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Identifying the Palisades of Vogt in Human Ex-vivo Tissue

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Abstract

Purpose: The Palisades of Vogt (POV) constitute the corneal epithelial stem cell niche, but identification of this region in ex-vivo tissue is difficult. Here we introduce a simple, direct method of identifying the POV in unsectioned, ex-vivo human tissue.

Methods: Twenty-two eyes were studied, four whole and eighteen rims. Orientation of whole eyes was determined and the eyes were marked to maintain their cardinal orientation prior to dissection. Samples were imaged with brightfield, linearly polarized light and transmitted circularly polarized light (CPL) and optical coherence tomography (OCT) volumes were acquired in all twelve clock hours around the limbus. Five samples were also fluorescently labeled to identify the epithelial basement membrane, and whole mounts were imaged with laser scanning confocal microscopy. Images were compared to confirm that the structures visible with polarized light were POV.

Results: Under CPL the POV presented as amber radial ridges visible in the superior and inferior regions of the tissue. Identification of POV was confirmed by correlating the structures seen under CPL, OCT and laser-scanning confocal microscopy.

Conclusions: CPL can be used to quickly identify POV regions in donor tissue. This technique can assist in targeted harvesting of stem cell regions for research and tissue for limbal transplant.

Keywords: palisades of Vogt; POV; limbus; cornea; corneal epithelium; stem cells; polarized light; corneal transplant; stem cell niche

Introduction

The palisades of Vogt (POV) form the corneal epithelial stem cell niche and are located predominantly in the superior and inferior corneal limbus[1]. Despite the importance of this region in maintaining corneal homeostasis, the POV remain difficult to identify in ex-vivo tissue, which complicates accurate harvesting of stem cells for culture, and tissue for limbal transplant. We have recently demonstrated that the POV can be visualized with optical coherence tomography (OCT), which facilitates in-vivo assessment of the limbus, however OCT is not ideally suited for ex-vivo applications because the required image processing is time consuming, and acquisition of OCT volumes requires equipment that is not readily available in the laboratory setting.

A simple, direct method of visualizing palisades ex-vivo could facilitate limbal transplants by allowing surgeons to accurately and quickly identify stem cell regions and would also allow stem cell researchers to more selectively harvest tissue from palisade-rich regions. Possible clinical application of this technique is suggested by recent ultra-structural studies of the cornea that have shown that the 'clear bubble' of the normal cornea is a complex structure with specific differences in thickness and density in particular anatomical regions[2] . Since palisades are preferentially located in the inferior and superior limbus, they could be used to determine the alignment of the corneal button prior to transplant so that the button could be aligned to the anatomic axis of the recipient, which might assist in improved post-surgical refractive correction and/or graft survival.

Collagen and elastin, the dominant structural components of the eye, have anisotropic molecular organization that retards light in characteristic ways [3,4,5] .That phase shift, caused by the birefringence of the tissue, can be seen as color changes under crossed polarizers, and the direction-independent character of circular polarization is an advantage when viewing biological

tissue[6]. Areas of different density or composition will cause a different phase shift, visible as a color change. Since the rippling basement membrane, which defines the POV is made primarily of collagen, we hypothesized that there might be enough change in birefringence in the POV to make them visible under polarization conditions.

Materials and Methods

Eighteen donor rims and four whole eyes were obtained (ages 29-69 years). The muscle insertion sites were identified on the whole eyes to locate the anatomical orientation of the globe, and the corneas were marked for orientation prior to dissecting the rims. All 22 corneal rims were imaged with transmitted light, crossed linear polarizers, and between crossed circular polarizers (Figure 1 A-C) light with a dissecting microscope. Radial amber ridges were seen in the limbus in opposing clusters which correspond to the known pattern of palisade ridges (Figure 1 D-E). OCT volumes were acquired around the circumference of the limbus with a spectral domain prototype OCT system that has been previously described, and volumes were reconstructed and segmented to identify POV regions[7]. Images from the polarized light and OCT image sets were compared to confirm that the structures visible with polarized light were POV, located in the superior and inferior limbus. To further confirm that these structures are palisades, we immunolabeled the same tissue for collagen VII to identify the basement membrane, and acquired stitched confocal volumes that were then segmented along the basement membrane to reveal the pattern of the POV. Correspondence between the CPL, OCT and confocal images demonstrated that CPL is showing palisades. (Figure 2A-H).

