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Histopathology of Internal Limiting Membrane Peeling In Traction Induced Maculopathies

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Abstract

Purpose: To compare the presence of cell fragments and entire cell bodies on the retinal side of the internal limiting membrane (ILM) after removal with and without dye assistance in traction maculopathies.

Methods: En-bloc removal of the ILM and epimacular tissue was performed during vitrectomy in 75 eyes without dye-assistance and in 45 eyes with assistance of either Brilliant Blue G (BBG) or Trypan Blue (TB). We included 79 eyes with macular holes (MH) and 41 eyes with other traction maculopathies. All ILM specimens were processed by serial sectioning preparation for light microscopy. Exclusively, if cellular elements of more than 2 µm in diameter were found on the retinal side of the ILM by light microscopy, specimens were prepared for transmission electron microscopy.

Results: 23 (19%) specimens of this series demonstrated such cell fragments and entire cell bodies on the retinal side of the ILM. Specimens removed from MH eyes demonstrated less frequently retinal cell debris than specimens removed from other traction maculopathies. If epiretinal cell proliferation was seen, cellular debris on the ILM was significantly more frequent, irrespective of BBG or TB assistance.

Conclusions: Removal of cellular structures on the retinal side of the ILM during ILM peeling appears to be associated with epiretinal cell proliferation. The presence of cell fragments and entire cell bodies on the retinal side of the ILM seems unrelated to the use of BBG and TB. Epiretinal membranes with wrinkling and folding of the ILM may contribute to structural changes that facilitate pulling off parts of cells or entire cell bodies during ILM peeling.

Keywords: Brilliant blue G; Electron microscopy; Epiretinal membrane; Histopathology; Internal limiting membrane; Macular hole; Retinal debris; Traction maculopathy; Trypan blue

Introduction

Although internal limiting membrane (ILM) peeling is widely accepted as an essentially safe surgical technique, removal of the ILM during macular surgery may sometimes lead to the presence of cell fragments or entire cell bodies on the retinal side of the peeled ILM. Microscopic and immunohistochemical studies occasionally demonstrated retinal cell debris on the ILM which was assigned to glial cells and neuronal debris, such as Müller cell endfeet and cell fragments of the retinal nerve fiber layer [1-6].

It is still under debate how frequent these findings are and whether these morphological observations have an impact on functional results after surgery. Only single reports related ILM peeling to impaired functional outcome [7]. Our group correlated visual outcome to the presence of extensive cellular debris covering large areas of the retinal side of the ILM in cases after the use of Indocyanine Green (ICG) as staining agent for ILM peeling [2,3]. However, subtle functional and morphological alterations were infrequently observed following macular surgery with ILM peeling, including changes of focal macular electroretinogram [8], dissociated optic nerve fiber layer appearance of the fundus [9-11], and visual field defects [12,13]. Recently, Lim and colleagues [14] correlated the presence of retinal cell debris of larger size with macular dysfunction on multifocal electroretinogram suggesting that major cellular fragments on the ILM are related to Müller cell damage with alterations of retinal function.

It is unclear whether the presence and the amount of retinal cell debris in ILM specimens are related to the procedure of ILM peeling itself or to modifications of the surgical technique, such as dye assistance for visualization of the ILM. Additionally, immunohistochemical investigations of surgically excised ILM specimens suggested that the presence of an epiretinal membrane may change the retinal cleavage plane during surgery [1].

Given this background, the aim of the present study was to investigate the presence of cell fragments and entire cell bodies on the ILM in 120 specimens according to (1) the underlying type of traction maculopathy, (2) the presence of epiretinal cell proliferation, and (3) the use of brilliant blue G (BBG) or trypan blue (TB). By light microscopy, specimens were screened for cell debris of large size, notably cell fragments of more than 2 µm in diameter and/or entire cell bodies. If cell fragments were present on the retinal aspect of the ILM, specimens were processed for transmission electron microscopy and analyzed using a standardized grading scale of these cellular elements. The presence of epiretinal cell proliferation was documented.

