


Ocular expression of cyclin-dependent kinase 5 in patients with proliferative diabetic retinopathy

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Keywords

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ABSTRACT

Aims/Introduction: Inhibition of peroxisome proliferator-activated receptor gamma (PPAR γ) phosphorylation mediated by cyclin-dependent kinase 5 (Cdk5) is one of the main mechanisms of action of antidiabetic drugs. In this study, we analyzed the ocular expression and activation of Cdk5 in patients with proliferative diabetic retinopathy (PDR).

Materials and Methods: The concentrations of PPAR γ , Cdk5 and its activating subunit (p35) were determined in the vitreous body of 24 PDR and 63 control eyes by enzyme-linked immunosorbent assay. In addition, the messenger ribonucleic acid and protein expression levels of PPAR γ , Cdk5 and p35 were measured in proliferative neovascular membranes from seven PDR eyes and non-neovascular epiretinal membranes from five control eyes by quantitative real-time polymerase chain reaction and immunohistochemical analysis.

Results: PPAR γ , Cdk5 and p35 concentrations in the vitreous body were significantly higher in the PDR group compared with the control group. There was also a positive significant correlation of Cdk5 with PPAR γ and p35 in the PDR group. Furthermore, the messenger ribonucleic acid expression levels of PPAR γ , Cdk5 and p35 in proliferative neovascular membranes were significantly higher in the PDR group compared with the control group. Immunostaining showed increased protein expression levels of PPAR γ , Cdk5 and p35 in proliferative neovascular membranes in the PDR group compared with the control group.

Conclusions: Cdk5 activation is involved in PDR pathogenesis through PPAR γ expression, and inhibition of Cdk5-mediated PPAR γ phosphorylation might be a new therapeutic target for treatment of PDR.

INTRODUCTION

One of the major complications of diabetes mellitus is diabetic retinopathy, which might lead to blindness due to retinal detachment or glaucoma caused by intraocular neovascularization, and aggressive treatment is necessary for advanced cases^{1,2}. To date, several studies have reported that various cytokines, such as vascular endothelial growth factor (VEGF), play an important role in the pathogenesis of proliferative diabetic retinopathy (PDR)^{3–5}, and anti-VEGF antibody has already been applied in clinical settings to treat intraocular neovascular diseases, such as PDR^{6–8}.

The transcription factor peroxisome proliferator-activated receptor gamma (PPAR γ) is abundant in the intraocular fluid of patients with PDR⁹. Additionally, the intraocular PPAR γ concentration is positively correlated with the VEGF concentration and the PDR stage⁹. Although PPAR γ is a nuclear receptor, it can be detected in the aqueous or vitreous humor, because it is liberated from vascular endothelial cells into the extracellular fluid in the form of exosomes^{9,10}. Furthermore, both the messenger ribonucleic acid (mRNA) and protein expression levels of PPAR γ increase in the proliferative neovascular membranes of patients with PDR, suggesting that PPAR γ is involved in PDR progression⁹.

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Cyclin-dependent kinase 5 (Cdk5) is a serine/threonine kinase mainly expressed in the nervous system. Cdk5 activation is mediated by binding with its regulators, p35 and p39^{11–14}. As p35 and p39 are mainly expressed in neurons, it was thought that Cdk5 activation is restricted to the nervous system^{13,14}. However, p35 and Cdk5 were recently detected in non-neuronal cells, such as pancreatic β -cells^{15,16}. Furthermore, Cdk5-mediated phosphorylation of PPAR γ at serine 273 regulates the expression of metabolic regulation genes, including adiponectin, and is related to the pathogenesis of insulin resistance¹⁷. In addition, the main mechanism of action of thiazolidinedione, a PPAR γ agonist antidiabetic drug, includes the inhibition of Cdk5-mediated PPAR γ phosphorylation¹⁸. These findings and the augmented ocular expression of PPAR γ in patients with PDR show that in addition to its role in insulin resistance, Cdk5 participates in PDR pathogenesis through PPAR γ . Studies have also found the Cdk5 regulatory subunit-associated protein 1-like 1 (*CDKAL1*) to be a susceptibility gene for type 2 diabetes mellitus; *CDKAL1* shows homology with Cdk5 regulatory subunit-associated protein 1 (Cdk5rap1), a neuronal protein inhibiting Cdk5 activation, suggesting the involvement of Cdk5 in diabetes mellitus pathogenesis^{19–23}. However, no studies have focused on the ocular expression of Cdk5. Furthermore, whether the ocular expression or activation of Cdk5 is enhanced in patients with PDR is unknown.

Therefore, in the present study, we explored the ocular expression and activation of Cdk5 in patients with PDR, as well as the association between Cdk5 and PPAR γ expression levels.

