbus. Thus, the expression pattern and levels of β4 and α3 integrins in the limbal explant and outgrowth cultured for 2 weeks on AM resembled corneal epithelial cells during the wound-repair process, suggesting that they are in a metabolically activated state and are probably ready to migrate and proliferate. Our results appear to indicate that an AM-based culture system is capable of supporting the propagation and preservation of the basal cells of the limbal epithelium. The repertoire of integrin expression may play crucial roles in promoting the epithelial outgrowth from limbal explants and in preserving limbal epithelial stem cells on AM.

Pellegrini et al. have suggested that p63 is a specific nuclear marker for human corneal and epidermal stem cells. They showed that the basal cells of human limbal epithelium are p63 positive (stem cells), proliferating cell nuclear antigen (PCNA) positive (old TA cells), or both p63- and PCNA positive (Ki-67 positive). This is discordant with findings in a recent report showing that p63 expression is confined to the basal cells of the human limbal epithelium. The reason for such a discrepancy could be cross-species differences and/or the sensitivity of the staining method used.

The 4A4 clone of the anti-p63 monoclonal antibody developed by Yang et al. has been used widely for the detection of p63 nuclear protein. This monoclonal antibody was designed to recognize six isoforms of p63. In this study, we have tested the specificity of this anti-p63 monoclonal antibody by Western blot analysis and found that it specifically recognizes the p63 isoforms in the lysates of rabbit corneal and skin (data not shown).

The expression patterns of p63 and Ki-67 in a limbal explant with an epithelial outgrowth cultured for 2 weeks on AM suggests that AM-based culture can preserve and allow the expansion of p63-positive cells. The copresence of Ki-67 indicated that these p63-positive limbal epithelial cells were highly proliferative. Although the exact role of p63 in the maintenance of limbal stem cells remains to be understood, our studies showed that an epithelial cell sheet grown from a limbal explant on AM retains important physiological, functional, and structural characteristics similar to normal limbal epithelium. Our results may serve to explain, at least partially, the successful clinical reconstruction of corneal surface with the ex vivo expanded limbal epithelial outgrowth on AM.

Acknowledgments

The authors thank the Division of Drug Biology of the Bureau of Food and Drug Analysis, Taiwan, for providing the New Zealand White rabbits, and Seng-Sheen Fan and Wei-Ting Chao, Department of Biology, Tunghai University, for providing assistance with confocal laser scanning microscopy (model LSM 510).

References


