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Diverse NF-κB expression in epiretinal membranes after human diabetic retinopathy and proliferative vitreoretinopathy

Chikako Harada,[1,2,3,4] Takayuki Harada,[1,2,3] Yoshinori Mitamura,[5,6] Hun-Meng A. Quah,[1] Kenji Ohtsuka,[5] Satoshi Kotake,[4] Shigeaki Ohno,[4] Keiji Wada,[2] Shinobu Takeuchi,[6] Kohichi Tanaka[1,7]

[1]Laboratory of Molecular Neuroscience, School of Biomedical Science and Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan; [2]Department of Degenerative Neurological Diseases, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan; [3]Department of Molecular Neurobiology, Tokyo Metropolitan Institute for Neuroscience, Fuchu, Tokyo, Japan; [4]Department of Ophthalmology and Visual Sciences, Hokkaido University Graduate School of Medicine, Sapporo, Hokkaido, Japan; [5]Department of Ophthalmology, Sapporo Medical University School of Medicine, Sapporo, Hokkaido, Japan; [6]Department of Ophthalmology, Toho University Sakura Hospital, Sakura, Chiba, Japan; [7]PRESTO, Japan Science and Technology Corporation (JST), Kawaguchi, Saitama, Japan

Purpose: Formation of epiretinal membranes (ERMs) after proliferative diabetic retinopathy (PDR) and proliferative vitreoretinopathy (PVR) results in progressive deterioration of vision, but its pathogenic mechanisms are still unknown. This study was conducted to examine the role of nuclear factor kappa B (NF-κB) in the formation of ERMs after PDR and PVR.

Methods: ERM samples were obtained by vitrectomy from 10 patients with PDR (aged 53±12 years with 14±5 years of diabetes), 20 patients with PVR, and 17 patients with idiopathic ERMs. Ten PVR and 17 idiopathic ERM samples were processed for reverse transcription-polymerase chain reaction (RT-PCR) analysis. In addition, 10 PDR and 10 PVR membranes were processed for immunohistochemical analysis.

Results: NF-κB mRNA expression levels were significantly higher (10 of 10 versus 9 of 17 subjects in idiopathic ERM, p=0.0119) in PVR subjects. Immunohistochemical analysis showed NF-κB protein expression in 8 of the 10 PDR samples as well as all 10 PVR samples, and NF-κB positive cells were partially double labeled with glial cell markers. Interestingly, NF-κB protein was also overlapped with angiogenic factor interleukin-8 (IL-8) in glial cells as well as vascular endothelial cells.

Conclusions: These results suggest that NF-κB is involved in the formation of both glial and vascular endothelial cell components, and that these two cell types might have functional interactions that lead to the enlargement of intraocular proliferative membranes.

Epiretinal membranes (ERMs) involving the macular or perimacular regions can cause a reduction in vision, metamorphopsia, micropsia, or occasionally monocular diplopia. The presence of ERMs has been associated with various clinical conditions including proliferative diabetic retinopathy (PDR) and proliferative vitreoretinopathy (PVR) [1]. The prevalence of ERMs in PDR is reported to be about 20% in Type 1 diabetes and about 5% in Type 2 diabetes [2,3]. PVR occurs when traction-generating cellular membranes develop in the vitreous and inner or outer surfaces of the retina after retinal detachment or major ocular trauma, and is the most common cause of failed repair of retinal detachment [4,5]. In general, PVR is considered to be a proliferative vitreoretinal disorder that is not related to retinal neovascularization. However, previous studies indicate that some PVR membranes were accompanied by a vascular component [6]. ERMs after PDR and PVR are composed of many cell types such as glial cells, retinal pigment epithelial cells, and vascular endothelial cells [6-8], but their pathogenic mechanisms are still unknown. However, various trophic factors and cytokines, such as basic fibroblast growth factor (bFGF), vascular endothelial cell growth factor (VEGF), and interleukin-8 (IL-8) were detected in vitreous fluid and ERM samples derived from PDR and PVR patients, and they are presumed to be involved in the pathogenesis of proliferative membranes [9-12].

