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NEW DIAGNOSTIC APPROACH TO PTERYGIUM-OPTICAL COHERENT TOMOGRAPHY AND *IN VIVO* LASER SCANNING CONFOCAL MICROSCOPY

V. Ivancheva, T. Marinova, S. Peeva, Y. Manolova, C. Grupcheva*

Department of Ophthalmology and Visual Science, Medical University, Varna, Bulgaria
Specialised Eye Hospital – Varna, Bulgaria

ABSTRACT

PURPOSE: To observe morphological changes in cornea with pterygium using *in vivo* laser scanning confocal microscopy (LSCM) and optical coherent tomography (OCT).

METHODS: Ten eyes of 8 patients with pterygium and 8 eyes of 8 healthy subjects were recruited. Anterior-segment OCT was used to measure the thickness of the pterygium, underlying and adjacent clear cornea. In vivo images at microstructural level were captured using LSCM. The density of basal corneal epithelial cells and keratocytes in the anterior stroma and the density of specific cellular structures of the pterygium were calculated. In the controls, the central cornea and nasal bulbar conjunctiva were analysed comparatively.

RESULTS: Optical coherence tomograms demonstrate destruction of the Bowman's layer and invasion of the connective tissue of pterygium's head in the space between Bowman's layer and corneal stroma with possibility of precise measurement. Using LSCM density of basal corneal epithelial cells and anterior keratocytes in pterygium was determined: 4761±62 cells/mm² and 419±19 cells/mm² respectively, which was lower than that in the controls. Dendritic cells were found in pterygia with density 114±11 cells/mm². The dendritic cell density in the pterygium was found higher than the density in the nasal bulbar conjunctiva of the normal corneas.Morphologic alterations of the sub-basal nerve plexus were observed in pterygium, as well as bright deposits at the level of the basal epithelial layer and also hyperreflective spots in the surface epithelium, corresponding to the clinically observed Fuchs' flecks.

CONCLUSIONS: OCT and *in vivo* LSCM are efficient methods for dynamic evaluation of the morphologic alterations of pterygium, allowing precise diagnosis, prognosis and monitoring of this disease. Study highlighted microstructural alterations and increased dendritic cells presumed to be characteristic of pterygium and adjacent clear cornea.

Key words: *Pterygium, in vivo laser confocal microscopy, optical coherent tomography, cornea*

INTRODUCTION

Pterygium is a wing-shaped formation of fibrovascular tissue invading the clear cornea (1). Most commonly, it occurs nasally in the inter-palpebral area. It is a degenerative lesion, associated with UV and blue light exposure (14, 17). Many other factors have been associated with the development of pterygia, including anti-apoptotic and immunologic mechanisms, growth factors, cytokines, extracellular matrix modulators, genetic factors and viral infections (2, 3, and 10). Evidence, based primarily on histological studies, has been inconclusive and the pathogenesis of pterygium remains unclear.

Previous studies are performed using surgically excised tissue samples and due to tissue processing and other artefacts did not allow for analysis of the microstructural alterations of both pterygia and the adjacent clear cornea of the human eye. Moreover anlimal models of preygium appear to be impossible to create.

Optical coherence tomography (OCT), especially anterior-segment OCT is a well

***Correspondence to**: *Prof. CN Grupcheva MD, PhD, DSc, FEBO, FICO(Hon), Medical University - Varna, BulgariaChair of the Department of Ophthalmology and Visual Science, Assoc Director, Specialised Eye Hospital - Varna, Bulgaria, 15 Doyran St. Varna 9002, 00359 89 9096709, fax. 0035 952 634096*

established tool for high-resolution cross sectional and three-dimentional imaging of transparent and semi-translucent tissues. Based on low-coherence interferometry, it measures the echo time delay and magnitude of backscattered or reflected light to build up two- and three-dimensional (3D) images of the cornea with a resolution of ≈20 μm (transversal) x 5 μm (axial). A few studies of pterygium have been performed so far, using OCT: for differentiation between ocular surface neoplasia and pterygia, for evaluation of results after pterygium surgery and for assessment of pingueculae and pterygia (11-13).

