• Basic Research •

Is Iba-1 protein expression a sensitive marker for microglia activation in experimental diabetic retinopathy?

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Abstract

● **AIM:** To investigate the changes of Iba-1 and other potential markers for microglia activation in experimental diabetic retinopathy (DR).

● **METHODS:** Male Sprague-Dawley rats were rendered diabetes *via* intraperitoneal injection of streptozotocin. The retinas were harvested at 1 to 24wk after diabetes onset. Hypoxia-treated mouse microglial cell line (BV2 cells) was employed as the *in vitro* model to mimic diabetic condition. The expressions of Iba-1, CD11b, ICAM-1 as well as the inflammatory factors were examined with real-time polymerase chain reaction, Western blot and immunofluorescence both *in vivo* and *in vitro*.

● **RESULTS:** Compared with age-matched normal control, the number of microglia (Iba-1 positive immunostaining) in diabetic rat retinas was increased from 1 to 24wk of diabetes, which was most obvious at 12wk of diabetes. Iba-1 protein expression detected by Western blot was increased slightly in diabetic rat retinas compared with that in age-matched normal control; however, there was statistically significant between two groups only at 2wk after diabetes onset. The mRNA expression of Iba-1 was decreased significantly at 2 and 4wk of diabetic rat retinas, and remained unchanged at 8 and 12wk of diabetes. In BV2 cells, there was no significant change for the Iba-1 protein expression between normoxia and hypoxia groups; however, its mRNA level was decreased significantly under hypoxia. To further characterize microglial activation, F4/80, CD11b and inflammatory factors were detected both *in vivo* and *in vitro*. Compared with normal control, the expressions of F4/80 and CD11b as well as the inflammatory factors, such as ICAM-1, iNOS, COX2, IL-1β and IL-6, were increased significantly both *in vivo* and *in vitro*.

● **CONCLUSION:** Iba-1 protein expression might not be a sensitive marker to evaluate the activation of microglia in experimental DR. However, Iba-1 immunostaining, in combination with other markers like CD11b and ICAM-1, could be well reflect the activation of microglia. Thus, it is of great importance to explore other potential marker to evaluate the activation of microglia.

● **KEYWORDS:** microglia; activation; Iba-1; diabetic rats; diabetic retinopathy

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INTRODUCTION

Diabetic retinopathy (DR) remains a major complication of diabetes and a leading cause of blindness among adults worldwide. The pathogenesis of DR is very complicated. It has been reported that microglia in diabetic retinas are activated and play an important role[1-8]. Iba-1 was reported as a microglia/macrophage-specific marker[9-10] and widely used for microglial detection[7,11-12]. However, microglial activation was mostly relied on the immunostaining of Iba-1 to characterize its morphology and distribution, and quantify its numbers, *etc*[7,12-13]. Whether increased expressions of Iba-1 protein (detected by Western blot) and mRNA could be used to reflect microglial activation sensitively remains debatable. It has been reported that the expression of Iba-1 is increased in activated microglia[13-15], suggesting that the increased expression of Iba-1 can be used as a marker for microglial activation[7,13-16]. However, some researchers have found that the activation of microglia in brain tissue is not always accompanied by increased expression of Iba-1. It is believed that Iba-1 can only label microglia and its expression level may not relate to microglia activation in brain tissue[17-18]. Whether the expression of Iba-1 is related to the activation of microglia in DR has not been well studied yet. As for the microglia activation in DR, many literatures reported microglial activation could be reflected by increased Iba-1 expressions examined with Western blot and real-time polymerase chain reaction (PCR) in retina, however no consistent data could be concluded, especially at the specific time points to reflect microglial activation in experiment DR[16,19-20]. In streptozotocin (STZ)-induced mice model, some studies reported that the retinal Iba-1 protein expression was only mildly increased by about 25% with significant difference 2mo after diabetes onset[2,19,21]; while others reported to be increased by about 72% at the same time points[22]. In STZ-induced diabetic rat model, the similar finding was found, *e.g.*, Zhang *et al*[8], using Sprague-Dawley (SD) rats, reported Iba-1 mRNA expression was increased four weeks after STZ injection, while Chen *et al*[5] found no increased in Wistar rats at the same time point. This indicated the species difference might influence a certain protein expression in diabetic rat model. As for the *in vitro* models of microglial activation, few studies with increased Iba-1 protein expression were reported to well mimic microglial activation as evidenced in DR. Most *in vitro* models of microglial activation were induced by lipopolysaccharides (LPS)[14,23-24].

To evaluate whether Iba-1 protein expression could be used as a sensitive marker for microglial activation, we characterized microglial activation both *in vivo* and *in vitro* by examining the expressions of Iba-1, CD-11b, F4/80, as well as inflammatory factors.

