Title: In vivo confocal microscopy features and clinico-histological correlation of limbal nerve corpuscles.

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Precis

In vivo confocal microscopy is a useful noninvasive tool to identify and image limbal nerve corpuscles and it can be used to investigate the role of these structures in normal and disease conditions.

Abstract

Aims: To describe the in vivo confocal microscopy (IVCM) features of human limbal nerve corpuscles (LNCs) and correlate these with the histological features.

Methods: We examined 40 eyes of 29 healthy living subjects (17 female, 12 male; mean age = 47.6) by IVCM. Four limbal quadrants were scanned through all epithelial layers and stroma to identify the LNCs and associated nerves. Ten fresh normal human corneoscleral discs from 5 deceased patients with a mean age of 67 years and 17 eye-bank corneoscleral rims with a mean age of 57.6 years were stained as whole mounts by the acetylcholinesterase (AChE) method to demonstrate LNCs and corneal nerves. Stained tissue was scanned in multiple layers with the Nanozoomer digital pathology microscope. The in vivo results were correlated to the histological findings.

Results: On IVCM, LNCs were identified in 65% of the eyes studied and were mainly (84%) located in the inferior or superior limbal regions. They appeared either as bright (hyper-reflective) round or oval single structures within the hyporeflective, relatively acelluar fibrous core of the palisades or were clustered in groups, often located anterior to the palisades of Vogt. They measured 36 μm in largest diameter (range 20-56 μm). The in vivo features were consistent with the histology which showed LNCs as strongly AChE positive round or oval structures.

Conclusion: The strong correlation with histology will enable use of IVCM to study LNCs in normal and disease conditions.

INTRODUCTION

The corneal limbus is unique in that it provides a niche for stem cells (SC) that support and maintain corneal epithelial turnover and wound healing. Anatomically, the limbus represents a transitional zone between the most anteriorly located clear cornea (corneal limbus) and the posteriorly located opaque sclera (scleral limbus). It has 10 to 12 layers of epithelium with melanocyte related pigmentation, which protects the SC from ultraviolet light damage. [1, 2]

The limbal microenvironment and SC niche are maintained by finely balanced interactions of innate immune-related cells, blood vessels, stem cells and nerves. The ocular dendritic cell population, Langerhans cells, is normally restricted to the corneal periphery and limbus. The limbus is endowed with a rich vascular network within the stroma of which these specialised immune cells reside and play a significant role in immune homeostasis of the cornea by participating in immune surveillance, and inducing antigen-specific immune reactivity and tolerance in a variety of corneal diseases.[3] The limbal epithelium has also been shown to mediate angiogenesis through the production of vascular endothelial growth factor (VEGF), which promotes corneal neovascularization following trauma or inflammation. [4]

Highly specialised SC niche structures, termed Limbal Epithelial Crypts (LECs), extend radially from the undersurface of the rete ridges into the conjunctival stroma or circumferentially along the limbus. [5-7] The role of LECs within the limbus is evidenced by the centripetal migration of epithelial cells during wound healing and corneal epithelial regeneration.[8] The innervation of the corneoscleral limbus consists of a dense network of highly branched tortuous nerve fibres that form an annular (ring-like) plexus around the cornea. [9] The corneal nerves terminate as free endings within the superficial epithelial cells. These receptors are known to exert sensory and trophic functions.[10] Until recently, it was believed that free nerve

terminals could only be differentiated through their function however, immunofluorescent studies on pig corneas suggest that these nerve endings can also be distinguished morphologically. This technique enables recognition of polymodal nociceptors and cold receptors through their expression of transient receptor potential cation channel subfamily V member 1 – immunoreactive (TRPV1-IR) and transient receptor potential cation channel subfamily M member 8 – immunoreactive (TRPM8-IR) respectively. This provides evidence that C- & Aδ- neurons, previously considered 'unspecialised', clearly have some specific function corresponding to their morphology and neurochemistry. [11]

Unlike corneal free nerve endings, histological studies have shown that limbal nerves terminate as subepitehlial compact nerve endings and are called "limbal nerve corpuscles" (LNCs). [12] LNCs are predominantly located within stromal invaginations between limbal rete pegs and LECs, suggesting that they are involved in the maintenance of the SC niche environment.

The aim of this work is to identify LNCs in normal living subjects and describe their morphological features and relationship to important limbal landmarks using in vivo confocal microscopy (IVCM) and histological examination.

METHODS

The research was conducted at University of Nottingham, Nottingham University Hospital NHS Trust, Queens medical centre, United Kingdom and approved by the East Midlands Research Ethics Committee (REC no. 06/Q2403/46).