MATERIALS AND METHODS

Vitreous samples and epiretinal membrane specimens

The PDR group comprised 24 eyes of 24 patients with PDR (10 men and 14 women, 3 patients with type 1 diabetes and 21 patients with type 2 diabetes) who were undergoing pars plana vitrectomy. The patients' average age was 57.8 ± 15.0 years, and the average duration of diabetes mellitus was 11.3 ± 8.4 years. All 21 type 2 diabetes patients with PDR were being treated with oral hypoglycemic agents, and six were on additional insulin treatment, whereas the three type 1 diabetes patients with PDR were being treated by insulin alone. The mean glycated hemoglobin was $7.3 \pm 1.15\%$.

The control group comprised 63 eyes of 63 patients (27 men and 36 women) who had undergone pars plana vitrectomy for idiopathic epiretinal membrane (ERM) and idiopathic macular hole, both of which are the eye diseases without intraocular neovascularization and any association with diabetes mellitus. The patients' average age was 68.1 ± 7.5 years. There were 13 patients with type 2 diabetes in the control group (mean glycated hemoglobin $6.88\% \pm 0.70\%$), but none had PDR. The mean age significantly differed between the PDR and control groups ($P < 0.001$).

All patients underwent pars plana vitrectomy at the Tokushima University Hospital, Japan, between January 2017 and August 2018. At the beginning of vitrectomy, before starting

intraocular infusion, undiluted vitreous humor was collected from the vitreous cavity using a vitreous cutter. There were no cases with a history of vitrectomy or ischemic retinopathy caused by disease other than PDR.

In addition, proliferative neovascular membranes were obtained from seven PDR patients (three men and four women, with an average age of 43.4 ± 15.0 years). Idiopathic non-neovascular ERMs were obtained from five control patients (two men and three women, with an average age of 68.8 ± 7.6 years). Vitreous humor and membrane samples were rapidly frozen and stored at -80°C until analysis.

The institutional review board of Tokushima University Hospital approved this study, and all patients provided informed consent. The procedures used complied with the Declaration of Helsinki.

Enzyme-linked immunosorbent assay

PPAR γ , Cdk5 and p35 concentrations in the vitreous humor were measured using enzyme-linked immunosorbent assay kits for human PPAR γ , Cdk5 and p35, respectively (LifeSpan Bio-Sciences Inc., Seattle, WA, USA) according to the manufacturer's recommended protocols. The detection limits of the assays were 0.16 ng/mL for PPAR γ , 0.3 ng/mL for Cdk5 and 78.2 pg/mL for p35. When the concentrations were undetectable, we used the detection limit for statistical analysis.

The total protein concentration in vitreous humor was determined using the Pierce 660 nm Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). The potential increase in PPAR γ , Cdk5 and p35 in the vitreous humor of the PDR group might not only reflect intraocular synthesis, but also the unspecific increase in total vitreal proteins because of the breakdown of the blood–retinal barrier and the leakage of plasma components into the vitreous cavity. To minimize the effect of this leakage, vitreous concentrations of PPAR γ , Cdk5 and p35 were adjusted for the vitreous total protein concentration^{9,24}.

Quantitative real-time polymerase chain reaction analysis

Total RNA in the proliferative neovascular membranes from seven PDR patients and in the idiopathic non-neovascular ERMs from five control patients were isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The resultant RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA), and complementary deoxyribonucleic acid was obtained using ReverTra Ace (Toyobo, Osaka, Japan). Quantitative real-time polymerase chain reaction was carried out using the ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, CA, USA) and the SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions. The abundance of each RNA was normalized to the amount of glyceraldehyde 3-phosphate dehydrogenase. The sequences of the primers were as follows: PPAR γ (sense: 5'-GCTGTGCAGGAGATCACAGA-3' and antisense: 5'-GGGCTCCATAAAGTCACCAA-3'); Cdk5

(sense: 5'-TGAGGTGGTCACACTGTGGT-3' and antisense: 5'-CTGGTCATCGACATCATTGC-3'); p35 (sense: 5'-ATGCCGACCCACACTACTTC-3' and antisense: 5'-AAACCCAGGAGAGACTGT-3'); and GAPDH (sense: 5'-CGACCACTTTGTCAAGCTCA-3' and antisense: 5'-AGGGGTCTACATGCAACTG-3').