Confocal microscopy allows non-invasive *in vivo* imaging of the superficial ocular tissues at cellular level. Its unique physical properties enable microscopic examination of all layers of the cornea and have been used to investigate numerous corneal diseases. It offers a new approach to study the physiological reactions of the cornea to different stimuli and the pathophysiologic events leading to corneal dysfunction in certain diseases. A few studies are performed using LSCM to observe pterygium in situ, they demonstrate potential uses of LSCM in pterygium research and suggest the role of dendritic cells in pathogenesis of pterygium (1).

Currently, in vivo studies of morphological alterations of pterygia remain limited. By combining OCT and *in vivo* LSCM we sought to perform a comprehensive investigation of the morphologic characteristics of the pterygium and the adjacent cornea, allowing precise diagnosis, prognosis and monitoring of this disease. Using LSCM we described and evaluated some specific features at microstructural level such as dendritic cells and changes in density of corneal epithelial cells and keratocytes in cornea invaded by pterygium.

MATERIALS AND METHODS

Study subjects:

Ten eyes of 8 patients with pterygium were examined. Eight eyes of 8 age-matched healthy subjects were included as controls. Patients with known systemic diseases associated with ocular surface disease, a history of ocular surgery, systemic or topical drug use, or contact lens use were excluded from both groups. Comprehensive eye examination including slit-lamp photo-biomicroscopy was performed on each subject.

Optical coherent tomography examination:

Optical coherent tomography was performed using 3D Topcon 2000 OCT with lateral resolution of ≤20μm and in-depth resolution of 5μm-6μm. Operating distance for anterior segment (AS) photography was 63,7 mm (with anterior segment special forehead rest); scan speed was 27 000 A-scans per second; scanning range on cornea 6x6mm. A standardized scanning protocol was performed for each patient. High resolution anterior segment OCT scans- 12 radial (1024 A line) and 3D (6x6mm, 512x128) were carried out. All exams were performed with centering on the visual axis.

In vivo laser scanning confocal microscopy examination:

In vivo confocal microscopy enables detailed analysis of the human cornea, allowing detailed visualization of the corneal microstructure. The LSCM uses a coherent high intensity light source and the laser beam is scanned over the back of the microscope objective by a set of galvanometer scanning mirrors. In this study we used Heidelberg Retina Tomograph/Rostock Cornea Module (Heidelberg Engeneering Gmbh, Germany), which is an applanating device. A 63x water immersion objective lens (Zeiss) is used with a 670 nm wavelength Class I diode laser as a light source to allow a scanning area of 300x300 μm (FOV-300 μm), with a lateral resolution of 1 μm/pixel and z-resolution of 4 μm and up to 800x magnification. The objective of the microscope was covered with a disposable sterile PMMA cap previously filled with eye gel (Corneregel) in order to provide immersion. One drop of topical anesthetic (Alcain 0, 5%) was applied in the inferior cul-de-sac of the eye to be examined. The patient was positioned in front of the instrument, his/her chin and forehead adjusted against the headrests. The instrument was justified using the inbuild software. The objective of the microscope was subsequently advanced to achieve a contact with the ocular surface, the position of the eye was monitored by a camera placed on the side of the objective. *In vivo* LSCM was performed on each eye with pterygium in 2 areas **(Figure 1)**: clear cornea adjacent to the pterygium (1) and pterygium body (2). Images were captured at the 2 points in the same sequence for all subjects. The central cornea and nasal bulbar conjunctiva (within 2 mm from limbus) were examined in healthy subjects. Clear images (without motion

blur or compression lines) from different observation points were selected for qualitative and quantitative analysis. The cells were counted using the inbuild Cell Count software (Heidelberg Engineering GmbH) in manual mode. The data are expressed as density (cells/mm²) ±SD (standard deviation).