Patients and Methods

From our archive of 2,349 surgically excised ILM and ERM specimens that were obtained during vitrectomy between 1999 and 2007 at the Department of Ophthalmology, Ludwig-Maximilians-University

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Munich, 120 ILM specimens from 120 eyes were chosen based to the following criteria. Specimens were included according to (1) diagnosis (macular holes (MH), macular pucker (MP), vitreomacular traction syndrome (VMTS), proliferative vitreoretinopathy (PVR), and diabetic macular edema (DME) associated with traction), (2) surgical technique of ILM peeling (unstained and stained specimens using BBG or TB), (3) specimen preparation method (preparation with 4% glutaraldehyde fixation solution and resin embedding in Epon 812), and (4) presence of the ILM within the specimen as demonstrated by light microscopy. We excluded specimens that have been used in other earlier electron microscopic studies published by our group.

This series consisted of ILM specimens removed from 79 eyes with MH and 41 eyes with other traction maculopathies. These were 18 specimens from eyes with MP, 13 specimens from eyes with VMTS, 6 specimens from eyes with DME associated with traction, and 4 specimens from eyes with PVR as presented in Table 1. Seventy five eyes were operated without intraoperative staining of the ILM or ERM. In 18 eyes, ILM removal was assisted by the use of brilliant blue G (BBG) (0.5 ml, 0.25%, Fluoron GmbH, Neu-Ulm, Germany). Trypan blue (TB) (0.5ml, 0.15%, DORC, Netherlands) was used in 27 eyes. Approval from the Institutional Review Board was obtained.

Four experienced surgeons performed a pars plana vitrectomy with peeling of the ILM and epiretinal tissue. The surgical procedure included the induction of a posterior vitreous detachment, if necessary, by suction with the vitrectomy probe around the optic nerve head. Removal of the ILM was performed by grasping the ILM en bloc with epiretinal tissue with an end-gripping forceps and peeling it off around the macular area. In case of a significant cataract, a combined procedure was performed. In eyes with MH, the vitreous cavity was finally filled with a mixture of 15% C_2F_6, and the patients were recommended to stay in face-down position for a minimum of four days at that time of surgery.

The specimens harvested during vitrectomy were immediately placed into phosphate-buffered 4% glutaraldehyde solution for fixation. Specimens were postfixated with Dalton's fixative, dehydrated in graded concentrations of ethanol, and embedded in Epon 812. For light microscopy, series of semithin sections of 750 µm were stained with an aqueous mixture of 1% toluidine blue and 2% sodium borax.

Overall, 1,524 semithin-sections obtained by serial-sectioning. These specimens were evaluated enclosing an average of 12.7 semithin sections per eye. If cell debris was present on the retinal side of the ILM, namely distinguishable cell fragments of more than 2 µm in diameter or entire retinal cell bodies, specimens were prepared for transmission electron microscopy by continuing serial-sectioning. Ultrathin sections of 60-70 nm were contrasted with uranyl acetate and lead citrate for electron microscopy. From each specimen, 35 ultrathin sections were analyzed on average. If cellular debris was smaller than 2 µm in diameter as demonstrated by light microscopy, specimens were not processed for further analysis.

Light microscopic analysis of tissue sections was performed using a Leica microscope DM2500 (Leica, Wetzlar, Germany). The morphologic features of both the vitreal and the retinal side of the ILM were evaluated in terms of cell distribution. Using a Zeiss EM 9 S-2 electron microscope (Zeiss, Jena, Germany), ultrastructural evaluation focused on the 23 specimens that were found with large cell debris or cell bodies on the retinal aspect of the ILM by light microscopy.

Results

Clinical features

Seventy nine woman and 35 men were included in this series, corresponding to 50 right eyes and 70 left eyes. Six patients underwent surgery on both eyes. The average age at time of surgery was 64 years (range 10 to 84 years). The total of 120 specimens was grouped according to diagnosis and dye assistance: specimens removed from eyes with MH (n = 79) and other traction maculopathies such as MP (n = 18), VMTS (n = 13), PVR (n = 4) and DME (n = 6), and specimens removed after using BBG (n = 18) or TB (n = 27) as presented in Table 1.