MATERIALS AND METHODS

Ethical Approval The animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and The Guides for the Care and Use of Animals (National Research Council and Tongji University). The protocol was approved by the Committee on the Ethics of Animal Experiments of Tongji University (No. TJHBLAC-2020-06).

Antibodies and Reagents The primary and secondary antibodies were listed in Table 1.

Diabetic Rat Model Male SD rats weighing approximately 120 g were purchased from Slaccas (SIBS, Shanghai, China). All rats were housed under a normal 12-hour light/dark schedule with *ad libitum* access to food and water.

The rats were randomly divided into two groups: normal control and diabetic group. Diabetes was rendered by intraperitoneally injected STZ (60 mg/kg, S0130, Sigma), and normal control received equivalent volume of normal vehicle (sodium citrate buffer, pH 4.6, Sigma). Establishment of diabetes was confirmed 24, 48, and 72h after STZ injection by measuring blood glucose levels using a glucometer (Precision PC; Medic, Cambridge, UK). The rats with blood glucose level exceeding 16.7 mmol/L for three consecutive times were considered as diabetic rats. The rats were anesthetized with intraperitoneal injection of sodium pentobarbital (40 mg/kg body weight), and then the rats were sacrificed and the retinas were harvested at the following time points: 1, 2, 4, 8, 12, and 24wk after diabetes onset for Western blot, real-time PCR, and immunofluorescence studies.

Microglial Cell line Culture Mouse microglial cell line (BV2 cells) was cultured in Dulbecco's modified Eagle medium (DMEM) high-glucose medium (Hyclone) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin and 1% streptomycin (Invitrogen). After the cells were cultured under normoxia (5%/95% carbon dioxide and atmospheric mixture) at 37℃ for 12h, they were randomly divided into two groups: normoxia group and hypoxia group. The cells in the hypoxic group were transferred to the hypoxic workstation (whitley H35 hypoxystation, DWS, UK) with 1% oxygen for 24h, and the cells in the normoxia group were further cultured in the normoxic incubator (Thermo) for 24h. The cell lysate was collected for Western blot and real-time PCR examination.

Western Blot The retinas and BV2 cells were lysed in a RIPA buffer (Beyotime Institute Biotechnology, China) for protein extraction. The protein concentration was determined using the bicinchoninic acid protein assay kit (Pierce). For Western blot analysis, 40 μg of total protein (15 μg for Iba-1 detection) was dissolved in 10% (15% for Iba-1) sodium dodecyl sulfatepolyacrylamide gels and transferred electrophoretically onto a nitrocellulose membrane (Millipore). The membranes

Table 1 The information for the primary and secondary antibodies

Antibody	Catalog number	Application (Conc.)	Host	Target species	Vendor
Anti-Iba-1	ab178846	WB (1:1000)	Rabbit	Rat/mouse	Abcam
Anti-Iba-1	019-19741	IF (1:500)	Rabbit	Rat	FUJIFILM Wako Pure Chemical Corporation
Anti-CD11b	NB110-89474	WB (1:500)	Rabbit	Rat/mouse	Novus
Anti-ICAM-1	AF583	WB (1:1000)	Goat	Rat	R&D SYSTEMS
Anti-ICAM-1	ab222736	WB (1:1000)	Rabbit	Mouse	Abcam
Anti-F4/80	27044-1-AP	WB (1:200)	Rabbit	Mouse	Proteintech
Anti-iNOS	ab3523	WB (1:400)	Rabbit	Mouse	Abcam
Anti-COX2	ab15191	WB (1:500)	Rabbit	Mouse	Abcam
Anti-β-actin	HB180823	WB (1:5000)	Rabbit	Rat/mouse	Yeasen
Anti-β-actin	HB180824	WB (1:5000)	Mouse	Rat/mouse	Yeasen
Anti-mouse IgG	925-32210	WB (1:5000)	Goat	Mouse	Li Cor Biosciences
Anti-rabbit IgG	925-68023	WB (1:5000)	Donkey	Rabbit	Li Cor Biosciences
Anti-goat IgG	925-32214	WB (1:5000)	Donkey	Goat	Li Cor Biosciences
Anti-rabbit IgG (Alexa 488)	ab150065	IF (1:500)	Donkey	Rabbit	Abcam

WB: Western blot; IF: Immunofluorescence.

were cut into several blots based on the size of the detected proteins and were blocked with 5% bovine serum albumin (BSA; Sigma) in TBST (50 mmol/L Tris, pH 7.6; 0.9% NaCl; and 0.1% Tween-20) for 30min at room temperature (RT). Then, the blots were separately incubated overnight at 4°C with the primary antibodies (Table 1). After washing thrice, the membranes were incubated with the respective secondary antibodies (Table 1) for 2h at RT. After extensive washing, we examined the blots using the Odyssey infra-red imaging system (LI-COR Biosciences, Lincoln, NE, USA). The densitometric values for the proteins of interest were normalized using β-actin.