In a previous study, we examined the expression of various neurotrophic factor receptors in ERMs following PDR, and found high expression levels of glial cell line-derived neurotrophic factor (GDNF) receptors [13]. GDNF family members mediate their actions through a multicomponent receptor complex composed of a transmembrane tyrosine kinase receptor, Ret, and one of the four glycosyl-phosphatidyl inositol (GPI)-linked GDNF family receptor α, designated GFRα1-GFRα4 [14]. Among them, GFRα2 mRNA was detected in 89% of PDR membranes, and GFRα2 protein was mainly observed in the glial cell component [13]. On the other hand, nuclear factor kappa B (NF-κB) p50 mRNA was detected in 91% of PDR membranes, and the active form of NF-κB p50 protein was observed in various cell populations [15]. However, we examined only the vascular endothelial cells, forsaking all other cell populations that were NF-κB positive. NF-

Correspondence to: Takayuki Harada, M.D., Ph.D., Department of Molecular Neurobiology, Tokyo Metropolitan Institute for Neuroscience, 2-6 Musashidai, Fuchu, Tokyo 183-8526, Japan; Phone: +81-42-325-3881; FAX: +81-42-321-8678; email: harada@tmin.ac.jp

κB is a transcription factor that can be activated by hypoxia, bacteria, viral proteins, various cytokines and trophic factors including tumor necrosis factor-α (TNF-α), interleukin-1β, and GDNF [16-18]. Interestingly, NF-κB is activated within 3 days after retinal detachment, in a time frame consistent with its potential for contributing to secondary cellular changes [19]. These results suggest the possibility that NF-κB, in combination with GDNF receptors, may be involved in the formation of the glial cell component of ERMs after PDR and PVR [15,18,20,21]. In addition, NF-κB expression in PVR membranes has yet to be examined.

In this study, we determined the coexpression of NF-κB p50 and GFRα2 proteins in PVR as well as PDR subjects. In addition, we found that NF-κB protein is overlapped with angiogenic factor interleukin-8 (IL-8) in both glial and vascular endothelial cells. We also proposed possible glia-vascular endothelial cell interactions in proliferative membranes.

METHODS

Subjects: This study was carried out in accordance with the tenets of the Helsinki Declaration. Informed consent was obtained from each patient for the collection of samples. Criteria for inclusion in the study were less than 80 years old, absence of renal or hematological diseases or uremia, absent administration of chemotherapy or life-support measures, and the fewest possible chronic pathologies other than diabetes. All PVR cases were grade C2 or worse according to the classification recommended by the Retina Society Terminology Committee [22]. The epiretinal membranes were surgically removed from consecutive eyes with secondary ERM after PDR (10 eyes) and PVR (20 eyes) or idiopathic ERM (controls, 17 eyes) undergoing pars plana vitrectomy and membrane peeling at Toho University Sakura Hospital. Membranes were dissected from the retinal surface with horizontal scissors or membrane pick. Samples derived from 10 of the PVR patients (age 45±23 years) and 17 control subjects (age 67±8 years) were processed for reverse transcription-polymerase chain reaction (RT-PCR) analysis. The remainder of the PVR samples (from subjects aged 43±17 years) and PDR samples (from subjects aged 53±12 years, duration of diabetes 14 ±5 years) were processed for immunohistochemistry. These samples were embedded in optimum cutting temperature (OCT) compound (Miles Laboratories, Naperile, IL), flash-frozen in liquid nitrogen, and then stored at -80 °C.

RNA extraction and amplification by RT-PCR: Total cellular RNA was prepared as previously reported [13,15]. RNA (0.1 μg) extracted from each sample was reverse transcribed into first-strand cDNA using the Superscript Preamplification System (Gibco, Paisley, Scotland) and oligo-dT primers.