Light microscopic features

Serial sections for light microscopic examination showed the ILM as a continuous strand in all 120 specimens that was often seen folded and wrinkled. The retinal side of the ILM was characterized by typical undulations allowing for a well-defined topographic assignment. As illustrated in Table 1, cell fragments of more than 2 µm in diameter and entire cell bodies on the retinal side of the ILM were seen in only 23 (19%) of all 120 specimens. In the group without dye assistance, retinal cell debris was found in 17 specimens composed of 7 specimens removed from eyes with MH, 4 specimens from MP, 2 specimens from VMTS, 3 specimens from traction diabetic macular edema and 1 specimen from PVR. In the group of dye assistance, retinal cell debris

No. of patients								
Diagnosis		**Total**	**ILM removed *without* dye assistance**			**ILM removed *with* dye assistance**		
			Large retinal cell debris	**Epiretinal cells**	**Total from diagnosis**	**Large retinal cell debris**	**Epiretinal cells**	**Total from diagnosis**
Macular holes	stage II	5	-	-	3	-	1	2
	stage III	33	1	13	24	1	4	9
	stage IV	11	2	3	5	2	3	6
	recurrent	19	3	8	13	-	5	6
	secondary	11	1	1	8	-	2	3
Macular pucker	primary	10	1	4	6	-	2	4
	secondary	8	3	4	5	-	1	3
Vitreomacular traction syndrome		13	2	5	6	2	6	7
Proliferative vitreoretinopathy		4	1	1	2	-	1	2
Diabetic macular edema with traction		6	3	3	3	1	2	3
Total		**120**	17	42	**75**	6	27	**45**

Table 1: Number of specimens that presented with retinal cell fragments of more than 2 µm in diameter or entire cell bodies on the retinal side of the ILM illustrated according to diagnosis and dye assistance with brilliant blue G or trypan blue.

was found in 6 specimens composed of 3 specimens removed from eyes with MH, 2 specimens from VMTS, and 1 specimen from traction diabetic macular edema.

Comparing the groups of diagnosis, there was a statistically significant difference in the presence of cell debris on the retinal side of the ILM. Specimens removed from MH demonstrated less frequently cell debris than specimens removed from MP, VMTS, PVR, and traction diabetic macular edema (Fisher's exact test, P = 0.015).

Epiretinal cellular or fibrocellular proliferation on the vitreal side of the ILM was found in 69 (58%) of all 120 specimens. In specimens where epiretinal cells were present, cell debris on the retinal side of the ILM was significantly more frequent (Fisher's Exact Test, P = 0.009). Specimens removed without dye assistance presented retinal cell debris in 23% of cases, whereas ILM specimens removed with dye assistance showed retinal cell debris in 13%. However, comparing these specimens removed with or without dye-assistance demonstrated no statistically significant difference in the presence of cell debris on the retinal side of the ILM (Pearson's chi-square test, P = 0.24). The presence of epiretinal cell proliferation within these groups with or without dye assisted peeling did not show a significant difference (Fisher's Exact Test, P = 0.71). Furthermore, there were no differences of structures on the retinal side of the ILM comparing age, gender, right or left eye, and surgeon.

Electron microscopic features

In 23 specimens that were found with cell debris or entire cell bodies on the ILM by light microscopy, transmission electron microscopy confirmed the presence of large cell fragments of more than 2 µm in diameter or entire cell bodies (Figure 1). In addition, small (<1 µm in diameter) round cell fragments, solitarily distributed and directly adjacent to the ILM (Figure 2A), and medium sized (1-2 µm in diameter) round cell fragments (Figure 2B) were mostly demonstrated independent of the neighborhood of large cell fragments or entire cell bodies in all 23 specimens. Cell fragments of more than 2 µm in diameter were frequently seen surrounded by masses of smaller cell fragments and multiple remnants of plasma membranes (Figure 2C). Entire retinal cell bodies with cellular organelles such as nucleus, mitochondria, Golgi complexes, and endoplasmatic reticulum were observed in direct contact to the ILM (Figure 2D).

Discussion

Removing the ILM during macular surgery is mandatory in most traction-associated maculopathies since numerous pathologic and clinicopathologic studies demonstrated an incomplete removal of vitreous collagen fibrils and epiretinal cells from the vitreal side of the ILM by ERM peeling alone. Thus, to relieve traction and to avoid fibrocellular re-proliferation with risk for recurrence of macular holes, macular pucker or other traction-associated retinal damage, the ILM has to be removed.

