Epiretinal membranes and neural plasticity of the retina
Oberstein, S.Y.L.

Citation for published version (APA):

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PVR: MIB1, ricin, vimentin, ezrin
Chapter 4

Cell proliferation in epiretinal membranes: characterization of cell types and correlation with disease condition and duration

S.Y. Lesnik Oberstein, G.P. Lewis¹, J. Byun², D. Herrera¹, E.A. Chapin¹, S.K. Fisher¹

Accepted for publication in Molecular Vision

¹ Neuroscience Research Institute and Dept. MCD Biology, University of California Santa Barbara, CA, USA.
² Mayachitra Inc., Santa Barbara, CA, USA
Abstract

Purpose: To quantify the extent of cellular proliferation, and immunohistochemically characterise the proliferating cell types, in epiretinal membranes (ERMs) from 4 different conditions: proliferative vitreoretinopathy (PVR), proliferative diabetic retinopathy (PDR), post-retinal detachment (ERMPRD) and idiopathic (iERM).

Methods: Twenty-eight ERMs were collected from human subjects during vitrectomy surgery and immediately fixed in paraformaldehyde. The membranes were immunolabeled with anti-MIB-1 or -SP6 to detect the Ki-67 protein in proliferating cells, in combination with -GFAP or -vimentin to identify glia, -ezrin to identify retinal pigment epithelial cells (RPE), ricinus communis to identify immune cells, and Hoechst to label nuclei. Digital images were collected using a laser scanning confocal microscope. The cell types were identified, their proliferative indices were tabulated as a percent of total number of cells in the membrane, and the number of dividing cells was related to the specific ocular condition and estimated disease duration.

Results: Epiretinal membranes of all 4 types were shown to be highly cellular and contained proliferating cells identified as glia, RPE, and immune. In general, the more rapidly growing membranes, such as in PVR, had more actively dividing cells compared to the other 3 conditions. The percentage of dividing cells was 0.55% for PDR, 2.9% for PVR, 0.35% for ERMPRD, and 0.65% for iERM. While all types of membranes had dividing cells, the number was relatively low compared to the total number of cells present.

Conclusion: The data demonstrate that the 4 types of ERMs examined here had different cell types actively dividing at the time of removal confirming that proliferation is a common event and continues over many months. The fact that the number of dividing cells was relatively low at the time of removal, however, suggests that proliferation alone may not be responsible for the problems observed with the ERMs, and that treatment strategies may need to consider the timing of drug administration, as well as the contractile and/or possibly the inflammatory characteristics of the membranes to prevent the ensuing effects on the retina.
Introduction

Epiretinal membranes (ERMs) are sheets of cells and extracellular matrix that occur on the vitreal surface of the retina. The most common are idiopathic epiretinal membranes (iERM) [1], where there is no known underlying pathology; they can become clinically significant when they cause a decrease in visual acuity or metamorphopsia. Membranes also occur as a result of disease or trauma of the eye such as in proliferative vitreoretinopathy (PVR), proliferative diabetic retinopathy (PDR), and after successful retinal detachment repair (ERMpRD). The epiretinal membranes that form post retinal detachment surgery (ERMpRD) occur in up to 6% of patients and behave differently to PVR membranes [2]. The different membrane types cause variable clinical symptoms like metamorphosia, blurred vision and micropsia or macropsia and problems such as traction on the retina or even traction retinal detachment. In all cases, however, the membranes appear to form initially as a result of cells from within the retina such as RPE, Müller cells, and astrocytes that begin proliferating and migrating onto the surface of the retina. Once this scaffold has formed, other cell types present at the vitreoretinal interface such as hyalocytes and macrophages are added and contribute to those cells undergoing proliferation in the membranes. The membranes become a serious problem when they become contractile, often resulting in a folding or detachment of the retina. It has been postulated by Machemer [3] and MacLeod that the formation of membranes in the eye is an aberrant form of healing response, with an initial proliferation phase after which the contraction phase occurs [4, 5].

Determining the exact cell types present in the membranes has been the focus of several previous studies [4,6-12]. Using light or electron microscopy, it has been shown that many cells seem to change morphological characteristics as the epiretinal membrane develops making it difficult to identify their origin [6-9]. In earlier studies cells were identified by morphology [6,13,14] and described as, myofibroblasts, hyalocytes, fibrous astrocytes, retinal pigment epithelium (RPE) and macrophages. With the addition of immunocytochemistry, however, it became apparent that the most obvious cells involved are glia, macrophages, RPE cells, and fibroblasts [8,11,15,16].