RNA Extraction and Real-time PCR Total RNA was extracted from rat retinas and BV2 cells. Reverse transcription was performed and real-time PCR was carried out by using SYBR Green Real-Time PCR master mix (Toybo, Osaka, Japan). The primers were designed by using Primer Premier Version 5.0 software and ordered from Sangon Biotechnology Co. Ltd. (Shanghai, China). The primers information was listed in Table 2.

Immunofluorescentce The rats were sacrificed after satisfactory anesthesia, and the eyeballs were removed and fixed with 2% paraformaldehyde (PFA) for 1h. The anterior segment of the eyeball, including the cornea, iris and lens, was dissected under a microscope (SMZ-168, Motic). The rest of the eyecup was fixed in 2% PFA overnight at 4°C, and was dehydrated in 30% sucrose (S1888, Sigma) for about 3h, and then embedded in optimal cutting temperature compound (Tissue Tek, Sakura, Japan) for cryosectioning. Serial sections (10 μm) were cut on a Leica microtome (Germany) and mounted on adhesion microscope slides (Citoglas Company, Taizhou, China). After dried, the sections were stored at -80°C until use. For immunostaining, the sections were incubated in phosphate buffer saline (PBS) for 10min and washed thrice in PBS; then, they were permeabilized and blocked in antibody buffer (1% BSA, 0.05% TritonX-100, 1×PBS) for 1h at RT. The sections were incubated overnight at 4°C with Iba-1 antibody as detailed in Table 1. The sections without primary antibody served as negative control. After being washed thrice in PBS, the sections were incubated with the appropriate secondary antibody for 1h at RT in the dark. After extensive wash, the sections were coverslipped and were examined under the confocal microscope (A1 R HD25, Nikon, Japan).

Statistical Analysis The data were presented as means± standard error (SE). The statistical analysis was performed using paired *t*-test (SPSS software, version 22.0, IBM Corp., Armonk, NY, USA). A *P* value of 0.05 or less was considered statistically significant.

RESULTS

Blood Glucose Level Increased and Body Weight Decreased in SD Rats After STZ Injection For normal control rats, the blood glucose level remains relatively constant (7.3±0.1 mmol/L) for the whole period, and the body weight was gradually increased with time progression (Figure 1). However, in STZ-injected rats, the blood glucose level was increased significantly 1d after STZ injection and reached a plateau 1wk after diabetes onset (30.2±0.8 mmol/L); the body weigh remains unchanged from 1 to 12wk, which was increase at 24wk after diabetes onset. For example, at 4wk of diabetes, the blood glucose level was increased significantly in diabetic rat compared with that in age-matched normal control (7.5±0.2 *vs* 31.9±0.8 mmol/L, n=12, P<0.001); the body weight was decreased significantly (379±6 *vs* 168±18 g, n=12, P<0.001).

Table 2 Primer information for real-time PCR

Gene	Species	Primer	Sequence (5'-3')	Base pair (bp)	Product size (bp)
Iba-1	Rat	Sense	TCTGAATGGCAATGGAGATA	20	231
		Anti-sense	GTTGGCTTCTGGTGTTCT	18	
CD11b	Rat	Sense	GACTCCGCATTTGCCCTACT	20	109
		Anti-sense	TGCCCACAATGAGTGGTACAG	21	
ICAM-1	Rat	Sense	AAACGGGAGATGAATGGTACCTAC	24	71
		Anti-sense	TGCACGTCCCTGGTGATACTC	21	
Actin	Rat	Sense	GTAAAGACCTCTATGCCAACA	21	227
		Anti-sense	GGACTCATCGTACTCCTGCT	20	
Iba-1	Mouse	Sense	TGAGGATCTGCCGTCCAAACTT	22	108
		Anti-sense	TTCTCCAGCATTCGCTTCAAGG	22	
F4/80	Mouse	Sense	CCTGCTGTGTCGTGCTGTTCA	21	120
		Anti-sense	GCCGTCTGGTTGTCAGTCTTGT	22	
CD11b	Mouse	Sense	AGCATCAATAGCCAGCCTCAGT	22	134
		Anti-sense	CCAGGTCCATCAAGCCATCCAT	22	
ICAM-1	Mouse	Sense	CGCTGTGCTTTGAGAACTGTG	21	62
		Anti-sense	ATACACGGTGATGGTAGCGGA	21	
iNOS	Mouse	Sense	TGGCTTGCCCCTGGAAGTTT	20	110
		Anti-sense	GCTGAGAACAGCACAAGGGG	20	
COX2	Mouse	Sense	CATCCCCTTCCTGCGAAGTT	20	178
		Anti-sense	CATGGGAGTTGGGCAGTCAT	20	
IL-6	Mouse	Sense	CGGAGAGGAGACTTCACAGAGG	22	161
		Anti-sense	GCAAGTGCATCATCGTTGTTCA	22	
IL-1β	Mouse	Sense	CTCGCAGCAGCACATCAACAAG	22	200
		Anti-sense	CGTCACACACCAGCAGGTTATC	22	
Actin	Mouse	Sense	AGGCGACAGCAGTTGGTTGGA	21	166
		Anti-sense	TTGGGAGGGTGAGGGACTTCCT	22	