Given the close anatomic situation, removal of the ILM during macular surgery bares the potential to damage inner retinal layers, thereby possibly inducing retinal dysfunction. Recent investigations supported this hypothesis by correlating retinal cell debris of large size, namely cell fragments of more than 2 µm in diameter, on the removed ILM with changes in electroretinogram [14]. The present study focused also on detecting large cell fragments of more than 2 µm and entire cell bodies in serial sections on the retinal side of the ILM by light microscopy and subsequent further analysis by transmission electron microscopy. Cellular debris of less than 2 µm in diameter is difficult to distinguish with certainty by light microscopy and was suggested not to interfere with function. Therefore, assessment of this study did not concentrate on smaller cellular debris as seen by light microcopy. Screening serial sections, large cell debris on the retinal side of the ILM was found in only 19% of all 120 specimens investigated. This cohort of specimens allowed comparison of cellular elements on the retinal side of the ILM (1) in five traction maculopathies, (2) in the presence and absence of epiretinal cell proliferation, and (3) with and without dye assistance using BBG or TB.

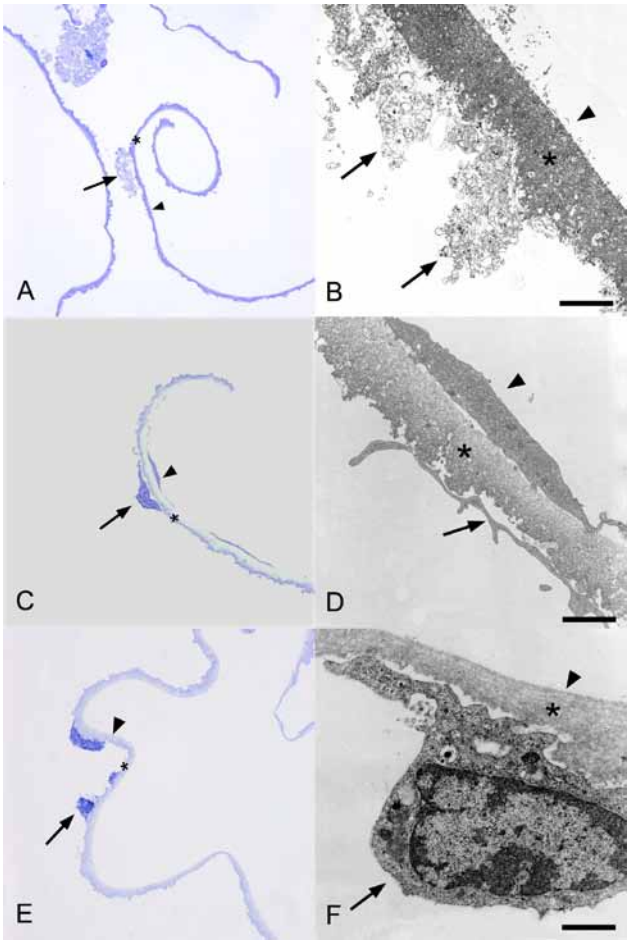

Figure 1: Light micrographs (LM) and transmission electron micrographs (TEM) of the same cellular elements but different sections present the internal limiting membrane (ILM) (asterisk) removed from eyes with diabetic macular edema, stage IV idiopathic macular hole, and proliferative vitreoretinopathy (from top to bottom). The ILM is characterized by an undulated retinal side and a smooth vitreal side.
(A, B) Cell membrane fragments (arrow) on the retinal side of the ILM. The vitreal side of the ILM (arrowhead) is devoid of cells and collagen. (Specimen removed for diabetic macular edema, original magnification: LM 40x; TEM x9,500; scale bar = 1.0 µm).
(C, D) Light micrograph shows a cell with nucleus on the retinal side of the ILM, EM shows one large cell fragment (arrow) in contact with ILM (asterisk), and a single cell on the vitreal side of the ILM (arrowhead) which shares features with fibrous astrocytes such as masses of intermediate type filaments, rough endoplasmic reticulum and electrondense particles. (Specimen removed for stage IV idiopathic macular hole, original magnification: LM 100x; TEM x4,800; scale bar = 2.0 µm).
(D, E) Entire cell body with cellular nucleus (arrow) located on the retinal side of the ILM (asterisk). The vitreal side of the ILM (arrowhead) is blank. (Specimen removed for proliferative vitreoretinopathy, original magnification: LM 100x; TEM x9,500; scale bar = 1.0 µm).