By using antibodies to the protein Ki-67, Heidenkummer et al. [11,15], examined cell proliferation in different types of epiretinal membranes. Using frozen and wax embedded sections they quantified the levels of proliferation and suggested that this could help predict membrane behaviour and could be of clinical significance to predict the risk of recurrence [11,15]. They developed a “proliferation index” (PI) (total number of cells divided by number of cells dividing) which could help clinically classify membrane types and “quantitatively
indicate the proliferation potential of the ERM”. However they did not correlate the PI to the underlying disease process in these studies, as they felt their numbers were too small to separate the inter-individual differences from the disease process differences [11,15].

In order to better understand the origins of the proliferating cell types and their relationship to the 4 different disease conditions, we used antibodies to the Ki-67 protein in combination with antibodies specific to proteins identified within glial, RPE and immune cells on whole membranes to quantify the dividing cell types and relate this number to the total number of cells present, as well as the type of ERM and the disease duration. We show here that proliferating glial, RPE and immune cells can be identified in all 4 types of ERMs although the relative numbers of dividing cells varied between disease condition and with the estimated duration the membrane was present in the eye. Implications for treatment strategies are discussed.

**Methods**

Twenty-eight membranes, classified as PDR (n=5), PVR (n=9), postRD (n=4) and iERMs (n=9), were collected at time of vitrectomy surgery from 27 patients. After removal from the eye, the membranes were immediately placed in fixative (4% paraformaldehyde in 0.1M sodium cacodylate buffer, pH 7.4; Electron Microscopy Sciences, Fort Washington, PA) and stored at 4°C until used. The membranes used in this study were from both males and females and the length of time between diagnosis and epiretinal membrane removal was variable from 1-25 months. (Table 1, Figure 1). The human surgery was conducted at the Academic Medical Center, Amsterdam, The Netherlands. All procedures had institutional research ethics committee approval and adhered to the tenets of the Declaration of Helsinki.

**Immunocytochemistry**

The ERMs were processed whole, without embedding or sectioning. Following fixation the tissue samples were rinsed in phosphate buffer (PBS) 3 x 5min, 1 x 1hr. Since the antibodies to Ki-67 require antigen retrieval to expose the epitopes, the tissue was incubated in citrate buffer (Dako, Carpinteria, CA) for 40 mins at 97°C. Following cooling for 20 min at room temperature, the tissue was rinsed in PBS 3 x 5 min and then incubated in normal donkey serum (1:20) in PBS, 0.5% BSA, 0.1% Triton X-100, and 0.1% azide (=PBTA) overnight at 4°C on a rotator. The following day the primary antibodies were added overnight at 4°C on a rotator in PBTA. The MIB-1 (mouse monoclonal, 1:100, Immunotech/Beckman Coulter, Fullerton, CA) or SP6 antibodies (rabbit polyclonal, 1:100, Abcam, Cambridge,
Two different markers were used to label Ki-67 because of the limitations in the triple label antibody combinations. Anti-MIB-1 was used in combination with antibodies to GFAP (rabbit polyclonal, 1:400; DAKO, Carpinteria, CA), an intermediate filament protein in glial cells, as well as biotinylated *ricinus communis* (ricin, 1:1000, Vector Labs, Burlingame, CA), a lectin that labels immune cells such as microglia and macrophages [17]. Anti-SP6

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Table 1. Table listing all the epiretinal membranes used in this study along with the age and sex of the patient, as well as the length of time between diagnosis and membrane removal (in months). PDR, proliferative diabetic retinopathy; PVR, proliferative vitreoretinopathy; ERMpRD, post-retinal detachment; iERM, idiopathic.
was used in combination with antibodies to ezrin (mouse monoclonal, 1:10,000, Sigma, St. Louis, MO) a cytoskeletal protein in the mirovilli of RPE, and either vimentin (chicken monoclonal; 1:2000, Chemicon, Temecula, CA), an intermediate filament protein present in glia, or biotinylated *ricinus communis*. Following rinsing of the primary antibodies in PBTA, the secondary antibodies were added, each at 1:200, in PBTA, overnight at 4°C on a rotator (donkey anti-mouse conjugated to CY3 for MIB1, donkey anti-rabbit conjugated to CY2 for GFAP, streptavidin CY5 for ricin, donkey anti-rabbit conjugated to CY3 for SP6, donkey anti-chicken conjugated to CY2 for vimentin and donkey anti-mouse conjugated to CY2 for ezrin). All secondary antibodies were purchased from Jackson ImmunoResearch, West Grove, PA. On the final day, the sections were rinsed in PBTA after which a Hoechst stain (1:5000; Invitrogen, Carlsbad, CA) for identification of cell nuclei was added to all samples. The membranes were mounted on glass slides in 5% n-propyl galate in glycerol and viewed on an Olympus FluoView 500 laser scanning confocal microscope (New York, NY). Images were collected as a “z” series of 5 to 10 images taken at 0.5 µm intervals. For quantitation, a single plane image was selected from the “z” series that contained the most number of anti-MIB-1 or -SP6 labeled cells. The number of labeled cells, as well as the total number of nuclei were counted manually from the digital images and normalized to the area of tissue. Labeling with the proliferating cell markers was correlated with the glial, RPE and immune cell markers while viewing the images in Photoshop where magnification could be increased and channels (colors) could be turned up or down. This ensured accurate determination which cell types were double labeled with the Ki-67 markers.