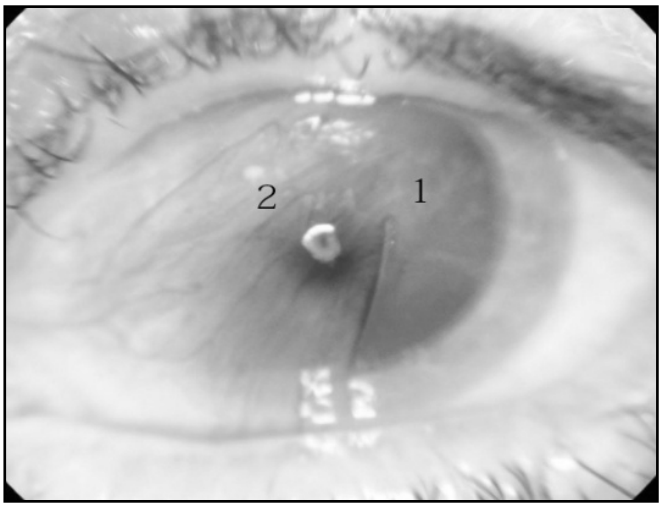

Figure 1. Clinical photography of pterygium, demonstrating the two areas (circles) where *in vivo* LSCM was performed: 1 adjacent clear cornea, 2 body of the pterygium.

RESULTS

The pterygium study group included 4 male and 4 female subjects with a mean age of 55,5 years. The control group included 4 male and 4 female subjects with a mean age of 58,5 years.

Pterygium on the AS-OCT images was defined as hyper-reflective elevated area on the cornea that corresponded to the clinically seen pterygium.

Analysis of the optical coherent tomograms:

Optical coherence tomograms demonstrate elevation of the corneal epithelium by a wedge-shaped mass of tissue separating the corneal epithelium from the underlying Bowman's membrane, which became irregular and disrupted. We observed satellite masses of pterygium tissue advanced under the epithelium beyond the clinically seen pterygium margins. Precise measurement of the thickness of the pterygium, underlying cornea and the adjacent clear cornea was facilitated by caliper tools of the OCT special software **(Figure 2).**

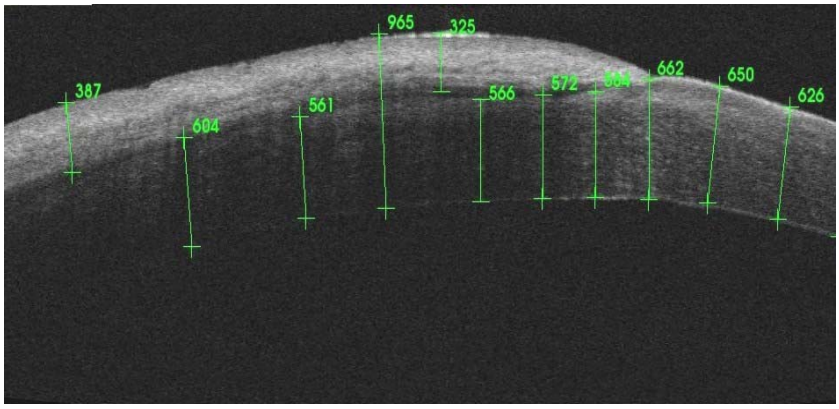

Figure 2. OCT image of anterior segment with advanced pterygium, demonstrating destruction of Bowman's membrane by the wedge-shaped mass of the pteygium head. The scan allows observation of the corneal-pterygium interface and precise measurement of the thickness of pterygium.

