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ORIGINAL RESEARCH PAPER

Upregulated CD200 in pre-retinal proliferative fibrovascular membranes of proliferative diabetic retinopathy patients and its correlation with vascular endothelial growth factor

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Abstract

Objective and design The objective was to determine the expression of CD200 in the pre-retinal proliferative fibrovascular membranes (PFVM) of patients with proliferative diabetic retinopathy (PDR) and to clarify its correlation with vascular endothelial growth factor (VEGF) and corresponding receptors.

Methods PFVM samples were collected by vitrectomy from 14 patients with PDR, and 11 non-diabetic patients who accepted vitrectomy for idiopathic epiretinal membranes removal. The expression of CD200, VEGF,VEGF-R1 and VEGF-R2 was measured via qPCR and immunofluorescent staining.

Results The mRNA level of CD200 was significantly higher in PDR patients than that in control patients. Meanwhile, CD200 and CD31 were found co-located and statistically associated in PFVM of PDR patients. The mRNA levels of VEGF, VEGF-R1 and VEGF-R2 were also significantly higher in PDR patients. Moreover, statistical association was found between CD200 and VEGF, VEGF-R1 in mRNA levels. But there was no significant correlationship between CD200 and VEGF-R2.

Conclusions These results suggest a significantly increased expression of CD200 in PFVM of patients with PDR and present a crucial association between CD200 and VEGF-involved pathway. It represents a potential therapy that interfering with CD200 may inhibit the VEFG expression and neovascular formation in PDR patients.

Keywords CD200 · Proliferative diabetic retinopathy · Proliferative fibrovascular membrane · Vascular endothelial growth factor

Introduction

Retinal neovascularization (RNV)-related diseases, such as diabetic retinopathy (DR) and retinopathy of prematurity (ROP), are the most serious blindness-causing diseases in the world [1]. DR can be divided into non-proliferative DR (NPDR) and proliferative DR (PDR) according to the level of microvascular degeneration and ischemic damage [2]. PDR is characterized by the growth of pre-retinal proliferative fibrovascular membranes (PFVM), which are made up of extracellular matrix and neovasculars. Pathologic angiogenesis and outgrowth of PFVM at the vitreoretinal interface turned out to cause vitreous hemorrhage or tractional retinal detachment are major contributors to serious vision loss [3]. Although the etiology of PDR is not fully understood, growing evidence suggests a key role for hyperglycemia and hypoxemia that result in angiogenesis and PFVM formation wherein vascular endothelial growth factor (VEGF) secretion is a drive-force for the neovascularization [4–7]. After persistent exposure to hyperglycemia and hypoxemia, VEGF is produced by 2.5- to 30-fold in retinal vascular endothelial cells, pericytes, retinal pigment epithelia, ganglion cells, and retinal glia in a reversible manner [8]. It binds and activates two tyrosine kinase receptors, VEGF-receptor (VEGF-R)1 and VEGF-R2, therefore to ignite the pathologic angiogenesis and to promote fibrovascular membranes that occur at the vitreoretinal interface. In recent years, as the revolutionary biological product comes out, anti-VEGF drug in clinical practice brought a significant change to the first-line therapeutic paradigm in the treatment of DR [9]. Nonetheless, most patients need repeated anti-VEGF injections because of its high incidence of recurrence, which

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will promote the side effects [10]. Thus, it is urgent to explore the upstream molecules of VEGF aiming to inhibit the PFVM formation in the patients with PDR.

Besides the ischemia-induced angiogenesis, chronic low-grade inflammation is also involved in the pathogenesis of PFVM [11, 12]. Strong evidence indicates that multiple pro-inflammatory chemokines induce angiogenesis and fibrosis in inflammatory sites, resulting in the formation of PFVM [13–15]. CD200, a novel immune-effective molecule, exists in a cell membrane-bound form, as well as in a soluble form in serum, which acts to inhibit acquired immune responses and inflammatory response [16]. Recent studies have shown the activation of CD200 has the potential to inhibit immunogenic inflammatory damage in the macrophage or microglia of retina [17, 18]. Furthermore, with the inhibition of CD200 in injured retinal tissue, microglia of retina can be significantly activated and the phagocytic ability of the microglia can be improved [17, 19]. What is the relationship between CD200 and VEGF? Our recent study has shown that CD200 level was increased in the vitreous samples of PDR patients, and it was significantly correlated with VEGF and its receptors (VEGFR-1 and VEGFR-2), and other pro-inflammatory cytokines [20]. Other studies showed that CD200-positive monocytes were increased in patients with neovascular age-related macular degeneration (AMD) [21]. Furthermore, Liu et al. revealed that melanoma tumors grown in CD200R-deficient mice exhibited increased expression of VEGF and HIF-1α genes with increased angiogenesis [22]. Moreover, Horie et al. revealed that CD200/CD200R signaling acts to suppress pro-angiogenic macrophage phenotype and the loss of such regulation leads to more severe ocular neovascularization in CD200R−/− mice than that in WT mice. Using the CD200R agonist, the neovasculars attenuated in a model of choroidal neovascularization [17]. All these studies revealed that CD200 might regulate the VEGF secretion in those retinal diseases, which were associated with retinal inflammation. However, whether CD200 regulated the expression of VEGF in PDR was still unknown.