RT-PCR analysis was carried out as previously described [13,23]. Complementary DNA reverse transcribed from total RNA was amplified by using primers specific for human NF-κB p50 (sense: 5'-CAC TTA TGG ACA ACT ATG AGG TCT CTG G-3'; antisense: 5'-CTG TCT TGT GGA CAA CGC AGT GGA ATT TTA GG-3'), GFRα1 (sense: 5'-AAG CAC AGC TAC GGG ATG CT-3'; antisense: 5'-GGT CAC ATC TGA GCC ATT GC-3'), GFRα2 (sense: 5'-ACG AGA CCC TCC GCT CTT TG-3'; antisense: 5'-GGG AGG CTT CGT AGA ACT CCT C-3'), and Ret (sense: 5'-TGG CAA TTG AAT CCC TTT TT-3'; antisense: 5'-ATG CCA TAG AGT TTG TTT TC-3'). PCR was done after initial denaturation at 94 °C for 3 min. Each cycle consisted of a heat-denaturation step at 94 °C for 15 s, annealing of primers at either 58 °C (GFRα2), 60 °C (GFRα1 and Ret), or 61 °C (NF-κB p50) for 2 min, followed by polymerization at 72 °C for 2 min. The expected sizes of the amplified cDNA fragments of NF-κB p50, GFRα1, GFRα2, and Ret were 406, 441, 339, and 439 bp, respectively. Human cDNA acquired from testis (Takara, Kyoto, Japan) was used as a positive control. Negative controls for PCR were made using "templates" derived from reverse transcription reactions lacking either reverse transcriptase or total RNA. After 35 cycles, 15 μl of each reaction mixture was electrophoresed on a 2% Tris borate-EDTA agarose gel and stained with ethidium bromide.

Immunohistochemistry: Frozen sections (7 μm thick) were cut by a cryostat, mounted on 3-aminopropyltriethoxysilane coated glass slides, and air-dried at room temperature. For immunohistochemical analysis, the sections were fixed in ice cold acetone and then washed with phosphate buffered saline (PBS). The sections were incubated with normal donkey serum for 30 min to block non-specific staining. They were incubated overnight at 4 °C with an affinity-purified rabbit polyclonal antibody raised against amino acids 350 to 363 mapping within the nuclear location signal (NLS) region of human NF-κB p50 [24] (2.0 μg/ml; Santa Cruz Biotechnology, Santa Cruz, CA). For double labeling immunofluorescence studies, they were then incubated with a goat polyclonal antibody against GFRα2 (1.0 μg/ml; Santa

Table 1. Patients that were positive for presence of mRNA

Sample	NF-κB	GFRα1	GFRα2	Ret	NF-κB GFRα1	NF-κB GFRα2
Proliferative vitreoretinopathy (n=10)	10 (100)	4 (40)	6 (60)	3 (30)	4 (40)	6 (60)
Idiopathic epiretinal membranes (n=17)	9 (53)	14 (82)	2 (12)	6 (35)	8 (47)	1 (6)
p value (Chi-square test)	0.0119	0.0393	0.0248	>0.9999		

NF-κB and GFRα2 mRNA expression in proliferative vitreoretinopathy was higher than those in idiopathic epiretinal membranes, while GFRα1 mRNA expression was higher in idiopathic epiretinal membranes.

Cruz Biotechnology), a mouse monoclonal antibody against vimentin (1x; Zymed, San Francisco, CA), GFAP (50x; DAKO, Glostrup, Denmark), glutamine synthetase (1.0 μg/ml; Chemicon, Temecula, CA) [25] or IL-8 (1.0 μg/ml; Santa Cruz Biotechnology). NF-κB p50 was visualized with FITC-conjugated donkey anti-rabbit IgG (200x; Jackson Immunoresearch, West Groove, PA) while GFRα2 and GFAP were visualized with Cy3-conjugated donkey anti-goat or anti-mouse IgG (800x; Jackson Immunoresearch), respectively. In the case of vimentin, glutamine synthetase and IL-8 which were visualized with FITC-conjugated donkey anti-mouse IgG (200x; Jackson Immunoresearch), NF-κB p50 was visualized with Cy3-conjugated donkey anti-rabbit IgG (1000x; Amersham Pharmacia Biotech, Piscataway, NJ). For double

