Expression of erythropoietin receptor in human epiretinal membrane of proliferative diabetic retinopathy

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Purpose: It is widely accepted that intravitreal levels of erythropoietin (Epo) are elevated in patients with ischaemic retinal diseases such as proliferative diabetic retinopathy (PDR). The aim of this study was to examine the expression of Epo and the Epo receptor (EpoR) in epiretinal membranes with and without diabetes.

Methods: Eighteen epiretinal membranes (PDR (n=10), idiopathic epiretinal membranes (IERMs) without diabetes (n=4) and inner limiting membranes (ILMs) (n=4)) were obtained during pars plana vitrectomy. Formalin-fixed and paraffin-embedded tissues were examined by immunohistochemistry with anti-Epo and EpoR antibodies.

Results: The histopathological findings demonstrated that PDR membranes consisted of a variety of endothelial cells forming a microvascular cavity with red blood cells and non-vascular stromal mononuclear cells. Membranous and cytoplasmic immunoreactivity for EpoR was strongly detected in endothelial cells and stromal cells in all PDR patients. Although microvessels were not observed in IERMs and ILMs, immunoreactivity for EpoR was noted in the cellular component of IERMs, and was weakly detected in ILMs. Epo was not expressed in any membrane.

Conclusion: EpoR was strongly expressed in microvessels of all PDR membranes. The *in vivo* evidence in this study suggests that Epo in the vitreous binds to EpoR in PDR membranes, which subsequently leads to the proliferation of new retinal vessels. EpoR immunoreactivity in non-vascular stromal cells in PDR membranes, and IERMs and ILMs might be indirectly correlated with ischaemia.

Diabetic retinopathy is the primary cause of blindness in working-age individuals in developed countries.¹ The visual disturbance primarily occurs from either the proliferation of new retinal vessels (proliferative diabetic retinopathy: PDR) or increased vascular permeability.² The vitreoretinal maculopathies resulting from traction by epiretinal membranes and the proliferation lead to significant visual loss.³ However, the proliferative mechanism in new retinal vessels and epiretinal membrane in PDR remains unclear.

Erythropoietin (Epo) was first described as a glycoprotein produced exclusively in fetal liver and adult kidney that acts as a major regulator of erythropoiesis.⁴ Recent ophthalmological studies have focused on elevated intravitreal levels of Epo in patients with ischaemic retinal diseases such as PDR.^{5–7} It is suggested that Epo plays an important role in the development of PDR, and its blockade can be beneficial for treatment.⁷ In fact, Epo derived from the vitreous fluid in patients with PDR was bioactive and stimulated proliferation of bovine retinal microvascular endothelial cells (BREC) *in vitro*.⁷

The Epo receptor (EpoR) is a member of the type I cytokine receptor family that includes cellular transmembrane receptors for factors such as growth hormones and interleukins.⁸ Epo shows angiogenic activity in vascular endothelial cells, stimulating proliferation, migration and angiogenesis *in vitro* by means of the EpoR expressed in those cells.^{9–10} These findings support the hypothesis that the Epo–EpoR pathway plays more important roles in the proliferation of new retinal vessels in PDR membrane than in membranes without diabetes. The purpose of this study was to examine the expression and immunolocalisation of Epo and EpoR in epiretinal membranes with and without diabetes.

MATERIALS AND METHODS

Ten patients (seven males and three females) with PDR underwent a pars plana vitrectomy between November 2005 and January 2007 at Hokkaido University Hospital, Japan. The clinical data of the patients are summarised in Table 1. Ages ranged from 50 to 73 (mean 63.1) years. Nine patients with PDR showed vitreous haemorrhage, and tractional retinal detachment was confirmed in five patients. For age-matched subjects without diabetes, we collected four idiopathic epiretinal membranes (IERMs) and four inner limiting membranes (ILMs) surgically removed from patients with macular hole (n=2) and macular hole retinal detachment (n=2). Ages in subjects without diabetes ranged from 55 to 88 (mean 67.8 years). The membranes peeled and removed from the retina were fixed in 4% paraformaldehyde and paraffin-embedded tissue sections were made for immunohistochemistry after informed consent was obtained. All studies conformed to the tenets of the Declaration of Helsinki and were approved by the