Therefore, the primary objective of this study was to analyze the expression and distribution of CD200 in the PFVM obtained from patients with PDR and idiopathic epiretinal membranes (IERM) in macular area from patients without DR, and further to discuss the correlation between the levels of CD200, VEGF and its receptors in PFVM of PDR patients.

Methods

Study population

This study was conducted in department of Ophthalmology, the First Affiliated Hospital of Xi'an Jiaotong University, from January 2014 to October 2014. The study was approved by the ethic review committee of Xi'an Jiaotong University and was conducted in accordance with the Declaration of Helsinki. We obtained informed consent from each subject for all examinations and procedures.

Fluorescein angiogram, standardized fundus color photographs and optical coherence tomography were used to assess the vitreoretinal conditions of PDR patients. If there was a dense cataract or vitreous hemorrhage (VH) resulting in opacity of the ocular fundus, the vitreoretinal conditions of PDR were assessed by ocular ultrasound.

Sample collection

A standard three-port pars plana vitrectomy was performed during surgery. The samples were surgically removed and collected. Then the samples were frozen at − 80 °C until the assay was performed. The PFVM in PDR are composed of vessels and fibrous tissues, while IERM is a fibrocellular membrane that proliferates on the inner surface of the retina at the macular area. It contains a variety of cell types, including one or more of the following: retinal glial cells (Müller cells, astrocytes, and microglia), hyalocytes, macrophages, retinal pigment epithelial cells, fibroblasts, and myofibroblasts [23, 24]. These are no vascular endothelial cells in the IERM and this is the essential difference between PFVM and IERM. So, IERM is the optimal tissue as a negative control as non-vascularized. Samples were collected from 14 patients with PDR and 11 patients with IERM (without DR). One sample was divided into two parts. One part was processed for qPCR analysis and the other part was processed for paraffin sections and frozen sections. They were performed for immunohistochemistry staining and immunofluorescent staining, respectively.

Quantitative PCR (qPCR)

Total RNA was purified from samples with TRIzol, and converted to complementary DNA (cDNA) using a cDNA first-strand synthesis system (Fermentas, Canada). qPCR was performed using the SYBR Green RT-PCR Master mix, according to manufacturer's protocol. The $2-\Delta\Delta Cq$ method [25] was used to calculate target gene expression. Expression of target genes was measured in triplicate and the cycle threshold values were normalized against β-actin. The PCR primers were designed based on the NCBI GeneBank database. The primers for human CD200, VEGF, VEGF-R1, VEGF-R2 and β-actin were as shown in Table 1.

Frozen section and immunofluorescence

Samples were fixed in 4% PFA for 2 h at room temperature. Next, the samples were dehydrated in 30% sucrose

and embedded in O.C.T. (Sakura Finetek, Torrance, California). Then they were fast frozen and cut into 7 μm-thick sections using a cryostat (Leica Microsystem, Buffalo Grove, IL). After being transferred to glass slides, the sections were washed with PBS, and permeabilized with 1% Triton X-100 for 15 min at room temperature before blocking with 1% BSA for 2 h. The sections were incubated with goat polyclonal antibody against CD200 (1:100; Abcam), mouse monoclonal antibody for CD31 (1:100; Abcam), VEGF (1:20; ThermoFish), VEGF-R1 (1:200; Abcam) and VEGF-R2 (1:200; Abcam) overnight at 4 °C. On the second day, after washing with PBS, sections were incubated with a mixture of fluorescein isothiocyanate- and Cy3-conjugated secondary antibody (1:500, Cell Signaling Technology) for 2 h at room temperature and were counterstained with DAPI for 10 min. The sections were examined by confocal microscopy (Zeiss 510; Carl Zeiss).