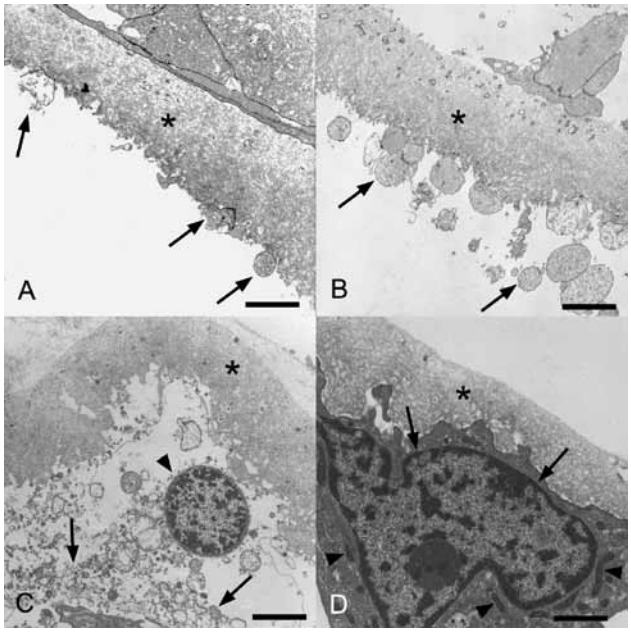

Figure 2: Quantification scale of retinal cell fragments on the ILM.
(A) Small (< 1 µm in diameter) and solitarily distributed round cell fragments (arrow) (specimen removed from a patient with persistent macular hole, original magnification: x9,500; scale bar = 1.0 µm);
(B) Medium sized (1-2 µm in diameter) round cell fragments (arrow) (specimen removed from a patient with persistent macular hole, original magnification: x4,800; scale bar = 2.0 µm);
(C) Large (> 2 µm in diameter) cell fragments derived from nerve fiber bundles and retinal cells, such as cell nucleus (arrowhead) (specimen removed for vitreomacular traction syndrome, original magnification: x4,800; scale bar = 2.0 µm);
(D) Entire cell body with cellular nucleus (arrow) and cellular organelles such as mitochondriae, endoplasmatic reticulum and Golgi complexes (arrowhead) (specimen removed for proliferative vitreoretinopathy, original magnification: x9,500; scale bar = 1.0 µm).

First, comparing the groups of diagnosis, there was a significant difference in the presence of retinal elements. Specimens removed from MH less frequently presented with cell fragments on the retinal side of the ILM than specimens removed from MP, VMTS, PVR, and diabetic macular edema associated with traction. Second, when cell proliferation on the vitreal side of the ILM was present, large cell debris on the retinal aspect of the ILM was more frequently observed than in specimens without epiretinal cell proliferation. Thus, the presence of cell fragments and entire cell bodies on the retinal side of the ILM is associated with the presence of epiretinal cell proliferation. Third, our results show that the presence of cell fragments or entire cell bodies on the retinal side of the peeled ILM is not related to the intraoperative use of BBG or TB, which were used in this series. This is in line with previous ultrastructural studies when ILM removal was assisted using BBG and TB [5,6]. In contrast, when in other studies indocyanine-green (ICG) was used, several reports demonstrated masses of large cell fragments on the retinal side of the ILM in the majority of specimens [2,3,5]. The morphology of this debris was different in that the amount of cell fragments was more extensive, and larger areas of the retinal side of the ILM were covered with masses of retinal debris after ICG-assisted peeling. It is of note that MH specimens of the present study demonstrated less frequently cellular debris than specimens from other traction maculopathies, whereas specimens removed with the use of ICG in macular hole surgery have mostly been reported with abundant cellular debris in small series [3]. Clinical and experimental studies suggested that ICG displaces the retinal cleavage plane into deeper retinal layers [2,3,15,16]. Since then, ICG has come under criticism and ICG related retinal toxicity was investigated with regard to dye concentration, exposure time, osmolarity, and light exposure [17,22].

Concerning the size of retinal debris on the ILM and their ultrastructural appearance, retinal fragments small in size might be of different origin than retinal fragments large in size. Small retinal cell debris of lamellar and round shape that are often seen in contact with undulations of the ILM most probably originate from Müller cell plasma membranes and fragments of Müller cell endfeet. These structures have already been seen in previous studies [5,6]. It was suggested that retinal cell debris on the removed ILM may represent histological evidence of retinal damage. In this context, several studies assumed that damage to retinal Müller cells or Müller cell endfeet may be related to dissociated optic nerve fiber appearance of the fundus after uneventful ILM peeling [9-11]. Furthermore, damage to retinal Müller cells was shown to cause transient changes in electroretinogram after ILM removal [8].

