Original Article

Histopathologic and Electron Microscopic Features of Internal Limiting Membranes in Maculopathies of Various Etiologies

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Purpose: To report micro- and ultrastructural features of internal limiting membranes (ILMs) in various maculopathies and to evaluate the effects of indocyanine green (ICG) and triamcinolone acetonide (TA) on epiretinal proliferations associated with ILM and on retinal cleavage plane.

Methods: ILMs from various maculopathies were evaluated regarding presence or absence of membrane-associated cells, type of cells and ILM thickness based on routine histopathology, immunohistochemistry and transmission electron microscopy (TEM).

Results: Thirty ILM specimens were enrolled; 25 of which were evaluated by histopathology and immunohistochemistry and 5 by TEM. ICG only had been used in 17 specimens, TA in 4, and both agents in one specimen. The majority of specimens were immunoreactive for glial fibrillary acidic protein and neuron specific enolase. No significant difference in specimen cellularity and alteration of cleavage plane was noted between ICG-stained and non-ICG-stained ILMs or between TA-assisted and non-TA-assisted ones. Excluding central retinal vein occlusion (CRVO) cases, acellularity was not observed in any of ILMs from diabetic macular edema (DME), cystoid macular edema (CME), and traumatic macular hole (TMH) eyes. TEM disclosed ILM thickening and cellularity in DME as compared to CRVO.

Conclusion: Acellular membranes from CRVO maculopathy may be a sequel of acute retinal ischemia. Thickened diabetic ILMs with high cellularity may be related to chronic activation of Muller cells. No obvious influence of ICG or TA on epiretinal cellularity was detected and the dyes seem to have no significant effect on cleavage plane.

Keywords: Interanl Limiting Membrane; Maculopathy; Central Retinal Vein Occlusion: Transmission Electron Microscopy

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INTRODUCTION

The internal limiting membrane (ILM) of the retina is a periodic acid-Schiff-positive structure 1-2 μm in thickness derived from Muller glial cells. The ILM is intimately associated with Muller footplates and combines with vitreous collagen fibrils.[1] Vitreoretinal surgery along with ILM peeling has been an effective therapeutic intervention for various maculopathies such as idiopathic macular hole (IMH),[2] diffuse diabetic macular edema (DME),[3] macular edema in

central retinal vein occlusion (CRVO)[4,5] and branch retinal vein occlusion (BRVO),[5,6] as well as cases with persistent cystoid macular edema (CME) following cataract surgery or uveitis.[7] It has been hypothesized that pathologic conditions of the ILM, such as thickening, vitreomacular traction and interstitial edema may contribute to the course and pathogenesis of various types of maculopathies.[5,8,9]

Several reports on the ultrastructural and immunohistochemical features of ILMs removed from IMH,[9-12] myopic and traumatic macular holes (TMH)[13] and diabetic maculopathy[8,12] have been published. It is believed that the retinal cleavage plane during ILM peeling might be affected by the nature of the maculopathy for which ILM peeling was performed. Demonstration of cellular fragments and Muller cell end-feet,[14,15] as well as glial and/or neuronal cells on the retinal surface of the ILMs after application of different types of dyes,[12] suggest that the retinal cleavage plane may be altered from the ILM to the innermost retinal layers.

Two published case series demonstrated that the use of indocyanine green (ICG) was associated with altered retinal cleavage.[14,16] In contrast, in another study[12] the influence of different types of dyes (ICG, trypan blue, and brilliant blue) on cleavage plane during ILM peeling was not confirmed. In a recent published report, immunocytochemistry was performed on flat-mounted ILMs demonstrating immunoreactivity for collagen type II, laminin and fibronectin in the area of vitreous attachment. Additionally, the report showed the presence of glial cells, retinal pigment epithelial cells, hyalocytes and macrophages within the areas of cellular proliferation of the ILM.[17]

To the best of our knowledge, none of the previous studies described the micro- and ultrastructural features of ILMs harvested from macular edema secondary to ischemic CRVO, macular hole formation following persistent CME or TMH. Furthermore, differences in immunohistochemical features among various forms of maculopathies have not been reported.