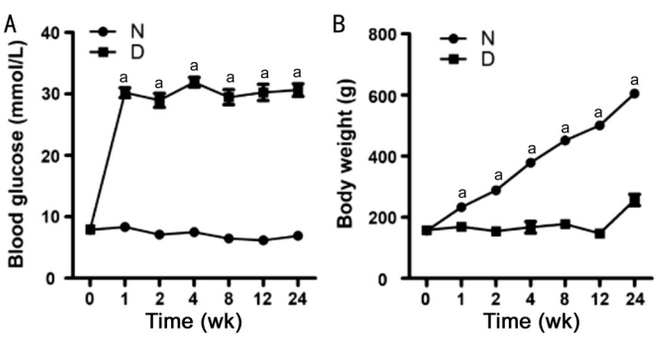

Figure 1 Blood glucose level and body weight in normal control and diabetic rats with time A: Blood glucose level; B: Body weight. n=12 for 0-8wk; and n=9 for 12 and 24wk. ^aP<0.05 versus age-matched control. N: Normal control; D: Diabetic rats.

Microglia Activated with Diabetes Progression Detected by Iba-1 Immunostaining To detect microglial activation in diabetic rat retinas, we used Iba-1 immunostaining. Under normal condition, microglia displayed a ramified morphology; while in diabetes, it became activated with amoeboid morphology (Figure 2). As shown in Figure 3, in age-matched normal control, Iba-1 positive cells were mainly distributed in the inner retina, *i.e.*, nerve fiber layer (NFL), ganglion cell layer (GCL), inner plexiform layer (IPL), rarely located in outer plexiform layer (OPL). The microglia were demonstrated as ramified morphology and the number of microglia remains constant throughout the detecting period. However, in diabetic rat retinas with disease progression, the microglial cells became activated rapidly with amoeboid morphology 1wk after diabetes onset and migrated from inner retina to outer retina with increasing cell numbers. The most obvious change of microglia was detected at 12wk of diabetes. The above data indicated that Iba-1 immunostaining could be used as a sensitive marker to detect microglial activation with cell number, characteristic morphology and territory distribution in diabetic retinas, which urged us to detect its protein and mRNA expressions to see whether or not the same changes was found.

Protein and mRNA Expressions of Iba-1 was Inconsistent with its Immunostaining Result in Rat Retinas with Diabetes Progression To detect whether or not the same changes with Iba-1 immunostaining was found in Iba-1 protein and mRNA expressions, we detected Iba-1 expression with Western blot and real-time PCR. At the same time, the protein

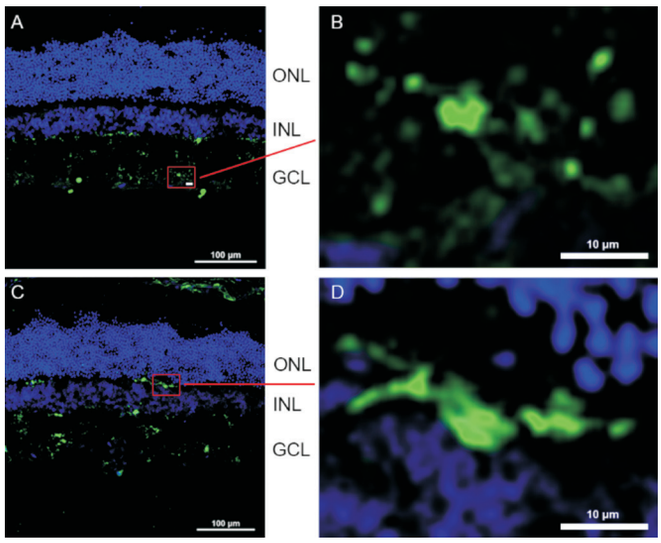

Figure 2 Different morphological characteristics of microglia in retina Microglia was immunostained with Iba-1 (green) in age-matched normal control (A and B) and 8-week diabetic rat retinas (C and D). The figures demarcated with rectangle in A and C were magnified in B and D accordingly, showing the ramified (B) and amoeboid (D) morphologies. ONL: Outer nuclear layer; INL: Inner nuclear layer; GCL: Ganglion cell layer. Scale bar: 100 μm in A and C, 10 μm in B and D.

