Interference microscopy delineates cellular proliferations on flat mounted internal limiting membrane specimens

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ABSTRACT

Aim: To demonstrate that interference microscopy of flat mounted internal limiting membrane specimens clearly delineates cellular proliferations at the vitreomacular interface.

Methods: ILM specimens harvested during vitrectomy were fixed in glutaraldehyde 0.05% and paraformaldehyde 2% for 24 h (pH 7.4). In addition to interference microscopy, immunocytochemistry using antibodies against glial fibrillar acidic protein (GFAP) and neurofilament (NF) was performed. After washing in phosphate-buffered saline 0.1 M, the specimens were flat-mounted on glass slides without sectioning, embedding or any other technique of conventional light microscopy. A cover slide and 4′,6-diamidino-2-phenylindole (DAPI) medium were added to stain the cell nuclei.

Results: Interference microscopy clearly delineates cellular proliferations at the ILM. DAPI stained the cell nuclei. Areas of cellular proliferation can be easily distinguished from ILM areas without cells. Immunocytochemistry can be performed without changing the protocols used in conventional microscopy.

Conclusion: Interference microscopy of flat mounted ILM specimens gives new insights into the distribution of cellular proliferations at the vitreomacular interface and allows for determination of the cell density at the ILM. Given that the entire ILM peeled is seen en face, the techniques described offer a more reliable method to investigate the vitreoretinal interface in terms of cellular distribution compared with conventional microscopy.

Since the advent of vitrectomy, ophthalmologists have focused their attention on epiretinal tissue removed during vitreoretinal surgery. Light microscopy and ultrastructural analysis were performed, thereby characterising cell types and extracellular matrix components, such as native and newly formed collagen.[1–3] Several studies have demonstrated that cellular proliferation accounts for tangential traction at the vitreoretinal interface, and removal of these cells together with the internal limiting membrane (ILM) of the retina has become a standard technique to relieve traction.[4–7]

Conventional work-up of retinal specimens depends on tissue sectioning. In the case of ILM, this means that a relatively large but comparably thin membrane is embedded and cross-sectioned, whereby only a minor part of the whole tissue is analysed. Recently, a preparation technique for ''bird's eye analysis of the ILM'' was proposed, and light and scanning electron microscopy was performed.[8 9] We were looking for an easy and reliable technique to show cellular proliferations at the ILM when flat mounted. Herein we present interference microscopy and potential applications of this technique in ILM specimens seen en face.

METHODS

Interference microscopy is able to detect changes in surface height. We used a modified microscope (Leica DM 2500, Germany), where the entering light is split into two beams which pass through the specimen and are recombined in the image plane where the interference effects make the transparent object details visible as intensity differences.

ILM specimens removed during vitrectomy were placed in a mixture of 2% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M phosphate-buffered saline (pH 7.4) for 24 h. Table 1 shows the total number and diagnosis of the specimens. In addition to interference microscopy, immunocytochemistry using antibodies against glial fibrillar acidic protein (GFAP) and neurofilament (NF) was performed according to protocols published previously.[10–12] Under a stereomicroscope (Leica MS 5, Germany) the specimens were then manipulated by using glass pipettes and unfolded to show the maximum area of their surface. Antifading mounting medium 4′,6-diamidino-2-phenylindole (DAPI; Dianova AKS-38448) and a cover slide were added.

RESULTS

Figure 1 shows an ILM specimen following flat mount preparation. In contrast to conventional light microscopy of sectioned tissue, the ILM can be seen en face, not sectioned. Interference microscopy clearly delineates cellular proliferations from ILM areas devoid of cells (fig 1A). DAPI staining demonstrates the distribution of cells attached to the ILM (fig 1B). Glial cells are stained using anti-GFAP (fig 1C). A retinal ganglion cell is stained by anti-neurofilament (fig 1D).

DISCUSSION

ILM peeling is performed in order to relieve vitreomacular traction. In macular holes, we remove the ILM in an area of 1.5 disc diameter surrounding the fovea. Theoretically, this may result in an ILM specimen of 7.07 mm^2 if the ILM was peeled in one fragment. At the macula, the ILM is 2–10 μm thick.[13] Assuming an ILM specimen of 7.07 mm^2 and 10 μm thickness is embedded, and the cross-section hits the maximum specimen diameter of 4.5 mm, the area seen under the microscope is 0.045 mm^2. This is 0.6% of

