The number of patients with diabetes is growing rapidly worldwide; it is estimated that 415 million adults had diabetes in 2015, and that the number of patients with diabetes will increase to 622 million by 2040.1 Because of the increased number of patients with diabetes, the number of patients with diabetic retinopathy (DR) is expected to increase.2,5 DR is a major vision-threatening disease in adults and constitutes an important microvascular complication caused by diabetes.3–6 Notably, DR is characterized by extensive vascular leakage, retinal neovascularization, and fibrovascular proliferative growth.7 Various factors (e.g., hyperglycemia, hypertension, and dyslipidemia) are reportedly involved in DR, and retinal ischemia is strongly related to DR severity.7–11 Diabetic macular edema (DME) involves accumulation of fluid in the macula due to diabetic retinopathy.5,12 DME is characterized by an accumulation of fluid in the macula due to diabetic retinopathy. Currently, anti-VEGF drugs are the standard treatment worldwide for DME. This study aimed to assess whether the existence of epiretinal membrane (ERM) affects anti-VEGF efficacy, due to reduced permeability of the antibody through the ERM.

**PURPOSE.** Diabetic macular edema (DME) is characterized by an accumulation of fluid in the macula due to diabetic retinopathy. Currently, anti-VEGF drugs are the standard treatment worldwide for DME. This study aimed to assess whether the existence of epiretinal membrane (ERM) affects anti-VEGF efficacy, due to reduced permeability of the antibody through the ERM.

**METHODS.** We retrospectively examined clinical data of DME patients who underwent anti-VEGF treatment and evaluated whether clinical differences existed between DME eyes with ERM and those without ERM. We then created an in vitro ERM model using MIO-M1, ARPE-19, and NTI-4 cells on Transwell membranes and evaluated antibody permeability through this in vitro ERM model using fluorescently labeled antibodies.

**RESULTS.** Central retinal thickness (CRT) change between before and 1 month after first anti-VEGF treatment, as well as final CRT and final visual acuity 12 months after first anti-VEGF treatment, significantly differed between DME eyes with ERM and those without ERM. The in vitro ERM model led to production of collagen I in a manner similar to that of human ERM specimens. Fluorescence intensity of the lower chamber of the in vitro ERM model was significantly reduced in a dose-dependent manner.

**CONCLUSIONS.** Clinical data analysis indicated that the existence of ERM in DME eyes lowered the efficacy of anti-VEGF treatment. Reduced antibody permeability through the in vitro ERM model suggested ERM presence was associated with resistance to anti-VEGF treatment in DME eyes with ERM.

**Keywords:** diabetic macular edema, epiretinal membrane, anti-VEGF antibody, Transwell
exist between DME eyes with ERM and those without ERM. Additionally, we constructed an in vitro ERM model using cell lines representative of human ERM specimens, and evaluated antibody permeability in the context of the existence of in vitro ERM.

**METHODS**

We retrospectively studied DME patients, and divided these patients into two groups as follows: “DME with ERM” and “DME without ERM”. The diagnosis of ERM was based on clinical, funduscopy examination by a retinal specialist (HK), as well as ocular imaging analysis. Related medical records were carefully reviewed, including duration of subjective metamorphopsia, simultaneous cataract, duration of symptoms, ophthalmologic treatments, and the presence of other retinal alterations. The inclusion criteria for patients were as follows: (1) diagnosis of DME (CRT ≥ 250 μm) that required treatment with ranibizumab; (2) absence of unexpected complications during anti-VEGF treatment for 12 months; (3) successful performance of optical coherence tomography (OCT) imaging (Cirrus OCT; Carl Zeiss Meditec, Dublin, CA, USA) during the study (signal strength ≥ 7). The exclusion criteria were as follows: (1) eyes with a history of vitrectomy for other diseases or corneal surgeries; (2) eyes in patients who had any ocular or systemic disorder that could affect retinal thickness (e.g., glaucoma, optic nerve diseases, or AMD); (3) eyes with VMT; (4) eyes that received other anti-VEGF drugs (i.e., not ranibizumab). The Nagoya University Hospital Ethics Review Board approved this retrospective analysis of patients’ data and waived the requirement for informed consent. We measured average retinal thicknesses using the Early Treatment Diabetic Retinopathy Study (ETDRS) chart before and 1 and 12 months after the initiation of anti-VEGF treatment.