and mRNA expressions of CD11b and ICAM-1 were detected to confirm microglial activation. As shown in Figure 4A, the protein expressions of Iba-1 was increased slightly in diabetic rat compared with that in age-matched normal control for the whole period, but the most obvious increase was detected at 2wk of diabetes (1.75±0.25 *vs* 1.15±0.19, n=6, P=0.029). For the mRNA expression of Iba-1, inconsistent with its protein result, it was decreased significantly at 2 (0.71±0.13 *vs* 1.00±0.06, n=6, P=0.037) and 4 (0.55±0.06 *vs* 1.00±0.11, n=6, P=0.033)wk of diabetic rat retinas, and remained unchanged at 8 and 12wk of diabetes (Figure 4B). For CD11b, compared with that in age-matched normal control, its protein expression was increased significantly at 12 (3.74±1.35 *vs* 1.10±0.18, n=10, P=0.036) and 24 (2.53±0.51 *vs* 0.98±0.18, n=10, P<0.001)wk of diabetes (Figure 4C), and its mRNA expression level was significantly increased at 8 (1.18±0.06 *vs* 1.00±0.07, n=6, P=0.028) and 12 (1.17±0.07 *vs* 1.00±0.07, n=5, P=0.039)wk of diabetes (Figure 4D). For ICAM-1, as shown in Figure 4E and 4F, its protein expression was increased in a time-dependent manner compared with that in normal control, which became statistically significant from 8wk of diabetes (2.80±0.31 *vs* 1.55±0.20, n=10, P<0.001), however, its mRNA expression was increased significantly only at 2wk after diabetes onset (1.56±0.17 *vs* 1.00±0.11, n=6, P=0.020). The above data indicated that the animal model of DR was successful, but the change of Iba-1 protein and mRNA expressions was not the same with its immunostaining in rat retinas with DR.

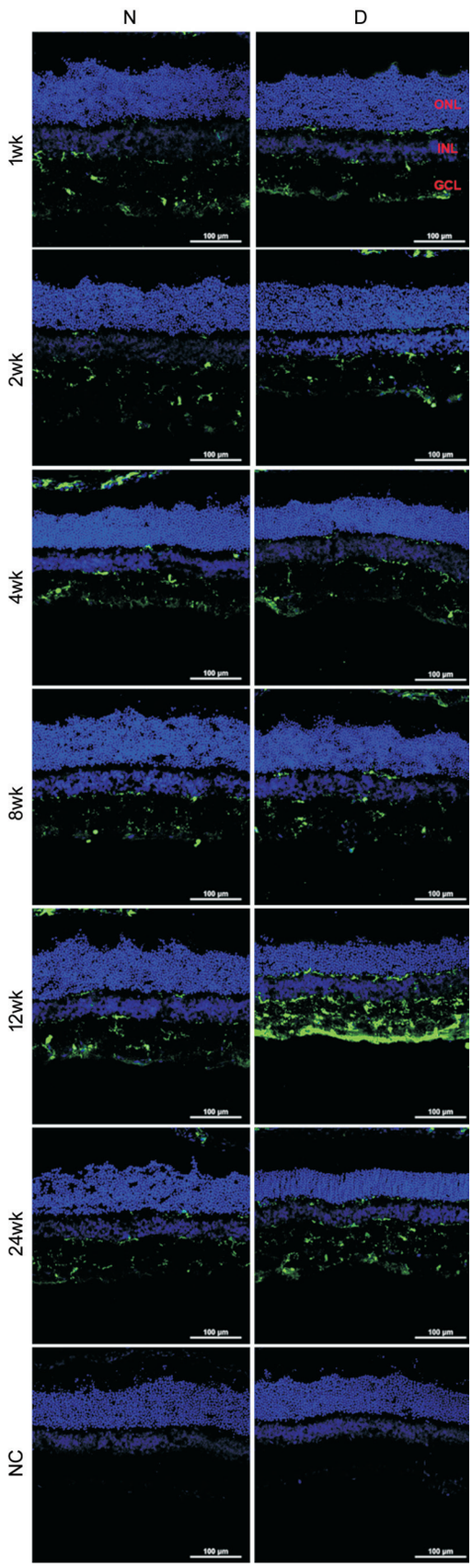

Figure 3 The changes of microglia in normal control and diabetic rat retinas The retina was immunostained with Iba-1 antibody (green). The nuclei were counterstained with DAPI (blue). N: Normal control; D: Diabetic rat; NC: Negative control; wk: Weeks after STZ injection; ONL: Outer nuclear layer; INL; Inner nuclear layer; GCL: Ganglion cell layer. Scale bar: 100 μm.