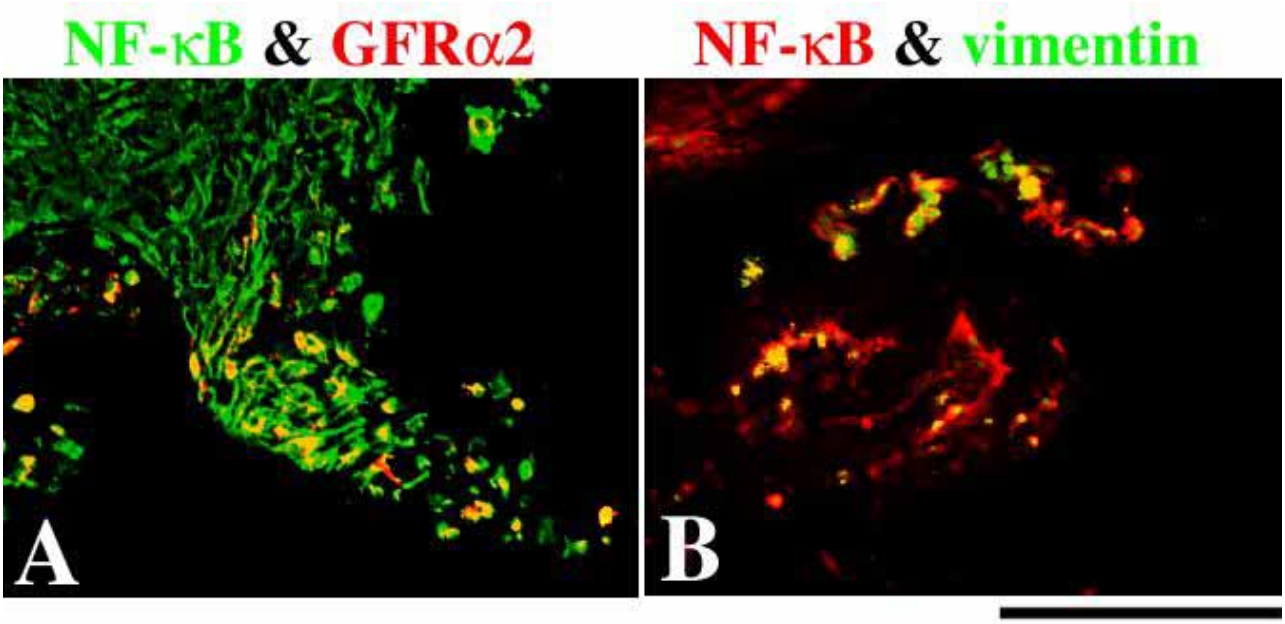

Figure 1. NF-κB expression in glial cells in PVR membrane. Expression of NF-κB and GFRα2 (**A**) and NF-κB and vimentin (**B**) in ERM derived from a 52 year old PVR patient. Double labeled cells are yellow. Bar represents 50 μm.

