The expression of LGR5 in healthy human stem cell niches and its modulation in inflamed conditions

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Purpose: The aims of this study are to investigate the expression of leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) protein in the normal human cornea and limbus and to analyze modifications of this expression under inflammatory conditions.

Methods: The expression of LGR5 was evaluated in seven limbal epithelial crypts (LECs), collected from healthy cadaver donors, and five inflamed LECs obtained from enucleated eyes. Central corneal buttons were used as controls. LGR5 protein distribution was determined by immunohistochemistry staining analysis.

Results: The cytoplasmic expression of LGR5 protein was observed in 100% of healthy LECs. Three out of five inflamed tissues analyzed were completely negative, while in the two remaining cases, we observed a moderate positivity in the basal cells of LECs. No relation was found between the expression of LGR5 and the grade of inflammatory cells.

Conclusions: These findings demonstrate the presence of LGR5-positive cells in human LECs and their decrease in inflamed conditions, which suggests a critical role of this protein during inflammation and its possible use as a marker in normal crypts.

Corneal stem cells (SCs) are located at the sclerocorneal limbus in the palisades of Vogt, which are highly pigmented structures with an abundance of melanocytes, antigen-presenting cells, and lymphocytes [1,2]. These cells, termed limbal epithelial crypts (LECs), are situated in an anatomically defined site of the human limbus called the “niche” [1,3]. The absence of a definitive biological or phenotypic marker contributes a degree of uncertainty related to the unequivocal isolation and characterization of limbal stem cells [3,4]. So far, a variety of SC markers have been proposed to identify this population of cells [4]. Among the major characteristics proposed for SCs are the following: small size, slow-cycling properties, expression of transporters (such as ABCG2, Na/K-ATPase, glucose transporter I), a transcription factor (p63), integrins (α9, β1, and β4), cytokeratin (K5/K14), cell cycle mediators (cyclin D, cyclin E), metabolic enzymes (α-enolase, cytochrome oxidase, carbonic anhydrase), and gap junction proteins (connexin 43) [3,4].

Recently, the expression of leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) by SCs was observed in multiple adult tissues [5], particularly in the intestinal crypt, stomach, hair follicle bulge, eye, and mammary gland [6,7]. This receptor, also known as glycoprotein hormone 38 (HG38), orphan G protein-coupled receptor 49 (GPR49), or novel putative G protein-coupled receptor expressed in follicles (FEX), was first reported as an orphan receptor with homology to the glycoprotein hormone receptor subfamily of the class A rhodopsin-like seven transmembrane domain [8]. In addition, Carmon and colleagues demonstrated that R-spondin, a family of proteins isolated as strong potentiators of Wnt/β-catenin signaling, functioned as ligands for LGR4 and LGR5 [9].

The presence of LGR5-positive cells in the eye was described for the first time by Krulova and colleague in BALB/c mice [4]. Limbal tissues from BALB/c mice were isolated on the Percoll gradient and a population with high expression of the SC marker ABCG2 and LGR5 was observed [4]. Recently, Brzeszcynska and colleagues showed that long-term organ culture–preserved corneal epithelial tissues have an heterogeneous population of cells that express genes typically expressed by SCs (mRNA encoding p63, ABCG2 and LGR5, and immunostaining for these markers) and cells with a differentiated phenotype (abundant expression of cytokeratins 12 and 3) [10]. These authors suggest that LGR5 expression can be related to cellular stemness and can be considered as a new phenotypic marker of residual human corneal limbal SCs [10]. The aim of our study is to investigate the presence of LGR5 in SCs of healthy human tissues using immunohistochemistry and to analyze possible changes in its expression due to inflammation.
METHODS

