Cellular Migration Associated With Macular Hole

A New Method for Comprehensive Bird’s-Eye Analysis of the Internal Limiting Membrane

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Objective: To elucidate the pathogenesis of macular hole formation, focusing in particular on the possible role of cellular migration on the cortical vitreous and internal limiting membrane (ILM) around the macular hole.

Methods: To gain a comprehensive overview of the ILM excised in macular hole surgery (n=36), the ILMs were carefully unfolded and spread out onto glass slides as continuous flat sheets that each contained a macular hole. The specimens were observed by light microscopy and transmission electron microscopy (n=9), and the cellular distribution was analyzed by scanning electron microscopy in a quantitative manner (n=27). Immunohistochemistry for glial fibrillary acidic protein and cytokeratin 18 was carried out for cellular characterization. Cellular proliferation was assessed by immunohistochemistry for proliferating cell nuclear antigen and Ki-67.

Results: Cellular migration was not apparent around the macular hole in the early stage of development of the macular hole (stage 2, 0 µm). As the macular hole passed through the later stages of development, cellular migration developed around the macular hole (stage 3, 84 µm) and the area of cellular migration gradually enlarged (stage 4, 420 µm). The immunophenotypic analysis showed that these cells were mainly glial fibrillary acidic protein–positive glial cells and cytokeratin 18–positive retinal pigment epithelial cells. The proliferating cell nuclear antigen and Ki-67 immunohistochemistry showed that some of these cells were proliferating on the ILM.

Conclusions: Cellular migration on the ILM is not necessary for the initial formation of a macular break. Cellular migration developed after the macular break occurred, and the migration and proliferation increased gradually from the macular hole.

Clinical Relevance: This study provides a new method for understanding the ultrastructural analysis of the pathogenesis of the macular hole.

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bers of various diameters. Other histological studies examining postmortem eyes with macular holes noted a high incidence of epiretinal formation in the macular area of the eyes. However, their detailed pathogenesis, especially the role and involvement of cellular migration on the ILM, still remains unclear.

We propose a new method for a topographic bird’s-eye analysis of the whole excised ILM. In this study, we describe detailed structures of the ILM around the idiopathic macular hole, the complex association of migrating and proliferating cells, and the extracellular matrix conferred by the developing stages of the macular hole. We examine the characterization and the proliferation of the distributing cells around the macular hole on the ILM.

**METHODS**

**ILM PEELING PROCEDURE**

Thirty-nine eyes of 38 patients who were diagnosed as having various stages of the idiopathic macular hole were prospectively studied clinically from January 2002 to July 2004. Biomicroscopical analysis of both the macular and vitreomacular relationships was carried out to identify the macular hole, and each was then graded according to the classification developed by Gass. A high-resolution optical coherence tomographic examination was used to confirm the state of PVD in each case. The stages of development of the macular holes were confirmed as stage 2 in 8 eyes, stage 3 in 13 eyes, and stage 4 in 15 eyes (total, 36 eyes). All of the data accumulation was carried out with approval from the ethics committee of Kyushu University, Fukuoka, Japan, and was performed in accordance with ethical standards in the 1989 Declaration of Helsinki. After informed consent was obtained from each patient, the patients underwent a standard 3-port pars plana vitrectomy. Balanced salt solution (BSS Plus; Alcon Laboratories, Fort Worth, Tex) was used as an irrigation solution. Triamcinolone acetonide (Kenakolt-A; Bristol Pharmaceuticals KK, Tokyo, Japan), a water-insoluble white corticosteroid, was used for visualizing the vitreous hyaloid as previously described. If necessary, posterior hyaloid detachment was induced by suction or forceps around the optic nerve head. The vitreous was removed and PVD was extended to the periphery. The ILM was then peeled off with ILM forceps intended to be 3 disc diameters surrounding the macular hole, and fluid-gas exchange was performed through an extrusion cannula over the optic nerve head and macular hole. Twenty-percent sulfurhexafluoride gas was then injected after closure of the scleral incisions. Postoperatively, patients were asked to keep a face-down position for at least 5 days.