Paraffin section and immunohistochemistry staining

Samples were fixed in an admixture solution consisting of PFA, dehydrated alcohol and glacial acetic acid for 24 h before being embedded with paraffin. Serial 6 μm sections were produced, and then the sections were dewaxed, rehydrated and rinsed in PBS. Antigen retrieval was performed in citrate buffer (10 mM, pH 6.0). Next, the sections incubated in the methanol solution containing 30% hydrogen peroxide for 20 min to block the endogenous peroxidase. Subsequently, primary antibody against CD200 (1:100) was added at 4 °C overnight after blocked the nonspecific binding sites with 1% BSA for 1 h at room temperature. On the following day, sections were rinsing with PBS and incubated with HRP-conjugated rabbit anti-goat IgG (1:1000) for 1 h at room temperature. Then, they were washed for three times and stained with diaminobenzidine (DAB). Images were observed with a microscopy (AxioCam MRC; Carl Zeiss).

Table 1 Primers of human CD200, VEGF, VEGF-R1, VEGF-R2 and β-actin for real-time PCR

Primers	Sequence(5′-3′)
CD200	
Forward	GTCTACCTACAGCCTGGTTTGG
Reverse	GCTGGGTAATGTTTATCTTGTCCTT
VEGF	
Forward	CTTCTGAGTTGCCCAGGAGA
Reverse	CTCACACACACACAACCAGG
VEGF-R1	
Forward	GTTACGTCACCTAACATCACTGT
Reverse	GTACGTTGCATTTGATATGATGAAG
VEGF-R2	
Forward	CCTCGGTCATTTATGTCTATGTTCA
Reverse	AGTAATGTACACGACTCCATGTTG
β-Actin	
Forward	ATCGTGCGTGACATTAAGGAGAAG
Reverse	AGGAAGGAAGGCTGGAAGAGTG

VEGF vascular endothelial growth factor, *VEGF-R* VEGF-receptor

Quantification

Immunoreactive cells were counted in five representative fields of each section, using an eyepiece in combination with the 40× objective. With this magnification, the positive cells present in each area were counted [26].

Statistical analysis

We used SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) for statistical analyses. Results are expressed as mean ± standard deviation. Two independent groups were compared by Student's *t* test and Chi square test. Spearman's correlation test was used to calculate the correlations between the variables investigated. All samples were tested in triplicate and P values < 0.05 were considered to be statistically significant.

Results

Clinical and demographic characteristics of the patients

The patients were enrolled consecutively from January 2014 to October 2014. Patients' backgrounds and demographics are summarized in Table 2. Samples from eyes obtained during a repeat vitrectomy were excluded and we also excluded eyes with prior anti-VEGF therapy. Consecutive type 2 diabetic patients with PDR ($n = 14$) had vitrectomies for PFVM, and patients with IERM ($n = 11$) had vitrectomies for IERM.

As shown in Table 2, the patients in PDR group were five females and nine males whose ages ranged from 53 to 78 years, with a mean of 64.79 ± 2.24 years. The duration of diabetes ranged from 6.23 to 13.45 years, with a mean of 8.92 ± 0.58 years. The patients in the control group were five females and six males ($P = 0.28$) whose ages ranged from 54 to 81 years, with a mean of 66.27 ± 2.49 years ($P = 0.6621$). The fasting blood glucose in the PDR group ranged from 6.3 to 15.4 mM, with a mean of 9.99 ± 0.73 mM. The fasting blood glucose in the control group ranged from 4.7 to 7.7 mM, with a mean of 6.27 ± 0.30 mM ($P = 0.0003$). Seven patients in the PDR group and five patients in the control group had hypertension ($P = 0.3$).

Table 2 Baseline patient characteristics from epiretinal membranes samples

	PDR group ($n=14$)	Control group ($n=11$)	P
Age (years)	64.79 ± 2.24	66.27 ± 2.49	0.6621*
Female gender, n (%)	9 (64.3)	6 (54.5)	0.28#
Duration of diabetes (years)	8.92 ± 0.58	N.A.	N.A.
Hypertension	7/7	5/6	0.3#
Fasting blood glucose (mmol/L)	9.99 ± 0.73	6.27 ± 0.30	0.0003*

Data are expressed as the mean ± standard deviation or the median and range. Indicates statistically significant result compared to the corresponding data in the control group as shown in Table 1 ($P<0.05$)

N.A. Not applicable, *PDR* proliferative diabetic retinopathy

*Student's *t* test

#Chi square test

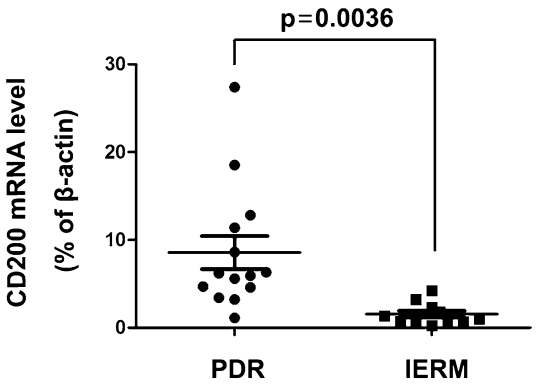

Fig. 1 The mean CD200 mRNA levels in membranes of PDR patients and IERM patients. Each point represents a measurement from a single patient

mRNA level of CD200 in PFVM and IERM

The mRNA level of CD200 was detected in the samples from 14 patients in the PDR group and 11 patients in the control group. qPCR results showed that comparing with the control patients, mRNA level of CD200 was significantly increased in the PDR group (Fig. 1; $P=0.0036$).