Sample preparation: Seven human eye bank corneal buttons with scleral rims (ages ranged from 73 to 80 years; mean age 76.4±2.702 years) and two corneas (ages ranged from 73 to 79 years; mean age 76±4.243 years) not suitable for transplantation were included in the study. The average death to enucleation time was 8 h (range from 4 h to 10 h). The mean storage time (Eusol-C, Alchimia Srl, Pordenone, Italy) between eye bank procedures and fixation was 26 h (range from 20 h to 48 h). In addition, five pathological corneoscleral tissue samples (ages ranged from 59 to 85 years; mean age 69.4±9.6 years) were collected at the time of enucleation of the eye. The enucleation was done because of uncontrolled infectious endophthalmitis affecting the cornea and the ocular surface, and the eye was fixed after retrieval. The etiology of endophthalmitis was originally related to corneal infection and included Stenotrophomonas maltophilia, Aspergillus flavus, Candida albicans, Pseudomonas aeruginosa, and one case of herpes simplex virus (HSV) necrotizing keratitis with cornea perforation and subsequent unidentified microbial superinfection. All pathological eyes presented variable degrees of limbal inflammation. In the normal donors, no evidence of any disease, desiccation, or damage was noted. All tissues were fixed in 4% formalin (Bio Optica, Milano, Italy) and then embedded in paraffin (Bio Optica). According to a previously published protocol [11] with minor modifications, 3 µm sections of the blocks were serially cut with a microtome (Leica Microsystems GmbH, Wetzlar, Germany) and monitored for the presence of LEC with hematoxylin and eosin stains. When a region containing the LEC was identified, the adjacent sections were collected for immunohistochemistry staining. The study adhered to the tenets of the Declaration of Helsinki.

Immunohistochemistry: Immunohistochemistry analysis was performed on sections where we observed the presence of LECs by hematoxylin and eosin staining. Each section was immunostained with LGR5 (1:10; Abcam, Cambridge, UK), p63 (1:200; Dako, Glostrup, Denmark), and ABCG2 antibody (1:20; Abcam). Formalin-fixed tissues were deparaffinized and pretreated by microwave antigen retrieval using buffered EDTA pH 9 (for LGR5 and p63) or buffered sodium citrate pH 6 (for ABCG2), followed by 10 min of protein block (Dako; required for LGR5). The anti-rabbit (for LGR5) or mouse (for p63 and ABCG2) En Vision system (Dako) was used before diaminobenzidine tetrahydrochloride (Dako) incubation. A negative control was performed for each antigen using the specific isotype antibody as control. All slides were counterstained with hematoxylin (Biocare Medical, Concord, CA). The slides were examined in a double-blind fashion, and digital images of representative areas were taken.

RESULTS

Expression of LGR5 in the healthy and inflamed human limbus: Expression of LGR5 was evaluated in seven healthy and five inflamed human limbi (Table 1). A strong cytoplasmic expression was observed in all LECs of the healthy limbus (Figure 1A), while no staining was observed in a specific isotype-negative control (Figure 1B). Two central corneal buttons were evaluated as controls and no positivity for LGR5 was observed (Figure 1C).

A moderate expression of LGR5 was observed in two samples of inflamed limbus, while no positivity was achieved in the remaining three cases (Figure 2A,B). No correlation was found between the number and subset of inflammatory cells and the LGR5 positivity. Moreover, phenotype analysis of the inflammatory cells by immunohistochemistry revealed that, in all the inflamed tissue samples analyzed (data not shown) they were predominantly macrophages, granulocytes, and lymphocytes.

To compare the expression of LGR5 with other putative SC markers, staining for p63 and ABCG2 was set up (Figure 3). A similar p63 expression was observed in both healthy and pathological samples (Figure 3A,B), as well as the positivity obtained for ABCG2 (Figure 3C,D).

| Tissues            | LGR5 positivity |
|--------------------|-----------------|
| Healthy cornea 1   | ++              |
| Healthy cornea 2   | ++              |
| Healthy limbus 1   | ++              |
| Healthy limbus 2   | ++              |
| Healthy limbus 3   | ++              |
| Healthy limbus 4   | ++              |
| Healthy limbus 5   | ++              |
| Healthy limbus 6   | ++              |
| Healthy limbus 7   | ++              |
| Inflamed limbus 1  | +/-             |
| Inflamed limbus 2  | +/-             |
| Inflamed limbus 3  | -               |
| Inflamed limbus 4  | -               |
| Inflamed limbus 5  | -               |

++: strong positivity, +/-: few positive cells, -: no positive cells
DISCUSSION

The corneal epithelium is maintained by a pool of SCs that resides predominantly in the corneoscleral limbus. The distribution of SC is not uniform along the limbus, but appears to be concentrated in defined anatomic structures termed limbal epithelial crypts [1,11,12]. Partial or total limbal SC deficiency resulting from damage to the limbus continues to pose a challenge in ocular surface reconstruction procedures aimed at restoring sight. Despite several studies, there is no single marker that can identify a limbal SC, and reliance is placed on a combination of different markers, some negative and some positive [13].

