·Clinical Research·

CD18 expression in granulocytes infiltrating the vitreous fluid in patients with diabetic retinopathy

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Abstract

• **AIM: To assess the levels of CD18 on the surface of granulocytes infiltrating the vitreous fluid in patients with diabetic retinopathy (DR).**

• **METHODS: Vitreous samples from twelve patients with non-proliferative DR with significant macula edema (group A), 33 patients with proliferative DR (grade 3 as group B, *n*=14, and, grade 4 as group C, *n*=19) were obtained during pars plana vitrectomy. Vitreous samples from 12 patients with macular hole as controls (group D) were analyzed together. The infiltrating of granulocytes and its surface level of CD18 were measured by flow cytometry. The level of CD18 was presented as the mean channel fluorescence (MCF) on a logarithmic scale.**

• **RESULTS: Granulocytes were detected in 6 of 12 vitreous samples from group A, 9 of 14 from group B, 15 of 19 from group C, and none of 12 from group D. MCF of CD18 on granulocytes from groups A, B, and C were 2.978±1.446, 3.201±0.692, and 4.072±0.837, respectively. The difference was significant (*F*=4.354, *P*=0.021). Subjects with more severe DR were more likely to have a higher level of CD18 MCF (trend test, χ^2=7.351, *P*=0.007). CD18 MCF was significantly associated with the development of DR (*r*=0.46, *P*=0.005 and β=0.147, *P*=0.035).**

• **CONCLUSION: Our results confirm the presence of granulocytes and the elevated levels of CD18 on the surface of them in the vitreous fluid from DR patients. These results may provide indirect evidence shown that granulocytes activation also has occurred in the retinal local compared to non-DR control.**

• **KEYWORDS:** CD18; diabetic retinopathy; granulocytes; inflammation; vitreous fluid
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INTRODUCTION

Diabetic retinopathy (DR) is a complication of diabetes in the microvasculature of the retina and a leading cause of adult vision loss [1]. It begins as non-proliferative abnormalities and progresses to proliferative DR (PDR). Non-PDR (NPDR) refers to microaneurysms, exudates, venous beading and intraretinal microvascular abnormalities (IRMA) in the retina. Proliferative disease is diagnosed when retinal neovascularization or with preretinal or vitreous hemorrhage is seen. According to the degree of retinal neovascularization, PDR were classified as grade 1-4[2].

The therapy for DR is based on laser treatment, steroid injections and surgery. Although these methods are proven to be effective to reduce the risk for visual loss in DR, they can not reverse the loss of visual function caused by DR[3]. There is an urgent need to clarify the mechanism under the development and progression of DR and then find the new therapeutic agents for its treatment.

In accordance with the latest views, the adhesion of leukocytes to the retinal vessels play important role in the development of DR[4]. Some of the earliest pathological signs in experimental DR included the adhesion of leukocytes to the retinal vessels and the vascular leakage due to leukocyte adhesion. The inhibition of the adhesion of leukocytes and retinal vascular endothelial cells could significantly reduce diabetic induced endothelial cell dysfunction and death and blood-retina barrier breakdown [5-9]. So leukocyte adhesion may be considered as a target of the treatment of DR, as described in literature[10-13].

A central role in leukocyte adhesion and migration is played by adhesion molecules especially integrins and immunoglobulin superfamily molecules such as intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). Integrins located on the surface of leukocytes are heterodimers containing β and (subunits. They are grouped into specific subfamilies, *i.e.* β1-, β2-, and β3 -integrins [14]. β2-integrins subfamily binding ICAM-1 located on the endothelium cells include LFA-1 (lymphocyte function-associated antigen, CD11a/CD18), Mac-1 (leukocyte adhesion receptor, CD11b/ CD18) and p150/95

transmembrane polypeptide (CD11c/CD18). Each of the β2-integrins has a common β-chain CD18[14]. It is considered that surface expression of β2-integrins is a marker of leukocyte activation[15].