In Vivo Confocal microscopy (IVCM): 40 eyes of 29 healthy living subjects (mean age = 47.6 years, range 21 to 68 years; 17 female and 12 male) were examined by laser scanning confocal microscope (Heidelberg Retina Tomograph II Rostock Corneal Module [RCM]; Heidelberg Engineering GmbH, Heidelberg, Germany). The device uses a class I diode laser (670-nm wavelength) with a 63X water-immersion

lens (Olympus, Tokyo, Japan) which captures 400X400 microns frames with 2 micron lateral resolution and 4 microns optical depth resolution. Image magnification on screen was 300X. IVCM was performed under topical anesthesia with MINIMS Proxymetacaine hydrochloride 0.5% (Bausch & Lomb Ltd, Surrey, United Kingdom). A digital camera mounted on a side arm furnished a lateral view of the eye and objective lens to monitor the position of the objective lens on the surface of the eye. A drop of 0.2% polyacrylic gel (Viscotears liquid gel; Novartis Pharmaceuticals Ltd., Surrey, United Kingdom) was used as a coupling medium between the objective lens of the microscope and the contact cap.

Four limbal quadrants were scanned through all the layers. Frames from epithelial and stromal layers containing LNCs and nerve fibres were selected for analysis. Qualitative morphologic evaluation of LNCs was then carried out and compared to their histological appearance. For the measurement of the size of LNCs, the widest diameter was considered.

Study of flat mounts: Ten fresh (within 72 hours of death) normal human corneoscleral discs from 5 deceased patients with a mean age of 67 years (range 55 to 73 years) and 17 eye-bank corneoscleral rims maintained in organ culture for eight days (range 3 to 15 days), with a mean age = 57.6 years (range 20 to 71 years) were used in this experiment. Due to the method of retrieving the corneoscleral discs, the orientation of the discs could not be ascertained and hence it was not possible to establish correlation of the nerve structures to a precise quadrant or clock-hour.

Acetylcholinesterase method for corneal nerve demonstration: The cholinesterase enzymes, found along the corneal sensory nerve axons, are believed to be responsible for the maintenance of the ionic gradient along the axons during propagation of the nerve impulse and post mortem, allow identification of nerves.[13]

This method has been described in detail previously.[9] Briefly, the corneoscleral discs were fixed in cold 4% formaldehyde (pH 7) for 4 hours and then rinsed overnight in phosphate buffered saline. Specimens were incubated in the stock solution containing acetylthiocholine iodide as a substrate for 16-24 hours at 37°C. The AchE enzyme in the nerves reacted with acetylthiocholine iodide in the substrate to produce a brown coloration of the nerves. The color was then intensified with a dilute solution of ammonium sulfide. Specimens were dehydrated by immersion in alcohol and were cleared in xylene. The specimens finally were mounted between a slide and cover slip and were prepared for image analysis.

Image analysis: The stained samples were imaged using a light microscope (Leica DM4000B; Leica Microsystems, Nussloch, Germany) and a Hamamatsu Nanozoomer digital pathology microscope system (Hamamatsu, Hamamatsu City, Japan). The areas of interest were serially imaged in the Z-axis starting from the most superficial layer in order to study the relationship of LNCs to the perilimbal nerve plexus and other limbal structures. The images were then stacked and merged to give a single, holistic, detailed anatomic view of the area. Merging the images was done through a Z-stacking software, AllFocus (AllFocus—Extended Depth of Field Software; Saphicon, Palo Alto, California, USA). Adobe Photoshop CS4 Extended (Adobe Systems, Inc, San Jose, California, USA) was required for additional image processing. For the measurement of the size of LNCs, the widest diameter was considered.

RESULTS

Histological findings

The histological features of limbal nerve corpuscles (LNCs) have already been described in detail in a previous work.[12] The LNCs were seen to form a 2 mm ring along the limbal circumference (figure1). The size varied from 20-50 μm. They were arranged in clusters and mainly gathered around the anterior border of the limbal

palisades of Vogt (POV), which were all innervated by fibres arising from the superficial limbal plexus anterior to POV (figure 2 and see online supplementary video 1). LNCs were found in all specimen examined, although skip areas were seen in the same sample where LNCs were absent (see online supplementary figure 1). Based on the histological clustering of LNCs around POVs, IVCM examination was especially targeted to the POVs.

