P63 staining on human ocular surface

CHENZHUO L, MD, PhD[1], MURUBE J, MD, PhD[2]

RESUMEN

Propósito: Recientemente se ha hipotetizado que la proteína nuclear p63 es el primer marcador de las células madres del epitelio corneal durante el desarrollo. La presente publicación determina la localización de las células queratolimbales madres del epitelio corneal por el patrón de expresión p63 en la superficie ocular.

Métodos: Se usó tejido queratolimbal humano para detectar por inmunohistoquimia el patrón de expresión de la proteína p63. Se desarrolló la hibridización in situ bidireccional para confirmar la expresión p63 mRNA en el anillo queratolimbal.

Resultados: La proteína p63 y ARNm se expresaron sólo en los núcleos de la capa basal de las células epiteliales limbales.

Conclusión: De acuerdo con nuestros hallazgos, la proteína p63 localizada en la células basales del epitelio queratolimbal es un buen marcador para detectar las células madres del epitelio corneal. La p63 podría utilizarse en práctica clínica para determinar la actividad de las células madres del epitelio corneal.

Palabras clave: P63, limbo corneal, células madre, epitelio corneal.

SUMMARY

Purpose: It has been recently hypothesized that transcription factor p63 may be the earlier marker of epithelial stem cells during development. This paper evaluates the p63 location of the corneal and limbal area to find out the p63 expression pattern in the ocular surface.

Methods: Human cornea and limbal tissue were used to detect p63 protein expression by immunohistochemistry. In situ hybridization with sense and anti-sense p63 probes were performed on the corneal and limbal tissue to confirm the p63 mRNA express pattern at the cornea and limbus.

Results: P63 protein and mRNA were expressed only in the nuclei of the limbal basal epithelial cells layer. There was no staining on the corneal epithelial cells and limbal superficial epithelial cells.

[1] Department of Mollecular and Cell Biology. Baylor College of Medicine. Houston, USA.
[2] Department of Ophthalmology. Hospital Ramón y Cajal. University of Alcalá. Madrid. Spain.

Correspondencia:
Juan Murube del Castillo
Fundación Rizal de Investigación Oftalmológica
Universidad de Alcalá de Henares
C/. Moralzarzal, 43
28034 Madrid
España

Conclusion: According to our findings, p63 which is located in limbal basal epithelial cells is a good marker for corneal stem cells, P63 could be used in the clinic practice to evaluate corneal stem cell activity.

Key words: P63. Corneal limbus. Stem cell. Epithelium.

The corneal epithelial stem cells are believed to be located at the limbus (1-3). The supporting data shows that: 1) The limbal basal cells lack the corneal epithelial differentiation-associated keratin pair K3 (4) and K12 (5); 2) The limbal basal epithelium contains proliferate characteristics such as slow-cycling cells identified as the «label-retaining cells» following pulse-chase labeling of all cells with DNA precursor, such as [3H]-thymidine or bromodeoxyuridine (BrdU) (6) and also the limbal basal epithelium exhibits high proliferative potential in culture (6-9); 3) Experimental studies and clinical observations show abnormal corneal epithelial wound healing as conjunctivalization, vascularization and chronic inflammation when the limbal epithelium is partially (10,11) or completely defected (12,13); 4) The limbal location of corneal epithelial stem cells can account for the relative preponderance of limbal neoplasms and the scarcity of corneal epithelial tumors, assuming that neoplasms arise mainly from relatively «undifferentiated» cells (14); 5) Limbal cells are essential for the long-term maintenance of the central corneal epithelium and they can be used to reconstitute the entire corneal epithelium in patients with limbal stem cell deficiencies (3,15). Collectively, these data leave little doubt that corneal epithelial stem cells are located at the limbal area.

A major challenge in stem cell biology is the ability to identify stem cells in situ. A variety of «limbal stem cells markers» have been suggested. The ability of a small population of cells located at the corneal limbus to retain tritiated thymidine label for long periods of time has been accepted as indicative of a stem cell population (16), but could not be accepted as a stem cell marker.

Very recently, the p63 nuclear protein, a member of the p53 family including p73, was suggested to be a marker of epithelial stem cells. Demonstrations show that p63 identifies basal epithelial cells (including stem cells) in the prostate (17), breast (18), bronchi (19), epidermis and corneal limbus (20). Previous work showed that p63 is highly expressed in the basal cells of many human epithelial cells, especially in progenitor or stem cell populations of epithelium tissues (21,22).

In this work we use an immunohistochemical method and in situ hybridization to evaluate the locations of p63 protein and mRNA express positive cells on the ocular surface in humans.

MATHERIAL AND METHODS

Human corneal and limbal tissue preparation

Normal human corneal and limbal tissues were collected from Texas Lion Eye Bank, Houston. Paraffin sections were made by the following procedure: human limbal tissues were fixed in 10% phosphate buffered formalin for one or two days, then transferred to 70% ethanol, dehydrated, paraffin embedded and 5 μm thick sections were cut. Haematoxylin-eosin staining, immunohistochemistry staining and in situ hybridization were done on the sections.

Immunohistochemistry staining

An antibody against human p63 (NeoMarkers) was used for immunohistochemistry. Sections were first boiled in 10 mM citrate buffer (pH 6.0) for 20 min, and then blocked with 10% horse serum in PBS for 1 h to decrease nonspecific antibody interactions.

This same solution was used to dilute the antibody at 1:1000. After rinsing in PBS, biotinylated anti-mouse IgG antibody was biotinylated with horseradish peroxidase reagent (Vectastain ABC kit, Vector Laboratories) and DAB to give a brown stain.

