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ORIGINAL ARTICLE

Histopathological Analysis of Internal Limiting Membrane Surgically Peeled From Eyes with Epiretinal Membrane

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ABSTRACT

Purpose: To investigate histopathological changes of internal limiting membrane (ILM) in patients with epiretinal membrane (ERM)

Materials and Methods: Forty-two eyes of 42 patients who were diagnosed as ERM and enrolled for vitreoretinal surgery were included in this study. Brilliant Blue G (BBG) was used to stain the ILM in all patients. ILM was peeled in all subjects and analyzed by light microscopy (methylene blue-Azur II × 40). ILM samples were then fixed in 2.5% glutaraldehyde solution and examined in JEOL-JEM 1400 and 2100F electron microscope and photographed by CCD camera (Gatan Inc., Pleasanton, CA).

Results: Remained ERM fragments were observed on 80% of ILM's. Vacuolization of ILM was observed in a patient with diabetic ERM. There were cells and cellular fragments observed mostly at retinal side of ILM which was likely to be a fragment of Muller cells of retina.

Conclusions: Most of the ILM's had residual ERM tissue and contained cells and cellular fragments at retinal side of ILM's. ILM peeling might have a role in decreasing ERM recurrence by removal of residual ERM tissues.

Keywords: Electron microscopy, epiretinal membrane, internal limiting membrane, retina, vitreoretinal surgery

INTRODUCTION

Epiretinal membrane (ERM) is a common retinal problem and associated with a number of ocular disorders.[1,2] It is mostly formed by proliferation of hyalocytes and glial cells. As it contains contractile cellular units, metamorphopsia, micropsia and decreased visual acuity could occur with the traction of macula.[3–6]

Vitreoretinal surgery with ERM removal is an accepted technique for ERM treatment.[7–9] However, there is a risk of ERM recurrence after surgery. It has been proposed that ERM recurrence might be the result of presence of myofibroblasts on remained internal limiting membrane (ILM) and incomplete ERM removal.[10,11] Remained ILM could cause ERM recurrence by serving as a scaffold for proliferation of myofibroblast and other cells.[12] It is widely accepted that peeling of ILM during ERM surgery decreases ERM recurrence by removing residual ERM.[13] However, during ILM removal there is also an increased risk for Muller cell damage because foot process of these cells has contiguity with ILM.

There is not enough histopathological studies in literature regarding changes of ILM in patients with ERM.[14] Thus, we investigated histopathological changes of ILM peeled surgically in patients with ERM.

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MATERIALS AND METHODS

This prospective randomized study was conducted at Ulucanlar Eye Research and Training Hospital in January–July 2013. Forty-two eyes of 42 patients who were diagnosed as ERM and enrolled for vitreoretinal surgery were included in this study. The study was conducted according to Declaration of Helsinki and informed consent was obtained from patients and local ethical committee approved the study.

Surgeries were performed with a 23 Gauge sutureless transconjunctival pars plana vitrectomy (TPV) by two expert vitreoretinal surgeon with the same technique. Following the core vitrectomy procedure, posterior hyaloid was separated and removed by vitreous cutter. Later on, infusion line was turned off and Brilliant blue G (0.024% and 0.25 mg/mL) was injected into vitreous cavity for 30 seconds and washed out immediately with an irrigation solution, then ERM was peeled off from the retina. After the ERM peeling, ILM was stained with the same fashion and peeled safely from the retina. The surgeries were ended with fluid-air and air-gas (C3F8 15% or SF6 20%) exchange.

Age, sex, best corrected visual acuity (BCVA), intraocular pressure, posterior segment ocular findings, spectral domain optical coherence tomography (SD-OCT) (Heidelberg Engineering, Heidelberg, Germany) findings of patients at preoperative and postoperative 3rd month were recorded. Patients were grouped in three groups according to the ERM etiology. Group 1 ($n = 9$) was composed of idiopathic ERM, Group 2 ($n = 14$) was composed of diabetic ERM and Group 3 ($n = 19$) was composed of ERM secondary to previous intraocular surgery.