In the current study, the immunohistochemical and ultrastructural features of ILMs and associated cells removed from eyes with IMH, persistent DME, macular edema secondary to ischemic CRVO, TMH and macular hole formation following persistent CME are reported. Additionally, in our series, the difference in membrane-associated cellularity and alteration in cleavage plane were compared between ICG-stained and non-ICG-stained ILMs and also between triamcinolone-assisted and non-traimcinolone-assisted ILMs.

METHODS

In this prospective consecutive case series, during standard three-port pars plana vitrectomy, the ILM in the macular area was grasped with end-gripping forceps, a large section of it was peeled and sent for histopathological studies. Out of the 30 ILMs removed from various forms of maculopathies, 25 underwent histopathologic and immunohistochemical studies and 5 were sent for transmission electron microscopy. Diagnosis of maculopathies included IMH (11 eyes), DME (10 eyes), macular edema secondary to ischemic CRVO (4 eyes), macular hole formation following persistent CME (4 eyes) and TMH (1 eye). Out of 10 DME ILMs, 6 specimens were evaluated by routine histopathology and immunohistochemistry and 4 were examined by TEM. Clinically, none of cases had apparent epiretinal membrane. In 18 out of 30 cases, the ILMs were removed after a 30-second exposure with <0.5 ml ICG with the concentration of 0.625mg/ml, including one case in which triamcinolone plus ICG was used. In 4 cases, ILMs were removed using triamcinolone acetonide. In 3 cases no dye was used. No complications were encountered during surgery.

Histopathology and Immunohistochemistry

Twenty-five ILMs were placed in an individual glass bottle containing 10% formalin and sent to the ophthalmic pathology laboratory. Seventeen out of twenty-five ILMs had been stained with ICG. After instillation of a drop of methylene blue solution to the container, the colorized specimen was then retrieved by filtering through a filter paper. After tissue processing and embedding in a paraffin wax block, thin tissue

sections were cut and stained with hematoxylin & eosin (H&E) and Periodic acid-Schiff (PAS). Immunohistochemistry studies were performed for glial fibrillary acidic protein (GFAP), CD68, neuron specific enolase (NSE), and alpha smooth muscle actin (α-SMA). The employed antibodies were mouse monoclonal antibodies, (Dako Diagnostics, Copenhagen, Denmark) which were used in conjunction with Labeled Strept Avidin Biotinylated Link (Dako Diagnostics, Copenhagen, Denmark). For both NSE and GFAP, sections from the retinal part of an enucleated eye were used as positive controls; sections from a chalazion and from a ciliary body leiomyoma were considered as positive controls for CD68 and α-SMA, respectively. One section from each ILM, which was not treated with the antibodies, was considered as the negative control. Finally all stained sections were examined with light microscopy (Olympus BX41, Tokyo, Japan). Membrane-associated cellularity was defined as presence of at least one cell adherent to the membrane on histopathologic examinations. Acellular membranes were those with no detectable cells in tissue sections. The presence or absence of glial, neural, myofibroblastic or macrophage-type cells associated with ILM was also recorded. The histopathologic and immunohistochemical results were recorded in a data table and the frequency and the percentage of immunoreactivity of ILM-associated cells for different cell markers was calculated. Then, the difference in membrane-associated cellularity and alterations in cleavage plane was compared between ICG-stained and non-ICG-stained ILMs and also between triamcinolone-assisted and non-traimcinolone-assisted ILMs using Fisher exact test. The presence of NSE-immunoreactive cells was considered as an alteration of cleavage plane towards inner retinal layers.