There were several studies related to the role of CD18 on the surface of leukocytes in the development of DR [6,7,9,15,16]. But among them most were related to experimental diabetes in animal models [6,7,9], fewer observations were made in people [15,16]. Among these observations made in people [15,16], they focused on the expression of CD18 on the surface of leukocytes from peripheral blood of DR patients. As opposed to peripheral blood, few studies provide data on the leukocyte and its surface expression of CD18 from the retina and vitreous of DR patients. Because leukocyte activation may primarily occur in areas of damaged microcirculation under the influence of local factors, the levels of CD18 on the surface of leukocytes from peripheral blood may not reflect that from the retina.

Granulocytes are the most abundant class of white blood cells, and typically the first type of leukocyte recruited to sites of inflammation. They are the major cells of the leukocytes which participant in the development of DR[17,18]. This study sought to evaluate the presence of granulocyte and its surface expression of CD18 in vitreous fluid from DR patients to indirectly ascertain whether or not they have changed in the retinal local compared to non-DR control.

SUBJECTS AND METHODS

Subjects Undiluted vitreous samples were collected from patients with full thickness macular hole, NPDR with clinically significant macula edema (CSME), and PDR. These patients were recruited consecutively from the Department of Ophthalmology of Xi'an No. 4 Hospital from November 2012 to September 2013, and were to undergo routine pars plana vitrectomy.

Ethical approval for the study was received from the local ethics committee at the hospital and written consent obtained from all participants. Clinical trials registration reference number of this study is ACTRN12611000440921 by http: //www.actr.org.au. Key inclusion criteria: to evaluate the levels of CD18 on the surface of granulocytes infiltrating the vitreous fluid in patients with NPDR with CSME (NPDR, group C), PDR (group D), and full thickness macular hole (FTMH) without diabetes (group A). Key exclusion criteria: the patients with persisting CSME involved the foveal centre at least 1 DD in size for <2y were included in this study. Exclusion criteria for patients with CSMO were: 1) posterior vitreous detachment diagnosed by the presence of a Weiss ring; 2) macular traction as evidenced by retinal striae involving the foveal center; 3) macular ischaemia as defined by an enlarged fovealar avascular zone (FAZ 41 000 mm) or significant perifoveal capillary loss on fundus fluorescein angiography (FFA); 4) coexistent retinal disease.

Methods The body mass index (BMI, kg/m^2) and waist-to-hip ratio (WHR) were calculated. Blood pressure was measured in all subjects in a sitting position on the right arm with standard mercury sphygmomanometer. Fasting plasma glucose (FPG), glycosylated hemoglobin (HbA1c), total cholesterol and its fractions, such as high density lipoprotein (HDLc), low-density lipoprotein cholesterol (LDLc), very-LDLc (VLDLc) and triglycerides (TG) were determined by venous samples.

Optical coherence tomography (OCT) were performed to assess the structural indices of the macula of patients with persisting CSME. The patients with persisting CSME involved the foveal centre at least 1 DD in size for <2y were included in this study.

The severity of retinopathy and the activity of neovascularization of PDR patients were graded by the surgeon intraoperatively according to the international clinical classification of DR and diabetic macular edema [2]. When there were neovascular tufts smaller than 0.5 PD somewhere in the retina but not in the optic disc was diagnosed as grade 1 PDR. When there were neovascular tufts larger than 0.5 PD somewhere in the retina outside the optic disc or smaller than 0.33 PD in the optic disc was diagnosed as grade 2 PDR. When neovascular tufts larger than 0.33 PD in the optic disc was diagnosed as grade 3. When vitreous hemorrhage was covering the whole retina or there was traction retinal detachment due to new vessels was diagnosed as grade 4 PDR.

Neovascularization was considered to be active when perfused preretinal capillaries were found, and to be quiescent if only nonperfused gliotic vessels or fibrosis were present.

