Confocal Scanning Laser Microscopic Findings of Excised Choroidal Neovascular Membranes of Age-Related Macular Degeneration and Their Comparison With the Clinical Features

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Purpose: To evaluate the histopathological characteristics of choroidal neovascular membranes excised from eyes of patients with age-related macular degeneration (AMD) and to correlate their characteristics with the clinical features of AMD.

Methods: Choroidal neovascular tissues were excised from 3 patients with AMD and examined by light and confocal scanning laser microscopy. The clinical features were obtained by fundus photography, fluorescein angiography (FA), and indocyanine green angiography (IA) and compared with the histopathological findings.

Results: Light microscopy showed the presence around the vascular structures of cells containing pigment. Confocal scanning laser microscopy revealed lipofuscin signals of the retinal pigment epithelium cells around the vascular tissue that was also confirmed by three-dimensional reconstructed views from serial optical sections. Clinical observations of the fundus by IA showed that all 3 cases had areas with hyperfluorescence in early phase on IA. A dark rim was observed around the area of hyperfluorescence in 2 cases, and the dark rim was located within the neovascular membrane. The patterns of fluorescence were heterogeneous in some phases on IA, which reflected the histological heterogeneity of the neovascular membrane.


Key Words: Age-related macular degeneration, choroidal neovascular membrane, confocal scanning laser microscopy, lipofuscin, retinal pigment epithelium.

Introduction

Age-related macular degeneration (AMD) is an important cause of adult visual impairment.1–3 Indocyanine green videoangiography (IA) improves the detection of choroidal neovascular membranes in patients with AMD when it is done in combination with fluorescein angiography (FA).4–10 However, the IA features of the choroidal neovascular membrane vary from case to case and further investigations are needed.

Choroidal neovascular membranes are induced and regress, interacting with the retinal pigment epithelium.11,12 The retinal pigment epithelial cells contain lipofuscin granules, which are the main source of autofluorescence in the ocular fundus.13–15 In the present report, autofluorescence of lipofuscin granules was used as a marker of the retinal pigment epithelium cells that might affect the development of choroidal neovascular membrane. Choroidal neovascular membranes associated with AMD were excised surgically and were examined by light and confocal scanning laser microscopy. By this method serial optical sections were obtained and three-dimensional structures could be reconstructed. Clinopathological features including the IA findings are discussed.
Materials and Methods

Three eyes of 3 AMD patients with submacular choroidal neovascularization were studied. Fundus examination by slit-lamp biomicroscopy, color fundus photography, FA, and IA were performed preoperatively. Five hundred milligrams of sodium fluorescein was injected for FA and 25 mg of indocyanine green, for IA. Fluorescent signals were recorded by a fundus camera (TRC-50IA; Topcon, Tokyo). Table 1 shows age, sex, laterality, preoperative visual acuity, duration of neovascularization, status of the lens,† surgical procedures, postoperative follow-up period, and postoperative visual acuity at the last visit. Preoperatively, signed informed consent forms were obtained from all subjects.

Removal of choroidal neovascular membranes was performed as reported previously.‡–§ Pars plana vitrectomy with a three-port system was performed using balanced salt solution (BSS Plus; Santen, Osaka) as irrigation fluid. After core vitrectomy, posterior vitreous detachment was carried out, followed by a small retinotomy with a microvitreoretinal blade. Irrigation fluid was then injected slowly with a 27-gauge cannula to separate the neovascular membrane and the retinal pigment epithelium from the neural retina. The neovascular membrane was grasped with vitreous forceps inserted through the retinotomy and the membrane was removed intact. Laser photocoagulation was performed around the retinotomy and fluid-air exchange was done. In patients with cataract, phacoemulsification through a self-sealing sclerocorneal frown and BENT incision were performed, and an intraocular lens was implanted before vitrectomy.

The excised specimens were fixed in 0.01 mol/L of phosphate buffered saline containing 2.5% glutaraldehyde and 4% paraformaldehyde for 24 hours, and mounted in Tissue-Tek OCT Compound (Sakura, Tokyo). Cryosections were made at a thickness of 10–20 μm. Sections were dehydrated and rehydrated, stained with hematoxylin-eosin and cleared. These samples were examined with a light microscope. A confocal scanning laser microscope (BX50; Olympus, Tokyo) with peak excitation at 568 nm and 488 nm was used to obtain serial optical confocal sections. Three-dimensional reconstruction of the specimens was performed with a computer-aided program (FVX1000; Olympus). Light field observations were performed with transmitted light using the same microscope to identify vascular structures.