ILM samples were cut into 5 μm sections using a microtome. Tissues were stained with Methylen Blue and examined using light microscopy (methylene blue-Azur II × 40).

ILM samples were fixed in 2.5% glutaraldehyde solution in phosphate buffer at pH 7.4 for 4 h. After further post-fixation, slicing and staining procedures, sections were examined in JEOL-JEM 1400 and 2100F electron microscopes and photographed by CCD camera (Gatan Inc., Pleasanton, CA) at Middle East Technical University and Hacettepe University laboratories. But, during transportation, cutting and slicing processes some ILM specimens were damaged, and thus histopathological examination was documented in only 10 of them.

Statistical Analysis

The software package used for statistical analysis was SPSS Version 15.0 for Windows (SPSS Inc, Chicago, IL). Statistical analysis included the difference in each parameter studied between groups. Shapiro–Wilk, Independest Samples "*t*", Mann–Whitney *U*, Paired-Samples "*t*" and Wilcoxon tests were used for statistical analysis in the study. A two-tailed probability of 0.05 was considered as statistically significant.

RESULTS

Forty-two eyes of 42 patients were included in this study. There was no significant difference regarding age, gender and eye lateralization between groups ($p > 0.05$). Preoperative and postoperative BCVA, ERM thickness and occurrence of posterior vitreous detachment in groups are outlined at Table 1. There was statistically significant difference regarding preoperative and postoperative BCVA in Group 1 and 3 ($p = 0.01$ and $p = 0.001$, respectively), although there was no significant difference in Group 2 ($p = 0.19$). ERM thickness was highest in Group 3 and lowest in Group 1, but there was no significant difference between groups regarding ERM thickness ($p = 0.31$).

Light Microscopy Results

ILM was in different thickness in each patient. Also, thickness of ILM at different localizations was not similar. Vitreal surface of the ILM's were smooth although retinal surface of the ILM's were irregular (Figure 1a and b). There were ERM fragments on some of the ILM's and fibroblast-like cells were observed inside the ERM tissues (Figure 1c). Similar to ILM, ERM thickness was not similar at different localizations (Figure 1d).

Electron Microscopy Results

There were cellular fragments on both sides of ILM in some specimens and only on thr retinal surface of ILM in other specimens (Figure 2a and b). Cells and cellular fragments on retinal surface of ILM were thought to be fragments of Muller cells damaged during peeling of ILM (Figure 2a and b). ILM's were not in the same thickness throughout the same specimens. (Figure 2b) ILM was in homogenous density and cellular fragments found mostly at retinal side of ILM. There were vacuolizations observed in ILM of a diabetic ERM patient (Figure 2c).

ERM's were composed of fibrillary structure and numerous cellular components were observed in them (Figure 2d). Remaining ERM pieces on peeled ILM's were mostly composed of multilayered collagen fibrils, and the collagen fiber layers were perpendicular to each other. There were fibroblast-like cells,

TABLE 1 Clinical features of groups.

	Group 1 ($n=9$)	Group 2 ($n=14$)	Group 3 ($n=19$)	p^*
BCVA (preoperative)	0.24 ± 0.21	0.17 ± 0.10	0.22 ± 0.18	$p>0.05$
BCVA (postoperative)	0.53 ± 0.24	0.27 ± 0.24	0.41 ± 0.30	$p>0.05$
p^{**}	$p=0.01$	$p=0.19$	$p=0.001$	
ERM thickness (preoperative)	20.0 ± 4.6 (13-27)	22.6 ± 5.9 (12-31)	27.1 ± 13.4 (15-66)	$p=0.31$
PVD(İncomplete/complete)	1/8	5/9	5/14	$p>0.05$

BCVA = Best corrected visual acuity, ERM = Epiretinal membrane, PVD = Posterior vitreous detachment. p^* = statistical difference between groups. p^{**} = statistical difference between preoperative and postoperative BCVA levels.