**Table. Summary of the 27 Excised Internal Limiting Membranes Analyzed by Scanning Electron Microscopy From Various Stages of Development of the Macular Hole**

<table>
<thead>
<tr>
<th>Specimen No.</th>
<th>Macular Hole Stage</th>
<th>Fixation</th>
<th>Immunohistochemistry†</th>
<th>Hematoxylin-Eosin Staining†</th>
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Abbreviations: CK18, cytokeratin 18; GA, glutaraldehyde; GFAP, glial fibrillary acidic protein; NA, not applicable; PCNA, proliferating cell nuclear antigen; PFA, paraformaldehyde.

*Twenty-seven specimens were confirmed as stage 2 in 5 eyes, stage 3 in 10 eyes, and stage 4 in 12 eyes. Successful expansion of the folded internal limiting membrane was obtained in 23 specimens, and immunohistochemistry and hematoxylin-eosin staining were carried out in 8 and 2 specimens, respectively.

†Ellipses indicate that the specimens were not analyzed by the method.

‡Specimen was analyzed by hematoxylin-eosin staining.
TRANSMISSION ELECTRON MICROSCOPY
OF THE EXCISED ILM

To carry out transmission electron microscopy, the 12 excised specimens (stage 2 in 4 eyes, stage 3 in 4 eyes, and stage 4 in 4 eyes) were immediately placed in 4% glutaraldehyde for fixation. Of the 12 specimens, 9 of them (stage 2 in 3 eyes, stage 3 in 3 eyes, and stage 4 in 3 eyes) were then postfixed in 2% veronal acetate buffer osmium tetroxide, dehydrated in ethanol and water, and embedded in Epon.\(^3\) Ultrathin sections were cut from blocks and mounted on copper grids. The specimens were observed with a JEM 100CX electron microscope (JEOL, Tokyo). Three specimens were further examined by flat-preparation transmission electron microscopy.

FLAT-PREPARATION TRANSMISSION ELECTRON MICROSCOPY

Three fixed specimens (stage 2 in 1 eye, stage 3 in 1 eye, and stage 4 in 1 eye) were dehydrated in ethanol and water, extracted as flat sheets with fine needles under a biomicroscope equipped with dark-field illumination (Nikon, Tokyo), and placed onto a glass slide. Then, the expanded ILMs were embedded in Epon. Ultrathin sections were cut from blocks and mounted on copper grids. The specimens were observed with a JEM 100CX electron microscope.

IMMUNOHISTOCHEMISTRY OF THE EXPANDED ILM

The ILMs were fixed in 4% paraformaldehyde in phosphate-buffered saline, extracted as whole sheets, and placed onto a glass slide (n=8). The specimens were air dried. The first antibodies against glial fibrillary acidic protein (Dako, Tokyo) (n=4), cytokeratin 18 (Chemicon, Temecula, Calif) (n=4), proliferating cell nuclear antigen (PCNA) (Chemicon) (n=4), and Ki-67 (Dako) (n=2) were used for 2 hours at room temperature. The second antibodies labeled with Cy5 (Zymed Laboratories, San Francisco, Calif) and rhodamine (Cappel, Aurora, Ohio) were used for 1 hour at room temperature. The specimens were also stained with 4,6-diamino-2-phenylindole dihydrochloride for nuclear staining and observed with a fluorescence microscope (Table). The immunohistochemical control experiments included a negative control and an isotype control using the specific IgG subtype. All of the specimens for immunohistochemistry were dehydrated in ethanol and water and then analyzed by scanning electron microscopy.