Transmission Electron Microscopy

Five out of thirty ILMs, including four non-ICG-stained ILMs harvested from DME cases and one ICG-stained ILM from an ischemic CRVO maculopathy, were individually fixed in 2.5% gluteraldehyde, and then processed for transmission electron microscopy (TEM). After examination of toluidine blue-stained, semi-thin sections of the ILM, ultrathin sections (70nm) were prepared and stained with uranyl acetate (1%). Three grids per specimen with 5-7 sections per grid were analyzed and an image was taken using a transmission electron microscope (EM 900, Zeiss, Germany). After taking photomicrographs of each case, the thickness of the ILM was measured in three separate areas from one or two photomicrographs of the same magnification (x3000). Then an average value was taken.

RESULTS

Thirty ILMs from thirty patients with a mean age of 56.8±12.01 (range, 9-73) years were enrolled in the study; 56.6% were male. Out of twenty-five ILMs processed for histopathologic examinations, diagnoses included IMH, 11 cases; persistent DME, 6 cases; macular hole formation following persistent CME, 4 cases; macular edema secondary to ischemic CRVO, 3 cases; and TMH, one case.

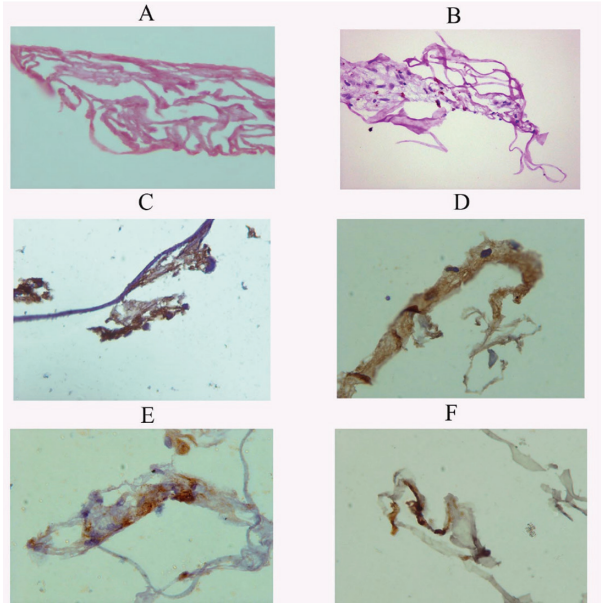

Figure 1. Convoluted ILM on Hematoxylin & Eosin (A) and Periodic acid-Schiff (B) stains (Magnification ×100 and 200, respectively) and immunoreactivity for GFAP (C), NSE (D), α-SMA (E), and CD68 (F) in the epiretinal proliferations attached to the ILMs harvested with ICG assistance from various maculopathies (magnification ×1000).

ILM, internal limiting membrane; GFAP, glial fibrillary acidic protein; NSE, neuron specific enolase; αSMA+, alpha smooth muscle actin; ICG, indocyanine green

Table 1. Results of histopathology and immunohistochemistry in 25 ILM specimens from maculopathies of various etiologies; ILM removal was performed using ICG (17 eyes), triamcinolone (4 eyes) and ICG plus triamcinolone (1 eye), while no dye was used in 3 eyes

IHC Results	GFAP +	GFAP -	NSE +	NSE -	αSMA +	αSMA -	CD68 +	CD68 -	acellular
IMH (n=11)	10 (9 stained by ICG)	0	8 (7 stained by ICG)	2 (2 stained by ICG)	2 (2 stained by ICG)	8 (7 stained by ICG)	1 (1 stained by ICG)	9 (8 stained by ICG)	1 (1 stained by ICG)
DME (n=6)	6 (2 ICG- & 4 TA- assisted)	0	5 (4 TA-assisted & 1 stained by ICG)	1 (1 stained by ICG)	4 (4 Tri-assisted)	2 (2 stained by ICG)	1 (1 stained by ICG)	5 (4 TA- &1 ICG-assisted)	0
CME (n=4)	4 (1 stained by ICG)	0	3	1 (1 stained by ICG)	1	3 (1 stained by ICG)	0	4 (1 stained by ICG)	0
CRVO (n=3)	1 (1 stained by ICG)	0	0	1 (1 stained by ICG)	0	1 (1 stained by ICG)	0	1 (1 stained by ICG)	2 (2 stained by ICG)
TMH (n=1)	1 (1 TA-assisted & stained by ICG)	0	1 (1 TA-assisted & stained by ICG)	0	0	1 (TA-assisted & stained by ICG)	0	1 (1 TA-assisted & stained by ICG)	0
Total (n=25)	22	0	17	5	7	15	2	20	3