Flow cytometric analysis Monoclonal antibody (mAb) against CD18 conjugated to phycoerythrin as well as isotype IgG control mAb was obtained from Becton, Dickinson and Co Immunocytochemistry Systems, San Jose, CA, USA. Undiluted vitreous samples (1 mL) were taken immediately at the start of vitrectomy prior to the start of infusion and were immediately placed in ice, centrifuged at 2500 rpm for 15min at 4℃ and separated into supernatants and cellular components. Cellular components were resuspended in staining buffer (phosphate-buffered saline plus 1% fetal calf serum plus 0.1% sodium azide) and incubated with 0.5 μL of 0.5 mg/mL isotype IgG control mAb and incubated for 5min on ice, then added to 400 μL of 20 mg/mL phycoerythrin-labeled CD18 antibody for 30min on ice.

A FACS scan flow cytometer (Becton-Dickinsonm, San Jose, CA, USA) and cell quest software were used for acquisition and analysis of the data. By evaluating the cell size (forward scatter) and granularity (side scatter), the acquisition areas of lymphocytes, monocytes, and granulocytes were identified. The granulocytes were analyzed for fluorescence intensity by

Table 1 Clinical and biochemical characteristics of the four groups

Variables	Group A	Group B	Group C	Group D	^1P	^2P
n (M/F)	12 (7/5)	14 (8/6)	19 (11/8)	12 (7/5)	1.00	0.90
Age (a)	49.30±3.74	50.21±2.72	52.14±2.72	49.18±6.61	0.33	0.17
BMI (kg/m^2)	21.90±1.91	22.06±1.54	22.50±1.56	20.46±1.37	0.03	0.65
WHR	0.93±0.09	0.92±0.11	0.92±0.59	0.86±0.12	0.29	0.92
FPG (mmol/L)	8.74±1.31	8.56±0.61	9.07±1.74	4.60±0.58	0.00	0.32
SBP (mm Hg)	130.60±13.14	127.93±8.92	133.43±9.72	112.11±15.76	0.001	0.39
DBP (mm Hg)	81.30±4.34	84.86±7.94	82.57±2.87	72.90±10.53	0.004	0.46
HbA1c (%)	8.24±1.37	8.61±1.776	8.55±0.92	5.67±0.28	0.00	0.71
Cholesterol (mg/dL)	190.60±32.16	191.85±21.16	197.21±24.99	166.16±12.16	0.01	0.78
HDLc (mg/dL)	44.80±7.43	45.42±5.09	46.00±5.37	45.14±5.06	0.01	0.89
LDLc (mg/dL)	104.80±21.22	98.35±17.79	108.21±19.44	99.25±16.62	0.49	0.40
TG (mg/dL)	218.10±60.36	253.21±67.44	236.07±50.72	127.00±18.53	0.00	0.37
VLDLc (mg/dL)	43.61±17.18	44.92±10.75	43.05±15.61	20.30±5.29	0.00	0.91

BMI: Body mass index; WHR: Waist-to-hip ratio; FPG: Fasting plasma glucose; SBP: Systolic blood pressure; DBP: Diastolic blood pressure; HbA1c: Glycosylated hemoglobin; HDLc: High-density lipoprotein; LDLc: Low-density lipoprotein cholesterol; VLDLc: Very-low-density lipoprotein cholesterol; TG: Triglycerides. *P* by χ^2 test; ^1P of the four groups after statistic analysis; ^2P of the three diabetic groups after statistic analysis.

Table 2 The mean CD18 MCF of the three DR groups

Groups	CD18 MCF	ANOVA		Trend test		LSD test
		F	*P*	χ^2	*P*	*P*
A	2.978±1.446	4.354	0.021	7.351	0.007	0.58[a]
B	3.201±0.692					0.01[b]
C	4.072±0.837					0.03[c]

[a]*P* of group A compared to group B; [b]*P* of group A compared to group C; [c]*P* of group B compared to group C.

using defined gates. Surface expression of CD18 was presented as the mean channel fluorescence (MCF) on a logarithmic scale.