Advantages of detecting dividing cells in tissue with antibodies to the Ki-67 protein rather than thymidine autoradiography include 1) the antibodies will label dividing cells in all phases of cell division except G0 (resting) unlike thymidine which is only incorporated in “S” phase [18-20], 2) autoradiography requires living tissue for the incorporation of

![Graph illustrating the average duration the epiretinal membranes were present in the eye from the initial diagnosis to removal (in months). PDR, proliferative diabetic retinopathy; PVR, proliferative vitreoretinopathy; ERMpRD, post-retinal detachment; iERM, idiopathic.](image)
Figure 2. Laser scanning confocal images of representative staining patterns on epiretinal membranes from patients with proliferative vitreoretinopathy (PVR). Anti-MIB-1 (A,B,E,F; red) or anti-SP6 (C,D; red) labelling was observed in anti-GFAP stained glia (green), ricin stained immune cells (blue) and anti-ezrin stained retinal pigment epithelial cells (green). Note that the amount of anti-GFAP labelled glia varied between membranes (A,B,F), the anti-ezrin labelling appeared to encircle the cells, and the ricin labelling was prevalent in all samples. Scale bars, 50 µm.
thymidine into the DNA and 3) the antibodies allow relatively straightforward labeling using multiple probes for the identification of multiple cell types.

**Results**

**Immunocytochemistry**

Immunocytochemical and other markers were used to identify RPE, immune, and glial cells, as well as all nuclei and those undergoing DNA synthesis. However, since the maximum number of fluorochromes we could use at one time was limited to 4, and the fact that not all antibodies could be combined because some were made in the same species, the membranes were divided into 2 main labeling groups: 1) anti-MIB-1 + anti-GFAP + ricin and 2) anti-SP6 + anti-ezrin + anti-vimentin. (Only a few membranes were labeled with anti-SP6 + anti-ezrin + ricin as shown in figure 2, but these were not included in the graphs due to the small number of samples.) Within both groups there were anti-MIB-1 and anti-SP6 labeled cells that did not label with any of our markers and they are listed as “unidentified” in the tabulated data (see below). These unidentified cells could be either 1) RPE cells in the first group 2) immune cells in the second group, or 3) cells not identified by our markers.

All membranes were found to be multi-layered and highly cellular, as illustrated by the...
Hoechst nuclear stain (Fig. 2, 3). Anti-MIB-1 and -SP6 labelling for cells undergoing DNA synthesis was found in all 28 epiretinal membranes examined (Fig. 2, 3). In addition, the membranes also labelled for proteins present in glial, immune (microglia and macrophages), and RPE cells, and examples from all these cell types were MIB-1/SP6 labelled, albeit at different levels. While the total number of Hoechst labeled nuclei far outnumbered the total number of anti-Ki-67 positive cells, most of the Ki-67 labeled cells appeared to also label

Figure 4. Graph showing the number of dividing cells/mm² (anti-MIB-1 labelled) for the 4 types of epiretinal membranes. Note that all 4 types contained dividing cells although the relative number was much lower compared to the total number of nuclei present. PVR membranes had the highest number of dividing cells. The unidentified cells were MIB-1 labelled but not labelled with either anti-GFAP or ricin. PDR, proliferative diabetic retinopathy; PVR, proliferative vitreoretinopathy; ERMpRD, post-retinal detachment; iERM, idiopathic.