Immunofluorescence

For immunofluorescence analysis, the proliferative neovascular membranes were embedded in Tissue-Tek OCT Compound (Sakura Finetechnical, Tokyo, Japan) and quickly frozen. Next, 7- μ m thick sections were cut on a cryostat at -20°C and subjected to immunostaining. After rinsing three times for 5 min each time with phosphate-buffered saline, the sections were incubated for 1 h in a blocking solution containing 10% horse serum and 1% Triton X-100 in phosphate-buffered saline (pH 7.4) at room temperature, and then incubated in primary antibodies in a blocking solution at 4°C overnight. The primary antibodies used were rabbit anti-PPAR γ (1:500; Cell Signaling Technology, Danvers, MA, USA), rabbit anti-Cdk5 (1:500; Cell Signaling Technology) and rabbit anti-p35/25 (1:500; Cell Signaling Technology). For double labeling, the primary antibodies were combined with an antibody against von Willebrand factor (vWF; goat anti-vWF, 1.0 $\mu\text{g}/\text{mL}$; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The sections were rinsed three times with phosphate-buffered saline, followed by incubation with the appropriate secondary antibodies, Alexa FluorTM 568 donkey anti-rabbit immunoglobulin G (1:1,000; Invitrogen, Carlsbad, CA, USA) and Alexa FluorTM 488 donkey anti-goat immunoglobulin G (1:1000; Invitrogen) in a blocking solution for 1 h at room temperature. In addition, the nuclei were counterstained with Hoechst. The samples were examined under a fluorescent microscope. ImageJ software (<http://imagej.nih.gov/ij/>; National Institutes of Health, Bethesda, MD, USA) was used to analyze staining intensities. Fluorescence intensity values were obtained after background subtraction from the intensity of the proliferative neovascular membranes and were normalized to the mean fluorescence intensity in the control group (ERMs).

Statistical analysis

The *t*-test was carried out to compare the protein concentrations between the two groups. The correlation between numerical parameters was analyzed using Pearson's correlation analysis. In addition, the mRNA expression and the immunofluorescent intensity were compared between the two groups using the Mann-Whitney *U*-test, because the number of samples was small and it was difficult to assume a normal distribution. All statistical analyses were carried out using JMP version 13.1.0 (SAS Institute Inc., Cary, NC, USA). A two-tailed *P*-value <0.05 was considered statistically significant.

RESULTS

The total protein concentration in the vitreous humor of the PDR group ($1.90 \pm 0.91 \text{ mg}/\text{mL}$) was significantly higher

compared with the control group ($0.62 \pm 0.42 \text{ mg}/\text{mL}$; $P < 0.001$; Figure 1a). The PPAR γ concentration per total protein concentration in the vitreous humor in the PDR group ($389.97 \pm 265.77 \text{ ng}/\text{mg}$) was significantly higher compared with the control group ($70.82 \pm 36.02 \text{ ng}/\text{mg}$; $P < 0.001$; Figure 1b). The Cdk5 concentration per total protein concentration in the PDR group ($4.83 \pm 5.43 \text{ ng}/\text{mg}$) was significantly higher compared with the control group ($0.82 \pm 0.88 \text{ ng}/\text{mg}$; $P < 0.001$; Figure 1c). The p35 concentration per total protein concentration in the PDR group ($367.13 \pm 520.70 \text{ pg}/\text{mg}$) was significantly higher compared with the control group ($188.86 \pm 123.92 \text{ pg}/\text{mg}$; $P = 0.014$; Figure 1d).

We focused on diabetes patients in the control group for further analysis. The PPAR γ and Cdk5 concentrations in control diabetes patients (69.34 ± 36.74 and $0.72 \pm 0.49 \text{ ng}/\text{mg}$, respectively) were significantly lower compared with the PDR group (389.97 ± 265.77 and $4.83 \pm 5.43 \text{ ng}/\text{mg}$, respectively; $P < 0.001$ and $P = 0.012$, respectively). The p35 concentration in control diabetes patients ($189.20 \pm 126.52 \text{ pg}/\text{mg}$) was also lower compared with the PDR group ($367.13 \pm 520.70 \text{ pg}/\text{mg}$), but the difference was not statistically significant ($P = 0.246$). This could be partly due to the small number of samples. In the control group, there were no significant differences in the PPAR γ , Cdk5 and p35 concentrations between control non-diabetes patients ($71.20 \pm 35.82 \text{ ng}/\text{mg}$, $0.86 \pm 0.96 \text{ ng}/\text{mg}$ and $188.77 \pm 123.24 \text{ pg}/\text{mg}$, respectively) and control diabetes patients ($69.34 \pm 36.74 \text{ ng}/\text{mg}$, $0.72 \pm 0.49 \text{ ng}/\text{mg}$ and $189.20 \pm 126.52 \text{ pg}/\text{mg}$, respectively; $P = 0.87$, $P = 0.64$ and $P = 0.99$, respectively).

Furthermore, we examined whether there was a significant association between the concentrations of each factor. Significant positive correlations were observed between PPAR γ and Cdk5 ($r = 0.44$, $P = 0.033$; Figure 2a), and between Cdk5 and p35 ($r = 0.70$, $P < 0.001$; Figure 2b), but there was no correlation between PPAR γ and p35 ($r = 0.306$, $P = 0.146$).

Next, we investigated the mRNA expression levels of PPAR γ , Cdk5 and p35 in the proliferative neovascular membranes of the PDR group and the idiopathic non-neovascular ERMs of the control group. The mRNA expression level of PPAR γ was significantly higher in the PDR group (0.79 ± 1.25) compared with the control group (0.033 ± 0.053 ; $P = 0.018$; Figure 3a). The mRNA expression level of Cdk5 was significantly higher in the PDR group (13.55 ± 14.17) compared with the control group (0.45 ± 0.29 ; $P = 0.028$; Figure 3b). The mRNA expression level of p35 was significantly higher in the PDR group (0.077 ± 0.070) compared with the control group (0.0051 ± 0.0032 ($P = 0.042$; Figure 3c).