Table 1 Clinical summary of patients with proliferative diabetic retinopathy examined in this study

Case	Age	Sex	L/R	preVA	postVA	TRD	VH
1	50	M	L	0.06	0.15	+	+
2	69	M	R	0.1	0.1	+	+
3	58	M	L	0.01	HM	+	+
4	70	F	R	0.09	0.5	–	+
5	64	M	R	HM	0.4	–	+
6	63	F	L	0.06	0.01	+	+
7	67	M	R	0.2	0.15	–	+
8	73	M	R	HM	0.4	–	+
9	66	M	R	0.4	0.2	–	+
10	51	F	R	0.06	0.6	+	–

M, male; F, female; L, left; R, right; preVA, preoperative visual acuity; postVA, postoperative visual acuity; HM, hand motion; TRD, tractional retinal detachment; VH, vitreous haemorrhage.

Abbreviations: BREC, bovine retinal microvascular endothelial cells; Epo, erythropoietin; EpoR, erythropoietin receptor; IERM, idiopathic epiretinal

ethics committee of the Hokkaido University Graduate School of Medicine, Japan.

YO-PRO-1 NUCLEAR STAINING

Formalin-fixed, paraffin-embedded tissue sections were cut at 6 μ m thickness. The slides were dewaxed, rehydrated and rinsed in phosphate buffered saline twice, and then were assessed for YO-PRO-1 for 5 minutes, as reported previously.^{11 12} The nuclei were then confirmed by laser scanning confocal microscopy (MRC-1024, Bio-Rad, Richmond, CA, USA; and LSM 510, Carl Zeiss, Oberkochen, Germany).

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Dewaxed paraffin sections were immunostained using the streptavidin-biotin peroxidase complex method. Formalin-fixed, paraffin-embedded serial tissue sections were cut at a thickness of 4 μ m, and endogenous peroxidase activity was inhibited by immersing the slides in 0.3% hydrogen peroxide in methanol for 30 min. As a pretreatment, microwave-based antigen retrieval was performed in 10 mM citrate buffer (pH 6.0). Then, non-specific binding of the primary antibody was blocked by incubating the slides in the blocking serum for 20 min. The slides were serially incubated with anti-Epo (dilution 1:50, R&D system) and EpoR (1:100, Santa Cruz Bioetch, Inc, California, USA) antibodies, overnight at 4°C, followed by the secondary antibody and biotinystreptavidin complex for 30 min each, at room temperature. Immunoreactions were visualised with diaminobenzidine and

the sections were counterstained with haematoxylin. To examine the specificity of immunostaining, the primary antibody was replaced with mouse normal IgG or Tris-buffered saline. Control slides were invariably negative for immunostaining. As a positive control, endometrial carcinoma of the uterus and Merkel cell carcinoma tissues of the eyelid were examined, in which cytoplasmic immunoreactivity for Epo and EpoR was detected in the tumour cells as previously described.^{13 14}

RESULTS

In YO-PRO-1 nuclear staining, a variety of cells formed a vascular cavity (fig 1A, arrowheads), whereas a few mononuclear cells without a cavity (fig 1A, arrows) were intermingled in PDR membranes. H&E staining demonstrated that the PDR membrane consisted of a variety of endothelial cells forming a microvascular cavity with red blood cells (fig 1B, arrows) and oval mononuclear cells without a cavity (fig 1B, arrowheads). The stroma was oedematous and/or fibrous. Membranous and cytoplasmic immunoreactivity for EpoR was strongly detected in endothelial cells and stromal cells (fig 1D,E) in all PDR patients. IERM consisted of oval or spindle mononuclear cells with thin eosinophilic collagen-like tissue.¹² Linear collagenous tissues were noted in ILMs that were surgically removed, where only a few mononuclear cells were found. Microvessels were not observed in IERM and ILM. Immunoreactivity for Epo was not detected in any membranes (fig 1C,D,F). In contrast, cytoplasmic and membranous