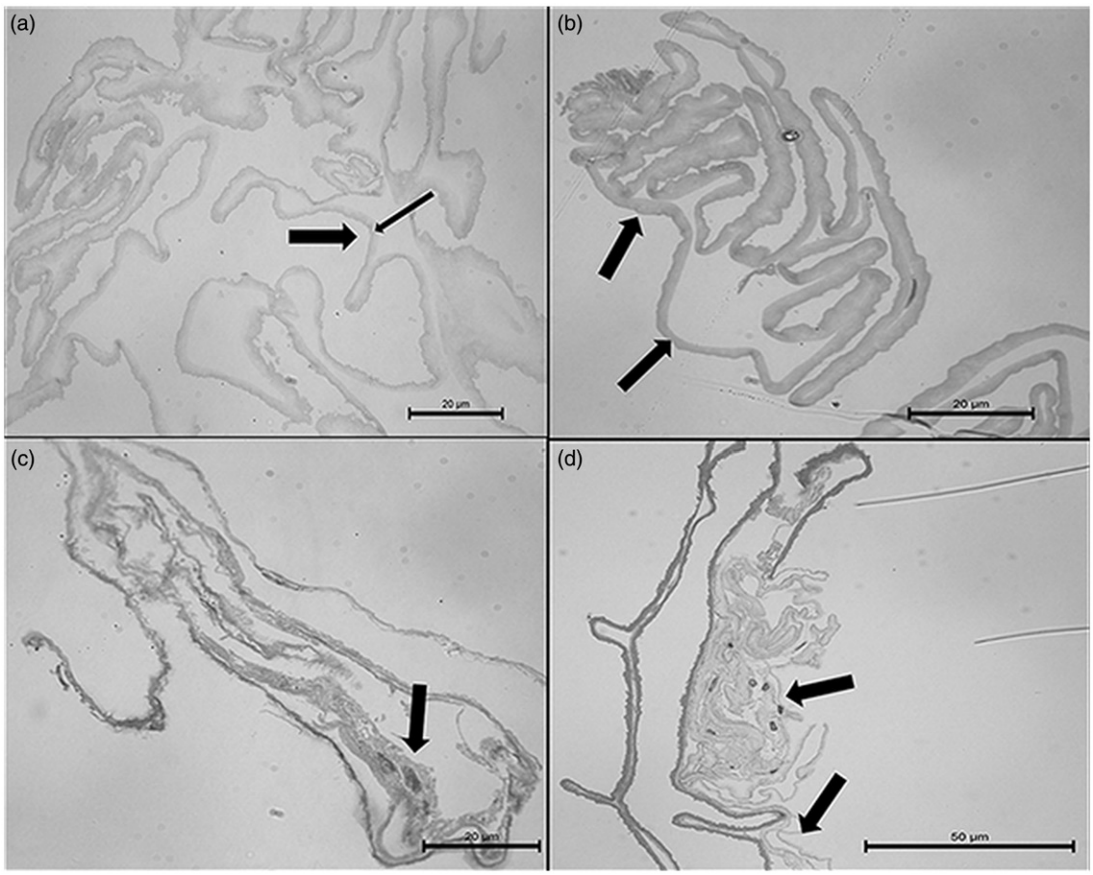

FIGURE 1 Light microscopy images of internal limitan membrane (ILM). (a) ILM composed of a rough-retinal surface (thin arrow) and smooth-vitreal surface (thick arrow). (b) ILM is not in the same thickness at different localizations (arrows). (c) Piece of epiretinal membrane (ERM) and fibroblast like cells in ERM. (d) ERM is composed of with different thickness (Methylen blue-Azur II, 40×).

glial cells and their fragments (Figure 3a and b). Fibroblast-like cells were in the fusiform shape with oval nucleus and lots of mitochondria and endoplasmic reticulum with granules containing cytoplasm (Figure 3c and d).