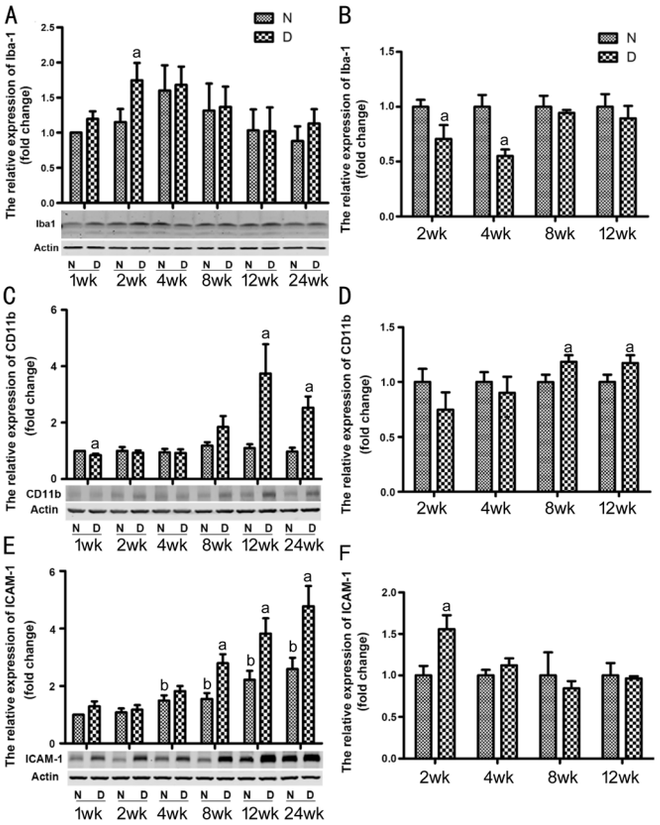

Figure 4 The expressions of Iba-1, CD11b and ICAM-1 in rat retinas with diabetes progression The protein and mRNA expressions of Iba-1 (A, B), CD11b (C, D) and ICAM-1 (E, F) were detected in rat retinas of normal control and diabetic rats from 1 to 24wk of diabetes. n=6 in A, B, D, and F; n=10 in C and E. [a]P<0.05 compared with the age-matched normal control; [b]P<0.05 compared with the normal control at 1wk. N: Normal control; D: Diabetic rat.

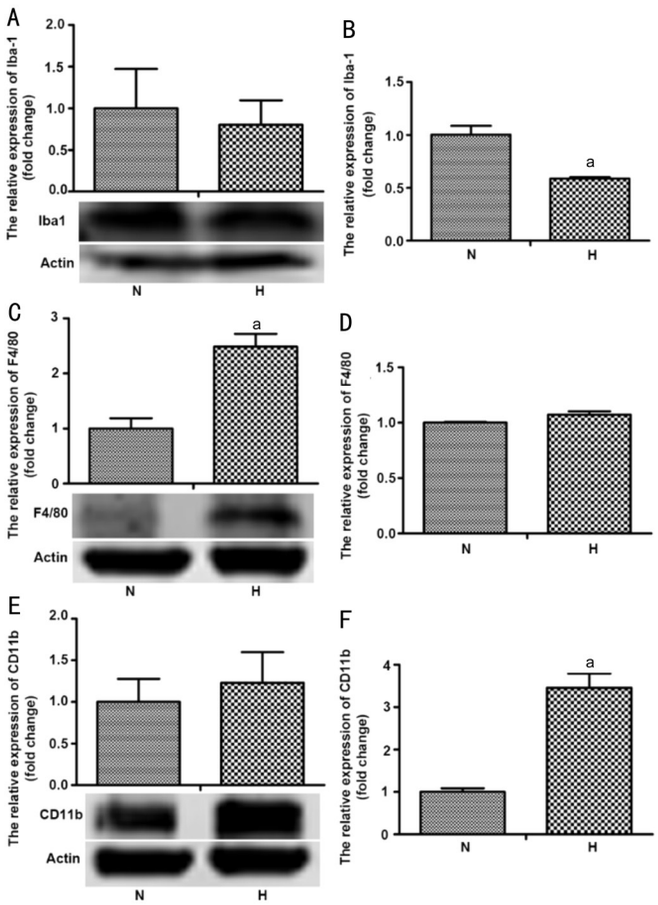

Figure 5 The expressions of Iba-1, F4/80, and CD11b in BV2 cells under normoxia and hypoxia The protein and mRNA expressions of Iba-1 (A, B), F4/80 (C, D) and CD11b (E, F) in BV2 cells under normoxia (N) and hypoxia (H). n=6 in A, C, and E; n=3 in B, D, and F. [a]P<0.05 compared with N.