**Sample Collection From Patients With DME and Immunostaining**

Each ERM specimen was collected during vitrectomy surgery for ERM. The tissue was immediately fixed with 4% paraformaldehyde and cryoprotected; then, 10-μm sections were obtained, as previously described. The sections were stained with hematoxylin and eosin, or immunostained with anti-alpha smooth muscle actin (αSMA) antibody (1:200; Sigma-Aldrich, St. Louis, MO, USA), anti-glial fibrillary acidic protein (GFAP) antibody (1:400; Cell Signaling Technology, Danvers, MA, USA), and anti-collagen type I antibody (1:200; Rockland Immunoclonicals, Inc., Limerick, PA, USA); they were then visualized with Alexa Fluor 488 and Alexa 594-conjugated antibodies (1:1000; Invitrogen, Carlsbad, CA, USA), as well as 4',6-diamidino-2-phenylindole (DAPI; Invitrogen). Images were acquired with a BioImaging Navigator fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). This study was conducted in accordance with the guidelines of the Declaration of Helsinki; the protocol was registered within the UMIN Clinical Trial Registry (registered number UMIN000024553) and approved by the Nagoya University Hospital Ethics Review Board. Written informed consent was obtained from all participating patients.

**In Vitro ERM Model and Antibody Permeabilization Assay**

Based on a previous study involving cultured RPE cells, we used a modified Transwell system (#353504 & #353495; Corning, Corning, NY, USA) in this study. The number of cells that were seeded on the membrane was determined on the basis of previous studies. Cultured MIO-M1 cells were purchased from E-lucid (University College London, London, UK), cultured ARPE-19 cells were purchased from the American Type Culture Collection (Rockville, MD, USA), and NT-4 cells were purchased from the Japanese Collection of Research Bioresource Cell Bank (Ibaraki, Osaka, Japan). The cells were grown in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) and supplemented with 10% fetal bovine serum (Gibco, Waltham, MA, USA) and 1% penicillin-streptomycin (Merck KGaA, Darmstadt, Germany). MIO-M1 alone or a combination of MIO-M1, ARPE-19, and NT-4 cells (each 4.7 × 10^4, or 1.4 × 10^5 in total) were seeded onto Transwell filters precoated with extracellular matrix (ECM, BD Biosciences, San Jose, CA, USA). Cells were cultured for 7 days prior to antibody permeabilization assays. To equalize the surface levels of medium in the upper and lower chambers, 300 μL of medium was placed in the upper chamber and 900 μL of medium was placed in the lower chamber. Ranibizumab was labeled with FITC using a conjugation kit (Fluoro Tag; Sigma-Aldrich), in accordance with the manufacturer’s protocol. Naked FITC, FITC-labeled ranibizumab, or IgG F(ab’)2 tagged with Alexa Fluor 488 (Alexa 488 Fab; 1:500; #A11070; Invitrogen) were added to the upper chamber. After incubation (1, 6, 24, 48, and 72 hours), the medium was removed from the upper and lower chambers (80-μL each), and fluorescence intensity was measured in each sample (PowerScan4 plate reader; DS Pharma Biomedical, Osaka, Japan).

**Statistics**

For DME patients, we expressed data as medians. In cases where one patient received treatment for both right and left eyes, we counted each eye individually (n = 2). We compared parameters between DME patients with and without ERM using the Mann-Whitney U test. For in vitro assays, we expressed data as means ± standard deviations (n = number of samples). F test and Student’s t-test or Welch’s t-test were used to compare results. P values < 0.05 were considered to be statistically significant in all analyses.