There were pieces of ERM found on peeled ILM in 80% cases (Figure 4a). There were glycogen droplets in glial cells and fibrillary structures containing fibroblast-like cells (Figure 4b and d). Chromatin condensation and cytolysis were observed in a fibroblast-like cell in ERM observed on peeled ILM (Figure 4c). So, there were both apoptosis and necrosis findings in same cell. There might be different type of dead pattern in these cells.

DISCUSSION

In this histopathological study, we revealed that there were cells and cellular fragments on both sides of ILM in ERM patients. Cells and cellular fragments on retinal side of ILM could be a sign of Muller cell damage or disrupted retinal nerve fiber damage during ILM peeling. There was also vacuolization in

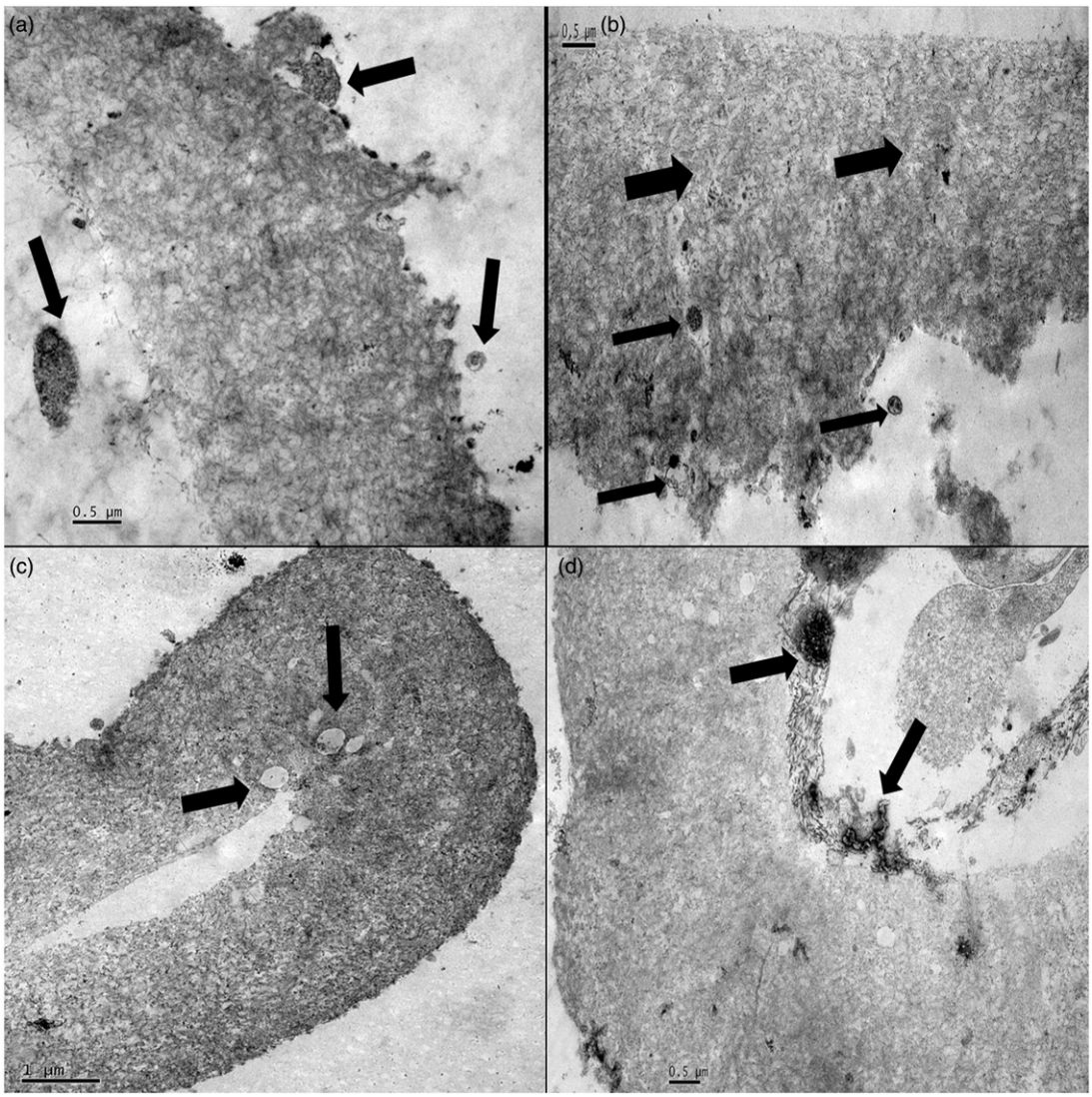

FIGURE 2 Electron microscopic images of the internal limiting membrane (ILM). (a) There are cells and cellular fragments at both sides of the ILM tissue. (b) Thickness of the ILM is not in the same thickness throughout the different parts (thick arrows). There are cells and cellular fragments at retinal surface of the ILM (thin arrows). (c) There are vacoules found in the ILM of the diabetic patient d. There are dense fibrillary structures and cells at the vitreal surface of the ILM in a diabetic patient (Uranyl acetate, lead citrate, ×25 000).

ILM of diabetic ERM patients. There was a high percentage of remained ERM pieces on peeled ILM's.