Analysis of the in vivo laser scanning confocal microscopy images:

The qualitative and quantitative microstructural characteristics of the pterygium, cornea, and the sub-basal nerve plexus were evaluated. In the clear cornea adjacent to the pterygium, the density of basal epithelial cells and keratocytes in the anterior

stroma was determined. The density of dendritic cells in the pterygium body was also evaluated. In the central cornea of control subjects, the density of basal corneal epithelial cells and keratocytes in the anterior stroma was determined. The density of dendritic cells was calculated in the nasal bulbar conjunctiva.

In vivo laser scanning confocal microscopy images of pterygium:
The *in vivo* LSCM images of the pterygium epithelium, showed tightly packed cells with light-gray bodies, dark borders, and punctiform hyper-reflective nuclei. Inflammatory cells, primarily dendritic cells, were distributed throughout the epithelium of the pterygium. Microcysts with hypo-reflective contents and hyper-reflective even borders were found in the sub-epithelium **(Figure 3a)**. Goblet cells, appearing as large hyper-reflective oval-shaped cells, were also observed in the pterygium's epithelium **(Figure 3b)**. The stroma of the pterygium had a dense fibrovascular structure with rich network of blood vessels filled with hyper-reflective blood cells and minimal infiltration of inflammatory cells in the surrounding tissue **(Figure 3c)**.

In vivo laser scanning confocal microscopy images of the clear cornea adjacent to the pterygium:
Basal corneal epithelial cells appeared as tightly packed cells with bright borders and dark core. They were found with density of 4761±62 cells/mm² in the clear central cornea adjacent to the pterygium and 6844±58 cells/mm² in the central cornea of healthy controls. The basal epithelial cell density in the control group was higher than that in the pterygium group.

At the pterygium-cornea junction, hyper-reflective pterygium epithelial cell clusters were found scattered within the basal corneal cell layer **(Figure 3d)**, presumed to correspond to the clinically seen Fuchs' flecks (6). The corneal basal cells located at the border were in some cases arranged in a columnar fashion **(Figure 3 e).**

Bright deposits, presumed to be melanin deposits, histologically proven by previous investigators (7), were sporadically found at the level of the basal epithelium **(Figure 3 f).**

Morphologic irregularities, including breakage, tortuosity, and localized looping and bulge formation, were commonly seen in the sub-basal nerve plexus of pterygium subjects **(Figure 3 g)**.

In the surface epithelial layer of the clear cornea were observed Goblet cells **(Figure 3 i)**.

The keratocyte density in the anterior stroma was 419±19 cells/mm² in the pterygium patients, which was lower than the density of 772±25 cells/mm² in the central cornea of the control group.

Distribution and density of dendritic cells:
Dendritic cells were found in the nasal bulbar conjunctiva of the control group with a density of 25±12 cells/mm². In the pterygium group, dendritic cells were found in the the pterygium body with a density of 114±11 cells/mm².

The dendritic cell density in the body of the pterygium was higher than the density in the nasal bulbar conjunctiva of healthy controls.

The majority of the dendritic cells in the pterygium displayed the branching dendritic shape. In some cases, the dendritic cells were so numerous that they took on a wire net appearance **(Figure 3 k)**. Interestingly the dendritic cells were also observed in deeper layers and demonstrated same characteristic appearance **(Figure 3 l)**.

DISCUSSION

Pterygium is a formation of fibrovascular tissue proliferation from the bulbar conjunctiva towards the cornea featuring complex etiology (14). Previous histopathologic studies based on surgically excised tissue have shown an invasion of fibroblastic tissue separating the basal corneal epithelial cell layer from the Bowman layer, with destruction of the underlying superficial corneal stroma (2-6). In our current study, we performed an in vivo dynamic examination of the structure of corneas with pterygium in situ using OCT and *in vivo* LSCM. Our findings were in accordance with those of other studies that used similar devices to report histopathologic structure alterations of the clear cornea adjacent to the pterygium (2-6, 11-13).