Results

The preoperative and intraoperative findings of case 1 are shown in Figure 1. A connective tissue formation, with hemorrhage, approximately one disk diameter (DD), was seen at the posterior pole; it included the macula. This formation was surrounded by subretinal hemorrhage spreading over about 3 DD (Figure 1A). At 40 seconds after fluorescein injection (Figure 1B), areas with faint hyperfluorescence were seen around the area of the subretinal connective tissue in a field of 3 DD with hypofluorescence resulting from blockage. At 256 seconds (Figure 1C), massive leakage of fluorescent dye was recorded in the subretinal connective tissue.

At 46 seconds on IA (Figure 1D), a neovascular membrane approximately 1 DD with a dark rim and faint hyperfluorescence was detected. The site of a photocoagulation performed previously was seen inferior to the neovascular membrane as a region of hypofluorescence (Figure 1D). Staining of the choroidal neovascular membrane with fluorescent dye was remarkable at 1054 seconds on IA (Figure 1E).

Light microscopic findings of the excised neovascular membrane of case 1 are shown in Figure 2. At low magnification (Figure 2A), cells containing pigment were seen especially in the peripheral regions of the membrane. At high magnification (Figure 2B), cells containing pigment were observed around luminal structures that contained erythrocytes in

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<th>Duration of Neovascular Membrane (Months)</th>
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*Grade: Emery’s grading.** IOL: Pseudophakia.
†PEA + IOL + VIT: phacoemulsification and aspiration, intraocular lens implantation, and vitrectomy. VIT: Vitrectomy only.
some sections. Confocal scanning laser micrographs of the excised neovascular membrane of case 1 are shown in Figure 3. Punctate signals of fluorescence were detected around vascular structures (Figure 3A). A stereoscopic view of the three-dimensional reconstruction (Figure 3B) showed that the punctate signals surrounded the vascular lumina.

The postoperative findings of case 1 are shown in Figure 4. At 58 seconds on IA (Figure 4A), the neovascular membrane with a dark rim, which was observed preoperatively, was not detected. The site of the photoocoagulation was seen as a hypofluorescent region. At 1215 seconds on IA (Figure 4B), the staining detected preoperatively was no longer observed. A color photograph of the fundus (Figure 4C) showed scarring at the preoperative photoocoagulation surrounded by hemorrhage and exudates superior to the fovea. The subretinal connective tissue was not observed. The preoperative visual acuity of hand motion improved to 0.01 at 23 months after surgery (Table 1).
Preoperative and intraoperative findings of case 2 are shown in Figure 5. Connective tissue formation, with hemorrhage, approximately 0.5 DD × 1 DD in size was seen at the posterior pole; it included the macula. This formation was surrounded by an area of 2 DD with exudates (Figure 5A). At 54 seconds on FA (Figure 5B), there was hyperfluorescence in the area with exudates due to dye leakage. At 185 seconds on FA (Figure 5C), the leakage of fluorescent dye became remarkable. Areas of hypofluorescence were noted, which were caused by a blockage of the dye (Figures 5B, 5C). At 21 seconds on IA (Figure 5D), an area of faint hyperfluorescence with a dark rim was observed, suggestive of a choroidal neovascular membrane. This area corresponded to the subretinal connective tissue in Figure 5A. At 378 seconds on IA (Figure 5E), the intensity of the fluorescence had not changed significantly when compared with that at 21 seconds (Figure 5D). Intraoperative recording showed that the removed subretinal tissue was heavily pigmented on its peripheral surface and that pigmentation had spread into the area of the dark rim (Figure 5F).

Light micrographs of the excised neovascular membrane of case 2 are shown in Figure 6. At low magnification (Figure 6A), cells containing pigment were seen especially in the peripheral regions of the membrane. At high magnification (Figure 6B), cells containing pigment were also observed around the vascular structures.

Confocal scanning laser micrographs of the excised neovascular membrane of case 2 are shown in Figure 7. Punctate signals of fluorescence were detected around the vascular tissue (Figure 7A). A stereoscopic picture (Figure 7B) shows that the punctate signals surrounded the vessels.