Polarized Light Microscopy: A dissecting microscope (Olympus SZ2/SZX2 Tokyo, Japan with Olympus DP80 camera) fitted with circular polarizing filters (Hoya 46mm Pl-Cir) was used to

examine and photograph the tissue. Rims were fixed in 4% paraformaldehyde (PFA) overnight, rinsed with phosphate buffered saline (PBS), and then kept in 1% PBS. Under transmitted light (Figure 1A) the structural pattern of the POV is hardly visible, and the intrinsic birefringence is not visible. Using linear polarization (Figure 1B), structures oriented at or near 45 degrees to the axes of polarization are discernable, but the palisade structure is not uniformly clear because of the orientation-specific nature of linear polarization. When viewed under circular polarization (Figure 1C), birefringent elements are revealed in all orientations and the pattern of the palisades is visible. Three of the rims were imaged pre and post fixation to make sure that the structures being seen were not a tissue fixation artifact.

OCT Imaging was conducted with a prototype system that has been previously described [6–8]. A 4x4 mm region of tissue was imaged with 400x400x1024 sampling. The scanner had a 100nm-wide light source centered at 870 nm, yielding a coherence length of 2 um in tissue. Image volumes were reconstructed and processed using FIJI (ImageJ software, developed by Wayne Rasband, National Institutes of Health, Bethesda, MD; available at http://rsb.info.nih.gov.ij.index.html) software.

Immunolabeling and Confocal Microscopy: Immunolabeling was performed on five of the corneal rims fixed in 4% paraformaldehyde. The tissue was bisected and relief cuts were made in the tissue to allow it to flatten out. The tissue was permeabilized with PBS-Tx overnight at 4C, then brought to room temperature and incubated in sodium borohydride, on a rocker, for ten minutes and rinsed in phosphate-buffered saline (PBS) (2 min x4). While still on the rocker, tissue was incubated 1 hr at RT in blocking buffer (containing 0.3% Triton X-100 and 10% heat-inactivated donkey serum), then washed with PBS and incubated for four hours with primary mouse monoclonal anti-human type VII collagen antibody 5D2 (from SundarRaj, University of

Pittsburgh, Pittsburgh, PA) and washed with blocking buffer (10 min x5) prior to 1 hr incubation with Alex-Fluor 488-conjugated donkey anti-mouse IgG secondary antibody, and washed with PBS (10 min x4). Finally, tissue was incubated in Hoechst and then washed in PBS (15min x4). The corneal rim halves were placed in a glass dish with a spacer and sealed with a glass coverslip. Stitched stack volumes were acquired and reconstructed with an Olympus FV1200 laser scanning confocal microscope with mosaic scanning and a 20x UPlan S-APO lens (numerical aperture 0.85) Fluoview software and median stack projections were built in FIJI to show the pattern of the basement membrane with FIJI software.

Results

CPL imaging showed more palisades and more detail than brightfield imaging, and OCT and confocal microscopy corroborated the accuracy of the identification. Sometimes the palisades were partially visible via brightfield when there was melanin present (Fig 2A), but this identification has been shown to be inconsistent[1]. Similarly, linear polarization also revealed palisades, but circular polarization provided the most information because it is non-directional and allowed more stable imaging of the full circumference of the limbus. It should be noted that this is a qualitative rather than quantitative technique and also that the structural birefringence of the palisades appeared as amber radial ridges in the limbus and should not be confused with the stress or strain birefringence seen in the punch region of the corneal rims, which is more dramatic but is found in the cut edge of the button harvest site.