Anterior-segment OCT allowed precise measurement of the thickness of the pterygium, underlying and adjacent clear cornea. That possibility is very important for taking the right decision for surgical treatment and monitoring the post-surgical period. Using *in vivo* LSCM we found some changes in

keratocyte volume and arrangements. We observed in vivo a columnar appearance of the basal corneal epithelium located at the pterygium-cornea junction, that was noted by previous investigators (2). Our study however, led to the conclusion that this may be due to the mechanical traction on the basal cells, during the invasion by the pterygium head between the epithelium and the Bowman membrane (2).

Different ex vivo studies have found increased numbers of various immunologic cells, such as mast cells, lymphocytes, plasma cells, CD4+ and CD8+T cells and dendritic cells in pterygium tissue samples(8). We calculated higher number of bright round and dendritic cells in pterygia than in healthy conjunctiva. This is suggestive of a chronic inflammation of the pterygium.

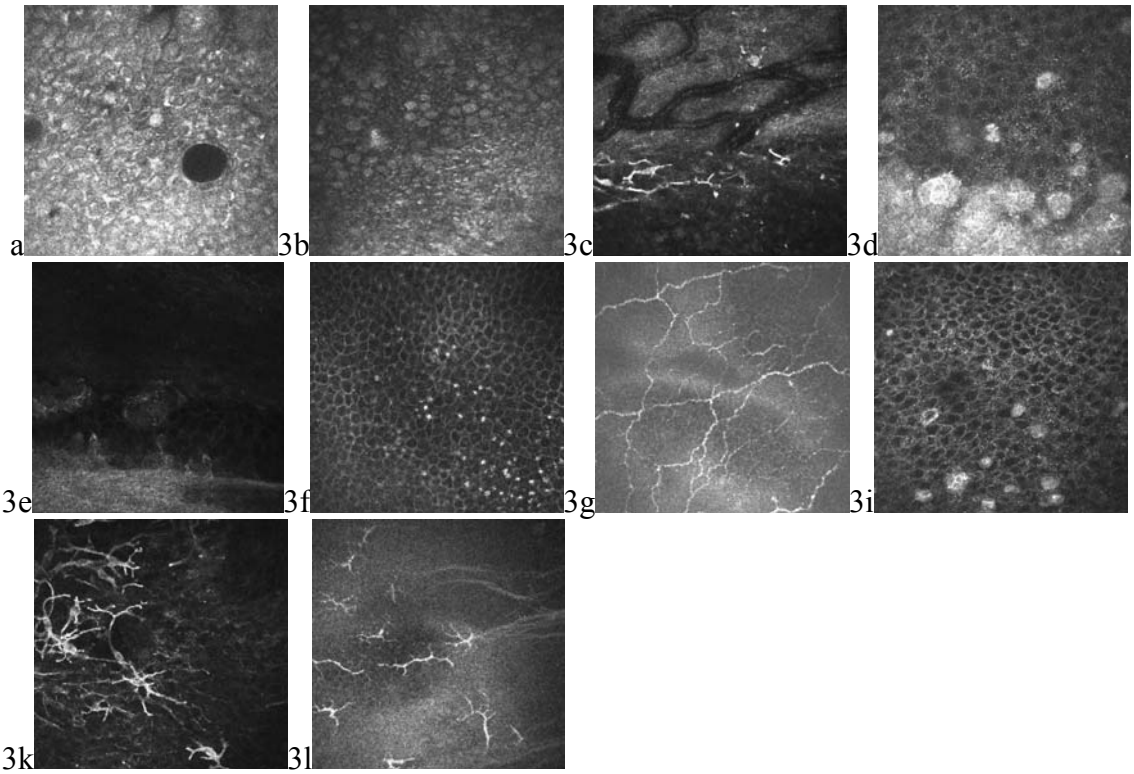

Figure 3. In vivo confocal microscopy demonstrating microstructural characteristics of pterygium altered tissues.
Microcysts with hypo-reflective core and hyper-reflective round borders in pterygium surface. (3a)
Pterygium surface featuring many Goblet cells. (3b)
Rich blood vessels network and dendritic cells with branching phenotype in pterygium stroma. (3c)
Pterygium head showing infiltration of hyper reflective spots in the corneal epithelium in front of the pterygium, corresponding to clinically seen Fuchs' flecks. (3d)
Columnar appearance of basal epithelial cells at the border of pterygium head and clear cornea. (3e)
Bright deposits in basal corneal epithelium, presumed to be melanin deposits. (3f)
Irregular sub-basal nerve plexus- tortuosity, localized bulge and looping formations. (3g)
Goblet cells in epithelial layer of adjacent clear cornea. (3i)
Dendritic cells with wire-net appearance in the epithelium of pterygium surface. (3k)
Dendritic cells with obvious dendritic appearance in sub-epithelial layers. (3l)

The wide distribution of dendritic cells throughout the pterygium suggests involvement of an immunologic mechanism in the formation of pterygia. Dendritic cells are specialized antigen-presenting cells, part of immune responses of the ocular surface. Langerhans cells are a type of dendritic cell found in the corneal and conjunctival epithelium (9). Mutation and migration of Langerhans cells in detail are still to be evaluated. Pro-inflammatory chemokines and cytokines such as IL-1, TNF-α, and CCR5 can mediate the recruitment of Langerhans cells into the cornea. Mediators of inflammation play an important role in the pterygium development (7, 8) and may also be involved