**RESULTS**

**DME Patients’ Characteristics and Their Association With ERM**

Of more than 500 first-visit DME patients in our hospital during the period from February 2014 to August 2018, only 43 eyes of 35 patients with DME were included in this study. The majority of DME patients were excluded because their anti-VEGF treatment had been changed within the study period. The demographic and clinical characteristics of the included patients are listed in Table 1. The median patient age was 61.0 years; there were 16 DME patients with ERM and 19 DME patients without ERM. Representative images of the color fundus outlined by ETDRS sectors and ETDRS-based retinal thickness, as well as horizontal OCT images before and 12 months after the initiation of anti-VEGF treatment, are shown in Figure 1. Regional retinal thicknesses, based on central sectors from ETDRS before and 1 and 12 months after the initiation of anti-VEGF treatment, as well as visual acuity and the number of intravitreal injections of anti-VEGF drug, are listed in Table 2. Changes in CRT before and 1 month after the initiation of anti-VEGF treatment, as well as final CRT and final visual acuity 12 months after the initiation of anti-VEGF treatment, significantly differed between DME eyes with ERM and those without ERM.
Cell Types in ERM Specimen in DME Eye

We obtained ERM tissue from a 63-year-old female DME patient with ERM. Phase-contrast microscopy showed pigmented cells in the tissue, indicating that the ERM specimen included migrated RPE cells. Immunohistochemical analysis also showed GFAP- and αSMA-positive cells. These images indicated that the ERM specimen included cells that had originated from RPE, Müller, and fibroblast cells (Fig. 2).

In Vitro ERM Model

Based on our immunohistochemical analysis and previous studies,40–44 we designed an in vitro ERM model (Fig. 3). ECM placed on the Transwell membrane served as internal limiting membrane (ILM); the cell complex (MIO-M1, ARPE-19, and NTI-4 cells) served as ERM. We first measured time-dependent change in Alexa 488 Fab-based fluorescence intensity in the lower chamber through ECM alone (no ERM). The fluorescence intensity of Alexa 488 Fab in the lower chamber through ECM alone increased in a time-dependent manner until the 48-hour time point, then decreased between the 48- and 72-hour time points (Fig. 4A). In addition, the fluorescence intensities in the lower chamber did not significantly differ between ECM(C0) and ECM(C+) cultures (86.67 vs. 80.83, n = 6, P = 0.42, Fig. 4B). These data indicated that the presence of ECM did not affect Fab permeability. Seeding MIO-M1, ARPE-19, and NTI-4 cells on the Transwell membrane led to production of collagen I (Fig. 4D) in a manner similar to that of human ERM specimens (Fig. 4C). In addition, MIO-M1, ARPE-19, and NTI-4 cells on the