By referencing the anatomical orientation of the tissue identified in the four whole eyes, we determined that the ridges we found were located in the inferior and superior regions of the limbus. In tissue recovered post-transplant, fibrovascular ridges were found opposing each other,

corresponding to the known location of the POV (red arrows, Figure 1D-E). The anatomical location and physical features of the POV were consistent with findings of previous studies[9 8]. The transition from corneal collagen to scleral collagen in the limbus is marked by an amber birefringence that is wider and more gradual in the palisade regions and individual palisades appear darker within that region. Since the inferior palisade ridge is known to be more linear in its configuration than the superior, this can be used to visually distinguish the superior from inferior orientation of donor tissue[11, 12]. This technique can be implemented by simply mounting the appropriate polarizers above and below the sample on a standard dissecting microscope with a transmitted light base, which is desirable when the goal is to harvest specific areas of the tissue.

Discussion

Characterization of the structure and function of the POV is vague, although it has been well documented that absence of this region produces limbal epithelial stem cell deficiency[13–16] . In addition, identification of a specific corneal epithelial stem cell marker has been elusive, and research has been complicated by difficulty identifying clear palisade regions in ex-vivo tissue prior to conducting immunofluorescent experiments. Recent studies have discussed visualization of POV with a slit-lamp, clinical confocal, or OCT[8,17,18] , but there is no simple, direct method to visualize palisades in ex-vivo corneal tissue to optimize harvesting of tissue for transplant or laboratory studies. This limitation is more than an academic problem. The challenges of imaging and analyzing for palisade location with OCT volumes are prohibitive in a standard laboratory setting and a present major barrier to localizing the area necessary for targeted harvesting of tissue from ocular epithelial stem cell regions. We present a simple, effective technique for easily identifying palisade-rich regions in ex-vivo tissue. Specific harvesting of palisade regions for

histological and immunofluorescent studies can facilitate research, and may also assist in further characterization of demographic differences in palisade structures[1,17,18] .

Currently, the cornea is transplanted without regard for orienting the donor tissue axis to the recipient. Recent work highlights the directional specificity of the cornea and demonstrates that a corneal transplant is a bioactive scaffold that has a distinct orientation and regional densities[2, 19]. Structural collagen in the human eye provides stability to the cornea, maintains intra-ocular pressure, and affects the degree of corneal astigmatism present[20, 21]. The preferential alignment of corneal collagen and variations in regional density and thickness has been shown to have physiological importance in stability and function, and maintaining alignment during transplantation may facilitate graft healing, survival or post-surgical refraction[19,20,22–24] . A technical drawback to testing this hypothesis has been that Eye Banks would need to mark the orientation of every cornea prior to removal from the globe, not only those corneas destined for corneal transplants. It is plausible that, in a clinical setting, matching the axis of the transplant tissue to the recipient by identifying the POV may facilitate better wound healing and post-surgical refractive correction.

A simple set of circular polarizers can be used to conduct this imaging with almost any dissecting microscope that has an illuminated base by placing one filter below the sample and the other above. The entire procedure takes less than a minute so it is not imperative that the tissue be submerged in media, although the configuration of a dissecting microscope can accommodate a vessel with media as well. Tissue must be viewed in a glass container because plastic containers invariably have strain birefringence which can interfere with visualizing palisades.

Conclusion: Laboratory application of CPL can be readily implemented to facilitate stem cell research. Clinical application of this technique is speculative at this point. Further investigation is necessary to determine whether aligning the axis of the corneal button to that of the donor is helpful, but this simple method of identifying corneal orientation will facilitate that work. This technique may also assist in determining good target regions when harvesting tissue for allogenic transplant.

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Figure Legends:

Figure 1: Whole corneal rim viewed under brightfield light (A), transmitted linear polarization (B), and transmitted circular polarization (C). 360 degree view of whole corneal rim with transmitted circular polarization (D-E) with superior (red) and inferior (green) regions magnified. Palisades appear as amber ridges in the limbus.

Figure 2: Whole corneal rims fixed in 4% PFA (A) and prior to fixation (E), with closeups of marked areas shown acquired with polarized light (B & F), OCT (C and G) and laser scanning confocal (D & H) imaging. Each of these modalities clearly shows the same region. The tissue in the left column shows melanin in the palisade region while the tissue in the right column does not. In addition, the tissue in the right column was imaged very shortly after harvest and has a more intact epithelium, hence the slightly different presentation under polarized light. Nevertheless, in each sample POV are visible.