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Regulatory T cells in the pathogenesis of type 2 diabetes mellitus retinopathy by miR-155

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Abstract. – OBJECTIVE: To explore the regulatory T cells (Treg) in the peripheral blood of diabetic retinopathy patients by microRNA-155 (miR-155), and investigate the mechanisms of regulatory T cells and miR-155 in the pathogenesis of diabetic retinopathy.

PATIENTS AND METHODS: The study explores the percentage of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells (Treg cells) and the expression of miR-155 in the peripheral blood of 20 cases with background diabetic retinopathy (BDR group) and 20 cases with proliferative diabetic retinopathy (PDR group). Flow cytometry and RT-PCR determined 18 cases with non-diabetic retinopathy (NDR group) and 20 cases of healthy control (NC group). ELISA determined the expression of TGF-β.

RESULTS: The percentages of Treg cells in the peripheral blood of patients in BDR group, PDR group, and NDR group had significantly decreased compared to that in the NC group ($p < 0.05$). The percentages of the Treg cells in the BDR and PDR groups were lower than those in NDR group ($p < 0.05$ in both cases). The percentage of Treg cells in the PDR group was lower than that in the BDR group ($p < 0.05$). The expression levels of miR-155 in the peripheral blood of the patients in the BDR group, PDR group, and NDR group had significantly increased compared to that in NC group ($p < 0.05$). The expression levels of miR-155 in the BDR group and PDR group were higher than that in the NDR group ($p < 0.05$ in both cases). The expression level of miR-155 in the PDR group was higher than that in the BDR group ($p < 0.05$). The expression levels of TGF-β in the BDR group and PDR group were significantly decreased compared to those in the NDR group and NC group ($p < 0.05$ in both cases). The expression of miR-155 was negatively related to the Treg cells and the expression level of TGF-β_2 ($r_1 = -0.835$, $p_1 = 0.000$, $r_2 = -0.771$, $p_2 = 0.000$).

CONCLUSIONS: In type 2 diabetes mellitus (T2DM) retinopathy, miR-155 may play an important role in the pathogenesis of T2DM retinopathy by regulating the Treg cells with TGF-β.

Key Words:
miR-155, Type 2 Diabetes Mellitus, Diabetic retinopathy, Regulatory T cells.

Introduction

Diabetes mellitus (DM)[1-3] is a metabolic disease, which is characterized by chronic elevated blood glucose. The damage to the body is due to various systemic complications. In various complications of DM, diabetic retinopathy (DR)[4-6] is a common microvascular complication. The DM patients with a disease course > 10 years usually have retinopathy of various degrees. However, the cause of DM has not been clarified. Recently, a new non-coding RNA, microRNA (miRNA) has been an important regulator of various biological functions. The change in the expression level of miRNA can not only cause chronic inflammatory responses in DM patients, but also lead to dysfunction and decreased number of pancreatic islet β cells with organized insulin resistance[7-8]. These changes promote dysfunction in other tissues, including the retina, kidney, heart, etc[9]. Therefore, miRNA plays important roles in diabetic complications[10]. Regulatory T cells are special immune-modulatory cells. Many studies demonstrated that the changes in the number and functions of Treg in the body were closely associated with the pathogenesis and development of various diabetic complications[11,12]. Moreover, many studies showed that miRNAs could be involved in the pathogenesis and development of various diseases through regulating the proliferation, functions, cytokines and intracellular signal transduction pathways in Treg cells[13]. This study analyzed the Treg cell percentage, the expression of miR-155 and TGF-β in the

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peripheral blood of T2DM retinopathy patients and explored the pathogenesis mechanism of T2DM retinopathy patients.

Patients and Methods

Patients

T2DM patients were divided into 3 groups based on ophthalmoscopy and fluorescein angiography: 20 cases of no apparent retinopathy (NAR), 20 cases of background DR (BDR) and 20 cases of proliferative DR (PDR). All T2DM retinopathy patients were in-patients who were diagnosed in the Diabetes Specialty Department in the hospital. The male/female ratio was 1:1, the age range was 38-70 years with a mean age of 52 years. Twenty healthy physical-examination persons who had no diabetes or ophthalmic disease history were in the NC group, with 10 male and 10 female; the age range was 35-65 years with a mean age of 50 years. All in-patients had no heart, liver or kidney disease history, or other allergic history, had not received any corticosteroids or any other immunosuppressive agents. There was no statistical difference in the age distribution in 4 groups.