TABLE 1. Demographic and Clinical Characteristics of DME Patients

<table>
<thead>
<tr>
<th></th>
<th>DME</th>
<th>DME + ERM</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of eyes</td>
<td>25</td>
<td>18</td>
<td>0.482</td>
</tr>
<tr>
<td>Age (y)</td>
<td>66.0 (59.0–71.0)</td>
<td>67.5 (62.5–71.8)</td>
<td>0.766</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>17/8</td>
<td>15/5</td>
<td>0.249</td>
</tr>
<tr>
<td>Duration of diabetes (y)</td>
<td>10.0 (8.0–20.0)</td>
<td>14.0 (10.0–25.0)</td>
<td>0.576</td>
</tr>
<tr>
<td>Hba1c (%)</td>
<td>6.8 (6.3–7.8)</td>
<td>7.1 (6.6–7.4)</td>
<td></td>
</tr>
<tr>
<td>Type of diabetes</td>
<td>0/25</td>
<td>0/18</td>
<td></td>
</tr>
<tr>
<td>Stage of DR</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>No DR (%)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Mild NPDR (%)</td>
<td>4 (16)</td>
<td>2 (11)</td>
<td>0.648</td>
</tr>
<tr>
<td>Moderate NPDR (%)</td>
<td>16 (64)</td>
<td>12 (67)</td>
<td>0.856</td>
</tr>
<tr>
<td>Severe NPDR (%)</td>
<td>5 (20)</td>
<td>4 (22)</td>
<td>0.860</td>
</tr>
<tr>
<td>PDR (%)</td>
<td>7 (28)</td>
<td>3 (17)</td>
<td>0.386</td>
</tr>
<tr>
<td>Previous laser surgery</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Macular laser (%)</td>
<td>18 (72)</td>
<td>14 (78)</td>
<td>0.668</td>
</tr>
<tr>
<td>PRP (%)</td>
<td>0 (0)</td>
<td>1 (5)</td>
<td></td>
</tr>
<tr>
<td>Previous cataract surgery</td>
<td>5 (20)</td>
<td>5 (28)</td>
<td>0.552</td>
</tr>
<tr>
<td>History of hypertension</td>
<td>9 (36)</td>
<td>10 (56)</td>
<td>0.203</td>
</tr>
<tr>
<td>History of kidney disease</td>
<td>4 (16)</td>
<td>6 (34)</td>
<td>0.184</td>
</tr>
<tr>
<td>Posterior hyaloid detached at baseline (%)</td>
<td>17 (68)</td>
<td>12 (67)</td>
<td>0.927</td>
</tr>
</tbody>
</table>

NPDR, nonproliferative DR; PDR, proliferative DR; PRP, panretinal photocoagulation.
Transwell membrane formed a multilayer configuration (Fig. 4F) in a manner similar to that of human ERM specimens (Fig. 4E). The fluorescence intensities of naked FITC did not significantly differ between ECM(−) and ERM(+) in the upper chamber (91.47 vs. 94.07, n = 5, P = 0.63) or lower chamber (54.17 vs. 53.75, n = 12, P = 0.88, Fig. 4G).

Association Between ERM and Antibody Permeabilization In Vitro

We next measured Alexa 488 Fab fluorescence intensity in the lower chamber of the in vitro ERM model using MIO-M1 alone or the combination of MIO-M1, ARPE-19, and NTI-4 cells (Figs. 5A, 5B). Compared with the ECM group (no cells, 101.33), the fluorescence intensities of Alexa 488 Fab in the lower chamber of the MIO-M1 alone group (61.72, P = 4.45 × 10^{-3}) and MIO-M1/ARPE-19/NTI-4 combined group (54.00, P = 2.18 × 10^{-7}) were significantly reduced (Fig. 5A). In addition, compared with the ECM group (no cells, 119.00), the fluorescence intensity of Alexa 488 Fab in the upper chamber of the MIO-M1 alone group (189.83) was significantly higher (P = 3.08 × 10^{-3}), and that of the MIO-M1/ARPE-19/NTI-4 combined group (172.17) tended to be higher (P = 1.31 × 10^{-2}, Fig. 5A). Furthermore, compared with the ECM group (no cells, 101.33), the fluorescence intensities of Alexa 488 Fab in the lower chamber of the MIO-M1/ARPE-19/NTI-4 combined group with 4.7 × 10^{4} cells (67.50, P = 1.33 × 10^{-6}) and in the group with 14.1 × 10^{4} cells (54.00, P = 2.18 × 10^{-7}) were significantly reduced in a dose-dependent manner (Fig. 5B). In addition, compared with the ECM group (no cells, 119.00), the fluorescence intensities of Alexa 488 Fab in the upper chamber of the MIO-M1/ARPE-19/NTI-4 combined group with 4.7 × 10^{4} cells (157.67, P = 4.23 × 10^{-5}) and in the group with 14.1 × 10^{4} cells (172.17, P = 1.31 × 10^{-5}) were significantly increased in a dose-dependent manner (Fig. 5B). Similarly, we measured FITC-labeled ranibizumab fluorescence intensity in the upper and lower chambers of the in vitro ERM model using MIO-M1 alone or the combination of MIO-M1, ARPE-19, and NTI-4 cells.