ERM is known to occur with glial cell proliferation and cause visual disturbances by macular distortion. ERM removal with TPV is an accepted treatment method for this disease.[11] There are conflicting reports regarding peeling of ILM in ERM patients. But accepted concept is that ILM peeling decreases recurrence risk of ERM.[13,15] In our study we aimed to investigate histopathological changes of ILM in patients with ERM. ILM was peeled in all patients and recurrence was observed in 3 of them. It was reported that residual myofibroblasts and incomplete ERM peeling during ERM surgery were the main reasons for ERM recurrences.[10,11,16] We have observed a 80% incidence of remained ERM on ILM and recurrence of ERM was observed in 3 patients with 3 months follow-up period. However, this is relatively a short period to give exact recurrence rate. In another study, the most important risk factors for recurrence of ERM were mainly not peeling ILM, preoperative low visual acuity and ERM in other eye.[17] It was speculated that residual myofibroblasts use ILM as a scaffold and proliferate on it and cause distortion and retractions of macula. These myofibroblasts are removed by peeling of ILM.[10,16] In our specimens remaining ERM tissues were mostly composed of multilayered fibrillary structure and numerous cells. Fibrillary layer was composed of two layers and these two layers were perpendicular to each other and fibrillary structures were not in same diameter. We speculate that there were different types of fibrillary structures in different layers. There were many cells in ERM tissues and these cells were mostly

