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Title: What biomarkers explain about pterygium OCT pattern

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ABSTRACT

Background: Optical coherence tomography (OCT) has become a very useful tool to study in vivo different ocular structures and to improve differential diagnosis and management of many ocular pathologies. This study aims to identify pterygium alterations that trigger characteristic OCT images and analyze if this pattern correctly demarcates lesion boundary.

Methods: Thirty-two patients, 22 men and 10 women, aged between 26 and 56 (mean age 40.5 ± 6.9) with symptomatic primary pterygium were recruited. After excision, lesion images were obtained by high definition OCT. Specimens were stained with hematoxylin-eosin (H&E); antivimentin for all mesenchymal origin cells and altered limbal basal cells; CD45 for lymphocytes and macrophages cells; CD1a for Langerhans cells; and S100 for melanocytes and Langerhans cells.

Results: The typical OCT wedge-shape hyperreflective mass was evident only by vimentin antibody and included, mainly, fibroblasts but also immune cells (verified by CD45) in a rich network of collagen fibers. The mass apex, often, extended centripetally as a thin subepithelial line, hyperreflective by OCT, formed by a row of fibroblasts under an apparently intact Bowman's layer, as vimentin samples revealed.

Hyperreflective epithelium overlying the mass showed a great number of vimentin positive infiltrated cells like melanocytes, Langerhans cells, and lymphocytes (identified by the other biomarkers). H&E staining revealed the presence of goblet cells.

Nevertheless, only vimentin staining revealed the presence of altered basal cells above partially dissolved or apparently intact Bowman's layer, coinciding in this last case with the fibroblast subepithelial line. In most of the cases (72%), the altered cells occupied a basal segment shorter than fibroblast subepithelial line but in some specimens, these cells exceeded fibroblast line length.

Conclusions: This study demonstrated the great visual accordance between pterygium OCT images and vimentin staining. Alteration in collagen arrangement, infiltration of inflammatory cells, and fibroblast subepithelial line in the lesion apex were the main histological changes responsible for the anomalous hyperreflectivity of the OCT pattern. By contrast, altered basal cells located in the basal epithelial layer of the pterygium head could not be detected by OCT, which could generate lesion size underestimation.

Keywords: pterygium, optical coherence tomography, vimentin, stem cells, immunocytological biomarkers.

INTRODUCTION

Pterygium is a common ocular disease described as a fibrovascular growth of bulbar conjunctiva that extends across the limbus invading the cornea. Nowadays, the exact pathogenesis of this lesion remains incompletely understood although different studies support the hypothesis that UV light could be the most important risk factor, damaging the limbal stem cells DNA [1-4]. Lesion excision is the main treatment and several approaches have been attempted to avoid postoperative recurrence, the most common complication of pterygium surgery. Although recurrence rate has diminished, mainly because the use of new surgical techniques and adjuvants, lesion regrowth still remains above 5% [6], [5]. Delimiting lesion edges before surgery would be essential for fighting pterygium recurrence [4, 7, 8] and a comprehensively interpretation of lesion tomographic images may help in this task.

Since appearing in the 1990, optical coherence tomography (OCT) has become a very useful tool to study in vivo different ocular structures [9-11] and to improve differential diagnosis and management of many ocular pathologies [12-14]. However, histopathological analysis is still necessary not only to really understand the nature of the lesion, but also to clearly interpret OCT images.

In this sense, Pterygium OCT-images show a typical hyperreflective wedge-shape mass (see Soliman & Mohamed) [15] that, coming from the conjunctival side of the eye, overruns the cornea. Remarkable similarity between pterygium OCT pattern and its corresponding hematoxylin-eosin (H&E) histopathology findings has been reported [13, 14]. Nevertheless, this widely used staining is quite unspecific and does not explain all the features of pterygium OCT pattern. To our knowledge, only H&E staining, has been compared with pterygium OCT

pattern. In contrast, immunohistochemical studies have revealed some particular aspects of pterygium pathogenesis and may provide additional information to better interpret OCT images.