Immunofluorescence analysis on membrane samples showed that compared with the control group, PPAR γ , Cdk5 and p35 showed stronger staining in the PDR group (Figure 4a–c). Quantitative analysis showed a significantly higher fluorescence intensity of PPAR γ in the PDR group (220.20 ± 49.06) compared with the control group (100 ± 40.92 ; $P = 0.003$; Figure 4d), a significantly higher fluorescence intensity of Cdk5 in

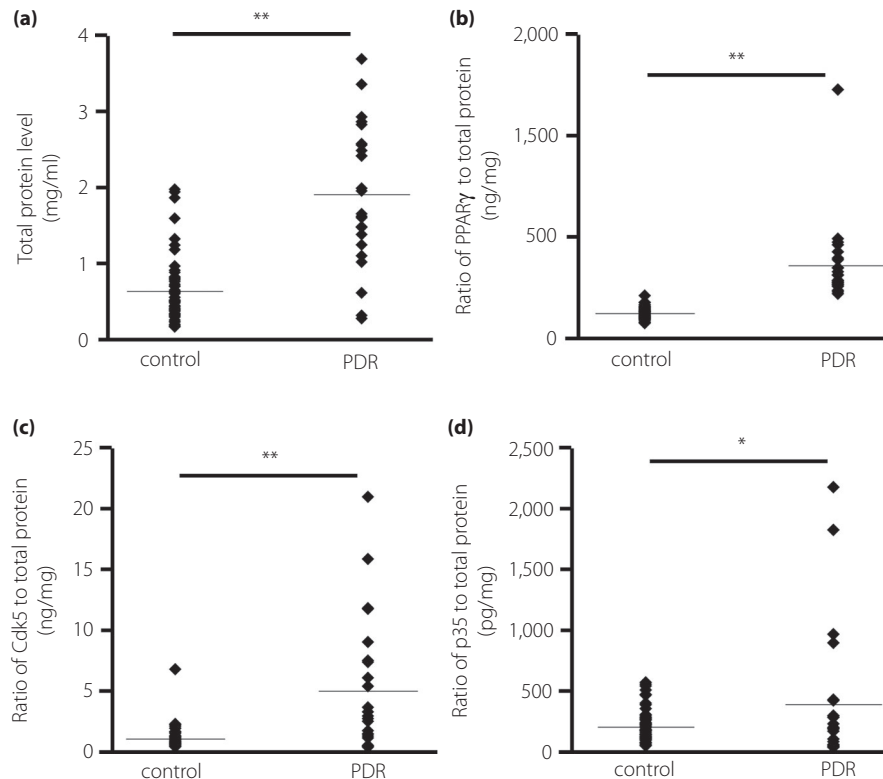


Figure 1 | Total protein, peroxisome proliferator-activated receptor gamma (PPAR γ), cyclin-dependent kinase 5 (Cdk5) and p35 concentrations in the vitreous humor. (a) The total protein level in the vitreous humor of the proliferative diabetic retinopathy (PDR) group was significantly higher compared with controls. (b) The PPAR γ : total protein ratio in the vitreous humor of the PDR group was significantly higher compared with controls. (c) The Cdk5 : total protein ratio in the vitreous humor of the PDR group was significantly higher compared with controls. (d) The p35 : total protein ratio in the vitreous humor of the PDR group was significantly higher compared with controls. Horizontal lines show the mean; * $P < 0.05$; ** $P < 0.001$.

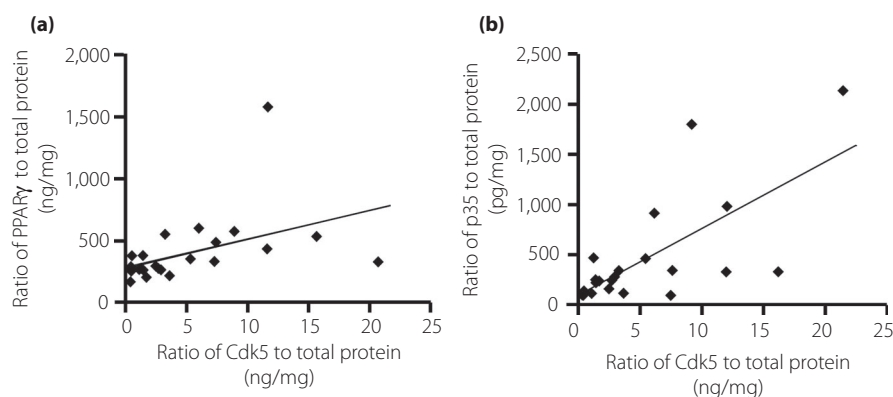


Figure 2 | Correlation among peroxisome proliferator-activated receptor gamma (PPAR γ), cyclin-dependent kinase 5 (Cdk5) and p35 concentrations in the vitreous humor. (a) Correlation between PPAR γ and Cdk5 concentrations. The PPAR γ : total protein ratio significantly correlated with the Cdk5 : total protein ratio ($r = 0.44$, $P = 0.03$). The solid line represents the best-fit linear regression line ($y = 21.905x + 288.083$). (b) Correlation between p35 and Cdk5 concentrations. The p35 : total protein ratio significantly correlated with the Cdk5 : total protein ratio ($r = 0.70$, $P < 0.001$). The solid line represents the best-fit linear regression line ($y = 40.992x + 67.447$).