A postoperative photograph of the fundus of case 2 is shown in Figure 8. Defects of the retinal pigment epithelium and subretinal hemorrhages were found at the retinotomy and at a site where the neovascular membrane had been located. The subretinal connective tissue was not present. The preoperative visual acuity of 0.03 improved to 0.1 at 25 months after surgery (Table 1).

Preoperative and intraoperative findings of case 3 are shown in Figure 9. The color fundus photograph (Figure 9A) shows a white-yellow lesion with massive exudates at the posterior pole. At 23 seconds on FA (Figure 9B), areas of hyperfluorescence were not detected. At 405 seconds on FA (Figure 9C), massive leakage of fluorescent dye was recorded at the posterior pole. At 58 seconds on IA (Figure 9D), two areas with dense hyperfluorescence were observed; one, about 1 DD, was located superior to the macula and the other, about 1/3 DD, was located inferiorly. An area with faint hyperfluorescence of approximately 3 DD was visible over the inferior arcade vessels, including the areas with the dense hyperfluorescence just described. At 656 seconds on IA (Figure 9E), the areas with dense hyperfluorescence were obscured but the area of about 3 DD with faint hyperfluorescence remained recognizable. Intraoperative recording revealed that a piece of subretinal tissue of about 3 DD had been removed from the area with faint hyperfluorescence observed in Figures 9D and 9E (Figure 9F).

Light micrographs of the excised neovascular membrane of case 3 are shown in Figure 10. At low magnification (Figure 10A), cells containing pigment were seen especially in the peripheral region of the membrane. At high magnification (Figure 10B), cells containing pigment were observed around the lumenal structures.
Confocal scanning laser micrographs of the excised neovascular membrane of case 3 are shown in Figure 11. Punctate signals of fluorescence were detected around the luminal structures (Figure 11A). A stereoscopic photograph (Figure 11B) showed that the punctate signals surrounded columnar vascular tissues. Scarring at preoperative photocoagulation with hemorrhage around it and exudates superior to fovea are seen. Subretinal connective tissue is not present.

Discussion

Indocyanine green has longer wavelengths of excitation and emission than sodium fluorescein. Therefore, it is used for imaging choroidal neovascular membranes. In this study, indocyanine green angiography revealed the location of subretinal connective tissue, which was not visible with fluorescein angiography. The preoperative visual acuity of 0.01 improved to 0.08 at 25 months after surgery (Table 1).
pre, the IA signals are less absorbed by the melanin and xanthophyll in the ocular fundus. This property enables IA to reveal occult choroidal neovascularization. Using IA in combination with FA improves the assessment of exudative AMD. However, the features of the choroidal neovascular membranes associated with AMD as visualized with IA vary from case to case, and more clinicopathological information concerning choroidal neovascularization and IA is needed.

Ishikawa and coworkers reported that the IA patterns of choroidal neovascular membranes were divided into four types. Nakajima et al divided the patterns of choroidal neovascular membranes into four types and reported their histological features. Ishikawa et al investigated the histological relationship between IA and choroidal neovascular membranes and reported that the retinal pigment epithelium cells surrounded a neovascular membrane in a different manner at different loci in the membrane.

In the cases presented here, IA demonstrated areas of hyper-, iso-, or hypofluorescence at different phases (Figures 1D, 1E, 5D, 5E, 9D, 9E). This indicates that the IA features of choroidal neovascular membranes are heterogeneous, which is correlated with the histological heterogeneity of the choroidal neovascular membranes of AMD.

In two cases in the present study, a dark rim was found around the choroidal neovascular membrane in IA. Light microscopy of the excised tissues revealed that many cells containing pigment were located at the peripheral regions of the membrane. Comparison of the light micrographs and the intra-

**Figure 6.** Light micrographs of excised neovascular membrane of case 2. (A) Low magnification. Retinal side is shown superiorly. Cells containing pigment are seen especially in the peripheral regions of membrane. (B) High magnification. Cells containing pigment are observed around luminal structures with erythrocytes in them (arrows). Bars = 100 μm.