The aim of this study is to identify the pterygium structures that trigger characteristic OCT pattern of the lesion and analyze whether this pattern correctly demarcates lesion boundary.

MATERIAL AND METHODS

Thirty-two patients, 22 men and 10 women, aged between 26 and 56 (mean age 40.5 ± 6.9) with symptomatic primary pterygium were recruited from Hospital de Terrassa-Consorci Sanitari de Terrassa (Barcelona, Spain). After obtaining informed consent, all patients were submitted to a complete examination to discard any other ocular anterior segment diseases, as exclusion criterion. The study was approved by the Ethics Committee of Consorci Sanitari de Terrassa and adhered to the tenets set out in the Declaration of Helsinki.

Anterior segment imaging was performed with the commercially available HD-OCT (Cirrus; Carl Zeiss Meditec, Inc., Dublin, CA). Five-Line Raster scanning protocol was used to obtain cross-sectional images of affected cornea and conjunctiva. The five-line raster option of a longitudinal section located at the apex of the lesion was chosen to describe OCT reflectivity patterns.

All patients underwent pterygium excision under local anaesthesia by a single surgeon (P.P.). Damaged tissue was fixed by immersion in 10% neutral buffered formalin and submitted to pathology department of Terrassa Hospital for staining process. After embedding in paraffin, serial sections of the samples were cut

along the longitudinal axis through the head and body of the pterygium, to make them comparable to OCT-images.

Sections were stained with H&E and 4 immunocytochemical biomarkers (Dako, Glostrup, Denmark) to identify and locate cells normally found in pterygium. Thus, mouse monoclonal antibodies specific to vimentine (clone V9, for all mesenchymal origin cells, tumoral cells, and limbal basal stem cells with anomalous motility) [7], CD45 (clones 2B11+PD7/26, for lymphocytes and macrophages cells) and CD1a (clone 010, for Langerhans cells), and rabbit polyclonal antibody specific to S100 (for melanocytes and Langerhans cells) were used. Goat anti-mouse IgG (Dako, Glostrup, Denmark) was applied as secondary antibody. Nuclei were counterstained with Mayer's hematoxylin. Samples were examined by light microscopy and digitally recorded at tiff resolution with a C-3030 zoom digital camera and the software Analysis Get it (Olympus Soft Imaging DTS).

RESULTS

Vimentin samples showed, among all the studied biomarkers, the highest visual accordance with pterygium OCT images (**Fig. 1a and b**). In that sense, the typical OCT wedge-shape hyperreflective mass was evident only by vimentin antibody. This staining also disclosed that the mass was formed by numerous cells immersed in a rich network of collagen fibers that had lost the particular orientation of healthy corneas, in accordance with H&E previously descriptions [13, 14] (**Fig. 1c and e**).

In OCT images and their corresponding vimentin stainings, the mass apex detached the epithelium from the underlying corneal layers and, often, extended centripetally as a thin subepithelial line. This line was hyperreflective by OCT, and was formed by a row of fibroblasts located under an apparently intact Bowman's layer, as vimentin samples revealed (**Fig. 1a and b**). The majority of cells within the wedge-shape mass were also fibroblasts but immune cells like lymphocytes and macrophages (verified by CD45 positive staining), were also found unevenly disposed, mainly around the mass vessels, but also in subepithelial clusters (**Fig. 1h**).

Epithelium overlying the mass was hyperelective near the conjunctival side of the pterygium OCT images, just where vimentin staining showed a great number of infiltrated cells (**Fig. 1a-d**). These cells corresponded to melanocytes, Langerhans cells and lymphocytes, stated by S100, CD1a and CD45 staining respectively (**Fig. 1g-j**). Samples stained with H&E also revealed the presence of goblet cells in this zone, less numerous and furthest from the lesion apex than infiltrated cells (**Fig. 1e and f**).