ILM, internal limiting membrane; IMH, idiopathic macular hole; DME, diabetic macular edema; CME, cystoid macular edema; CRVO, central retinal vein occlusion; TMH, traumatic macular hole; IHC, immunohistochemistry; ICG, indocyanine green; TA, triamcinolone acetonide; GFAP+, positive immune reactivity for glial fibrillary acidic protein; GFAP-, negative immune reactivity for glial fibrillary acidic protein; NSE+, positive immune reactivity for neuron specific enolase; NSE-, negative immune reactivity for neuron specific enolase; αSMA+, positive immune reactivity for alpha smooth muscle actin; αSMA-, negative immune reactivity for alpha smooth muscle actin

Histopathology and Immunohistochemistry

Histopathologic examination disclosed fragments of a PAS-positive convoluted membrane attached to oval and spindle shaped cells, with no apparent lymphocytic infiltration (Figure 1). Although these epiretinal proliferations were observed histopathologically, they were not apparent clinically. Overall, three ILMs (two from CRVO and one from IMH) were found to be acellular. None of the ILMs from DME, persistent CME and traumatic macular hole were acellular.

Three out of twenty-five ILMs disclosed no cells in routine or immunohistochemistry studies (Table 1 and Figure 1) and of the remaining ILMs, immunoreactivity for GFAP was observed in 100% (22 out of 22), for NSE in 77.3% (17 out of 22), for α-SMA in 31.8% (7 out of 22) and for CD68 in 9% (2 out of 22). Out of seven ILMs with α-SMA immunoreactivity, four were from DME, two from IMH and one from macular hole formation following persistent CME. In only two cases (one from DME and one from IMH) immunoreactivity for CD68 was observed. In only one of the three ILMs from macular edema secondary to ischemic CRVO, a few cells immunoreactive for GFAP and NSE were detected. No significant difference in membrane-associated cellularity or alteration in cleavage plane was noted between ICG-stained and non-ICG-stained ILMs or between triamcinolone-assisted and non-traimcinolone-assisted ILMs ($P<0.999$, and $P<0.999$ respectively.

Of cases in which triamcinolone had been used for ILM peeling (5 eyes), four were from DME and one from traumatic macular hole. In DME cases, with the exception of CD68 immunoreactivity found only in one specimen, all cases were immunoreactive for GFAP, NSE and α-SMA. In the only ILM from the case of TMH, immunoreactivity for all studied antibodies was observed in ILM-associated cells.

Transmission Electron Microscopy

Transmission electron microscopy was performed on 5 specimens that included 4 cases of DME and one case of ischemic CRVO. ILMs from DME cases disclosed a monolayer

of astrocyte-like cells attached to the ILM on one side and to collagen fibrils of the vitreous on the other (Figure 2). ILM thickness ranged from 4,270 to 5,650 (mean 4,947.5±465.8) nm. The only ILM from CRVO cases that underwent TEM, was acellular; however, a multilayered membrane was observed to be focally attached to the ILM (Figure 3) with ILM thickness ranging from 1,200 to 3,730 (mean 2,170 ±1,113.9) nm.