F4/80 and CD11b was Increased in BV2 Cells Under Hypoxia To detect the change of Iba-1 expression in BV2 cells under hypoxia, we used Western blot and real-time PCR. At the same time, two markers of microglial activation (F4/80 and CD11b) were also detected. As shown in Figure 5, there was no significant increase for Iba-1 protein expression in BV2 cells under hypoxia; however, its mRNA level was decreased significantly under hypoxia (0.59±0.01 *vs* 1.00±0.08, n=3, P=0.039) with unknown reasons (Figure 5A and 5B). For F4/80, compared with normoxia group, its protein expression was increased significantly (2.48±0.23 *vs* 1.00±0.19, n=6, P=0.001), while its mRNA level remained unchanged (Figure 5C and 5D). For CD11b, its protein expression was increased slightly under hypoxia, but its mRNA expression was increased dramatically (3.46±0.34 *vs* 1.00±0.08, n=3, P=0.012, Figure 5E and 5F). The above data indicated that microglia were activated under hypoxia, which cannot be well reflected only by Iba-1 protein expression.

Increase of Inflammatory Factors Confirmed the Activation of Microglia Under Hypoxia To further confirm the activation of microglia under hypoxia, we detected several inflammatory factors including iNOS, COX2, ICAM-1, IL-1β and IL-6. For BV2 cells under hypoxia, the protein and mRNA expressions of iNOS, COX2, and ICAM-1 were increased significantly (Figure 6), *i.e.*, ICAM-1 (1.97±0.52 *vs* 1.00±0.20, n=6, P=0.031 for protein and 1.90±0.14 *vs* 1.00±0.11, n=3, P=0.007 for mRNA), iNOS (5.38±0.48 *vs* 1.00±0.25, n=6, P=0.002 for protein and 48.73±9.28 *vs* 1.00±0.14, n=6, P=0.004 for mRNA), and COX2 (2.29±0.16 *vs* 1.00±0.11, n=6, P=0.003 for protein and 15.69±2.12 *vs* 1.00±0.09, n=3, P=0.019 for mRNA). We also detected the mRNA expressions of IL-1β and IL-6 in hypoxia-treated BV2 cells. As shown in Figure 6, the mRNA levels were about 2.63- (IL-1β) and 3.54-fold (IL-6) of that in normal control (n=3, P<0.05).

DISCUSSION

Microglial activation was reported in many fundus diseases, like DR and age-related macular degeneration (AMD)[1,5-6,25], in which it played a detrimental effect on neuronal cells. So, it is important to study the mechanisms of microglial activation and to find effective treatment in these diseases. In this study, we attempted to find out the correlation between Iba-1 expression and microglial activation, but found Iba-1 protein expression