Reagents and Instruments

Ficoll human lymphocyte separation solution (Tianjin Haoyang Biological Products Technology Co., Ltd.), human regulatory T cell kit (including FITC-CD4, APC-CD25 antibody, PE-Foxp3 antibody, PE-mouse IgG2a isotype control antibody, fixation solution, permeation solution, normal mouse serum (eBioscience, San Diego, CA, USA), Trizol reagent, PrimeScript™ RT reagent kit (Perfect Real Time), SYBR® Premix Ex Taq™ II (Tli RNaseH Plus) (all purchased from Takara, Otsu, Shiga, Japan). Shanghai Sangon synthetized the primers. RNase-free purified water (Ailaipu Biotechnology Co., Ltd. Tianmen, China), CFX96™ Real Time PCR Detection System amplifier, flow cytometer (BD, San José, CA, USA).

Methods

A quantity of 4 mL venous blood was collected with a vacuum blood tube (heparin anticoagulant tube for flow cytometry, EDTA anticoagulant tube for RT-PCR), diluted with equal volume of phosphate buffered saline (PBS), added the blood into a 15 mL centrifuge tube, which had been previously added to 3 mL lymphocyte separation solution and centrifuged horizontally at 2000 rpm for 20 min. Then the middle cloud layer was transferred to another centrifuge tube with a micro pipettor, washed with PBS for 3 times, adjusted the cell concentration to $1*10^7$/ml, and stored at 4°C.

Then we added 100 µl above mentioned peripheral blood mononuclear cells (PBMC) suspension in each tube: blank tube (1), isotype control tube (2) and treatment tube (3). Followed by 200 µl pre-mixed CD4/CD25 antibody in tube 2 and tube 3, but not in tube 1, and incubated at 4 for 30 min. It was then twice washed with FACS staining solution. Freshly prepared 1 mL fixation/permeation solution was added to each tube, mixed well and incubated at 4 for 45 min and then washed twice with permeation solution. The cells were suspended and 2 µl normal mouse serum was added to tube 2 and tube 3, but not in tube 1, and then incubated at 4°C for 15 min. A quantity of 20 µl Foxp3 antibody was added to tube 3, and 20 µl mouse IgG2a antibody was added to tube 2, but not in tube 1, then incubated at 4°C for 45 min. The cells were washed with permeation solution twice and suspended and then loaded onto a flow cytometer.

A quantity of $1*10^7$ PBMCs was added in a 1.5 mL Eppendorf (EP) tube, and the total RNA was purified with Trizol reagent. The RNA concentration and purity was determined with a UV spectrophotometer, and gel electrophoresis determined the RNA integrity. The RT-PCR was performed as required in PrimeScript™ RT reagent kit and SYBR® Premix Ex Taq™ II kit. Shanghai Sangon synthetized all primers. The TGF-β level in the plasma was determined by ELISA, which was performed as required in the kit instruction.

Statistical Analysis

Data analysis was performed using the SPSS 17.0 software (SPSS Inc., Chicago, IL, USA), and the measurement data in the normal distribution was represented by $x \pm s$. The *t*-test was used for group to group comparison, ANOVA or rank sum test was used for the comparison between multiple groups, and the Pearson correlation analysis was used for correlation analysis. $p < 0.05$ indicated statistical significance.

Results

The Treg percentage and TGF-β expression in the peripheral blood in each group, as shown in Tables I and II.

Table I. The percentage of Treg in the peripheral blood in each group (%, $\bar{x} \pm s$).

Group	$CD4^+ CD25^+ Foxp3^+$
NDR group (n = 18)	5.379 ± 0.388^a
BDR group (n = 20)	$4.673 \pm 0.535^{a,b}$
PDR group (n = 20)	$3.406 \pm 0.5931^{a,b,c}$
NC group (n = 20)	2.744 ± 0.636

Note: In comparison with NC group, $^a p < 0.01$; in comparison with NDR group, $^b p < 0.01$; in comparison with BDR group, $^c p < 0.01$.