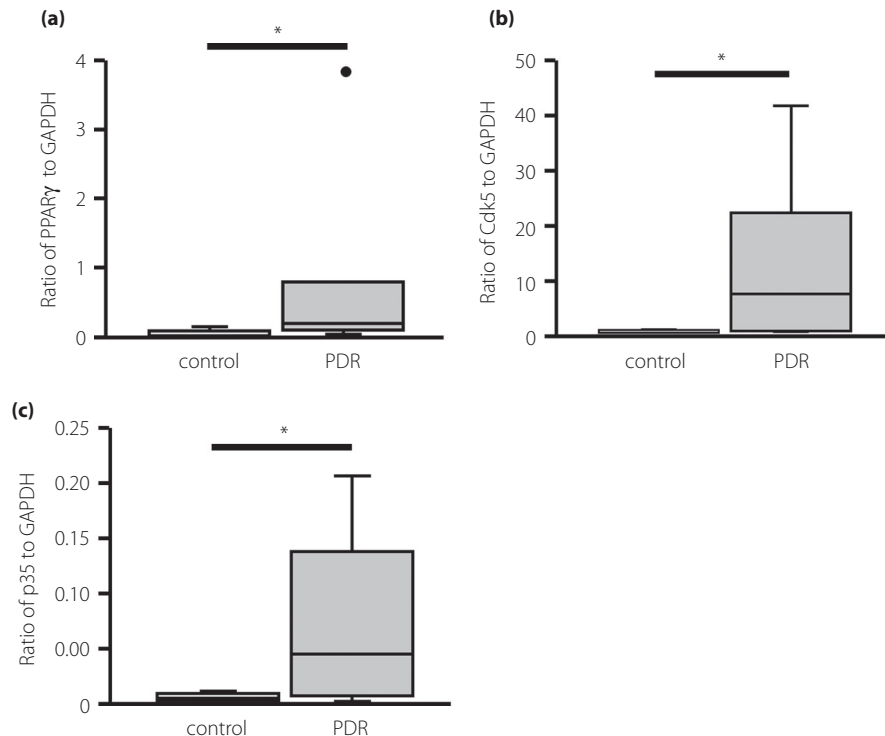


Figure 3 | Messenger ribonucleic acid (mRNA) expression of peroxisome proliferator-activated receptor gamma (PPAR γ), cyclin-dependent kinase 5 (Cdk5) and p35 in idiopathic non-neovascular epiretinal membrane samples from the control group and in proliferative neovascular membrane samples from the proliferative diabetic retinopathy (PDR) group. (a) Expression of PPAR γ mRNA was significantly higher in the PDR group compared with controls. (b) The PDR group showed significantly higher expression of Cdk5 mRNA compared with controls. (c) The PDR group showed significantly higher expression of p35 mRNA compared with controls. Scale bars show standard deviations; * $P < 0.05$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