Immunohistochemical analysis of PFVM and IERM

No staining was observed in the negative control slides (Fig. 2a). All of the PFVM from PDR patients showed a strong nuclear immunoreactivity for CD200 (Fig. 2b, c) (Fig. 2c was partial enlarged detail of Fig. 2b), with a mean number of 19.86 ± 14.56 (range 3–54). However, no patients with IERM showed immunoreactivity for CD200. And it showed that the mean number of CD200-positive nuclear density in PFVM from patients with PDR was significantly higher than those from patients with IERM (Fig. 2d, $P=0.0004$).

Localization and expression of CD200 in PFVM and IERM

Double-labeling immunofluorescent staining was employed to determine the location and expression of CD200 in PFVM from patients with PDR and IERM from patients with IERM. Co-localization of CD200 with CD31, a vascular endothelial cells (VECs) marker (Fig. 3a), showed that CD200 protein was selectively produced in VECs. Furthermore, counting the CD200-positive nuclear density, it showed that CD200 was strongly presented in the PFVM of PDR patients (Fig. 3b; $P=0.0005$). When the whole PDR group was considered, a significant correlation was detected between positive nuclear density of CD200 and CD31 ($r=0.7691$, $P=0.0013$, Fig. 4c).

mRNA levels of VEGF and its receptors (VEGF-R1 and VEGF-R2) in PFVM and IERM

qPCR was performed to analyze the mRNA levels of VEGF and its receptors, including VEGF-R1 and VEGF-R2. Mean mRNA levels of VEGF, VEGF-R1 and VEGF-R2 in PFVM from PDR patients were significantly higher than those in IERM from control group ($P<0.0001$; $P=0.0024$; $P=0.0044$, respectively; Fig. 4).

Relationship of CD200 with VEGF, VEGF-R1 and VEGF-R2 in PFVM of patients with PDR

When the whole PDR group was considered, significant correlations were detected between CD200 and VEGF ($r=0.9033$, $P<0.0001$; Fig. 5a), between CD200 and VEGF-R1 ($r=0.8177$, $P=0.0004$; Fig. 5b). In contrast, no significant correlations were detected between CD200 and VEGF-R2 ($r=0.4901$, $P=0.0752$; Fig. 5c).