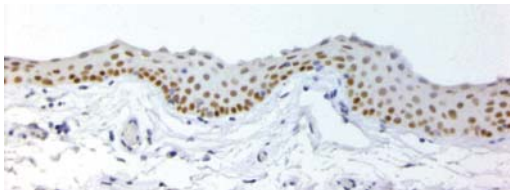

Fig. 1: P63 detected at the limbal basal epithelial cells.

In situ hybridization

Transcript containing p63 sequence were detected using [^{35}S]UTP labeled riboprobes. The 2.0 kb p63a probe was generated from plasmid p63-Bluescript KS and synthesized using EcoRI digestion and T3 RNA polymerase (Promega) for the anti-sense probe and HindIII digestion plus T7 RNA polymerase (Promega) for the sense probe. Hybridizations were done on 5 μm thick ocular sections and were exposed to photographic emulsion for 6-10 days before developing and haematoxylin counterstaining.

RESULTS

P63 proteins were only immunodetected in the nuclei of limbal basal epithelial cells. There was no staining on the corneal epithelial cells and limbal superficial epithelial cells (fig. 1). To confirm the p63 expression pattern in the limbal and corneal area, in situ hybridization of p63a was performed on corneal and limbal tissue. As a control, there is no staining of p63 in the corneal and limbal areas with p63 sense probe (fig. 2a). While using anti-sense riboprobe showed that the p63 mRNA is clearly located only on the basal limbal epithelial cells nuclei; there was no signal in the superficial limbal epithelium and cornea (fig. 2b). These results indicated that TAp63-a mRNA was strongly expressed only in the limbal basal epithelium.

DISCUSSION

Anatomically, the limbus acts as a junction or the transition zone between the corneal and conjunctival epithelia. Phenotypic expression is intermediate between the corneal and conjunctival epithelium.

The specific location of corneal epithelial stem cells in the limbus provides several functional advantages (ChenZhuo L, 1998): 1) Limbal basal cells are heavily pigmented and are thus well protected; 2) Limbal epithelium is extremely resistant to shearing forces, and in many species displays a highly undulating epithelial-stromal junction (Hogan MJ, 1971) with pegs of stroma extending upward that are interconnected with anchoring fibrils linking to the basement membrane (23); 3) Limbal epithelium is adjacent to a rich vascular network that provides ample nutrients and other supportive factors. The blood vessels form part of the

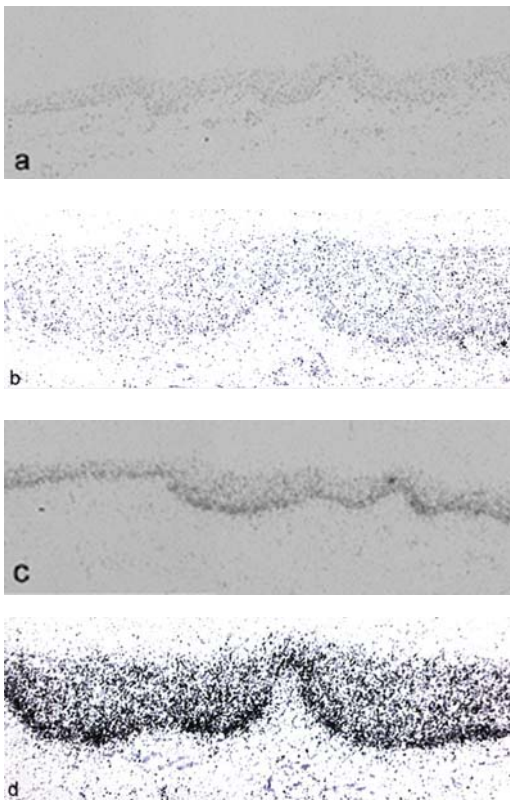

Fig. 2: In situ hybridization with p63a riboprobe on limbal area. With sense riboprobe (a, c): no signal on the limbal area. With antisense riboprobe (b, d): strong p63 mRNA express on the limbal basal epithelium.

palisades of Vogt which allow close approximation between blood vessels and the epithelium, potentially providing increased levels of nutrition and blood-borne cytokines to the cells at the limbus (23); 4) Limbal epithelium serves as the junctional epithelium to prevent conjunctival epithelial ingrowth onto the corneal surface during the healing of a large corneal epithelial defect (12).

The p63 transcription factor belongs to a family that includes two structurally related proteins, p53 and p73. Whereas p53 plays a well-stablished role in tumor suppression, p63 and p73 play unique roles in morphogenesis and key regulators in the development of neuronal and pheromonal (24) and p63 in limbus, epithelial, and craniofacial development pathways (22,25,26). To date, p63 is the first gene product definitely distinguishing stem cells from their transient amplifying progeny in stratified squamous epithelia. Identification of p63 as a stem cell marker is consistent with the phenotype of $p63^{-/-}$ mice (22,25). It was known that $p63^{-/-}$ mice lack stratified epithelia and contain clusters of terminally differentiated keratinocytes on the exposed dermis (25), and that p63 is expressed in the nuclei of keratinocytes with proliferative potential (27). In addition, it has been demonstrated that p63 expression is gradually reduced from the basal cells to the terminally differentiated keratinocytes (20,28). These findings correspond to those observed in our study: strong p63 is specifically expressed in limbal basal cell, and lack of p63 staining in corneal basal cells and limbal superficial epithelial cell. It strongly suggests that the phenotype of $p63^{-/-}$ mice could contribute to a failure to maintain stem cells (25) rather than to the inability of the $p63^{-/-}$ ectoderm to form epithelial lineages during development (22). We show here that p63 is not expressed in the basal TACs of corneal epithelium, but only in limbal basal epithelial cells. These observations show that possession of p63 is not simply a property of multiplying cells (27), but a property of stem cells.

Generally, in this work, the findings strongly suggest that p63 may be the best currently identified marker for corneal/limbal stem cells.

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