RESULTS

VERTICAL ANALYSIS OF PEELED ILM

Light microscopical examination of semithin sections showed characteristic sinusoidal folds of complexly folded ILM (Figure 1A). Transmission electron microscopy
showed collagen membranous tissue with a smooth inner (vitreous) surface and an irregular outer (retinal) surface (Figure 1B). In the early stage of the macular hole (stage 2), cellular migration was rarely seen (Figure 1B). Some cells, namely, glial cells, retinal pigment epithelial cells, and origin-unknown fibroblast-like cells, were seen on the inner surface of the ILM in the middle stages (stage 3) (Figure 1C) of the macular hole and were seen often in the later stages (stage 4) (Figure 1D). Some cellular membranes and organelles derived from underlying Muller cells were occasionally seen on the outer surface of the ILM; however, there were no changes according to the stage of development of the macular holes (Figure 1B-D). We could not identify the location of the macular hole in complexly folded ILM by ultrathin cross sections.

HORIZONTAL ANALYSIS OF EXPANDED ILM

Light microscopical examination of the excised whole ILM (Figure 2A) spread out onto a glass slide showed the intact ILM sheet containing an ILM defect corresponding to the area of the macular hole (asterisk) (Figure 1B). The complexly folded excised ILM (A) extended onto a glass slide showed the intact ILM sheet containing an ILM defect corresponding to the area of the macular hole (asterisk) (Figure 1B). A dark-field illumination image showed that the ILM has the characteristic multilinear pattern associated with the underlying nerve fibers of the ganglion cells (original magnification ×10). C. The hematoxylin-eosin–stained section showed migrating cells around the macular hole (asterisk) (original magnification ×200). E. Some of the cells on the ILM were pigmented cells (arrows) (original magnification ×400).

Immunohistochemical analysis revealed that most of the dispersed cells on the ILM were glial fibrillary acidic protein–positive glial cells (Figure 3A). There were also cytokeratin 18–positive retinal pigment epithelial cells among the glial cells (Figure 3A). These cells formed a continuous cellular sheet around the macular hole on the ILM. To investigate whether these cells were migrating and/or proliferating on the ILM, we examined their proliferation by 2 different proliferating cell markers, PCNA and Ki-67. Proliferating cell nuclear antigen is a 36-kd proliferation-associated antigen, and Ki-67 is a large nuclear antigen preferentially expressed during all of the active phases of the cell cycle but absent in resting cells. Nuclear staining by 4′,6-diamino-2-phenylindole dihydrochloride showed chromosomes of dividing nuclei (arrows) (original magnification ×1000). Immunohistochemistry of proliferating cell nuclear antigen (C) and Ki-67 (D) showed proliferating cells (arrowheads) among the distributed cells on the internal limiting membrane (original magnification ×400).

IMMUNOHISTOCHEMICAL ANALYSIS

Light microscopical examination of the excised whole ILM spread out onto a glass slide showed the intact ILM sheet containing a macular hole (Figure 2B). Dark-field illumination microscopy showed that the ILM had the characteristic multilinear pattern associated with the underlying nerve fibers of the ganglion cells (Figure 2C). The hematoxylin-eosin–stained section showed migrating cells around the macular hole (stage 4) (Figure 2D). A few pigmented cells were seen among the distributing cells around the macular hole on the ILM (Figure 2E).

VERTICAL ANALYSIS OF EXPANDED ILM

Light microscopical examination of semithin sections of the expanded ILM showed a linear shape of the ILM.
On the expanded ILM, few Azur II–stained cells were shown around the ILM defect corresponding to the area of the macular hole at stage 4, providing better spatial understanding of the ILM. Transmission electron microscopy demonstrated the expanded linear ILM and migrating cells (Figure 4B) as well as dense collagen fibers, namely, residual posterior hyaloid between the ILM and migrating cells (Figure 4C).

**SCANNING ELECTRON MICROSCOPY**

Scanning electron microscopy revealed a smooth inner surface (Figure 5A) and rough outer surface (Figure 5B) of the ILM. This dense collagen layer is a basement membrane of Muller cells, namely, the ILM, and demonstrates the characteristic smooth surface. Neither fibrous collagen nor fibrillar vitreous collagen are smooth surfaced by scanning electron microscopy. In the early stages of development of the macular hole, migrating cells were not apparent on the inner surface of the ILM around the macular hole (Figure 5A). Notably, in the later stages, migrating cells were clearly visible around the ILM defect corresponding to the area of the macular hole (Figure 5C), indicating that these cells were migrating away from the macular hole. Cellular migration occurred on the ILM, showing flat and sticking morphological features and spreading filopodia on the ILM (Figure 5D). A topographic image of the expanded ILM showed no cellular migration around the ILM defect corresponding to the area of the stage 2 macular hole (asterisk) (original magnification ×75).