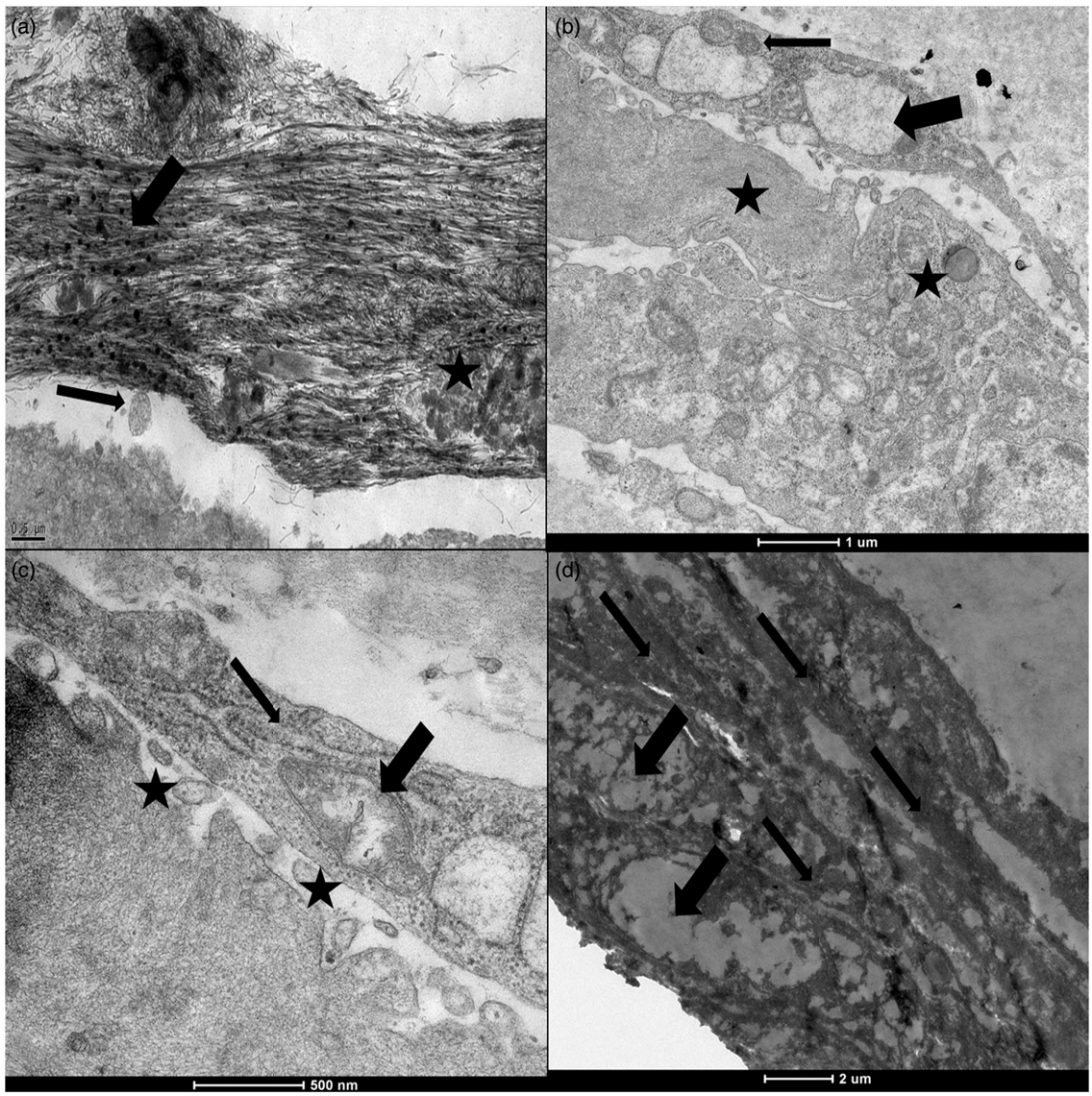

FIGURE 3 Electron microscopic images of internal limiting membrane (ILM) with epiretinal membrane (ERM) tissue found on them. (a) There are fibroblast like cellls (star), fragments of nucleus and cells (thin arrow), and collagen fibrils extending different directions (thick arrow) on ERM tissue. (b) There are mitochondria (thin arrow), rough endoplasmic reticulum (thick arrow) of fibroblast like cells, glial cells and cellular fragments seen at ERM (stars). (c) There are cellular fragments (stars), mitochondria (thick arrow) and rough endoplasmic reticulum (thin arrow) of fibroblast like cells. (d) There are multiple cellular layers (thin arrows) and fuciform shaped fibroblast like cells with euchromatic shaped nucleus (thick arrows) at ERM (Uranyl acetate, lead citrate, ×25 000).

fibroblast-like cells and glial cells with large mitochondria and enlarged endoplasmic reticulum. Large percentage of ERM tissues observed on peeled ILM's in our series show importance of ILM peeling. Fibroblast-like cells and fibrillary structures of remained ERM on peeled ILM's might be a cause for ERM recurrence.