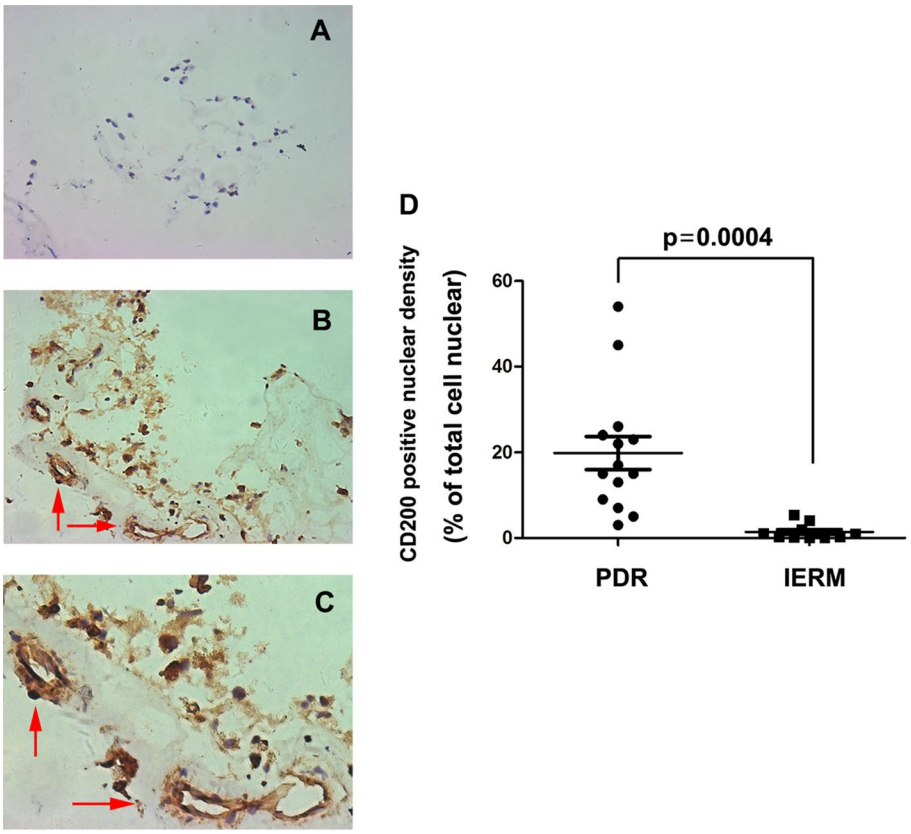

Fig. 2 The expression of CD200 in membranes of IERM patients (**a**) and PDR patients (**b**); **c** by immunohistochemical staining. The mean CD200-positive nuclear density in membranes of PDR patients and IERM patients was counted. Each point represents a measurement from a single patient (**d**)

Location of CD200, VEGF, and its receptors in PFVM from patients with PDR

Double-staining immunohistochemistry revealed that CD200 immunoreactivity was co-localized in VEGF-, VEGF-R1-, and VEGF-R2-positive endothelial cells in PFVM from patients with PDR (Fig. 6).

Discussion

In our previous study, CD200 level was found dramatically elevated in the vitreous samples of PDR patients; however, it was failed to be validated in venous blood samples [20]. These results indicated that CD200 did not leak into the vitreous from leaked blood vessels. Nevertheless, whether CD200 was secreted by retina, PFVM or some other tissues in the eyes of PDR patients were still unknown. The present study investigated CD200 expression levels and its location in PFVM of PDR patients and analyzed the relationships between CD200 with CD31, VEGF and its receptors (VEGF-R1, VEGF-R2). The major findings are: (1) The mRNA level of CD200 was significantly higher in PFVM of PDR patients than that in IERM of control group. (2) Counting the positive nuclear density in sections, the expression of CD200 was significantly elevated in PFVM of PDR patients comparing with those from IERM patients. (3) Double-labeling immunofluorescent staining showed that CD200 and CD31 proteins were co-localized in PFVM sections, thus CD200 protein was selectively produced in VECs. (4) Pearson correlation tests demonstrated that the CD200-positive nuclear density was related significantly to the CD31-positive nuclear density in the PFVM of PDR patients. (5) The mRNA level of CD200 was found to be positively correlated with increased VEGF and VEGF-R1 levels in PFVM from PDR patients, but there was no significant correlationship between CD200 and VEGF-R2. (6) CD200 protein showed co-localization with VEGF, VEGF-R1, and VEGF-R2 protein in PFVM of PDR patients. In conclusion, all these results suggested that CD200 might be a pivotal player in the pathogenesis of PFVM in PDR patients.

CD200, a novel immune-effective molecule, is a cell membrane-bound protein and exerts critical function in acquired immune responses [27]. It is widely expressed in neurons, endothelia, smooth muscle cells and myeloid cells, including macrophages, granulocytes and dendritic cells, and also expressed on T and B lymphoid cells, and natural killer cells [28, 29]. The CD200–CD200 receptor (CD200R) axis is known as an inhibitory axis and is critical in controlling excessive inflammatory responses in case of inflammation or infection [30, 31]. It plays a