**CELLULAR MIGRATION FROM THE MACULAR HOLE**

The distance of cellular migration from the ILM defect corresponding to the area of the macular hole gradually increased through each stage of development of the macular hole (Figure 6). The cellular migration occurred from the edge of the macular hole and developed to the peripheral area of the ILM (Figure 6A–C). In most cases, cellular migration was observed as a continuous sheet of cells around the macular hole (Figure 6B and C).
This study demonstrates that cellular migration around the macular hole develops after the macular break occurs and that cellular migration occurs from the macular hole and enlarges on the ILM. To our knowledge, this is the first article clearly showing that cellular migration on the ILM is not necessary for the initial formation of a macular break.

COMPREHENSIVE TOPOGRAPHIC ANALYSIS OF EXCISED ILM

Some previous histological studies demonstrated cellular migration on the ILM around the macular hole; however, this was based on findings from the ultrathin partial cross sections of the ILM, leaving the origin and distribution of the cells largely unknown, as shown in Figure 1. We propose a topographic analysis of the whole excised ILM that enables understanding of cellular migration, especially its origin, frequency, distribution, and relationship with the macular hole (Figure 6). Whereas a cross section gives a snapshot of a limited area of excised complexly folded ILM at a particular time point (Figure 1), horizontal observation enables a comprehensive analysis of spatial distribution that offers a temporal perspective of cellular migration around a macular hole in its process of development (Figure 6).

DISTRIBUTED CELLS AROUND THE MACULAR HOLE

The ultrastructural studies of migrating cells and epiretinal membranes have found them to be glial cells, retinal pigment epithelium, myofibroblasts, and so on. Our light microscopical studies demonstrate that some of the migrating cells were pigmented cells (Figure 2E), and immunohistochemical studies also demonstrate a mosaic-like migration pattern of glial fibrillary acidic protein–positive glial cells and cytokeratin 18–positive retinal pigment epithelial cells (Figure 3A). These cells were intermingled to form an epiretinal membrane as a continuous cellular sheet on the ILM (Figure 2E, Figure 3A, Figure 4B, and Figure 5C). To further estimate the cellular proliferation on the ILM, we examined their proliferation by 2 different proliferating cell markers, PCNA and Ki-67. Although the proliferating cells dispersed around the distributed cells around the macular hole, an obvious proliferating front was not observed in the specimens.

ROLE OF CELLULAR MIGRATION IN THE PATHOGENESIS OF THE MACULAR HOLE

Figure 6A shows a complete lack of cellular migration in the early stage (stage 2) of development of the macular hole. In contrast, Figure 6B shows cellular development around the edge of the macular hole, and Figure 6C shows a large amount of cellular migration from the macular hole to the peripheral area. Notably, in the later stage of development of the macular hole, some specimens showed no cellular migration around the macular hole (Table). Our results confirm that the initial break of the macular hole is not dependent on cellular migration around the macular hole (Figure 6D). In contrast, cellular migration developed after the macular break and expanded from the edge of the macular hole to the periphery (Figure 4A and Figure 6A-C), finally forming an...
epiretinal membrane on the ILM. This cellular migration and its contraction of the extracellular matrix on the ILM (Figure 1B-D and Figure 4) might lead to further progression of the macular hole (stage 3) and might keep the macular hole open even after PVD (stage 4).

The role of cellular migration in the pathogenesis of the macular hole remains quite unclear. We provide a comprehensive bird’s-eye analysis of the ultrastructure of the ILM and demonstrate cellular migration and proliferation in a quantitative manner, proposing the association of cellular migration to the pathogenesis of a macular hole.

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