ILM lies between vitreous and retinal surface and has a smooth vitreal surface and irregular retinal surface. It has been proposed that tight connection between Muller cells foot process and ILM is the main reason for this irregularity. It has been showed that there were cellular fragments on retinal surface of ILM and these cells are belongs to Muller cells.[18–20] Haritoglu et al. have studied ILM tissue of macular hole patients and have shown that there were round cellular fragments on retinal surface of ILM and they proposed that these cellular fragments belonged to inner layer of Muller cells.[21] Muller cells play an important role in homeostasis and functions of retina.[22–24] These Muller cells modulate neuronal activity by controlling concentration of neuroactive substances.[25] In our study there were always cells and cellular fragments of cells on retinal side of ILM, but only in some parts of ILM there were cells and cellular fragments on vitreal side of ILM. We thought these cells and cellular fragments at retinal side of ILM were Muller cell origin. Muller cell damage may disturb homeostasis and functions of retina, and thus neurosensory retinal damage might occur after ILM peeling. In a different study there were cells with electro dense nucleus and origin of these cells were not defined. Tari

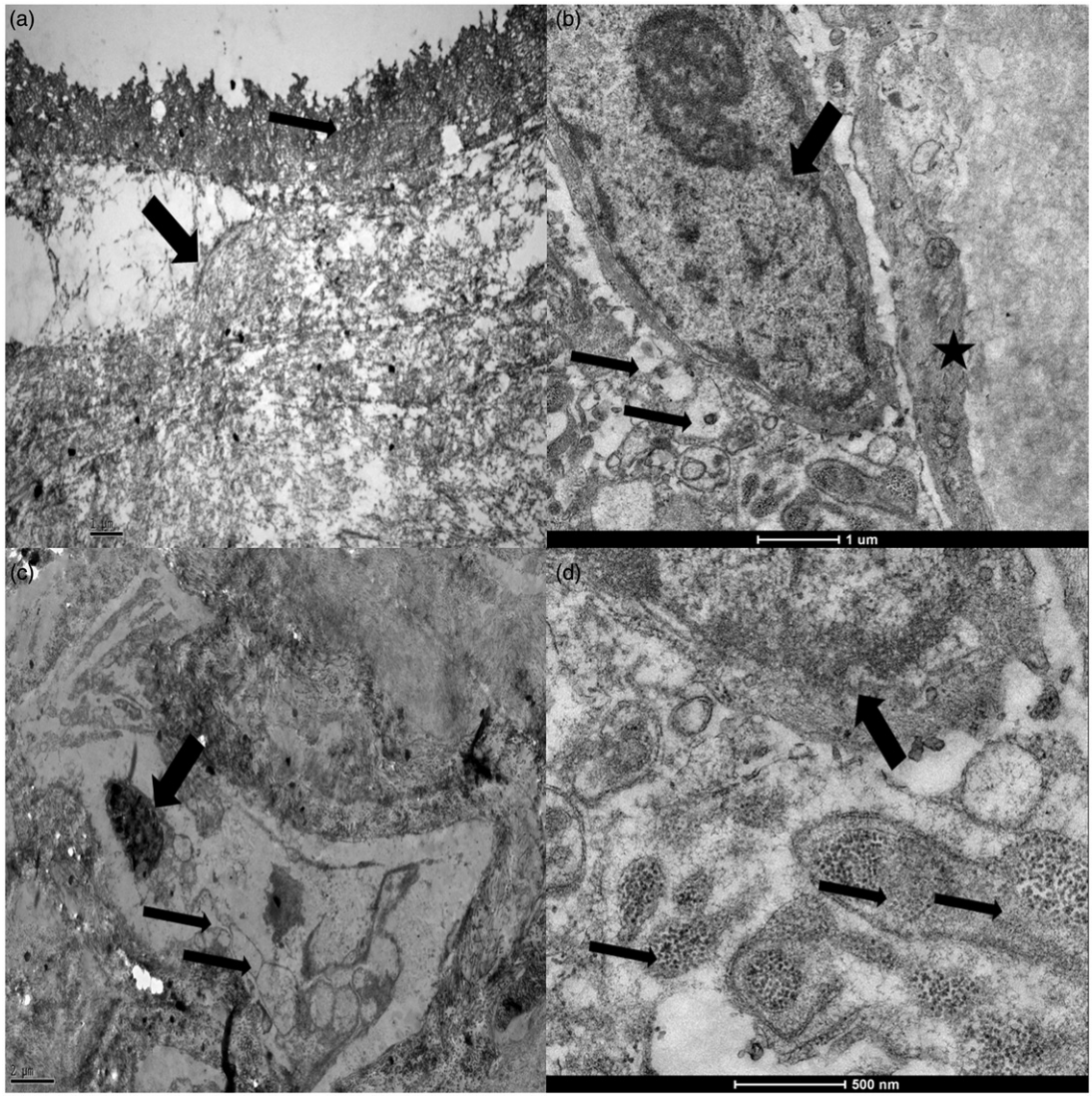

FIGURE 4 Electron microscopic images of internal limiting membrane (ILM) with epiretinal membrane (ERM) tissue and cells found on them. (a) ILM and a piece of incompletely seperated ERM. (b) Fibroblast like cell with euchromatic nucleus (thick arrow), glycogen droplets in cytoplasm of glial cells (thin arrows) and cellular extension of a fibroblast like cell (star). (c) Prominent chromatin condensation in nucleus of the fibroblast like cell (thick arrow), cytoliz findings in cytoplasm of the fibroblast like cell (thin arrows) and enlarged mitochondrias with different sizes in cytoplasm (black arrow). (d) Fibroblast like cells with euchromatic nucleus (thick arrow), glycogen droplets in cytoplasm of the glial cells (thin arrow) (Uranyl acetate, lead citrate, ×25 000).

et al. have found that there were transport vesicular like structures on ILM of macular pucker patients and showed that these structures were belong to foot processes of Muller cells.[26]