The epithelial reflectivity gradually diminished in OCT images and reached normal corneal hyporelective appearance, which was consistent with the decrease and vanishing of the infiltrated cells (**Fig 1a-c**). However, this non-infiltrated epithelium revealed some altered basal cells only when vimentin staining was used (**Fig. 1b, g and k**). These polygonal shaped vimentin positive cells appeared in the basal epithelium layer, above partially dissolved or apparently intact Bowman's layer, coinciding in this last case with the fibroblast subepithelial line. In most of the cases (76%), the length of the segment occupied by the altered basal cells was equal or shorter than fibroblast subepithelial line.

However, in some specimens, some altered basal cells exceeded fibroblast line length (**Fig. 1k**) although this is not visible in OCT images (**Fig. 1l**).

DISCUSSION

The results of this study demonstrated the great visual accordance between the pterygium OCT images and vimentin staining. The alteration in collagen arrangement, the infiltration of inflammatory cells, and the fibroblast subepithelial line in the lesion apex were the main histological changes responsible for the anomalous hyperreflectivity of the OCT pattern. By contrast, altered cells located in the basal epithelial layer of the pterygium head could not be detected by OCT, which could generate lesion size underestimation, in some cases.

The stromal findings agree with previously descriptions [16, 17]. The regularly arranged collagen lamellae, typical of healthy corneal, were invaded by pterygium loose connective tissue, much more disorganized, vascularized and with inflammatory cells. It is worth mentioning that, similar stromal OCT patterns were also described in other pathologies with collagen changes, as neoplasia or conjunctival lymphoma [12-14], and, particularly, in those where inflammatory cells infiltration occurred, as in bacterial keratitis [18, 19].

Epithelium reflectivity in OCT images varied throughout the lesion. However, staining such as H&E did not allow observing remarkable epithelial changes [7, 13], except for the presence of goblet cells. Nevertheless, these cells could not cause reflectivity changes, according to Evans and co-workers study [20].

In contrast, vimentin staining revealed the presence of numerous inflammatory infiltrated cells in the affected corneal epithelium near the conjunctival side of the lesion. To our knowledge, this is the first time that information of pterygium

vimentin staining was complemented with those of other biomarkers to identify inflammatory cells.

In the same location, OCT images exhibited the higher epithelial reflectivity. Lymphocytes and Langerhans cells infiltrates, identified by CD1a and CD45 stains, respectively, were related to the immune response produced by chronic inflammation associated with pterygium [17, 21, 22]. Probably, the presence of melanocytes in the epithelial basal layer (highlighted by S100 stain) was not related to the pathology, but the degree of patients' pigmentation. It should be noted that pterygium prevalence is higher in areas with strong solar irradiation [23, 24], where healthy population have high melanocyte density in conjunctiva [25]. As has been shown in several OCT-studies, both inflammatory cells in microbial keratitis [18] and melanin granules in melanosomes of retinal pigment epithelium [26] provided hyperreflectivity. Consequently, they could be responsible, in part, of pterygium epithelial pattern. In fact, healthy conjunctival epithelium appears more hyperreflective by OCT compared to corneal epithelium [27, 28, 29] due to the different cells disposition but also to the presence of inflammatory cells and melanocytes.

Pterygium vimentin staining also disclosed that, the more evident sign of corneal epithelial conjunctivalization was infiltrated cells. Dushku and co-workers [7, 8, 30] previously described the presence of goblet cells as indicator of conjunctivalization. However, these cells were less numerous and appeared furthest from the lesion apex than epithelial infiltrated cells. In the same way, corneal epithelium seems to manifest the characteristic hyporefectivity of healthy corneas in OCT images when all these infiltrated cells disappeared.

In addition, vimentin revealed the presence of altered cells only in epithelial basal layer above apparently intact or partially altered Bowman's layer of pterygium specimens. Unlike infiltrated cells, altered basal cells were not detected by OCT, since lesion pattern showed normal hyporeflexivity epithelial basal layer in this specific localization.