Large retinal cell fragments appear to originate from different structures, including proliferating glial cells or microglial cells. A similar phenotype of cells has previously been reported as multilayered glial cell proliferation underneath the ILM in eyes with macular pucker [23]. Retinal glial cells are believed to play an important role in the formation of epiretinal membranes by responding to tissue stretch [24,25]. Traction forces are thought to stimulate epiretinal cell proliferation by glial cell activation, migration of glial cells from retinal layers through pores of the ILM onto its vitreal side, and proliferation of glial cells on the vitreal surface of the ILM. Thus, it might be hypothesized that large retinal cell debris as demonstrated in this study may represent migrating and proliferating glial cells. Our findings are in accordance with a recent immunohistochemical study of retinal cell debris on the retinal side of the ILM that demonstrated anti-glial fibrillary acidic protein (GFAP) positive retinal debris in correlation with the presence of epiretinal membranes [1]. Positive immunoreactivity of GFAP and vimentin was also reported in epiretinal cell proliferation of ILM specimens removed during macular surgery [26-28].

Epiretinal cell proliferation is known to be associated with various vitreomacular traction disorders [29-34]. One might hypothesize that the presence of epiretinal cell proliferation may alter cell-cell or cell-matrix adhesion interactions on both sides of the ILM and that epiretinal membranes possibly enhance the rigidity of the ILM. As a consequence, the presence of epiretinal cell proliferation potentially might facilitate the removal of cell fragments during the procedure of ILM peeling that is rather due to pulling forces than to a displacement of the retinal cleavage plane. However, a second hypothesis should also be taken into consideration. Given recent immunohistochemical evidence that vitreous derived cells, namely hyalocytes, are involved in epiretinal membrane formation [26], the presence of cell fragments and entire cell bodies on the retinal side of the ILM may represent a secondary event underneath the ILM following epiretinal membrane formation with traction. In that case, ILM removal would pull off parts of this reactive cell proliferation.

The study's main limitation is due to conventional cross sectioning preparation procedures for light microscopic analysis. Cross sections of ILM specimens obtained by serial sectioning were analyzed and still represent a minor part of the whole specimen. However, flat mount preparation of ILM specimens that was proposed for better cell quantification does not allow for topographical analysis with regard to the retinal and vitreal side of the ILM. The design of this study was not aimed at analysis of retinal function but exclusively on the analysis

of morphologic features of the retinal and vitreal aspect of surgically removed ILM specimens. For further analysis of the functional aspect of such morphologic findings a prospective study including multimodal imaging and functional tests together with further electron microscopic analysis is necessary.

The surgically excised ILM and ERM specimens included in this study were chosen from our archive of 2,349 specimens that were obtained during vitrectomy between 1999 and 2007. During this long period of 8 years surgeons' habits may have changed over time with regard to intravitreal dye administration and the choice of dye. The decision whether brilliant blue G or trypan blue was used to stain the ILM was dependent on the surgeons' evaluation of the vitreomacular interface. Thus, a selection bias is most probably related to the intraoperative situation using dye in more difficult cases, such as thicker epiretinal membranes. Based on this consideration, specimens removed with dye assistance might be expected to present more cellular fragments on the retinal side of the ILM than specimens removed without dye assistance. Given that the contrary was found (although the difference was not significant), administration of brilliant blue and trypan blue appears to facilitate ILM peeling without harm to inner retinal layers. This finding is in accordance to previous research of our group [6]. In this context, one might assume that dye-assisted ILM peeling leads to less "grabs" on the retina or less forceps micro-trauma by better visualization of the ILM.

In summary, in this study the presence of cell fragments of more than 2 µm diameter and entire cell bodies on the retinal side of the peeled ILM was not correlated to the use of BBG or TB, but to the presence of epiretinal cell proliferation. Based on serial sectioning of the ILM, our findings suggest that the presence of large retinal cell fragments in ILM specimens removed during macular surgery primarily depends on the underlying disease and on the different pathologic features of traction maculopathies.

Summary Statement

Removal of cellular structures on the retinal side of the Internal Limiting Membrane (ILM) during ILM peeling is associated with epiretinal cell proliferation. The presence of cell fragments and entire cell bodies on the retinal side of the ILM seems unrelated to the use of BBG and TB.

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The authors have no proprietary interest in any aspect of this study.

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