In vivo confocal microscopy findings

LNCs were identified in 65% of the eyes studied and were mainly (84%) located in the inferior or superior limbal regions. They appeared either as bright (hyper-reflective) round or oval single structures within the hypo-reflective, relatively acelluar fibrous core of the palisades (figure 3) or were gathered in a large group, often located anterior to POV (figure 4). LNCs were measured at 36 μm (range 20-56 μm). The limbal nerve plexus consisted of deep and superficial layers. The deep nerves were often seen as straight large nerve trunks, while the superficial plexus appeared as tortuous bright structures that were easily differentiated from the blood vessels on IVCM (figure 5). Blood vessels had wider and darker lumen, which contained numerous bright cells (figure 6).

In addition to LNCs, Langerhans cells and melanocytes appear as hyper-reflective structures in the human corneal limbus on IVCM. They can be differentiated on the basis of their location, morphology and relation to other limbal landmarks.

Both Langerhans cells and melanocytes normally resides within the basal epithelial cells of the limbal rete pegs, whereas LNCs are often observed within the hypo-reflective stromal core of the POV.

Langerhans cells have characteristic dendritic morphology. They possess relatively large slender cell bodies and long inter-digitating dendritic processes in the corneal periphery (figure 7A), whereas the cells located in the central cornea are smaller and have relatively fewer and shorter dendrites.[14, 15] Langerhans cells can be clearly

differentiated from large ovoid hyper-reflective LNCs. Melanocytes, however, appear as numerous round hyper-reflective bodies in the basal cells of the POV in moderately pigmented subjects (figure 7B) and as compact sheets of hyper-reflective round bodies within the rete pegs in heavily pigmented individuals (figure 7C).[16, 17] IVCM volume scanning often reveals limbal stromal nerves terminating in LNCs, which is a unique feature (figure 7D). Langerhans cell processes too can be seen in contact with stromal nerves (figure 7D).

DISCUSSION

In vivo confocal microscopy (IVCM) is a reliable method of imaging the cornea at the cellular level giving results that are comparable to histological appearances allowing for direct comparison.[18] We were able to use it to obtain clear and reproducible images of limbal structures in considerable detail.[19] The histological demonstration of LNCs established these structures as an integral part of the limbal innervation though their function is not fully elucidated. In this study we were able to demonstrate IVCM features of LNCs in the human eye including their location and morphology. They appeared as hyper-reflective round or oval structures located within or anterior to POV. Within POV, LNCs were often isolated with no apparent connection to a nerve terminal. However, in a few sections, fine terminal nerve fibres were seen to make connection with the LNCs. This could be related to the plane of imaging with IVCM. When the LNC and the nerve are in the same en-face plane, IVCM will demonstrate both structures. However, if the nerve is at a different depth, the connection of the nerve to the LNC can be easily missed. In contrast, LNCs anterior to POV were arranged in clusters and of hyper-reflective bodies of apparent different sizes, which too could be an artifact of the orientation of the LNCs in relation to the plane of examination. Though we have measured the diameter of the structures this may not be truly representative of the mean size as described above.

IVCM examination localised the LNCs predominantly to the inferior and superior limbus. This corresponds to the distribution of POV, which too are more prominent in these regions. Taken together with the close association of LNCs and the POV, especially the LEC, it suggests that LNCs may be making an important contribution to the SC niche environment.

Though histologically LNCs were demonstrated in the limbus of all donor corneal discs, by IVCM they were detected in only 65% of the living subjects examined. The inability to image these structures in a third of our subjects can be attributed to several factors. Individual variations with some having more ‘skip areas’ could have caused us to miss areas with LNCs by the examination protocol used.[12] It is well known that the limbal and conjunctival epithelium are brightly hyper-reflective, which could mask the presence of LNCs with similar reflectivity. Previous studies have shown that pigmented POV produce high contrast images on IVCM compared to non-pigmented POV. [17] When the reflectivity of the structure of interest is similar to the surrounding structures or background, detection becomes difficult.[17] Additionally, IVCM technique has its own limitations. The small uniplanar field of examination and image degradation due to involuntary micro-saccadic eye movement can make it difficult to identify and track structures within the limbus, especially if they are randomly distributed.[19-21]

Nerve receptors in the central and peripheral nervous systems play a major role in interoception, exteroception and proprioception. Interoception refers to the signaling and perception of internal bodily sensations.[22, 23] Interoception is distinguishable from exteroception (perception of the external environment or stimuli) and proprioception (unconscious perception of the position of the body in space).[24]

Although, corneal intraepithelial nerve terminals in humans largely exhibit exteroceptive function responding to various external stimuli (e.g. nociceptive and thermoceptive), encapsulated nerve endings, which were found in the region of the iridocorneal angle of cetaceans, are believed to function as pressure receptors, possibly to regulate intraocular pressure. [25]