Statistical Analysis All analyses were performed using the SPSS statistical package for Windows version 17.0. One-way analysis of variables (ANOVA) was used for between-group comparisons of continuous variables, and the χ^2 test was used for categorical variables. Fisher's least significant difference *t*-test was performed for multiple comparisons. The relationship between CD18 MCF and DR stage was analyzed by Pearson's correlation coefficients analysis and multiple stepwise linear regression analysis. The relationships between CD18 MCF and other clinical parameters were estimated by multiple stepwise linear regression analysis. The stage of DR was entered into the model using continuous variable. $P<0.05$ was considered as significant.

RESULTS

We studied the levels of CD18 on the surface of granulocytes from the vitreous fluid sample in 12 cases of NPDR with CSME (NPDR, group A, M/F=7/5) and 33 cases of PDR. According to the grade of the PDR, the 33 cases of PDR were consisted of 14 cases of grade 3 (group B, M/F=8/6) and 19 cases of grade 4 (group C, M/F=11/8). Among the 33 PDR patients, 8 patients from group B and 10 patients from group C had been considered as active PDR. Twelve cases of FTMH without diabetes patients were considered as control group (group D; *n*=12, M/F=7/5).

The clinical and biochemical characters of the patients were showed in Table 1. The 4 groups were matched for age, gender, WHR, and LDL. Higher BMI, FPG, systolic blood pressure, diastolic blood pressure, HbA1c, Cholesterol, high-density lipoprotein, TG, and VLDLc levels were found in diabetic patients in comparison with no diabetic controls. All of the clinical and biochemical characters were comparable among the three diabetic groups.

Granulocytes were detected in 6 (50%) of the 12 vitreous samples from group A patients, in 9 (64%) of the 14 vitreous samples from group B patients, in 15 (78%) of the 19 vitreous samples C patients, and none of the 12 vitreous samples from group D patients. In both groups B and C, 66% of the patients with CD18 expression had active PDR.

Because no granulocyte was detected in group D, we only compared the levels of CD18 on the surface of granulocytes from vitreous fluid among the diabetic groups.

The level of CD18 MCF was summarized in Table 2 according to the groups. The MCF of CD18 from groups A, B, and C were 2.978±1.446, 3.201±0.692, and 4.072±0.837, respectively. The difference of CD18 MCF among the three groups was significant (F=4.354, P=0.021). Subjects with more severe DR were more likely to have a higher level of CD18 MCF (trend test, χ^2 =7.351, P=0.007). After analyzed by Fisher's least significant difference *t*-test, the level of CD18 MCF from group C was significantly different from that of groups A and B (P=0.01 and 0.03, respectively), but

Table 3 Multiple regression analysis between CD18 MCF and other clinical parameters

Parameters	β	*P*
DR stage	0.147	0.035
FPG	0.483	0.002
SBP	0.029	0.011
HDLc (mg/dL)	0.057	0.009

DR: Diabetic retinopathy; FPG: Fasting plasma glucose; SBP: Systolic blood pressure; HDLc: High-density lipoprotein.

the difference of CD18 MCF between group, A and B was comparable (P=0.58).

In order to know the condition of retinal neovascular to the influence of CD18 level, we compared the levels of CD18 between active and quiescent PDR patients. The MCF of CD18 on granulocytes from active PDR was higher than that from quiescent PDR patients (4.611±0.721 *vs* 4.142±0.877), but the difference was not significant (F=1.171, P=0.301).

The development of DR was significantly associated with the levels of CD18 MCF not only by Pearson's correlation coefficients analysis (r=0.46, P=0.005) but also by stepwise multiple linear regression analysis (β=0.147, P=0.035). Other clinical parameters, including FPG, SBP, and HDLc, were also significantly associated with CD18 MCF by stepwise multiple linear regression analysis (P=0.002, 0.011, and 0.009, respectively; Table 3).

DISCUSSION

There is growing evidence from experimental diabetes in animal models shown that leukocytes are involved in capillary nonperfusion, retinal vascular leakage, and endothelial cell damage in DR [5-9]. The possible role of leukocytes and leukocytes induced capillary occlusion in DR cannot be studied, at least in the short term, by noninvasive methods in humans. Analysis of the vitreous fluid obtained from DR patients subjected to vitreoretinal surgery is a useful means of indirectly exploring the events that are taking place in the retina.