**Table 2. Central Retinal Thickness, Visual Acuity, and Number of Intravitreal Injections of Anti-VEGF in DME Patients**

<table>
<thead>
<tr>
<th></th>
<th>DME (n = 25)</th>
<th>DME + ERM (n = 18)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CRT</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>baseline</td>
<td>475.00 (419.00–580.00)</td>
<td>440.00 (375.25–521.25)</td>
<td>0.23</td>
</tr>
<tr>
<td>1 mo after treatment</td>
<td>330.00 (287.00–360.00)</td>
<td>385.00 (299.50–454.00)</td>
<td>0.18</td>
</tr>
<tr>
<td>12 mo after treatment</td>
<td>511.00 (281.00–547.00)</td>
<td>582.00 (354.25–520.25)</td>
<td>0.01</td>
</tr>
<tr>
<td><strong>CRT change</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 mo/baseline</td>
<td>74.22 (57.63–84.81)</td>
<td>88.12 (70.52–94.35)</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Visual acuity</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>baseline</td>
<td>0.30 (0.22–0.52)</td>
<td>0.40 (0.30–0.68)</td>
<td>0.47</td>
</tr>
<tr>
<td>12 mo after treatment</td>
<td>0.22 (0.05–0.30)</td>
<td>0.40 (0.22–0.77)</td>
<td>0.04</td>
</tr>
<tr>
<td><strong>Number of injections</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>During 12 mo after treatment</td>
<td>4.00 (2.00–5.00)</td>
<td>2.00 (1.25–4.75)</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Median (Q1–Q3).
to exclude the possibility that goat IgG F(ab')2 and ranibizumab react differently to in vitro ERM model that is composed of human cells. Compared with the ECM group (no cells, 68.79) the FITC intensities of FITC-labeled ranibizumab in the lower chamber of the MIO-M1/ARPE-19/NTI-4 combined group with 4.7 $\times$ 10^4 cells (54.17, $P$ = 4.12 $\times$ 10^-5/C0) and in the group with 14.1 $\times$ 10^4 cells (38.67, $P$ = 9.19 $\times$ 10^-5/C0) were significantly decreased in a dose-dependent manner (Fig. 5C). In addition, compared with the ECM group (no cells, 200.79), the fluorescence intensities of FITC-labeled ranibizumab in the upper chamber of the MIO-M1/ARPE-19/NTI-4 combined group with 4.7 $\times$ 10^4 cells (221.79, $P$ = 2.92 $\times$ 10^-5) and in the group with 14.1 $\times$ 10^4 cells (237.79, $P$ = 1.59 $\times$ 10^-5) were significantly increased in a dose-dependent manner (Fig. 5C). These results indicated that the existence of ERM component cells (MIO-M1/ARPE-19/NTI-4) reduced antibody permeabilization in the in vitro ERM model.

**DISCUSSION**

In this study, change in CRT after first ranibizumab injection, final CRT, and final visual acuity strongly suggested that DME eyes with ERM exhibited worse responses after anti-VEGF treatment, compared with those without ERM. Previous studies have revealed conflicting results regarding the influence of VMIA on anti-VEGF treatment in DME. The Diabetic Retinopathy Clinical Research Network reported that treatment with ranibizumab is effective in cases of DME involving younger patients, lower grade of retinopathy, and eyes without retina wrinkle. In our study, there were no significant differences in age or grade of retinopathy; therefore, these factors presumably did not affect differences in CRT and final visual acuity. Yoon et al. reported that DME eyes with VMIA had smaller visual improvement after three injections of anti-VEGF drugs. Wong et al. reported that the presence of ERM
restricted functional (vision) and anatomic (CRT) improvements in DME. The prior findings are thus consistent with those of our study. Conversely, a retrospective cohort study showed that eyes with evidence of VMA (except for VMT) at baseline exhibited better visual acuity improvement, compared with those without VMA, at 6 months after treatment with ranibizumab.\(^4^5\) However, in that previous study, seven of 26 patients with VMA exhibited PVD within the evaluation period. Although there was no statistically significant difference relative to the findings in the 17 patients who did not show changes in VMA, the eye with PVD was reported to exhibit better BCVA improvement and CRT reduction.\(^4^5\) In our current study, changes in PVD were not recognized within the study period, and the ERM did not show robust changes.