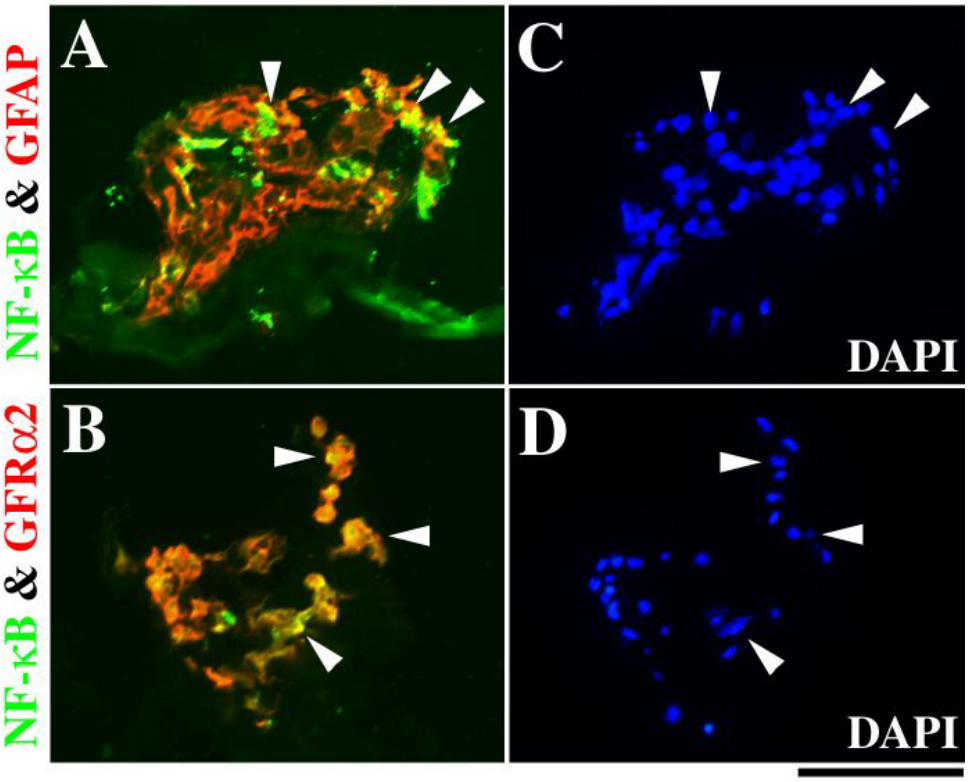

Figure 2. NF-κB expression in glial cells in PDR membrane. Expression of NF-κB and GFAP (**A**), and NF-κB and GFRα2 (**B**) in ERM derived from a 52 year old PDR patient with a 13 year history of diabetes. Double labeled cells are yellow (arrow heads). Panels **C** and **D** show DAPI staining of the sections adjacent to the sections shown in Panels **A** and **B**, respectively. Bar represents 50 μm.

labeling immunofluorescence study of IL-8 and GFAP, the sections were incubated with a mouse monoclonal antibody against IL-8 (1.0 μg/ml; Santa Cruz Biotechnology) and a rabbit polyclonal antibody against GFAP (500x; DAKO). IL-8 was visualized with FITC-conjugated donkey anti-mouse IgG (200x; Jackson Immunoresearch) while GFAP was visualized with Cy3-conjugated donkey anti-rabbit IgG (1000x; Amersham Pharmacia Biotech). The sections were examined by a confocal laser scanning microscope (Olympus, Tokyo, Japan). Primary antibody preabsorbed by blocking peptide (Santa Cruz Biotechnology) was used for negative controls. The adjacent section of each specimen was stained with 4,6-diamidino-2-phenylindole (DAPI).

Statistics: Data are presented as mean±SEM except as noted. The χ^2 test was used to test for significance of the difference between presence of various mRNAs examined in PVR versus controls. Statistical significance was accepted at $p<0.05$.

RESULTS

Expression of NF-κB p50 and GDNF receptors in ERMs after PVR was examined by RT-PCR analysis. As shown in Table 1, NF-κB p50 mRNA was detected in all 10 (100%) PVR patients, but in 9 of 17 (53%) idiopathic ERM (control) patients (a statistically significant difference in p50 mRNA expression between PVR and control patients; p=0.0119). GFRα1 mRNA was detected in 14 of 17 (82%) control subjects, but in 4 of 10 (40%) PVR patients (p=0.0393). On the other hand, GFRα2 mRNA was detected in 6 of 10 (60%) PVR patients, but in only 2 of 17 (12%) control subjects (p=0.0248). Ret mRNA was detected in 3 of 10 (30%) PVR patients and 6 of 17 (35%) controls (p>0.9999). We also examined the relationship between NF-κB p50 and GFRα mRNA expressions in both PVR and control patients (Table 1). NF-κB positive and GFRα1 positive patients were found in 4 of 10 (40%) of those with PVR, and 8 of 17 (47%) control subjects. On the other hand, NF-κB positive and GFRα2 positive patients were found in 6 of 10 (60%) PVR subjects, but only 1 of 17 (6%) control patients.