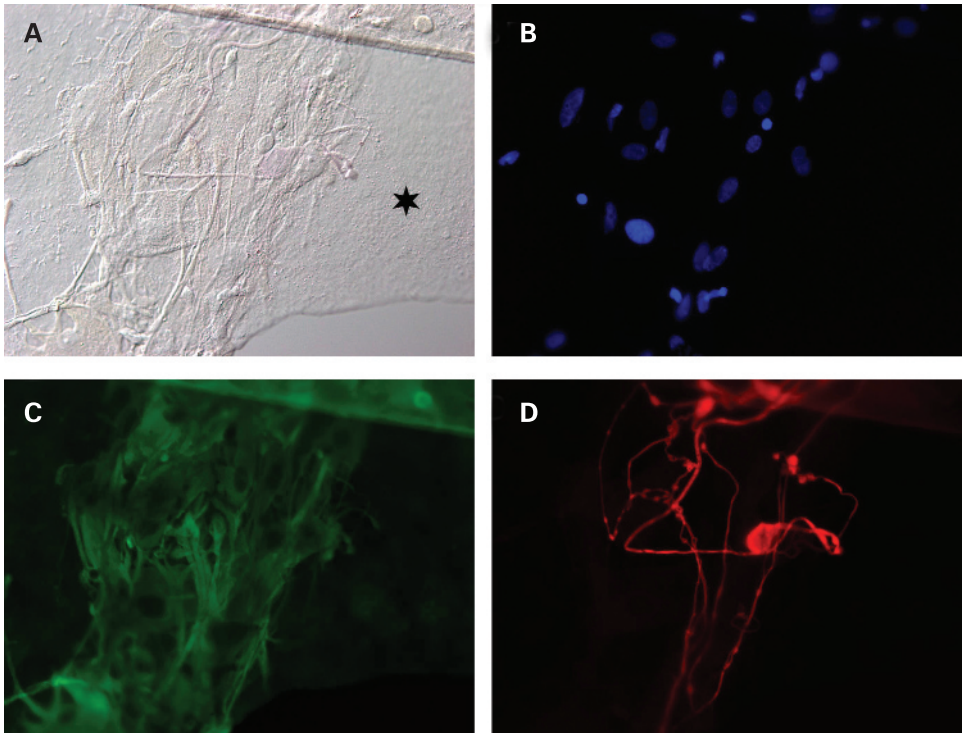

Figure 1 Flat mount preparation of the internal limiting membrane removed from an eye with a macular hole. (A) Interference microscopy delineating cellular proliferations and helps to distinguish them from internal limiting membrane areas without cells (asterisk). (B) 4′,6-Diamidino-2-phenylindole staining of cell nuclei. (C) Cellular proliferations stained with antibodies against glial fibrillar acidic protein. (D) Anti-neurofilament staining of a retinal ganglion cell. Magnification: 400×.

the total area of the specimen. This percentage is even less if not the centre of the specimen, but a more peripheral part is sectioned. Moreover, if 10 serial sections were analysed in this specimen, the area under investigation would only be 6% compared with the area of the peeled membrane. In conclusion, cross-sectioning of ILM specimens shows a very small part of the membrane only.

If fibrocellular proliferation was uniformly distributed on the vitreal side of the ILM, it would not matter which part of the specimen was analysed. Cells and extracellular matrix, however, are rarely arranged in a regular manner, and ILM specimens commonly contain areas without cellular proliferation and those with cells and collagen.[4–6] Depending on the area sectioned, the pure ILM or ILM with fibrocellular proliferation can be seen, and it is not clear which situation is the more representative one in this case. From an ILM specimen without cells and collagen, one might get the wrong impression that fibrocellular proliferation was not present, and vice versa. Given that ILM peeling is performed to relieve vitreoretinal traction, and traction is generated by cells such as myofibroblasts and others, it is likely that misleading conclusions are drawn from non-representative membrane sections.

Interference microscopy in combination with flat mount preparation of the ILM as presented herein overcomes several limitations of conventional light microscopy of tissue sections. The major advantage is related to the whole area of the membrane which can entirely be analysed by using different techniques. Cellular proliferations can be delineated by interference microscopy without the need for tissue staining. This saves processing time to prepare the specimens compared with conventional embedding method. Immunocytochemistry techniques can be applied to flat mounted ILM specimens without changing the protocol compared with cross-section microscopy, and glial and retinal cells can be characterised. Staining of the nuclei by using DAPI enables ophthalmopathologists to determine the exact number of cells on the ILM, and helps to calculate the density of cells attached to ILM specimens. Due to the thinness of the ILM, confocal microscopy is not necessary. Finally, after analysis, the specimen can be processed for transmission electron microscopy (not shown) according to previous protocols.

Interference microscopy of ILM specimens gives new insights into the pathology of the vitreoretinal interface in different diseases, raising new questions. Does the cell density correlate with clinical signs of vitreomacular traction, such as surface wrinkling or macular puckering? Are retinal cells, in particular ganglion cells, avulsed and removed when the ILM is peeled off? Are there any differences in the amount of ganglion cell removal depending on the underlying disease or the extent of vitreomacular traction? Do these findings correlate with functional changes, such as visual impairment or paracentral scotomata? We are looking forward to addressing these questions in further studies, and we want to encourage retinal surgeons and ophthalmopathologists to perform a flat mount preparation of the ILM to shed more light on the whole area of the vitreomacular interface which we remove during macular surgery.

Table 1 Number of specimens and diagnosis

Diagnosis	No of specimens
Macular hole	69
Macular pucker	14
Proliferative vitreoretinopathy	2

Competing interests: None.

Ethics approval: Ethics approval was provided by Ethikkommission der LMU München.

Patient consent: Obtained.

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