Previous reports [7, 8] located vimentin positive cells in corneal and adjacent conjunctival basal epithelial layer, assigning them an altered stem cell condition. These studies revealed that pterygium limbal epithelial basal cells present an anomalous motility that allow them to move out radially, hypothetically, in all directions, thus altering differentiation process and epithelial corneal regeneration. Although the present study confirms pterygium corneal localization of altered basal cells, no vimentin positive basal cells were detected in the conjunctival epithelium of the 32 specimens analysed. This discrepancy may be due to the different staining method used. Similarly, Kato and co-workers [31], using the same method that previous mentioned studies, only describe altered basal cells in the pterygium head. Nevertheless, it is worth mentioning that antibody high concentration in the tissue could produce artifactual suprabasal and surface layer staining [7]. Further studies are required to elucidate whether these altered basal cells could be present in conjunctival basal layer.

In addition, Chen and co-workers [21] found "round, flattened epithelioid cells" in conjunctival impression cytology specimens, that positive stained with vimentin, considering them as "presumed pterygium cells", that is, altered limbal basal cells. In our modest opinion, these rounded vimentin positive cells distributed diffusely over the pterygial body could be likely, inflammatory infiltrative lymphocytes. Until now, cells that originate pterygium have been only identified

in epithelial basal layer [7, 8, 30, 31] and the presence of lymphocytes in the lesion body have been corroborated by CD45 staining in our study.

Altered limbal basal cells, move onto corneal epithelium dragging the adjacent conjunctival epithelium and stroma [7, 30]. They would be responsible for the growth and invasion of pterygium over the cornea [4]. If these cells could not be detected by OCT, their invasion would be as invisible as under slit lamp biomicroscope. However, these altered cells always appear above the fibroblast subepithelial line, which is seen as a thin hyperreflective line in OCT images. This line often extended beyond the altered basal cells and, therefore, it would represent an objective assessment of lesion boundary in most pterygia. Unfortunately, altered basal cells exceeded fibroblast line in some lesions. Under these conditions, OCT images could underestimate lesion edges. Hence, lesion removing beyond the called grassy cap, related to fibroblast accumulation [29], seems to be a good strategy to minimize lesion recurrences in most cases but not all. Further assessments with a higher number of serial scans and histological sections will be necessary to confirm the percentage and differential characteristics of these cases and the relationship between presence of altered basal cells and lesion recurrence rate.

In conclusion, the present study demonstrated the great accordance between pterygium OCT images and vimentin staining. This fact, together with the complementary information of the other biomarkers allowed identifying all the lesion structures that trigger characteristic OCT pattern. Pterygium boundary could be well demarcated in most but not all the cases. The lack of altered basal cells detection by OCT seems to generate underestimation of the injury extent in some eyes. This fact must be taken into account for a total lesion excision.

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ETHICAL APPROVAL: This study was in accordance with the ethical standards of the Ethics Committee of Consorci Sanitari de Terrassa and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

INFORMED CONSENT: Informed consent was obtained from all individual participants included in the study.

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Figure 1. Sample imaging of pterygium lesion by OCT and light microscopy. Visual accordance between OCT pattern (a) and vimentin staining (b) in patient 21. Vimentin vs H&E staining (c-d and e-f, respectively). Higher magnification of the squared areas highlights numerous infiltrated cells (d) and some goblet cells (f) in patient 5. Complementary immunocytochemical biomarkers staining (g-j) in patient 29. Brown cells express positive vimentin (g), CD45 (h), CD1a (i) and S100 staining (j). Detail of fibroblast subepithelial line and altered basal stem cells stained with vimentin (k) vs OCT pattern (l) in patient 14. Arrow: Bowman's layer. Arrowhead: subepithelial line. Circle: vimentin positive altered stem cells. Ellipse: CD45, CD1a and S100 positive infiltrated cells. High concentrations of antibody produced round artifactual stainings in corneal epithelium (i).