As the data was in percentages, which was not in the normal distribution, the Kruskal-Wallis rank sum test ($\chi^2 = 60.478$, $p = 0.000 < 0.01$) was used. The percentages of the Treg cells ($CD4^+$ $CD25^+$ $Foxp3^+$ T cells/$CD4^+$ T cells %) in the BDR group, PDR group, NDR group and NC group were different. Further comparison by Bonferroni method indicated that any group to group comparison between the BDR group, PDR group, NDR group and NC group was statistically significant ($p < 0.05$). Note: in comparison with NC group, $^a p < 0.01$; in comparison with NDR group, $^b p < 0.01$; in comparison with BDR group, $^c p < 0.01$.

The relative expression level of miR-155 in each group, as shown in Figure 1.

The expression level of TGF-β, as shown in Table II.

The correlation analysis of miR-155 and Treg cells as well as TGF-β in the peripheral blood in DR patients: miR-155 was negatively related to Treg cells (r = -0.835 $p = 0.000$) and TGF-β (r = -0.771, $p = 0.000$).

Discussion

With the change of lifestyle and an increasing obese population and elongation of mean lifespan, the incidence of diabetes has been gradually increasing, especially for T2DM. According to the latest figures by International Diabetes Federation (IDF), there will be at least 552 million diabetic patients by 2030 worldwide[14]. Of the various diabetic complications, DR is the most common and has been a major cause of blindness. The prevalence of DR increases with the course of diabetes. After 20 years of DR, most T1DM patients and more than 60% T2DM patients have DR[1]. In 2010, it is estimated that 93 million patients have DR globally, and 28 million patients face the threat of impaired vision due to DR. This number will continue to increase with the ageing of the population, obesity and fast prevalence of diabetes. This makes DR a global public health and economic problem[15,16].

Table II. The expression level of TGF-β in each group (pg/ml, $\bar{x} \pm s$).

Group	TGF-β
NDR group (n = 18)	154.37 ± 63.19
BDR group (n = 20)	243.78 ± 70.10
PDR group (n = 20)	374.64 ± 79.39
NC group (n = 20)	119.47 ± 50.63

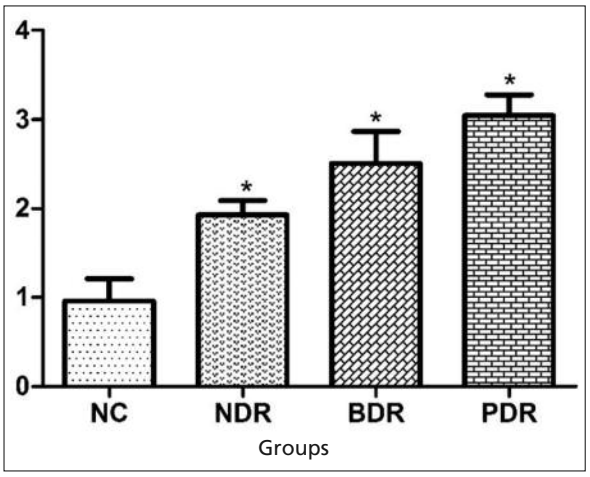

Figure 1. The relative expression level of miR-155 in each group. *Note:* *In comparison with NC group, $p < 0.01$ in all cases; $p < 0.01$ for group-group, comparison.

The basic pathological change of DR is retinal neovascularization and fibrosis hyperplasia. Many recent studies indicate that DR is an inflammatory disease. It was reported that a new non-coding gene, the microRNA was involved in the post-translational regulation. MicroRNA is an important regulator of various biological functions. There are more than 2000 human miRNA listed in miRBase that can regulate up to 60% known gene transcription[8]. The number and targets of the miRNA are so many that miRNA is involved in the pathogenesis and development of many diseases, including DR. Various post-translational levels of miRNA can regulate unbalanced inflammation by regulating inflammatory factors and signal pathway. The miR-155 is a typical multifunctional miRNA and essential to the immune response. It was reported that miRNA-155 could regulate some cytokines secreted