Figure 5. Graph showing the number of dividing cells/mm² (anti-SP6 labelled) for the 4 types of epiretinal membranes. Note that iERM did not have dividing RPE cells and ERMpRD did not have dividing glia. PVR membranes had the highest number of dividing cells. The unidentified cells were SP6 labelled but not labelled with either anti-ezrin or -vimentin. PDR, proliferative diabetic retinopathy; PVR, proliferative vitreoretinopathy; ERMpRD, post-retinal detachment; iERM, idiopathic.
with one the cell specific antibodies used here. Examples of typical staining patterns observed in 6 different membranes from the PVR group, are illustrated in figure 2. In general, the glia and the RPE cells appeared within independent domains, whereas the immune cells usually appeared dispersed throughout the membrane. In addition, while the anti-GFAP staining appeared to fill the cytoplasm of the glia (Fig. 2A,B), the ezrin staining appeared to encircle the RPE cells, which had both an elongated (Fig. 2C,D) or circular morphology (Fig. 2E). The fact that anti-ezrin, which normally labels the apical microvilli, appears here to encircle the cells, most likely represents microvilli that have collapsed and now appearing along the surface of the cells. In some cases the membranes contained anti-Ki-67 labelled cells that were not identifiable using this set of antibodies (Fig. 4, 5). Examples of common staining patterns from pRD, iERM and PDR membranes stained with anti-MIB-1, -GFAP and ricin, are shown in figure 3. The MIB-1 labelling alone is shown in the bottom half of each image, without the anti-GFAP and ricin labelling to better visualize the dividing cells (arrows are placed for reference points). All three images illustrate the presence of dividing cells as well as glia and immune cells. Anti-ezrin labelling of RPE was observed in pRD and PDR membranes (data not shown) but not in iERM membranes.

**Quantitation**

All membranes contained many cells as determined by the number of Hoechst-labelled nuclei, far more than were identified by the antibodies used in this study (Fig. 4, 5). The number of nuclei ranged from approximately 1800 to 5700 nuclei/mm². In general, the PVR membranes had the greatest number of dividing glia, immune and RPE cells/mm² (Fig. 4, 5). Fewer but consistent numbers of dividing cells were found in the other 3 groups. The percentage of dividing cells from was 0.55% for PDR, 2.9% for PVR, 0.35% for ERMpRD, and 0.65% for iERM. Of particular interest was the observation that very few immune cells, and no RPE cells, were dividing in the iERM group although active glial cell proliferation was observed. Active glial cell proliferation was also observed in all groups except the ERMpRD group, although glial cells were present as evidenced by the anti-GFAP labelling. Immune cells were present in all 4 groups, some of which were undergoing DNA synthesis, indicating an inflammatory component to ERM formation.

The duration that the membranes were present in the eyes before removal revealed that the PVR membranes were of the shortest duration but had the highest proliferation rates and greatest number of nuclei, with an average duration of less than one month (0.25-6 months range; Table 1, Fig. 1). The ERMpRD had an average duration of 3 months (range 1-5 months) and the iERMs had the longest duration with an average of 11 months from
the start of clinical symptoms (range 4-25 months). In the iERM group there was a certain patient delay, as often patients had noticed a problem with one eye, but did not present to the ophthalmologist until the symptoms disturbed binocular vision. It was more difficult to assess the duration of the diabetic proliferative membranes. In this case, we used either a decrease in vision indicating active neovascularisation with traction or bleeding, or the first clinically noted fibrovascular proliferation noted by a clinician. This gave an average duration of 6.5 months (range 2-14 months).

**Discussion**

Antibodies to MIB-1 and SP6 are routinely used to show DNA synthesis and presumably cell division [18-20]. However, these antibodies have only been used in sectioned ERMs [11,15] and have not previously been used in whole mounts of epiretinal membranes to show proliferation rates in different ERMs compared to the total cellularity or to identify the different cell types. Using these antibodies, we show that there is active cell division in all 4 types of epiretinal membranes studied here regardless of estimated duration. The data do, however, indicate that the amount of cell division varies. We observed that the more rapidly growing membranes, as in PVR, had a higher total number of cells and a significantly higher number of anti-MIB-1 stained cells, as has been shown previously in sectioned membranes [11,12,15,21,22]. This indicates that proliferation is most likely a major contributor in the expansion of these membranes. The older membranes had a much lower level of Ki-67 labelling indicating that these may be less “reactive”. This is in contrast to the study by Zhang et al. where no difference in proliferation rates between membrane types was observed [12]. Two reasons for this apparent discrepancy could be that only 2 iERMs were used in the Zhang study, and that they used sections of membranes rather than entire membranes processed as a flat-mount, as used in this study. This clearly demonstrates the need for using large sampling procedures in a study such as this where relatively small numbers of cells may be involved.