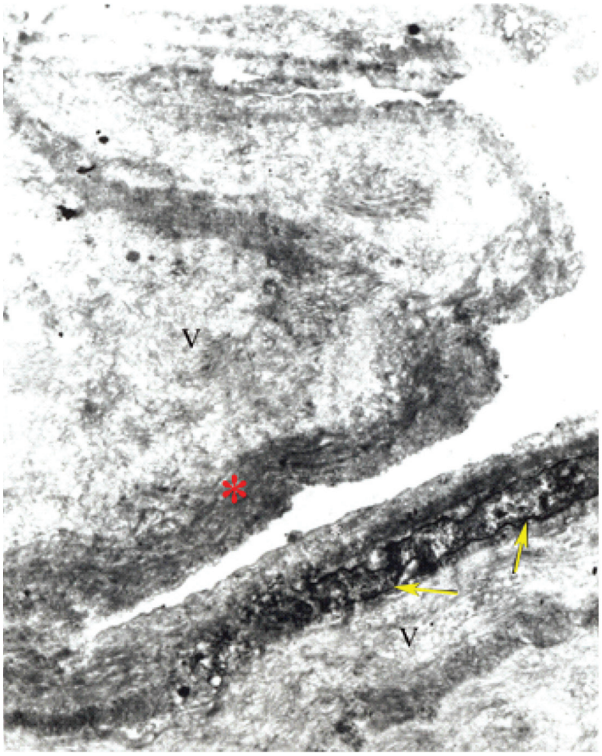

Figure 2. Note an astrocyte-like cell (arrows) attached to the internal limiting membrane (asterisks) on one side and to collagen fibrils of the vitreous (V) on the other side (magnification ×3,000).

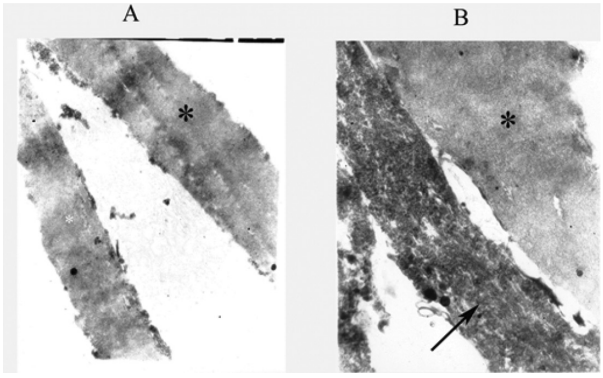

Figure 3. A) The absence of cells associated with an internal limiting membrane (asterisks) from an ischemic central retinal vein maculopathy (magnification ×20,000). B) Focal presence of an electron-dense multilayered membrane (arrow) attached to the internal limiting membrane on transmission electron microscopy (magnification ×30,000).

DISCUSSION

Our study demonstrated low expression of macrophages and predominant presence of GFAP-reactive cells attached to the ILMs harvested from maculopathies of various etiologies. NSE-immunoreactive cells were the second most widely observed cell. Interestingly, ILMs from cases with ischemic CRVO were not associated with cells. Conversely, in DME cases, ILMs were associated with epiretinal proliferation predominantly of myofibroblastic differentiation. Furthermore, using electron microscopy, ILM thickness in DME cases was greater than the one specimen from ischemic CRVO.

Glial fibrillary acidic protein is a marker for glial cells. In a study by Gandorfer et al,[18] glial cells were the main cells present in ILMs and epiretinal membranes removed from eyes with idiopathic macular pucker. In our series, the presence of a higher number of such cells associated with ILMs from various maculopathies and fibrous astrocytes on the vitreal side of diabetic ILMs seen on transmission electron microscopy, may be the result of the migration of retinal glial cells to the inner surface of the ILM[19] and might be relevant to the possible formation of tangential traction at the vitreomacular interface.

Observation of NSE-immunoreactive cells in 17 out of 22 ILMs (77.3%) from all maculopathy groups, indicates an altered cleavage plane from the ILM to the innermost retinal layers which may be due to retinal damage during ILM peeling in both ICG-assisted and non-ICG-assisted eyes. This observation and the presence of large cellular fragments and Muller cell end-feet noted in other studies[9,15,16] oppose the idea of induction of ILM detachment by ICG use.