In addition to analyzing clinical data, we also examined the association between ERM and anti-VEGF drugs through biological experiments using cultured cells. Immunostaining of ERM from DME eyes with ERM showed GFAP-positive, αSMA-positive, and pigmented cells, suggesting that Müller, fibroblast, and RPE cells were present; this was consistent with the findings of previous reports.\(^4^0,4^3,4^4,4^6\) Idiopathic ERM is reportedly formed by the growth of glial cells,\(^4^2\) suggesting that it may be derived from Müller glia cells.\(^4^5\) Moreover, ERM has been reported to contain myofibroblasts,\(^4^0,4^3,4^4,4^6\) and RPE cells have been observed in ERM after retinal breaks.\(^4^0,4^1\) Therefore, we chose to use three different types of cells as membrane that simulates ERM as follows: ARPE-19 cells (RPE), MIO-M1 cells (Müller glia), and NTI-4 cells (myofibroblasts).

The in vitro ERM model in this study, described in the prior paragraph, significantly reduced fluorescence intensity in the lower chamber; in contrast, it enhanced fluorescence intensity (remnant) in the upper chamber as the number of cells increased. In addition, when the total number of cells was similar, the antibodies showed a reduced tendency to permeate through the in vitro ERM model comprising a mixture of MIO-M1, NTI-4, and ARPE-19 cells, compared with the in vitro ERM model comprising MIO-M1 cells alone; notably, this difference was not significant. Because the size of the Alexa 488 Fab used is smaller than the pore size of the Transwell filter, the Transwell filter did not directly interfere with antibody permeabilization. In addition, the fluorescence level of Alexa 488 Fab was unlikely to be attenuated at 24 hours (Fig. 4A) and ECM was unlikely to directly interfere with antibody permeabilization (Fig. 4B). Therefore, in our in vitro ERM model, the reduced antibody permeabilization was caused by the ERM itself. Additionally, our in vitro ERM model produced collagen type I in a manner similar to that of human ERM samples, and may be useful as a novel in vitro model to study ERM.

The limitations of this study were as follows: (1) The Alexa 488 Fab comprised an antibody derived from an animal. Therefore, we cannot exclude the possibility that nonhuman IgG had unexpected biological reaction with human cells. (2) The ERM diagnostic criteria were not strictly controlled. A previous report noted that ERM was recognized by OCT as thin, hyperreflective bands anterior to the retina, or bright red bands in a pseudocolored image.\(^4^7\) The diagnosis of globally adherent ERM by spectral-domain OCT is based on a difference in brightness (or in bright red color on pseudocolored image) of the surface tissue; this is more evident in spectral domain OCT than in conventional OCT.\(^4^8\) However, there are various types of ERM, and we did not strictly define or categorize ERM in this study. (3) Although the results from previous clinical studies indicated that the effect of ERM on the anti-VEGF treatment is due to the adhesion of ERM as well as its traction, our in vitro ERM model produced collagen type I in a manner similar to that of human ERM samples, and may be useful as a novel in vitro model to study ERM.

In conclusion, based on the results of our clinical and biological analysis, we suspect that the resistance of DME to anti-VEGF treatment is partially due to increased resistance to antibody permeabilization through the ERM.
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The authors thank Shu Kachi, Tadasu Sugita, and Masatoshi Nagaya for important clinical and scientific suggestions.

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References