In the present study, we used flow cytometry to analyze vitreous fluid from patients with DR for the presence of granulocytes, as well as the expression of the molecule CD18 on the surface of them. While granulocytes were absent in the vitreous fluid of nondiabetic subjects with macular holes, most of the vitreous fluid from the DR patients contained granulocytes with CD18 expression. Similar data was also reported by Cantón *et al* [19]. In a study to investigate T cells infiltrating the vitreous fluid in patients with PDR, they found that T lymphocytes were detected in most of diabetic patients but in none of the non diabetic patients. The reason for these findings may be that vitreous body is an immune privileged site protected from systemic circulation by the blood-retinal barrier [20]. Although there is much evidence showing that the adhesion of leukocyte and retinal vascular endothelium played causal role in the blood-retinal barrier damage of DR from experimental animal mode[5-9], there is little information about the role of leukocyte in the development of early stage of DR in human. This probably is the first report documenting the presence of granulocytes in the vitreous body of non proliferative stage DR patients. This finding indirectly indicated that granulocytes may participate in the development of early stage DR of human.

It is considered that surface expression of CD18 is a marker of leukocyte activation. In this study, we found that there were significant differences of CD18 MCF among the diabetic groups. The development of DR was significantly associated with the levels of CD18 MCF. These findings were in line with our previous results obtained in the peripheral blood of DR patients, and hence provided further evidence for our previous view, that is, the levels of CD18 on the surface of granulocytes may be a marker of the development of retinopathy [16]. Although CD18 knockout mice have significantly fewer adherent leukocytes in retinal vasculature than that in the wild type diabetic mice, they still have some as reported by Joussen *et al*[9]. So CD18 may only play part of the role beneath other factors such as (4 integrin/CD49d[11].

During the process of this study, we observed a tendency that it was easier to detect granulocytes in the vitreous fluid from active PDR patients than that from quiescent PDR patients (16/18 *vs* 8/15). We considered that it must relate to the large amount of vitreous hemorrhage in active PDR patient. One interesting finding observed in the present study is that the difference of CD18 levels between active PDR group and quiescent PDR patients were not significant. It indicated that the condition of retinal neovascular may be not associated with the levels of CD18 on the surface of granulocytes.

Although there was study shown that there was no correlation between leukocyte activation and metabolic parameters in patients with microangiopathy [16], but in our study, we found that the levels of CD18 were correlated with metabolic unbalance in diabetes, such as FBG and HDLc, or with blood pressure values. Considering the important role of CD18 expression in the development of DR, it is important to keep the homeostasis of metabolism.

One of the major problems in any technique for studying vitreous cells is to obtain an adequate number for analysis. Many cells in the vitreous fluid are already nonviable, and the remainder can disintegrate very quickly after collection of the sample. Flow cytometry was used to study T cells infiltrating vitreous fluid of patients with DR [19], but to our knowledge, it has not been previously applied to analyze granulocytes in the vitreous fluid of patients with DR. The experimental approach used (vitreous fluid immunofluorescence, performed immediately after collection) allows us to simulate the *in vivo* scenario as closely as possible.

In conclusion, our results confirm the presence of granulocytes and the elevated levels of CD18 on the surface of them in the vitreous fluid from non-prolifetave and proliferative stage of DR patients. These results may provide indirect evidence shown that granulocytes activation also has occurred in the retinal local compared to non-DR control. Agents that block the activation of granulocytes or the expression of CD18 may be beneficial to the treatment of DR, especially during its early stage.

The limitation of our study was the lack of histomorphology data such as cytospins of the vitreous samples stained with conventional CD18 immunohistochemistry which will provide information about the *in situ* distribution of CD18 on different cell populations and will give additional hints by staining intensity. Moreover a simple identification of granulocyte by their characteristic nuclei is possible. These may become our future agenda for the supplement of the data obtained in this current paper.

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