SCs are characterized by their small size, low rate of replication, expression of certain markers such as a transporter ABCG2, p63, integrin α9α6β1/β4, cytokeratin 19 and 15, laminin 5, N-cadherin, and vimentin [3,4]; the expression of some markers by LSCs, including cx43, is still debated [11,12], while other molecules such as desmoglein 3 are considered negative markers of stemness [3,12]. Two in vitro studies in mice and humans reported the expression of LGR5 markers in limbal SCs [4,10]. Moreover, in the SC niche of the murine hair follicle, LGR5 was identified in actively cycling cells, and it has been shown that LGR5-positive cells maintain all cell lineages over long periods of time and build new hair follicles [14].

Figure 1. LGR5 immunohistochemical staining in the human healthy stem cell niche and in the cornea. A: The expression of leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) was localized in the cytoplasm of limbal cell niche. B: No positivity was observed with specific isotype control. C: LGR5 was not detected in the cornea. Each section was counterstained with hematoxylin. Bar scale=50 micron. Magnification X200. * Indicates the limbal side of the specimen.

Figure 2. Expression of LGR5 in inflamed human limbal stem cell niches. Leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5) was variously expressed in limbal epithelial crypts (LECs; arrows). A: Three out of five samples were negative. B: In two cases, we observed a moderate grade of positivity. No correlation was found with the number of inflammatory cells (arrowheads). Each section was counterstained with hematoxylin. Bar scale=50 micron. Magnification X200. * Indicates the limbal side of the specimen.
This study showed the expression of LGR5 in healthy human LECs by immunohistochemistry for the first time. We observed that 100% of LECs analyzed were positive for the LGR5 marker, suggesting that this molecule can be considered a new molecular marker of stemness. Moreover, we analyzed, the expression of LGR5 in inflamed LECs for the first time. Interestingly, the positivity was completely abolished in 60% of analyzed samples, while in 40% of cases it decreased significantly. In contrast, as previously shown [3], the expression of other putative SC markers such as p63 and ABCG2 was still evident regardless of the inflammatory condition. These results indicate that LGR5 may be considered a stemness marker that is affected by inflammatory damage at an early stage. The data obtained via immunohistochemical analysis of normal and inflamed tissues support the hypothesis that LGR5 can be used as one of the putative SC markers for healthy human LECs. There have been no reports in previous studies of LGR5 expression in inflamed human eye structures.

Several studies showed that oxidative DNA damage of LGR5-positive epithelial cells in the gastric mucosa was increased in patients with gastric cancer and Helicobacter pylori infection, but not in gastric cancer patients without H. pylori infection [15,16]. Uehara and colleagues suggested that oxidative damage of LGR5-positive epithelial cells may be mediated by the interaction of H. pylori with host susceptibility factors that affect the response of LGR5-positive epithelial cells in gastric cancer patients [15]. Similarly, the presence of inflamed cells around LECs affected LGR5-positive cells, causing them to become negative. Of course, it will be of interest to confirm these data with a larger number of inflamed tissues, despite the difficulty in collecting these kind of samples.

Figure 3. p63 and ABCG2 positivity in both healthy and pathological condition. A: Nuclear p63 staining in healthy tissues was shown. B: p63 expression was evidenced also in pathological cases. C: Cytoplasmic ABCG2 positivity was observed healthy samples. D: ABCG2 expression was achieved also in inflamed tissues. Arrowheads indicate inflammatory cells. Each section was counterstained with hematoxylin. Bar scale=50 micron. Magnification X200. * Indicates the limbal side of the specimen.
To the best of our knowledge, this study provides the first immunohistochemical illustration of the expression of LGR5 in healthy and inflamed corneoscleral limbi and LECs. Such studies will help us to understand the mechanisms involved in the survival and destruction of SCs in response to inflammation. LGR5 can be used as a marker of human corneal epithelial limbal wellbeing and provides great promise for future cornea regenerative pharmaceutical strategies.

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