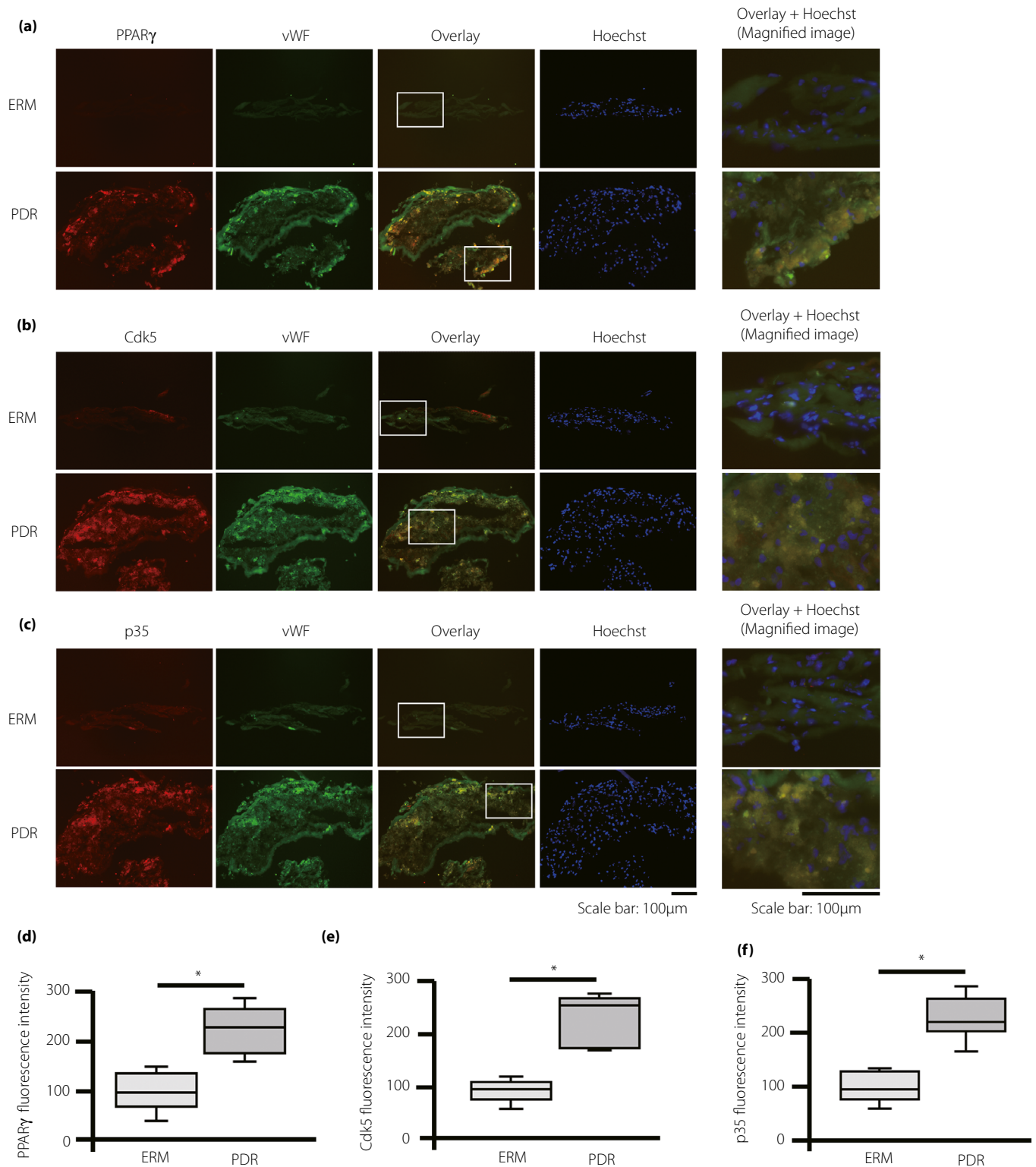
the PDR group (230.86 ± 46.50) compared with the control group (100 ± 21.36 ; $P = 0.003$; Figure 4e) and a significantly higher fluorescence intensity of p35 in the PDR group (231.91 ± 15.93) compared with the control group (100 ± 13.26 ; $P = 0.003$; Figure 4f).

Double labeling with an anti-vWF antibody suggested predominant expression of PPAR γ , Cdk5 and p35 in the endothelial cells of the PDR membrane.

DISCUSSION

To the best of our knowledge, this is the first study to show Cdk5 overexpression in intraocular samples from patients with PDR. We also found increased p35 expression, an activator subunit of Cdk5, suggesting that ocular Cdk5 activation occurs in patients with PDR. Considering the significant correlation observed between Cdk5 and PPAR γ expression, we believe Cdk5 might participate in PDR pathogenesis through PPAR γ .

Figure 4 | Double-labeling immunofluorescence for (a) peroxisome proliferator-activated receptor gamma (PPAR γ), (b) cyclin-dependent kinase 5 (Cdk5) and (c) p35 with von Willebrand factor (vWF) in membrane samples derived from control patients with epiretinal membranes (ERMs) and patients with proliferative diabetic retinopathy (PDR), together with magnified images of square areas. Nuclear Hoechst staining was carried out with the same sections. (a) Membrane samples derived from the PDR group, but not controls, showed intense staining of PPAR γ protein. Most of the PPAR γ -stained cells of membranes were double-labeled with anti-vWF antibody. (b) Membrane samples derived from the PDR group, but not controls, showed intense staining of Cdk5 protein. Most of the Cdk5-stained cells were double-labeled with anti-vWF antibody. (c) Membrane samples derived from the PDR group, but not controls, showed intense staining of p35 protein. Most of the p35-stained cells were double-labeled with anti-vWF antibody. (d) Quantitative analyses of (a). Fluorescence intensity of PPAR γ was significantly higher in the PDR group compared with controls. (e) Quantitative analyses of (b). Fluorescence intensity of Cdk5 was significantly higher in the PDR group compared with controls. (f) Quantitative analyses of (c). Fluorescence intensity of p35 was significantly higher in the PDR group compared with controls. Data are normalized to the mean intensity in the control group (ERM). Scale bars show the standard deviations; * $P < 0.01$.