**Figure 7.** Confocal scanning laser micrographs of excised neovascular membrane of case 2. (A) Punctate signals of fluorescence (arrows) are detected around vascular tissue (asterisks). (B) Stereo pair of three-dimensional reconstruction. Punctate signals (arrows) surround vascular structures (asterisks). Bars = 100 μm.
Figure 8. Color photograph of postoperative fundus of case 2. Defects of retinal pigment epithelium and subretinal hemorrhage are seen at retinotomy and at site where neovascular membrane was located. Subretinal connective tissue is not observed.

operative recordings of the neovascular membranes revealed that the dark rims corresponded with the heavily pigmented areas of the choroidal neovascular membranes (Figure 13). Cases that show this dark rim in IA have been reported. The retinal pigment epithelium cells in conjunction with the choroidal neovascular membranes may form the thick pigmented layer (Figure 13, middle). This pigmented region can be removed by surgical excision of the neovascular membrane (Figure 13, bottom). It is possible that the dark rim around the choroidal neovascularization observed by IA (Figure 13, top) is formed by the blocking of the background fluorescence of the choroid by these pigmented layers. Thus, in areas where the neovascular membrane penetrates the retinal pigment epithelium, the thick pigmented layers block fluorescence from the choroid, resulting in the dark rim.

If this is the case, the surgical removal of the choroidal neovascular membrane may create defects in the retinal pigment epithelium in the region of the dark rim. When the macula is involved in the dark rim, the distance between the macula and the outer edge of the dark rim will affect postoperative visual acuity. In two of the cases presented, the entire macula was involved in the area of the dark rim in Case 1 (Figures 1D, 1E), and the macula was at the edge of the dark rim in case 2 (Figures 5D, 5E). The postoperative visual acuity was better in case 2 than in case 1 (Table 1).

Figure 9. Preoperative and intraoperative findings in case 3. (A) Color photograph of fundus. White-yellow lesion with massive exudates is seen in posterior pole. (B) Fluorescein angiography (FA) at 23 seconds following dye injection. There is no area with hyperfluorescence. (C) FA at 405 seconds. Massive leakage of fluorescent dye is recorded at posterior pole. (D) Indocyanine green angiography (IA) at 58 seconds. Two areas with dense hyperfluorescence are observed; one is about 1 DD, and the other is about 1/3 DD. Area of faint hyperfluorescence of approximately 3 DD is visible over inferior arcade vessels including areas with dense hyperfluorescence. (E) IA at 656 seconds. Areas of dense hyperfluorescence are obscured but an area of about 3 DD with faint hyperfluorescence remains recognizable. (F) Intraoperative photo. Superior side of eye is located inferiorly in photo. Subretinal tissue of about 3 DD was removed from the area with faint hyperfluorescence observed in Figures 9D and 9E. Removed tissue was pigmented in central and peripheral regions.

Figure 13 illustrates diagrammatically the spreading of the neovascular membrane into the area of the dark rim. Intraoperative recordings showed that the neovascular membranes were large enough to involve the area of a dark rim. This potential masking
The effect of the proliferated retinal pigment epithelium should be considered when evaluating the size of a choroidal neovascular membrane of AMD by IA.\textsuperscript{25,26} In a report of an experimental animal model of laser-induced choroidal neovascularization,\textsuperscript{26} it was shown that the proliferated retinal pigment epithelium cells around neovascular vessels coincided with the dark rim. The present article confirmed this observation in clinical cases of AMD. Moreover, the possible relationship between the location of the dark rim and the visual outcome in the current report is consistent with a previous article.\textsuperscript{28} Thus, the location of the dark rim observed in IA may be useful for predicting outcome.

It has been reported that the retinal pigment epithelium is involved in the induction and the regression of the choroidal neovascular membrane. Yamagishi et al\textsuperscript{11} reported that, in an experimental

**Figure 10.** Light micrographs of excised neovascular membrane of case 3. (A) Low magnification. Retinal side is shown at top. Cells containing pigment are seen especially in peripheral region of membrane. (B) High magnification. Cells containing pigment are observed around luminal structures (arrows). Bars = 100 \(\mu\)m.

**Figure 11.** Confocal scanning laser micrographs of excised neovascular membrane of case 3. (A) Punctate signals of fluorescence (arrows) are seen around vascular structures (arrowhead). (B) Stereo pair of three-dimensional reconstruction. Punctate signals surround columnar vascular lumen (arrowhead). Bars = 100 \(\mu\)m.