in dendritic cell migration. However, dendritic cells were distributed along the sub-basal nerve plexus in corneas with pterygium, suggesting possible "via the nerve" route to the cornea. We found bright deposits in basal epithelium of clear cornea, presumed to be melanin deposits, histologically proven by previous investigators (7). They found positive correlation between mast cell count and presence of melanin in pterygium. In the surface epithelial layer of the clear cornea we observed Goblet cells, which is a sign of stem cells deficiency, according to some investigators (15).

Morphological irregularities in the sub-basal nerve plexus and decreased corneal epithelial cell and keratocyte density were other interesting findings of the current study. Corneal innervation has important trophic function to the cornea (19, 20), so irregularities in sub-basal nerve morphology may play a role in reducing corneal epithelial cell and keratocyte densities in corneas with pterygium. The average keratocyte density in the anterior stroma of healthy human central cornea was reported to be much higher than that in the posterior stroma (18). In our study, we found an obvious decrease of the keratocyte density in anterior stroma. Increased levels of IL-1 are thought to upregulate other matrix metalloproteinases and mediators of inflammation, potentially contributing to pterygium development (21). One could consider that inflammatory mediators such as IL-1 may also contribute to the decreased keratocyte density in the anterior stroma by triggering keratocyte apoptosis.

In this study, *in vivo* LSCM clearly demonstrated characteristic alterations of the pterygium and adjacent cornea at micro structural level. Other studies have also shown *in vivo* LSCM to be an effective way to observe the morphologic characteristics of the limbus in vivo(16). Because of the focusing effect of the cornea, UV light is concentrated onto the limbus and this is the Coroneo popular theory for limbal stem cell damage from excessive UV exposure (17). Future studies using *in vivo* LSCM to investigate the relationship between limbal changes and pterygium would be beneficial for proper management.

In conclusion, our study utilized OCT and *in vivo* LSCM to demonstrate in situ morphological alterations in corneas with pterygium. OCT provided high-resolution images of the pterygium showing clearly the anatomical relationship between the corneal tissues and the affected areas. The use of these new modality of imaging may help to decrease the current recurrence rates after pterygium, providing detailed topographic characteristics before and after surgery.

Microstructural observations of the cellular elements in pterigium and adjacent structure provide a new insight for future pterygium research. In vivo LSCM could be used in studies to observe histopathologic alterations throughout the progression of pterygium, to study the characteristics of recurrent pterygium, and to monitor the effects of different treatment strategies for pterygium. Such studies would also improve the current understanding of the pterygium pathogenesis.

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