PPAR is a nuclear hormone receptor superfamily of ligand-activated transcription factors related to retinoids, steroids and thyroid hormone receptors, which includes three isoforms: PPAR α , PPAR β/δ and PPAR γ ²⁵. PPAR is activated in the

presence of specific ligands through the formation of a heterodimer with the retinoid X receptor, which binds to target deoxyribonucleic acid in specific regions called peroxisome proliferator response elements, and induces PPAR-dependent gene

expression^{25,26}. PPAR γ is important in the homeostasis of lipids and glucose, and it is also a target protein for the antidiabetic drug, thiazolidinedione^{27,28}. In high-fat/high-sucrose-fed mice, insulin resistance was induced by increased PPAR γ phosphorylation at serine 273 in adipose tissue by Cdk5, and the PPAR γ ligand blocked Cdk5-mediated phosphorylation^{17,18,28}. PPAR is expressed heterogeneously in the eyes of mammals and constitutively in the entire retina^{29,30}.

Several studies have shown the effects of PPAR γ ligands on PDR and angiogenesis^{31–35}. Intravitreal injection of troglitazone and rosiglitazone (RSG), which are PPAR γ ligands, inhibited choroidal neovascularization in a rodent model of laser photocoagulation-induced choroidal neovascularization³¹. Telmisartan induced adiponectin secretion and improved insulin resistance by inhibiting PPAR γ phosphorylation in adipocytes³², and intraperitoneal administration of telmisartan improved the ocular pathological condition by blocking leukocyte adhesion to retinal vessels and suppressing intercellular expression of adhesion molecule-1 and VEGF in mice models of diabetes³³. Higuchi *et al.*³⁴ showed that thiazolidinedione suppresses neovascularization in the eyes of mice with PDR through regulation of adiponectin-mediated tumor necrosis factor- α production. *In vitro*, RSG and pioglitazone inhibited the angiogenesis-promoting effect of basic fibroblast growth factor and VEGF in a chicken chorioallantoic membrane model³⁵.

PPAR γ ligands protect retinal ganglion cells (RGCs) from glutamate-induced cytotoxicity³⁶. Glutamate is a major neurotransmitter in the retina, but induces RGC death at high concentrations. The glutamate concentration is elevated in the retina of PDR model rats³⁷. Troglitazone protects RGC-5 cells (an established transformed rat RGC line) from glutamate toxicity and shows anti-oxidant properties³⁶. PPAR γ ligands are also involved in fibrotic changes that play a crucial role in PDR pathogenesis³⁸. Transforming growth factor beta (TGF- β) signaling plays an essential role in fibrotic changes. Ocular TGF- β overexpression was detected in patients with PDR, suggesting that TGF- β is involved in the ocular formation of proliferative neovascular membranes in patients with PDR³⁹. Hatanaka *et al.*³⁸ reported that the expression of fibrosis markers, such as phalloidin, α -smooth muscle actin and fibronectin, in retinal pigment epithelium cells co-cultured with TGF- β increases; their expression decreases in the presence of pioglitazone. Thus, by suppressing TGF- β signaling, PPAR γ ligands seem to inhibit the fibrotic changes required for ocular proliferative neovascular membrane formation in patients with PDR. Shen *et al.* carried out a case-control study of 282 diabetes patients, and their results supported these experimental findings⁴⁰. Specifically, the progression from non-PDR at baseline to PDR over a period of 3 years occurred in 47.4% patients in the control group and 19.2% patients in the PPAR γ ligand RSG group. The authors suggested an almost 60% reduction in relative risk in the RSG group⁴⁰. Recently, a new antidiabetic compound (SR1664) has been identified as a PPAR γ ligand, which does not induce adipogenesis^{18,41}.

SR1664 selectively blocks Cdk5-mediated PPAR γ phosphorylation and has strong antidiabetes activity. It prevents the common adverse effects of PPAR γ ligands, such as fluid retention and weight gain¹⁸. In light of these facts and the observation of ocular Cdk5 activation in patients with PDR in the present study, we believe that Cdk5-mediated phosphorylation of PPAR γ plays an important role in PDR pathogenesis. In addition, specific phosphorylation inhibitors of PPAR γ , such as SR1664, might be useful for ameliorating PDR; however, further studies are required to confirm this.

Surprisingly, PPAR γ phosphorylation at serine 273 increased and insulin resistance worsened in adipose-specific Cdk5-knockout mice⁴². Specifically, extracellular signal-regulated kinase (ERK) directly phosphorylates serine 273 of PPAR γ , whereas Cdk5 suppresses ERK through mitogen-activated protein kinase/ERK kinase (MEK)⁴². In the obese state, phosphorylation at serine 273 is driven by both Cdk5 and ERK, and Cdk5 suppresses ERK. Cdk5 deficiency increases PPAR γ phosphorylation by ERK, and ERK/MEK inhibition suppresses this phosphorylation⁴². The authors suggested that MEK/ERK inhibitors might be effective in treating type 2 diabetes.