Immunohistochemical analysis was performed to identify the activated form of NF-κB p50 and GFRα2 protein expressions in ERMs after PVR. NF-κB protein was detected in all 10 samples examined (Figure 1A,B), and many NF-κB positive cells were double labeled with GFRα2 (yellow in Figure 1A). Since GFRα2 protein expression is increased in the glial component of ERMs after PDR, we examined whether NF-κB is coexpressed with a glial cell marker, vimentin [13]. PVR membranes contained many glial cells, and some of them were double labeled with NF-κB (yellow in Figure 1B). Similar results were obtained in experiments using other glial cell markers such as glutamine synthetase or GFAP (data not shown). NF-κB or GFRα2 immunoreactivity was completely abolished when the primary antibody was preabsorbed with blocking peptide (data not shown).

We previously demonstrated that NF-κB p50 mRNA is expressed in 20 of 22 ERMs derived from PDR patients (91%) [15], but did not examine whether its protein is distributed to

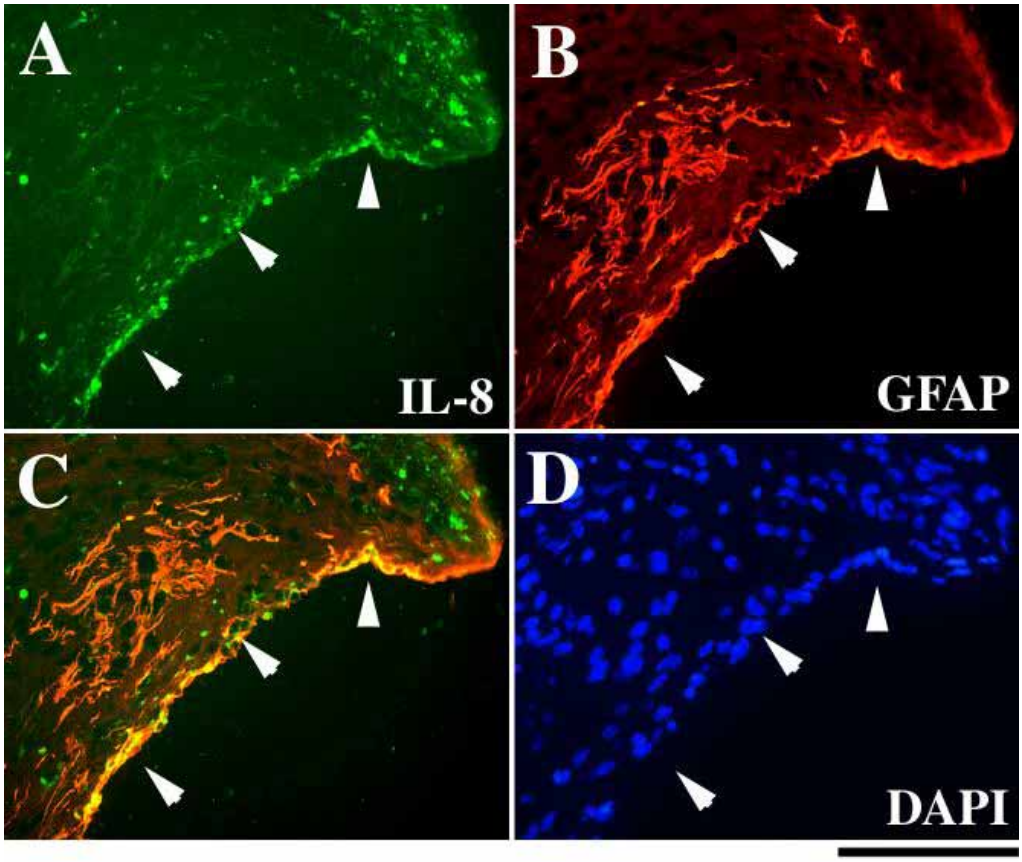

Figure 3. IL-8 expression in glial cells in PDR membrane. Expression of IL-8 (**A**) and GFAP (**B**) in a section of an ERM derived from a 52 year old PDR patient with a 10 year history of diabetes. **C**: Double labeling method demonstrates the co-expression of IL-8 and GFAP protein (arrow heads). **D**: DAPI staining of an adjacent section. Bar represents 50 μm.

glial cells like PVR membranes (Figure 1). To examine this possibility, we carried out double labeling immunohistochemistry for the activated form of NF-κB p50 protein and glial cell markers. NF-κB protein was detected in 8 of 10 PDR subjects examined, and many NF-κB positive cells were double labeled with GFAP (arrowheads in Figure 2A) as well as GFRα2 (arrowheads in Figure 2B). Similar results were obtained in experiments using other glial cell markers such as glutamine synthetase or vimentin (data not shown). NF-κB or GFRα2 immunoreactivity was completely abolished when the primary antibody was preabsorbed with blocking peptide (data not shown).