In our study we have observed an increased vacuolization in ILM of diabetic patients. To the best of our knowledge, there is no published study showing vacuolization of ILM. We hypothesized that increased generation of reactive free radicals and lipid peroxides in patients with diabetes may be a possible cause of vacuolization of ILM by diabetes-mediated oxidative stress. Autophagic vacuolization was shown to be a preceding morphological feature for type 2 authophagic cell death.[27] Thus, vacuolization of ILM might be a sign of increased oxidative injury observed in diabetic patients. Tamura et al. have found numerous inflammatory cells on retinal side of ILM in diabetic macular edema patients and ILM was thicker in diabetic macular edema patients.[28] But, according to our study, ILM thickness was not the same at different localizations, thus it is not possible to compare thicknesses of ILM's in different patients. To compare ILM thickness, one should compare exactly same part of ILM and it does not seem applicable.

ILM is optically clearly tissue and peeling of ILM might be difficult in some cases and ILM dyes could be used in these cases. There are some studies showing ILM staining decreases risk of ERM recurrence.[29] One of the first used dye for ILM is indocyanine green (ICG).[30,31] But increased number of retinal toxicity was observed with ICG usage.[32,33]

Trypan blue (TB) has been showed as more safer for intraocular usage but ILM staining power was low.[34] Recently, BBG has been introduced and it has no toxic effects on human retina.[35–37] It was also shown that BBG did not induce any apoptosis on retinal cells at in vitro study.[17] In our study we have used BBG to stain ILM in all patients.

There were some limitations of our study. We could not have performed immune histochemical staining to define these cells. If we had used an immunohistochemical study on these cells, results of this study would be a more significant. Another limitation was that we have used BBG in all cases to stain ILM, although no toxicity of BBG on retinal cells have been observed. It would be more appropriate if we have examined ILM's with/out stained. Most of the ILMs were disturbed during transportation, staining and cutting processes for histological analysis. So, we could documented and photographed only some of the specimens.

As a conclusion, histopathological analysis of ILM's in ERM patients revealed that most of the ILM's had residual ERM tissue and there were numerous fibroblast-like cells on this tissues. Although during ILM peeling there is a risk for damaging Muller cells and retinal nerve fibers, ILM peeling in ERM surgery has an important role by removing residual ERM tissues and decreasing the chance of reproliferation of myofibroblasts and reproduction of ERM. Further large prospective histopathological studies with immunohistochemical examinations should be performed to clarify these cells and show retinal neuronal changes.

DECLARATION OF INTEREST

The authors declare no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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