**Figure 12.** Color photograph of postoperative fundus of case 3. Subretinal connective tissue has disappeared. Defects of retinal pigment epithelium are visible at retinotomy and in part of area where neovascular membrane was located. Subretinal hemorrhage is observed around defects.
model of subretinal choroidal neovascularization, damage to the retinal pigment epithelium during the development of neovascularization promoted the proliferation of neovascular tissue. This showed that the retinal pigment epithelium was responsible for regression of choroidal neovascularization. On the other hand, Yamagishi et al\textsuperscript{12} reported that when the retinal pigment epithelium was damaged before the induction of neovascularization, no evidence of neovascular formation was observed, suggesting that the retinal pigment epithelium is implicated in neovascularization. Thus, the retinal pigment epithelium cells may play a role in both the induction and the regression of choroidal neovascular membranes.

To investigate the histological relationships between the choroidal neovascular membrane of AMD and the retinal pigment epithelium, the authors focused on the fluorescent lipofuscin. Lipofuscin is a pigment that is contained in the retinal pigment epithelium cells. Histological analysis has revealed that lipofuscin is the most potent fluorescent substance in the aging retinal pigment epithelium.\textsuperscript{13–15} To detect the fluorescent signals of lipofuscin, the authors used a confocal scanning laser microscope. Confocal scanning laser microscopy makes possible the optical confocal sectioning of tissues and the reconstruction of their three-dimensional organization.\textsuperscript{29–32} The histological organization was visualized by using glutaraldehyde, which also has autofluorescence, as a fixative.\textsuperscript{33} As a result, lipofuscin granules were detected as punctate signals in the background fluorescence of the tissues (Figures 3A, 7A, and 11A). The three-dimensional reconstruction demonstrated that the lipofuscin signals surrounded the vascular tissues (Figures 3B, 7B, and 11B). There have been several reports on the autofluorescence of the fundus observed in vivo.\textsuperscript{15,34–38} However, histological examination of autofluorescence of neovascular tissue has not been described to date. The present article is the first report of the detection of autofluorescence in the surgically excised neovascular membranes of AMD. Significantly, all three cases in this study had lipofuscin granules in the choroidal neovascular membranes. This finding is consistent with the proposition that the retinal pigment epithelium cells play a role in the induction and the regression of choroidal neovascularization. The thickness of the cryosection was set at 10–20 $\mu$m in the present study; thicker preparations may improve stereoscopic visualization. Lipofuscin granules are a potent fluorescent substance in the aging retinal pigment epithelium.\textsuperscript{13–15} However, some macrophages and melanocytes may contain lipofuscin granules from phagocytosis, and thus some of the fluorescent signals may come from sources other than the retinal pigment epithelium. This possibility cannot be excluded by confocal scanning laser microscopy alone. The retinal pigment epithelium cells in a choroidal neovascular membrane, on the other hand, can form papillary or luminal structures. Immunohistochemical study of the patterns of retinal pigment epithelium cells, lipofuscin granules, and vascular structures in choroidal neovascular membranes will yield more information.

In case 1, the areas of hyperfluorescence on FA were demonstrated to extend outside the excised neovascular membrane (Figures 1B, 1C, and 1F). These areas may be the frontier of a developing neovascular membrane. It is possible that the developing neovascular membrane was excised with its

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**Figure 13.** Schematic representations of indocyanine green angiography (IA) (top panel), choroidal neovascular membrane with retinal pigment epithelium (middle panel), and excised tissue (bottom panel). Choroidal neovascular membrane and retinal pigment epithelium overlap to varying degrees and may block background choroidal fluorescence on IA, forming dark rim (top). These thick pigmented layers can become attached to surgically excised tissue (bottom). CNV: choroidal neovascular membrane, N: neural retina, P: retinal pigment epithelium, B: Bruch’s membrane, C: choroid.
most peripheral region left behind and that the remains of the membrane regressed spontaneously because their feeder vessels had been removed. Hsu et al.\textsuperscript{39} reported a thin fibrovascular membrane beneath the retinal pigment epithelium in postmortem eyes obtained after submacular membrane resection of AMD patients, and speculated whether this membrane might represent a clinically unrecognized choroidal neovascular membrane that had not been removed. Considering that photocoagulation of feeder vessels of the choroidal neovascular membrane has some effect on arresting the development of a membrane,\textsuperscript{40} partial removal of the neovascular membrane to preserve the retinal pigment epithelium may be an alternative to total excision.

In summary, the present article supports the argument that the retinal pigment epithelium cells play a special role in the induction and regression of choroidal neovascular membranes in AMD. Clinicopathological study should be carried out in conjunction with IA to achieve accurate diagnosis and more effective treatment of AMD.

References