Previous studies have shown that IL-8 induced by NF-κB plays a key role in retinal angiogenesis after PDR [9]. In addition, we recently found that IL-8 is expressed in the neovascular component of PDR membranes, but did not examine its expression in other cell types [15]. Since NF-κB is expressed in glial cells (Figure 2A), we examined whether IL-8 protein is also distributed to the glial cell region. Interestingly, IL-8 (Figure 3A) and GFAP (Figure 3B) proteins are partially overlapped in PDR membranes (yellow in Figure 3C). Such IL-8 immunoreactivity was completely abolished when the primary antibody was preabsorbed with blocking peptide for IL-8 (data not shown). These results suggest a possibility that IL-8 protein might be produced in glial cells in PDR membranes. Similar results were also observed in PVR membranes (data not shown).

DISCUSSION

This study shows high expression levels of NF-κB and GFRα2 mRNAs in ERMs after PVR as well as PDR [13]. In addition, NF-κB and GFRα2 or angiogenic factor IL-8 proteins were coexpressed in glial cells in both PDR and PVR membranes. Together with our previous findings that NF-κB is expressed in vascular endothelial cells [15], NF-κB might be involved in the formation of both glial and neovascular components. On the other hand, glial cells can produce and secrete several factors that stimulate the proliferation of other cell types in ERMs [26-29]. Thus, inhibition of NF-κB activation and IL-8 production in glial cells may modify glia-vascular cell interactions and prevent the formation and enlargement of proliferative membranes. We have suggested a similar concept in the case of retinal degeneration [28,29].

GDNF and neurturin are structurally-related and distant members of the transforming growth factor-β (TGF-β) superfamily [14]. Recent studies have shown that TGF-β upregulates its own gene expression as well as genes that code for other polypeptide growth factors, such as interleukin-1 and bFGF, which are crucial for wound healing [30]. In addition, we recently found that exogenous GDNF increases brain-derived neurotrophic factor (BDNF), bFGF, and GDNF productions in retinal Müller glial cells [29,31]. Since these factors are secreted from retinal injury sites, one possible cause of ERM formation after ocular trauma and vitreoretinal surgery is the increase in release and concentration of these growth factors. In this context, PVR membranes can be considered as an "overhealing" disease. On the other hand, PVR is characterized by a breakdown of the blood-retinal barrier (BRB) [9]. Igarashi et al. recently demonstrated that barrier function of the BRB is regulated by GDNF and neurturin secreted from glial cells [32]. These results suggest functional implications of GDNF, neurturin, and their receptors in both the initiation and the process of PVR membrane formation. Although GDNF might induce NF-κB activation [16], NF-κB positive cells in PVR and PDR samples are not always GFRα2 positive (Figure 1A and Figure 2B). In addition, NF-κB and GDNF receptor expressions were also detected in some idiopathic ERM samples (Table 1). Thus, the role of NF-κB and GFRα2 in the process of ERM formation should be determined in more detail. For example, quantitative analysis of these genes may support our present results. We are currently planning to use real-time PCR analysis and examine their roles in secondary ERMs after various diseases other than PDR and PVR.

Our present findings suggest the possibility that inhibiting biological activity of NF-κB and/or GFRα2 in the glial component may be useful in preventing the formation of intraocular proliferative membranes. Recent studies have shown that transfection of IκB mutant, which blocks NF-κB activation [33], can inhibit angiogenesis by suppressing the production of IL-8 [34,35]. Thus, utilizing angiogenic factor blockers in combination with NF-κB-specific blocker and/or antisence oligonucleotide could lead to possible synergistic inhibition of the formation of intraocular proliferative membranes. However, before determining this possibility, further investigations into the precise role of NF-κB on retinal cells and all cell types in ERMs will be needed.

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