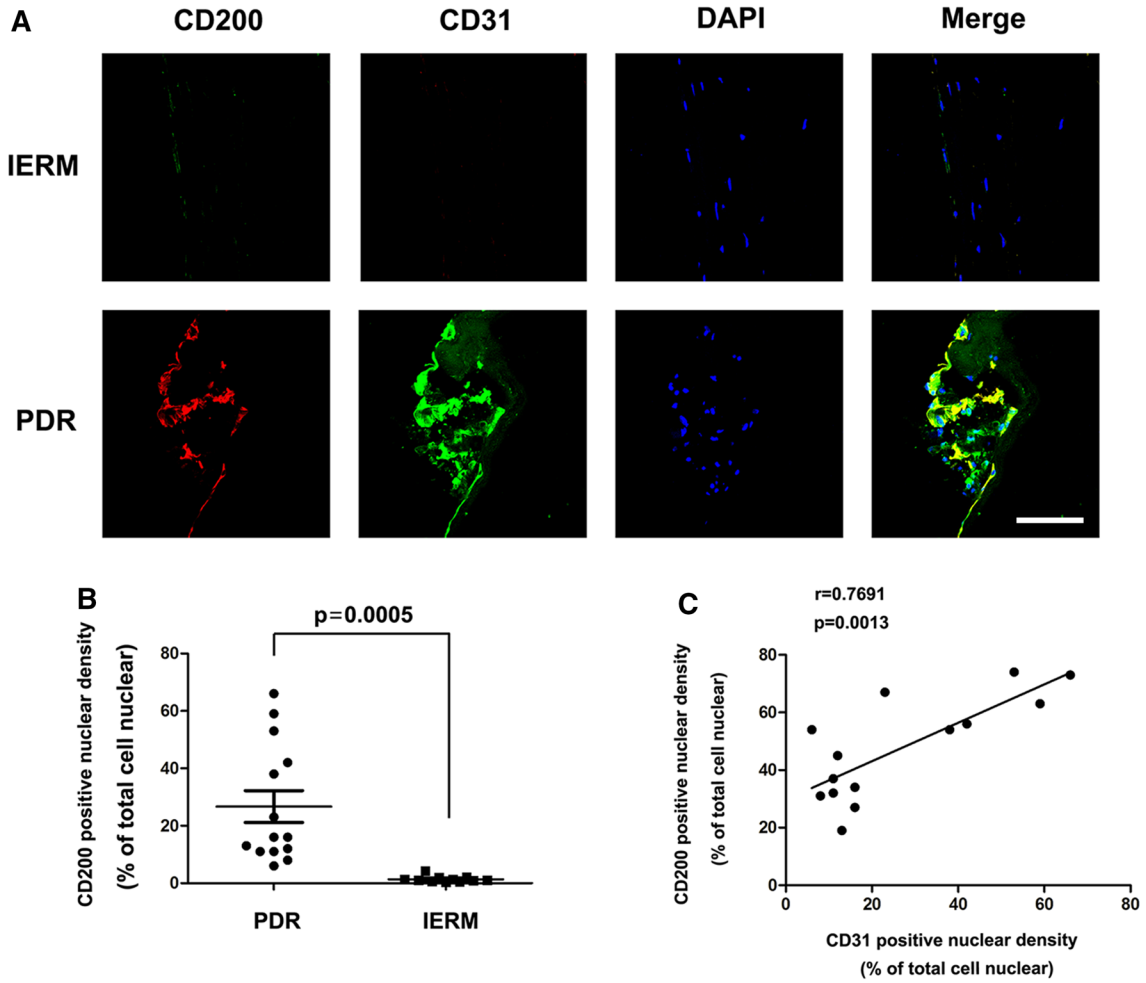

Fig. 3 The expression of CD200 and CD31 in membranes of IERM patients and PDR patients by double-labeling immunofluorescent staining (**a**). The mean CD200-positive nuclear density in membranes of PDR patients and IERM patients were counted (**b**). The correlation between CD200 and CD31 in the PDR group was calculated (**c**). Each point represents a measurement from a single patient

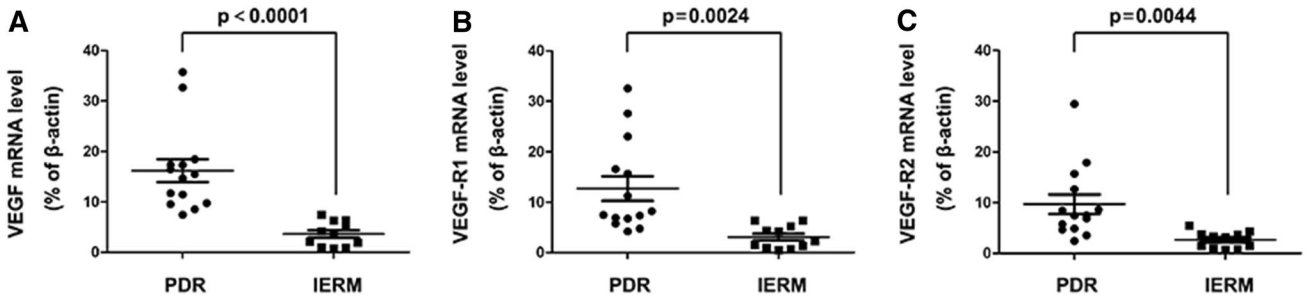

Fig. 4 The mean VEGF (**a**), VEGF-R1 (**b**), VEGF-R2 (**c**) mRNA levels in membranes of PDR patients and ERM patients. Each point represents a measurement from a single patient

role of immunomodulatory action, such as induction of immune tolerance, regulation of cell differentiation, adhesion and chemotaxis of various cell populations [32]. Furthermore, it is involved in cytokine and chemokine release from leukocyte subsets [31]. Recent studies reported that CD200 could ameliorate neuroinflammatory diseases such as multiple sclerosis [33], and also in Alzheimer disease [34] as well as in the aging brain [35]. Retina shares the