In the present study, we did not explore the correlation between Cdk5 and VEGF. In an endothelial/interstitial cell co-culture assay, Biscetti *et al.*^{43,44} reported that activation of PPAR α and PPAR γ induces endothelial tube formation in a VEGF-dependent manner. Consistent with their results, we previously showed a significant positive correlation between the intraocular concentrations of PPAR γ and VEGF in patients with PDR⁹. In addition, several studies have reported a relationship between Cdk5 and angiogenesis^{45–49}. Liebl *et al.*⁴⁵ showed that VEGF and basic fibroblast growth factor activate Cdk5, regulating endothelial cell migration and angiogenesis by remodeling the actin cytoskeleton through Ras-related C3 botulinum toxin substrate 1. Sharma *et al.*⁴⁸ showed that Cdk5 is highly expressed in proliferating bovine aortic endothelial cells, whereas its expression is low in quiescent bovine aortic endothelial cells. They showed that angiostatin, an angiogenesis inhibitor, downregulates Cdk5 expression and inhibits the proliferation of vascular endothelial cells⁴⁶. Xie *et al.*⁴⁷ reported suppressed VEGF expression after Cdk5 activity inhibition in rat pituitary cells. Herzog *et al.*⁴⁸ reported that inhibition of Cdk5 in vascular endothelial or hepatocellular carcinoma cells reduces the expression of hypoxia-inducible factor 1 α and transcription of its target genes, such as VEGF-A. *In vivo*, the authors showed angiogenesis impairment by inhibition of Cdk5 activity through reduction in the abundance of hypoxia-inducible factor 1 α ⁴⁸.

Cdk5 also plays an essential role in the pathogenesis of fibrosis^{50–52}, which is important for the ocular formation of proliferative neovascular membranes in patients with PDR⁵³. Bai *et al.*⁵² showed that Cdk5 promotes tubulointerstitial fibrosis through the ERK1/2 and PPAR γ signaling pathways in diabetic nephropathy, and that elevated p35 might predict the severity of diabetic nephropathy fibrosis. In addition, Cdk5 might be

activated by several cytokines, including tumor necrosis factor- α ¹⁷. In PDR, tumor necrosis factor- α is elevated in the vitreous humor and vascular endothelial cells in the proliferative neovascular membrane, and is critical for the proliferative phase of PDR^{54–58}. Choi *et al.*¹⁷ showed that tumor necrosis factor- α , interleukin-6, and free fatty acids activate Cdk5 and promote PPAR γ phosphorylation at serine 273. Thus, Cdk5 might play a crucial role in endothelial cell function, angiogenesis and fibrosis in PDR. In the present study, Cdk5 expression was observed predominantly in vascular endothelial cells in the ocular proliferative neovascular membranes in patients with PDR, and Cdk5 might target vascular endothelial cells, thus supporting the results of previous studies.

The present study had a few limitations. First, because of the relatively small sample size, patients with PDR could not be analyzed in detail in relation to their disease stage or treatment history. Second, the patients' average age significantly differed between the PDR and control groups; nevertheless, it is unclear whether there is a difference in Cdk5 or PPAR γ expression, depending on age. Third, it was not possible to analyze multiple biomarkers in vitreous humor samples due to the low protein content of the samples obtained during vitrectomy. Therefore, the relationship with VEGF could not be examined, but as described before, we believe that there is a correlation between ocular Cdk5 and VEGF in patients with PDR. Fourth, as the samples were obtained during vitrectomy, the study was cross-sectional. Therefore, changes over time could not be determined, and comparisons before and after treatment could not be made. Finally, although we detected elevated ocular Cdk5 levels in patients with PDR, the results of the present study do not provide direct causal evidence that Cdk5 is involved in PDR pathogenesis.

The establishment of an endothelial cell model to elucidate the molecular mechanism underlying Cdk5-PPAR serine phosphorylation and increased endothelial cell proliferation is required to support the clinical observations in the present study. Thus, further research is necessary. For example, we need to examine whether human umbilical vein endothelial cell culture with a high-glucose concentration leads to aberrant activation of Cdk5 and whether treatment with Cdk5 inhibitors or SR1664, a selective inhibitor of PPAR γ phosphorylation, suppresses ocular angiogenesis in PDR animal models.

In conclusion, the ocular concentrations of Cdk5 and its activator subunit p35 are significantly upregulated in patients with PDR. Furthermore, there is a significant correlation between Cdk5 and PPAR γ expression in patients with PDR. The present data suggest that Cdk5 might play a role in PDR pathogenesis, such as angiogenesis, through PPAR γ . Therefore, our study suggests the possibility that inhibiting Cdk5-mediated PPAR γ phosphorylation might be a new target for treating PDR.

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DISCLOSURE

The authors declare no conflict of interest.

Approval of the research protocol: The study was approved by the institutional ethics committee of Tokushima University Hospital.

Informed consent: All informed consent was obtained from the participants.

Approval date of registry and the registration no. of the study/-trial: No. 1084, 6 April 2018.

Animal studies: N/A.

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