Interestingly, morphologically identical receptors have been shown to serve a function specific to the tissue they are found in; for example, ‘taste receptors’ located in the meningeal lining sense pH of CSF; ‘bitter taste receptors’ found in air passages can facilitate bronchodilation and beating of cilia in the lung, and ‘olfactory receptors’ play roles in both sperm chemotaxis and muscle cell migration.[26-28] These studies bring a novel perspective on the possible role of LNCs. Though LNCs are encapsulated and share some similarities to previously described ‘touch receptors’ in the skin, their role in the limbus could be unrelated to their morphological appearance.[12]

Our study provides useful information on the precise location, morphology and organisation of LNCs in normal living subjects. The ability to identify and image these structures in vivo will promote further research to investigate their role in health and disease, especially in limbal pathology and glaucoma, should they have a role in pressure sensing.

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E. Contributorship statement

Mouhamed Al-Aqaba conceived of the study, examination of subjects with IVCM, data analysis and drafting the manuscript. Fady Anis, Imran Mohammed and Anjali Yapa carried out the processing of the histological samples. Winfried Amoaku and Imran Mohammed contributed to data analysis, revision of the manuscript. Harminder Dua contributed to design of the work; analysis and interpretation of data, drafting the work. All authors read and approved the final manuscript.

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Figure Legends

Figure 1. En face light micrograph of whole mount Acetylcholinestrase (AchE) stained normal cornea showing limbal nerve corpuscles (LNCs), as dark brown ovoid structures, in one limbal quadrant. Scale bar = 1 mm.

Figure 2. En face light micrograph of whole mount Acetylcholinestrase (AchE) stained cornea showing four limbal nerve corpuscles (arrows) that reside within the palisades of Vogt (P), which are all connected to fibres (arrowheads) arising from the superficial limbal plexus anterior to the palisades. Bar = 100μm.

Figure 3. In vivo confocal microscopy images (A, B) and light micrographs (C, D) of limbal nerve corpuscles (LNCs) residing within the palisades of Vogt. On IVCM,

LNCs appear as hyper-reflective ovoid or elongated structures (arrows) within the hyporeflective fibrous core of the palisades (A & B). Figure B inset shows a LNC and its terminal branch (arrowhead). On histology, LNCs appeared as dark brown structures with their terminal branches visible in some sections (C, D). These were specifically located in the fibrous core of the palisades between inter-palisade rete ridges. Bar = 100μm. Frame depth; A=36 μm, B=42 μm.

Figure 4. In vivo confocal microscopy images of limbal nerve corpuscles (LNCs) correlated with histology (A to F). Both IVCM and histology show localization of LNCs as clusters. A sequence en face IVCM images showing a cluster of LNCs in the inferior limbus at the level of basal epithelial cells (A), and extending posteriorly to Bowman's zone (B). Sub-basal nerve fibres are also seen. A corresponding light micrograph of Acetylcholinestrase (AchE) stained whole mount corneoscleral disc showing a group of LNCs and their nerve branches (C). Another sequence IVCM images of a group of LNCs extending deep into limbal stroma (D & E). A corresponding light micrograph of AchE stained whole mount corneoscleral disc showing numerous LNCs located at different depths within the limbal stroma (F). The ones, which were out of focus, were lying deeper within the stroma. Bar = 100μm. Frame level; A = 62μm, B = 66μm, D = 55μm, E = 58μm.

Figure 5. In vivo confocal microscopy images and corresponding light micrographs of the superficial limbal plexus of nerves (A to D). This plexus appeared as an intricate network of fine convoluted branches of nerves. Bar = 100μm. Frame level; A = 57μm, C = 67μm.

Figure 6. En face IVCM images of anastomosing limbal blood vessels filled with bright cells. Venules are wider in diameter (A). Arterioles are of a smaller diameter

and demonstrate a thicker wall (B). These are clearly different from the limbal nerves shown (C) which appear as solid hyper-reflective lines. Bar = 100μm. Frame level; A = 104μm, B = 115μm, C = 288μm.

Figure 7. In vivo confocal microscopy images of normal human corneoscleral limbus. Langerhans cells appear as hyper-reflective slender cell bodies (arrowheads) with dendritic cytoplasmic processes (A). Melanocytes appear as numerous round hyper-reflective bodies in the basal cells of the palisades of Vogt in a moderately pigmented subject (arrowheads) (B) and as compact sheets of hyper-reflective round bodies within the rete pegs in a heavily pigmented individual (C). Limbal stromal nerves are seen terminating in limbal nerve corpuscles (arrows) (D). Langerhans cell processes too can be seen in contact with stromal nerves (arrowheads) (D). Bar = 100μm. Frame level; A = 43μm, B = 52μm, C = 59μm, D = 57μm.