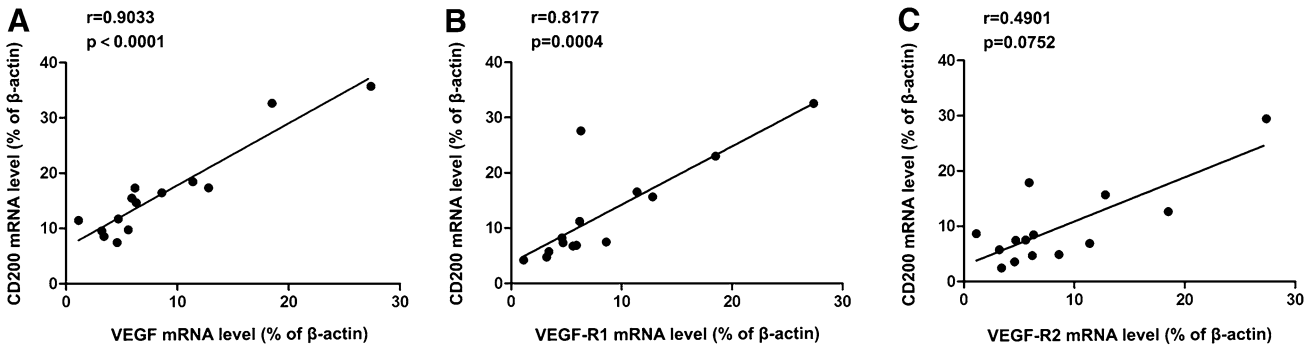

Fig. 5 The correlation between CD200 and VEGF (**a**), VEGF-R1 (**b**), VEGF-R2 (**c**) in the PDR group was calculated. Each point represents a measurement from a single patient

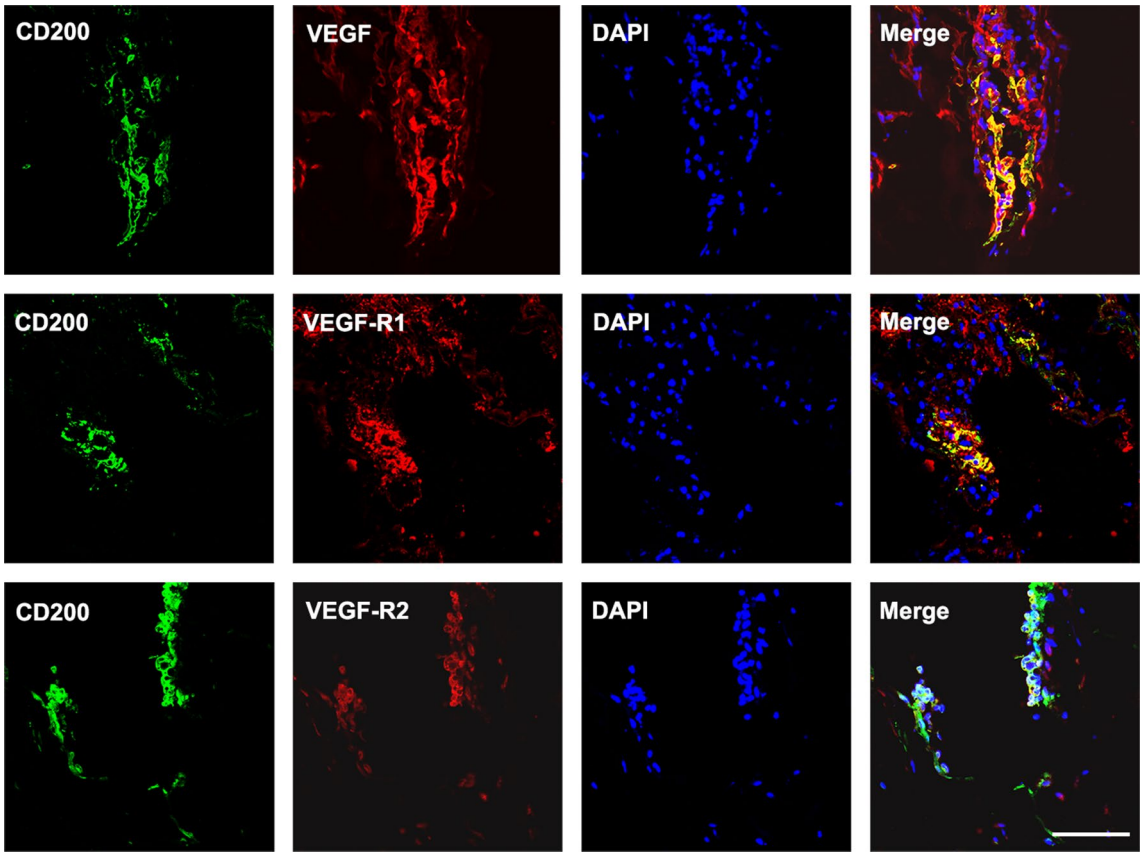

Fig. 6 The expression of CD200 and VEGF, VEGF-R1, VEGF-R2 in PFVM of PDR patients by double-labeling immunofluorescent staining