We also show that the membranes contain thousands of nuclei, indicating they are much more cellular than previously thought [8, 11, 15]. Indeed this appears to be the first study to attempt an estimate of the total number of cells comprising these membranes using Hoechst staining and counting cells per mm², rather than estimating cell counts from sections. We have not found any previous note of Hoechst staining of epiretinal membranes or of the high number of nuclei stained. We find a high cellularity of between approximately 3000 to 6000 cells per mm² (Figs. 4, 5). Our immunostaining data indicate that the greatest
accumulation of proliferating RPE cells occurred in the PVR and ERMpRD membranes while very few occurred in the PDR and iERM membranes. It is reasonable to hypothesize that these results represent the fact that retinal tears are present in the PVR and ERMpRD retinas, allowing migration of the RPE through these openings [6,14]. Interestingly, in eyes with PDR membranes and no history of retinal tears, either before or during surgery, some dividing RPE cells were observed. This suggests that perhaps the RPE cells had migrated through the intact retina as suggested by previous studies [23,24] although we cannot prove that this was the case in our study. Another finding that came from staining with this combination of antibodies is that, while glia were found in all membrane types, no dividing glia were observed in ERMpRD group. This suggests that the proliferative phase may have ended early after the detachment occurred and what we are observing here is the growth of processes arising from Müller cells within the retina spreading over the retinal surface of the membrane. Indeed, following experimental retinal detachment in animal models, most of the proliferation that is observed occurs in Müller cells within the inner nuclear layer during the first 3-4 days after detachment [25,26]. As detachment (or reattachment) time increases these cells grow and expand on either surface of the retina [27]. This may also suggest that in later stages of ERM formation, contraction of these cells, rather than proliferation, may be the cause of the wrinkling and subsequent re-detachment of the retina. There also appears to be a significant cellular inflammatory component in all epiretinal membranes, as shown by the ricin labelling, which may play a role in the development and continued expansion of the membrane. The presence of macrophages, lymphocytes and monocytes in ERMs has been noted and assessed previously [28-32]. They observed the different types of immune cells and immune factors and in which ERMs these occurred [28,29]. However, they did not quantify the number of immune cells.

Whether this immune activation is in response to the initial (micro) trauma of the disease or posterior vitreous detachment [33] or a self perpetuating reaction, possibly caused by a breakdown of the blood retina barrier, it is not clear. However, the immune cells we observed were not only found in all membranes, but are also actively proliferating in all membrane types. Finally, while the number of total nuclei far outnumbered the total number of dividing cells, most of the cells appeared to be labeled with one of the antibodies used here. The unidentified cells in our study could be either 1) RPE cells in the first group 2) immune cells in the second group, or 3) cells not identified by our markers. This suggests that other cell types such as fibroblasts, and different types of immune cells not labeled by our antibodies, may make up a minor component of the membranes, although further examination is required to confirm this.
As has been postulated previously, there seems to be a parallel between wound healing and epiretinal membrane formation, where there is an early proliferative phase with a higher cell division rate in more reactive membranes and a slower cell division rate in the older, less reactive membranes, after which there might be a later contractile phase with the deposition and contraction of extracellular matrix [3,4,5]. All membranes of all types we stained showed active proliferation of the 3 cells types most commonly found in epiretinal membranes, glia, RPE and immune cells. We did however, have a group of cells we could not identify, either because they did not stain with the antibodies we used, or due to the restriction we had by using four markers and not being able to count all cell types at the same time. However, it was also observed that epiretinal membranes, even in the active phase of PVR, showed less cell proliferation than might be expected. This suggests that perhaps much of the cellular proliferation is an early event in the membrane formation and indeed may even occur within the retina prior to actual membrane formation, and that deposition of extra cellular matrix and contraction of the membrane, which causes the clinical symptoms, is a secondary event. This would imply that, for possible clinical treatments aimed against membrane formation, the treatment would need to be given as early as possible. Once the membrane has formed, anti-contractile agents may then be more effective than anti-proliferative drugs.

In conclusion, by using whole mounts of ERMs from different disease conditions combined with nuclear staining, we have shown that the cellularity of ERMs is higher than previously shown using sectioned ERMs. We have identified the different cell types proliferating and find that the proportions of cell types proliferating differ between the different diseases. We also show that the proliferation rate is higher in the more reactive membranes of shorter duration, but that the proliferating cells are still only a small proportion of the total cell number. This implies that the proliferative phase of the ERM may occur before the clinical aspect of the ERM formation, which has clinical and therapeutic implications.
References