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Expression and tissue distribution of p63 isoforms in human ocular surface epithelia

Satoshi Kawasaki[a,*], Hidetoshi Tanioka[a], Kenta Yamasaki[a], Che J. Connon[b], Shigeru Kinoshita[a]

[a]*Department of Ophthalmology, Kyoto Prefectural University of Medicine, 465, Kajii-cho, Hirokoji-agaru, Kawaramachi-dori, Kamigyo-ku, Kyoto 602-0841, Japan*
[b]*School of Optometry & Vision Sciences, Cardiff University, Cardiff, UK*

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Abstract

The functional significance of p63 in regulating cell proliferation in various stratified epithelial cells has previously been proposed. More than six isoforms have been reported for this protein; however, it is not yet clearly understood how functionally different these isoforms are. To investigate how these isoforms are used in ocular surface epithelia, we studied the spatial distribution of p63 isoforms within human ocular surface epithelia. Individual layers (basal, intermediate, and superficial) of the human ocular surface epithelia (cornea, limbus, and conjunctiva) were selectively obtained using a laser micro-dissection device. These samples were equally amplified and subjected to RT-PCR analysis with primer pairs, which specifically amplify each of five isoform-determining regions or each of six p63 isoforms. Regarding the N-terminal region, the TA domain was not detected in all samples, while a ΔNp63 specific region was detected in the basal–intermediate region of all types of epithelia and in the superficial layer of the limbus. Regarding the C-terminal region, an α-isoform specific region was detected in all layers of the conjunctiva and limbus, as well as in the basal to intermediate layers of the cornea. A β-isoform specific region was detected in the basal to intermediate layers of the limbus. A γ-isoform specific region was detected in almost all layers of all epithelia. Among the six p63 isoforms, only ΔNp63α was detected in the basal to intermediate layers of the limbus and conjunctiva. These results suggest that ΔNp63α is the most dominant isoform within human ocular surface epithelia. This isoform may contribute, at least in part, to the maintenance of cell proliferative capacity within the ocular surface epithelia.
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Keywords: p63; ΔNp63α; alternatively spliced variant; ocular surface epithelia; cell proliferation; laser micro-dissection; RT-PCR

1. Introduction

p63 was first cloned from a rat circumvallate taste papilla cDNA library as a highly homologous gene to p53 (Schmale and Bamberger, 1997), a key player in anti-cancer systems. However, in spite of the significant sequence similarity between these two proteins, p63 is not used in tumour suppression, but in development. The function of p63 was elegantly shown using knockout (KO) mice, in which the absence of p63 results in severe anomalies in limb and craniofacial morphogenesis, as well as severe hypoplasia of squamous epithelia (Mills et al., 1999; Yang et al., 1999).

From these previous studies, a significant role of p63 in maintaining squamous epithelia has been presumed. Yang et al. (1999) proposed a model in which p63 may help epithelial stem cells retain their proliferative capacity. Later, Pellegrini et al. (2001) reported the expression of p63 in young transient amplifying (TA) cells and stem cells of the epidermal and corneal epithelia, proposing that this protein is a specific marker for keratinocyte stem cells. Since then, p63 has been used as a putative stem-cell marker in limbal and cultivated corneal epithelial cells (Harkin et al., 2004).

More than six isoforms have been reported for the p63 protein (Yang et al., 1998). The N-terminus region exhibits two alternative transcription initiation sites, giving rise to two different N-termini, including the TA isotype, which

* Corresponding author. Satoshi Kawasaki, Department of Ophthalmology, Kyoto Prefectural University of Medicine, 465, Kajii-cho, Hirokoji-agaru, Kawaramachi-dori, Kamigyo-ku, Kyoto 602-0841, Japan.
E-mail address: skawasak@eye.ophth.kpu-m.ac.jp (S. Kawasaki).

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contains/is the *trans*-activating domain, and the ΔNp isotype, which lacks this domain. The C-terminus region exhibits three alternative splicing patterns, giving rise to three types of C-termini, including α, β, and γ isotypes. The combination of the two N-termini and three C-termini produces a total of six isoforms for the p63 protein. In addition, special types of p63, designated as TA*p63 (Yang et al., 1998), which has an additional 39 amino acids in the N-terminus, and ΔNp73L, which corresponds to ΔNp63 lacking exon 4, have also been discovered (Foschini et al., 2004).