Alpha-smooth muscle actin (α-SMA) is an immune marker for myofibroblastic cells that were reported as the predominant cell type in epiretinal membranes removed from vitreomacular traction syndromes.[20] In the study

by Gandorfer et al,[18] a proportion of cells present in ILMs and epiretinal membranes from cases with idiopathic macular pucker were immunoreactive for α-SMA. Such cells were reported to be capable of exerting tangential traction at the vitreomacular interface. In the current study, such cells ranked only third amongst cell types attached to ILMs of various maculopathies, but were predominantly observed in DME cases followed by idiopathic macular hole.

CD68 is an immune marker for macrophages, which were the least commonly observed cells associated with ILMs in the current study. We observed macrophages in only two cases; one with IMH and one with DME. Furthermore, we did not find any inflammatory cell attached to the ILMs. However, inflammatory cells were reported on the vitreous side of ILMs in cases of diffuse DME,[21] which by producing inflammatory cytokines may be involved in enhancing the maculopathy. Therefore, it seems that although inflammation can possibly affect the pathogenesis of DME and macular hole, it may be involved only in the early stages of the disease. We suppose that in late stages of evolution of macular hole formation and in DME, inflammation may not be a salient histologic feature.

We found that ILM thickness in the four DME cases studied by TEM was greater when compared to the single case of CRVO. Matsunaga et al similarly reported increased ILM thickness in DME cases as compared to IMH. [8] A similar report is present in the study by Tamura et al,[21] in which ILM thickness in the diabetic retinopathy group was significantly greater than the non-diabetic retinopathy. This may be due to an increased accumulation of various extracellular matrices in DME[8] resulting from functional disturbance of water movement between the vitreous and the retina due to diabetic maculopathy. Additionally, the activation of Muller cells may be a possible contributing factor for ILM thickening in DME cases.

Of the four ILMs from CRVO cases, three were not associated with cells and only in one, a few cells immunoreactive for GFAP and NSE were detected. The number of cells in ILMs from CRVO cases in the study by Tamura et al[21] was significantly lower than those in the diabetic retinopathy group. Acute-onset ischemia and high density of cytotoxic by-products in CRVO may contribute to the absence of cells associated with such ILMs. Based on the aforesaid findings, it may be concluded that acute ischemia in CRVO may cause cellular death, and conversely, chronic ischemia in DME may induce proliferative changes leading to a predominant presence of fibroglial and myofibroblastic cells.

ILM removal is usually a challenging maneuver, however, it may be essential for a successful surgery in various macular disorders.[22] Controversy still surrounds the issue of whether to stain or not to stain the ILM for its surgical removal. While triamcinolone and vital dyes such as ICG may help surgeons for safer and easier ILM removal, their adverse effects on the inner retina and alteration of cleavage plane from the ILM to the inner retinal layers are the concern of investigators.[12,16,22-24] In the current study, no significant difference in membrane-associated cellularity and alteration of cleavage plane were observed between ICG-stained and non-ICG-stained ILMs, including ILMs that were removed by triamcinolone. Therefore, it can be concluded that an experienced surgeon employing a less traumatic technique for ILM removal plays a prominent role in preventing undue interference by the eye.

In conclusion, differences in histopathologic and ultrastructural features of ILMs may be the result of the underlying cause and subsequent pathophysiologic changes. Acuteness of the onset of the ischemia and chronicity of the evolved condition might contribute to the observed changes. It seems that the use of vital dyes for ILM removal during vitrectomy may have no significant effect on ILM-associated cellularity and retinal cleavage plane. The absence of cells associated with ILMs in CRVO maculopathy may be due to acute retinal ischemia. Thickened diabetic ILMs associated with epiretinal proliferations is possibly related to chronic activation of Muller cells. Low expression of macrophages in our series suggests that inflammation may not be a prominent feature in later stages of evolution of macular hole and DME.

Conflicts of Interest

None.

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