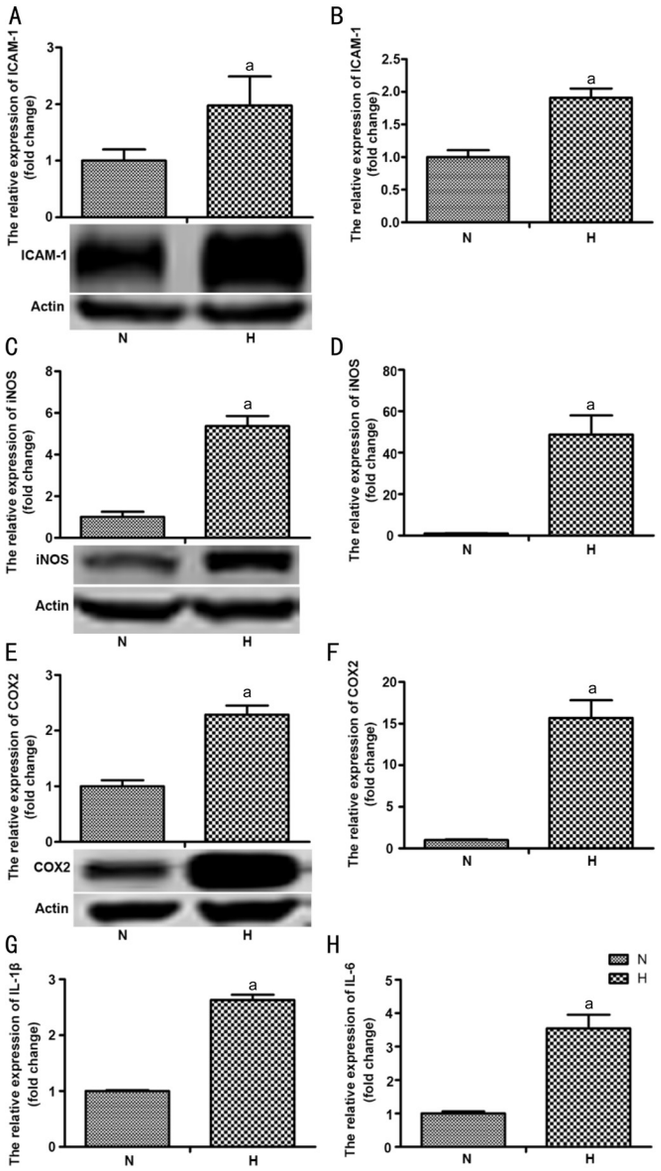

Figure 6 The expressions of inflammatory factors in BV2 cells under normoxia and hypoxia The protein and mRNA expressions of ICAM-1 (A, B), iNOS (C, D) and COX2 (E, F) in BV2 cells under normoxia and hypoxia. The mRNA expressions of IL-1β (G) and IL-6 (H) in BV2 cells under normoxia and hypoxia. N: Normoxia, H: Hypoxia. n=6 in A, C, D, and E; n=3 in B, F, G, and H. $^aP<0.05$ compared with N.

in the diabetic rat retinas was not really correlated to microglial activation. This finding was consistent with the previous studies in brain tissue[17-18], possibly due to less abundance of Iba-1 protein. To establish an *in vitro* model to mimic microglial activation in DR, we tried several culture systems to treat BV2 cells, including high glucose, glyoxal, $CoCl_2$, and hypoxic incubation, but no significant increase of Iba-1 protein was detected. The puzzle results urged us to detect other markers with different techniques to study microglial activation both *in vivo* and *in vitro*.

The *in vivo* results of this experiment indicated that Iba-1 immunostaining could be reflect microglial activation with morphological change, distribution and increasing numbers with diabetes progression, consistent with previous reports[5,26-27]. In our study, the most obvious change of microglia was detected at 12wk of diabetes which was basically consistent with CD11b protein expression. As for Iba-1 mRNA expression, however it was inconsistent with the protein changes detected with Western blot and its immunofluorescence. The most significant increase of Iba-1 protein expression was detected at 2wk of diabetes. However, its mRNA expression was decreased significantly at 2 and 4wk of diabetes. These results indicated that Iba-1 immunostaining could be used as a sensitive marker to detect microglial activation with cell number, characteristic morphology and territory distribution in diabetic retinas, but its protein expression might not be a sensitive marker to evaluate the activation of microglia in experimental DR.

To confirm the activation of microglia, we detected CD11b (microglial mareker) and ICAM-1 expressions *in vivo*, and found the protein as well as mRNA expressions were increased with diabetes progression, especially the increase of ICAM-1 with a time-dependent manner. ICAM-1 was also an inflammatory factor, and was reported to be produced and secreted by activated microglia[21,28-29], which mediated the breakdown of inner blood-retinal barrier.

In order to study the activation of microglia, we established an *in vitro* model, in which BV2 cells were cultured under hypoxia (1% oxygen). With this system, we detected the microglial activation markers as well as inflammatory molecules. Unexpectedly, the Iba-1 protein expression did not alter, while its mRNA level was decreased significantly. However, other parameters, like F4/80, CD11b and inflammatory factors (ICAM-1, iNOS, COX2, IL-6, IL-1β) were significantly increased compared with the normal control. The inconsistent result with Iba-1 between *in vivo* and *in vitro* suggested that Iba-1 alone might not be a good marker to evaluate microglial activation in experimental DR.

Based on the above experimental results, we speculated that Iba-1 could be used to label microglia/macrophages specifically, but under the condition of microglial activation in experimental DR, the increase of Iba-1 protein was very limited to be well recognized. Another possibility might be the low abundance of Iba-1 protein due to relatively less number of microglial cells in the retina.

Due to weak adhesion of BV2 cells, the acquisition of Iba-1 immunostaining for the activated microglia were largely limited. It merits further study to characterize microglial activation *in vitro* by using primary microglial cells and the other immortalized cell lines, such as HMC3 and EOC2. In addition, serial biomarkers and the optimal combination for microglia activation warrant further study.

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