Precise analysis of p63 expression within ocular surface epithelia, including detailed information regarding these isoforms, will contribute to understanding how this protein is used in the maintenance of ocular surface epithelia. At present, several antibodies raised against different portions of p63 isoforms are commercially available. However, as described above, regions that determine each specific p63 isoform are located separately at the N-terminus and C-terminus, making it unlikely that a single antibody is able to detect a specific type of p63 isoform. Therefore, using pairs of PCR primers to distinguish the individual p63 isoforms, we investigated the expression of individual p63 isoforms by RT-PCR analysis against the individual layers of human ocular surface epithelia. We found that ΔNp63α is the most dominant type of p63 isoform within human ocular surface epithelia and that this isoform is specifically located within the basal to intermediate layers of the limbal and conjunctival epithelia.

2. Materials and methods

2.1. Human subjects

This research was conducted after a thorough review and approval by the Committee for Ethical Issues on Human Research of Kyoto Prefectural University of Medicine and in accordance with the tenets of the Declaration of Helsinki. Permission to use corneas for research was obtained from all of the families of the deceased.

Three human corneo-scleral tissues were obtained from three donors (a 79-year-old male and two 54-year-old females) approximately 6 hr after death at the North–West Lion Eyebank (Seattle, WA, USA). Each donor was free of any ocular surface disorder or history of eye surgery. Before embedding, the surface conditions of each of the corneo-scleral tissues were inspected using a binocular slit lamp and were found to be free of apparent epithelial damage.

2.2. RT-PCR analysis for laser micro-dissected samples

The three corneo-scleral tissues were snap-frozen in Tissue-Tek® OCT compound (Sakura Finetechnical, Tokyo, Japan) with liquid nitrogen. Corneo-scleral sections of 10 μm were cut and placed onto either a silanated or a specialized glass-slide (Penfoil slide; Leica Microsystems, Tokyo, Japan) for immunostaining or laser micro-dissection, respectively. Individual epithelial layers (basal, intermediate, superficial) from the three ocular surface epithelia were dissected using a laser micro-dissection device (AS LMD; Leica Microsystems, Wetzlar, Germany). Keratin 12 is known as a corneal epithelial specific keratin (Kurpakus et al., 1990, 1994) and is thought not to be expressed in limbal basal stem cells. Therefore, only cells negative for keratin 12 were collected for the limbal basal cell samples using the laser micro-dissection device (Fig. 1). This process was achieved by viewing serial adjacent sections that were immunostained with keratin 12. Total RNA was extracted from each of the laser micro-dissected samples with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's guidelines. The RNAs were reverse-transcribed and amplified using a Super SMART PCR cDNA synthesis kit (BD Bioscience, Tokyo, Japan) according to the manufacturer's guidelines. Briefly, cDNAs were synthesized from the extracted RNAs using 0.84 μM 3′ SMART CDS Primer IIA [AAGCAGTGGTATCAACGCAGAGTACT(30)VN; V = A or C or G] and 0.84 μM SMART IIA oligonucleotide (AAGCAGTGGTATCAACGCAGAGTACGCGGG) in a buffer containing 1× first-strand buffer, 2 mM dithiothreitol (DTT), 0.2 mM dNTP, 1 U/μl RNase inhibitor and 1/20 volume of PowerScript reverse transcriptase. After purification using standard column chromatography, the cDNAs were amplified by PCR with 0.24 μM 5′ PCR Primer IIA (AAGCAGTGGTATCAACGCAGAGT) in 100 μl of reaction buffer containing 1× Advantage 2 PCR buffer, 0.2 mM dNTP and 1× Advantage 2 polymerase mix. The thermal conditions used were an initial denaturation step (95°C for 1 min), followed by an amplification cycle (95°C for 5 sec, 65°C for 5 sec, and 68°C for 3 min). The cycle number was optimized for each sample as follows. First, each sample was amplified with 15 cycles using the above thermal conditions. Then the thermal cycling was paused and a 70-μl aliquot was removed from each sample and stored at 4°C before the following optimization process was completed. Next, the thermal cycle was resumed and a 5-μl aliquot was removed from each sample after cumulative cycle numbers 18, 21, 24, 27, 30, and 33. All aliquots from all samples were electrophoresed in 2% agarose gel to estimate the number of cycles required to reach the plateau phase of amplification for each sample. The optimized cycle number was determined as one cycle fewer than that needed to reach the plateau. Then each stored sample was amplified again up to the individual optimized cycle number. The PCR products were electrophoresed in 2% agarose gel to assure the cDNA amplification and to estimate their amounts. The amplified cDNA samples were diluted to normalize their

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