principles of CD200 to control neuroinflammation as a part of the neural system. Recent research revealed that retinal microglia in CD200-knockout mice displayed normal morphology, but unlike the wild type mice, they tend to have higher expression of pro-inflammatory cytokines, such as nitric oxide synthase (NOX) 2 [36]. And in experimental autoimmune uveitis, CD200 was involved in the polarization of microglia [37]. Furthermore, Taylor et al. demonstrated that activation of CD200 may reduce retinal cell loss in experimental glaucoma [18]. The character of CD200 varies in different diseases. As of now, the underlying mechanism of PDR remains unclear. Accumulated evidence suggests that low-grade chronic inflammation is a major cause involved in the pathogenesis of PDR. Our previous study showed that CD200 was increased in the vitreous samples of PDR patients, and correlated significantly with VEGF and its receptors (VEGFR-1 and VEGFR-2), and other pro-inflammatory cytokines [20].

In this study, we found that the CD200 expression was significantly elevated in the PFVM of PDR patients compared with that of nondiabetic patients with IERM. These results further demonstrated that CD200 was involved in the pathogenesis of PDR. As mentioned before, CD200 is widely expressed on various cells. However, CD200 receptor displays a restricted tissue distribution, including activated T and B cells [38]. Recent studies showed that CD200 was expressed on blood vessel endothelium in human cutaneous squamous cell carcinoma [39], and in corneal endothelial cells [40]. Our results showed that CD200 was co-localized with CD31, a vascular endothelial cells marker. This was consistent with previous researches. Furthermore, this was the first time that we found CD200 was expressed on vascular endothelial cells in PFVM of PDR patients. However, the signal pathways involving in the neovascularization and inflammation are very complicated, and CD200 may not be the only molecule that was altered. Previous studies indicated that interleukin (IL) family members, such as IL-6, IL-8 and IL-10, might involve in this signal pathway [20]. Thus, further works are needed.

A large number of inflammatory molecules are involved in the pathological process of angiogenesis in PDR. A key player in this process is VEGF and the exertion of VEGF functions on endothelial cells is dependent on VEGF receptors, including VEGF-R1 and VEGF-R2. A widely accepted theory is that the activation of VEGF-R1 influences the metabolism of pro-angiogenic molecules, while the activation of VEGF-R2 promotes endothelial cells migration and proliferation [41, 42]. These receptors are the decisive signaling transducers for angiogenesis under pathologic conditions, such as cancer and DR [42]. Our previous studies demonstrated the vitreous levels of VEGF, VEGF-R1, and VEGF-R2 in PDR patients were obviously higher than non-diabetic patients. Moreover, in the vitreous of PDR patients, the expression of VEGF and its receptors was significantly correlated with CD200 [20]. The present study showed similar results. In the PFVM of PDR patients, the expression of VEGF and its acceptors was dramatically increased and significant correlations were detected between CD200 and VEGF, VEGF-R1 in PFVM from patients with PDR. Although there was no significant correlation between CD200 and VEGF-R2, but double-staining immunohistochemistry revealed that CD200 immunoreactivity was co-localized in VEGF-R2-positive endothelial cells. In our opinion, CD200 was not correlated with VEGF-R2, this may be due to the following reasons: (1) the sample size of this study was relatively small. (2) CD200 was only the upstream regulator of VEGF/VEGF-R1 pathway, which was irrelevant with VEGF-R2. So further works are needed to demonstrate these hypotheses. Findings of previous investigations and the present study suggested that CD200 might be involved in the VEGF-induced inflammation in the pathogenesis of PDR.

Despite the amazing results, several imperfections existed in our present study. First of all, the sample size was relatively small. Furthermore, we have not investigated the correlation between CD200 expression and severity of PDR. Finally, we could not draw a causal relationship between CD200 and PDR through the finding of increased CD200 expression in PFVM and vitreum of PDR patients and its positive correlations with VEGF and VEGF receptors.

In conclusion, our study provided evidences, for the first time, that CD200 was selectively produced in VECs and the expression of CD200 was increased in the PFVM of patients with PDR. Furthermore, CD200 was significantly correlated with VEGF and its receptors (VEGF-R1), while CD200 was only co-localized with VEGF-R2-positive cells. CD200, which was produced by VECs, acted to regulate the release of VEGF. Consequently inhibited inflammatory response and retinal angiogenesis in PDR. Further studies are needed to investigate the possible molecular mechanisms of CD200 that contribute to retinal angiogenesis in hypoxia-induced retinal microvascular endothelial cells and in mouse model, including DR mice and CD200/CD200R-knockout mice and it will help us to further understand the role of CD200 in the pathologic angiogenesis in PDR.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

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