by T cells. Moreover, miRNA-155 is a Foxp3-dependent miRNA[17,18]. Regulatory T cells are characterized by the Foxp3 expression and the secretion of TGF-β and Interleukin-10 (IL-10). It can play anti-inflammatory roles through contact inhibition, and is a special T cell sub-population of low response and immune suppressive function. With the stimulation of antigens, the Treg cells can hardly proliferate, inhibit the activation and proliferation of $CD4^+$ T cells and $CD8^+$ T cells, and play important regulatory roles in many inflammatory diseases. Both the differentiation and function maintenance of Tregs are dependent on the expression of the Foxp3, which is considered the key transcriptional factor in Treg cells[19-21]. Yao R[22] found that although miR-155 had no regulation roles in the immune suppressive function of the Treg cells, miR-155 could regulate the Treg cell differentiation and Foxp3 expression. The up-regulation of miR-155 was important to the increased Treg cell response. Moreover, Anagha et al[15] found that miR-155 could influence CD62L, cause decreased CD62L level and further change the phenotype of the Treg cells[23-25].

This study demonstrated that the Treg cell levels in DR patients were significantly lower than those in healthy controls and varied dynamically with the development of the disease. The Treg cell count decreased significantly in PDR, which indicates that the change of autoimmune factors are due to abnormal Treg cells which were involved in the pathogenesis and development of DR. With the change in the Treg cell level, the secretion pro-inflammatory factors and the expression of transcriptional factor TGF-β were down-regulated accordingly. Moreover, the expression of miR-155 in PBMC in DR patients had increased, which was consistent with other reports[7]. The expression of miR-155 was negatively related to both Treg cells and TGF-β[26-28]. It was reported that the low expression level of miR-155, Treg cells and TGF-β were closely related to the pathogenesis and development of DR. This indicated that the higher miR-155 expression level, the more possible the occurrence of DR in diabetic patients. However, in the case of Treg cells and TGF-β, the correlation was negative. TGF-β is a multifunctional growth factor and can be secreted by many immune cells. It can regulate cellular development, differentiation, apoptosis and immune response based on the cell types and internal environment, and play a critical role in the various cellular processes[29,30]. There are 3 types of TGF-β in mammals, i.e., TGF-β1, TGF-β2, and TGF-β3. TGF-β1 is the main expression form in the immune system. After binding to the receptor, the TGF-β can phosphorylate the transcriptional factor Smad downstream, and regulate the transcription of the target gene by binding the DNA with other transcriptional factors. Moreover, TGF-β can activate transcriptional factor STAT5 through IL-2. STAT5 binds to Foxp3 the promoter and promotes the differentiation of iTreg. Both the development of Treg cells and its transcriptional factor Foxp3 are dependent on the presence of TGF-β. TGF-β can induce the increase of Foxp3 gene expression in peripheral naïve $CD4^+$ $CD25^-$ $Foxp3^-$ T cells, which can develop into $CD4^+$ $CD25^+$ $Foxp3^+$ Treg cells. It was reported that in transplantation model, specific TGF-β antibody could significantly decrease the level of Treg cells in recipients. It was reported that miR-155 could antagonize Smad signal pathway by regulating JAK/STAT (Janus Kinase/Signal Transducer and Activator of Transcription) signal pathway. Thus it was speculated that miR-155 may inhibit the biological activity of TGF-β. The regulation of Treg cell proliferation by increased miR-155 had certain immune suppressive effect. Meanwhile, relatively low level of TGF-β could not effectively promote Treg cell development. As a result, Treg cell functional deficiency aggravated. With the dual effects of pro-inflammation and immune suppression, this promoted the pathogenesis of DR. Thus, it was speculated that the regulation of TGF-β and Treg cells by miR-155 may be a pathway in the unbalanced regulation network of DR, however, the precise mechanism and signal pathway of the regulation effect were unknown and required in-depth investigation.

Conclusions

This study explored the roles of miR-155, Treg cells and TGF-β in the pathogenesis of DR, and provided new viewpoints for the mechanism of the pathogenesis of DR. The early diagnosis of DR at genetic, cellular and molecular levels could be feasible, and may be a new target for the intervention of the pathogenesis, development and treatment of DR.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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