Figure 1 YO-PRO-1 nuclear staining in epiretinal membrane of proliferative diabetic retinopathy (PDR) (A), H&E staining (B), and immunodetection of erythropoietin (Epo) (C, F, H) and Epo receptor (EpoR) (D, E, G, I) in epiretinal membranes of patients with PDR (A–E), idiopathic epiretinal membranes (IERMs) (F, G) and inner limiting membranes (ILMs) (H, I). In YO-PRO-1 nuclear staining, a variety of cells form a vascular cavity (A, arrowheads) in PDR membranes, where a few oval mononuclear cells without a cavity (A, arrow) are also detected by confocal microscopy (A). The PDR membrane consists of endothelial cells forming a microvascular cavity (B, arrows) and stromal oval mononuclear cells without the cavity (B, arrowheads). Epo is not expressed in PDR membranes (C). Immunoreactivity for EpoR is strongly detected in endothelial cells as well as in stromal cells (D). At high magnification, the immunoreactivity is localised in the cytoplasm and vascular lumen. The bar indicates 20 μ m (E). Epo is not expressed in IERMs (F) and ILMs (H). Cytoplasmic and membranous immunoreactivity for EpoR is detected in the cellular component of IERMs (G). A weak immunoreactivity for EpoR is observed in a few mononuclear cells in ILMs (I).

immunoreactivity for EpoR was noted in a cellular component of IERMs (fig 1E). Only a few EpoR-expressing cells were intermingled in ILM tissues (fig 1G).

DISCUSSION

We clearly detected immunoreactivity for EpoR in all PDR membranes. It is indisputable that vitreous Epo levels were significantly higher in patients with PDR than in those with non-ischaemic ocular diseases, including IERM and macular hole.^{5,7} Taken together, it is presumed that a functional Epo–EpoR pathway plays more important roles in ischaemia-associated pathologies in PDR membranes than in patients without diabetes. In contrast, immunoreactivity for Epo was not detected in any membrane, including PDR membranes, consistent with a recent report.¹⁵ It is suggested that increased Epo levels in the vitreous fluid of PDR patients are due to increased local production in the retina,^{5,7} but not in the proliferative membrane itself.

In this study, membranous and cytoplasmic immunoreactivity for EpoR was strongly detected in many endothelial cells of all PDR membranes. The binding of Epo to EpoR leads to the activation of transcription factors, which then induce mitosis of erythroid precursor cells.¹⁶ Epo stimulates proliferation of BRECs in a dose-dependent manner *in vitro*.⁷ Taken together, the evidence obtained in this study suggests that a relatively large amount of Epo in the vitreous binds to EpoR in the PDR membrane, which subsequently induces proliferation of new retinal vessels.³

Immunoreactivity for EpoR was detected in not only endothelial cells but also oval mononuclear cells in membranes of PDR patients, the latter of which is mainly derived from glial cells.¹⁷ In addition, despite no microvascular formation in IERMs and ILMs, unexpectedly, EpoR was apparently expressed in cellular components of IERMs, probably derived from glial cells.¹² It has been reported that EpoR is also expressed in various non-haematopoietic cell types, including neurons, retinal photoreceptors and tumour cells.^{13,14,18,19} Sugawa *et al* demonstrated that EpoR was expressed in glial cells and stimulation of the signalling-induced glial cell maturation.¹⁹ Thus, it is indicated that EpoR expression in non-vascular stromal cells in PDR membranes, IERMs and ILMs are indirectly correlated with ischemia, and further studies are needed to clarify the role of EpoR.

Katsuya *et al* proposed that the therapeutic use of recombinant human Epo (rhEpo) should be reconsidered in PDR cases.⁶ It was also reported that Epo treatment had a favourable impact on retinopathy in patients with diabetes.²⁰ However, our data indicate that the Epo–EpoR pathway may stimulate the proliferation and development of new retinal vessels in PDR membranes. Indications for the administration of rhEpo should be carefully evaluated in patients with diabetes with epiretinal membranes.

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