Th1/Th2 cytokine expression in diabetic retinopathy

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ABSTRACT. Diabetic retinopathy (DR), an important complication of diabetes mellitus (DM), is not well understood. T helper cell balance (Th1/Th2) is involved in various autoimmune diseases; however, its role in DR is not understood. This study explores changes in Th1 and Th2 cytokine expression during DR. Blood samples were collected from 25 healthy volunteers (normal control group), 35 patients with type 2 DM (T2DM group) without DR, and 30 cases of T2DM patients with DR (DR group). Real-time PCR was used to measure mRNA expression of IL-2 and TNF-α, secreted from Th1 cells, and of IL-4 and IL-10, secreted from Th2 cells. We used ELISA to detect cytokine expression in serum to analyze the correlation between Th1 and Th2 cytokines. IL-2 and TNF-α mRNA and protein expression
levels in the T2DM and DR groups were significantly higher than in the normal control group (P < 0.05). Compared with the T2DM group, the DR group had higher IL-2 and TNF-α levels (P < 0.05). IL-4 and IL-10 levels were lower in the DR group compared with the normal and T2DM groups (P < 0.05), while T2DM showed no difference compared with the normal control (P > 0.05). IL-2 and TNF-α were negatively correlated with IL-4 and IL-10 in the DR group, respectively. We found that Th1 cytokine secretion was higher and Th2 cytokines secretion was lower during DR, leading to a Th1/Th2 imbalance, suggesting that Th1/Th2 imbalance is a side effect for DR occurrence and development.

Key words: Diabetic retinopathy; Th1; Th2

INTRODUCTION

Following economic level improvement and lifestyle changes, the incidence of diabetes mellitus (DM) increased and the disease includes both type 1 DM (T1DM) and type 2 DM (T2DM), among which T2DM has the highest incidence in our country (Alexander et al., 2015; Wang et al., 2015b). Diabetic retinopathy (DR), a common ophthalmologic disease that causes blinding eye disease worldwide, is a serious and common diabetic microvascular complication and occurs in more than 30% of DM patients, especially in T2DM patients. According to the World Health Organization (WHO) survey, DR, due to its high incidence, can cause permanent damage, such as neovascular macular degeneration and polypoidal choroidal vasculopathy, if not controlled effectively and timely (Zhao et al., 2015). DM is an autoimmune and chronic progressive inflammatory disease. Additionally, the main inflammatory changes of DR include leukocyte infiltration and adhesion in the retinal microvascular system, leading to complex pathophysiological processes, such as capillary basement membrane thickening, blood-retinal barrier leakage, capillary cell apoptosis, and retinal detachment (Wilkinson-Berka et al., 2006). Inflammatory cytokines and inhibiting-inflammatory cytokines, such as TNF-α and interleukin-10 (IL-10), are secreted abnormally in DR patients, causing organ dysfunction and further inducing disease progression (Boynton et al., 2015; Garcia et al., 2015).

T helper cells (Th) are divided into two subgroups, Th1 and Th2, according to the secreted cytokines (Song et al., 2015a). Recent studies found that Th1 and Th2 cells regulate and suppress each other by mediating cytokine secretion and that maintaining an appropriate Th1/Th2 balance is important for normal immune function (Vargas-Rojas et al., 2015). Th1 cells mainly secrete IL-2, TNF-α, and other cytokines, while Th2 cells mainly secrete IL-4 and IL-10 (Tian et al., 2015). Th1/Th2 maintains a steady state by adjusting cytokine secretion, which is important for regulating cellular and humoral immunity (Kornet et al., 2015). T1DM occurrence is closely associated with T cell mediated organ specific autoimmune disease due to viruses, bacteria, or drug effects (Wang et al., 2015a). T1DM occurrence and pathogenicity are closely related to cytokine level, which causes CD4+ T cells to differentiate to Th1 subgroup (Anand et al., 2014; Kong et al., 2015). However, the role of Th1 and Th2 cytokines in T2DM patients with DR is complicated and has not been fully elucidated. This study explores Th1 and Th2 cytokine expression and balance in DR.
MATERIAL AND METHODS

Patients

Sixty-five T2DM patients in Affiliated Hospital of Weifang Medical University, between January 2012 and December 2014 were enrolled, including 35 T2DM patients (T2DM group) and 30 T2DM patients with DR (DR group). There were 18 males and 17 females in the T2DM group, with an average age of 42.1 ± 13.7 (32-67) years old and an average body mass index (BMI) of 23.2 kg/m². In the DR group, there were 16 males and 14 females, with an average age of 40.1 ± 15.2 (35-65) years old and an average BMI of 22.1 kg/m². Study inclusion criteria conform to the T2DM and T2DM with DR diagnostic criteria published by the WHO in 2014 (Yordanova et al., 2014). Exclusion criteria included patients with other endocrine system or autoimmune diseases. Another 25 cases of healthy volunteers were selected as the normal control group, which included 13 males and 12 females, with an average age of 41.3 ± 11.5 (31-66) years old and an average BMI of 22.6 kg/m². The oral glucose tolerance test was normal in the control group, and other immune related diseases were ruled out. No obvious differences in gender, age, or BMI were found among the three groups (P > 0.05). This study was approved by the Medical Ethics Committee in our hospital and all subjects gave signed informed consent.

Reagents

TRIzol, RNA extraction kit, RT-PCR primers, reverse transcription kit, and real-time PCR reagents were bought from Invitrogen. IL-2, TNF-α, IL-4, and IL-10 ELISA kits were obtained from eBioscience (San Diego, CA, USA). A Labsystems Version 1.3.1 microplate reader was obtained from Bio-Rad (Hercules, CA, USA).

Blood sample collection

Blood was extracted from the elbow superficial vein from the patients within 24 h of achieving a fasting state. We used 5 mL blood for biochemical index detection. The other 5 mL blood was centrifuged at 3000 g for 15 min, and the serum was stored at -80°C.

Blood glucose detection

Fasting blood glucose (FBG), 2 h postprandial blood glucose (P2hG), and glycosylated hemoglobin (HbA1c) were detected with automatic biochemical analyzer, (BioSino, Beijing, China), according to the manufacturer instruction.

Real-time PCR for the evaluation of Th1 and Th2 cytokine expression

Total RNA was extracted from peripheral blood using TRIzol and purity was detected by ultraviolet spectrometry photometer according to the instruction (Beckman Coulter, Brea, CA, USA). Total RNA was reverse transcribed to cDNA according to the manufacturer protocol by using primers designed by Primer Premier 6.0. The primers were synthesized by Sangon and are listed in Table 1. The real-time PCR cycle consisted of 52°C for 1 min, followed by
35 cycles of 90°C for 30 s, 58°C for 50 s, and 72°C for 35 s. Gene expression was quantified relative to GAPDH expression using an optimized comparative Ct ($2^{-\Delta \Delta Ct}$) value method.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward 5’-3’</th>
<th>Reverse 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>AGTGCCAGCTCGTCTCATAG</td>
<td>CCGTTGAACTTGCGGTAG</td>
</tr>
<tr>
<td>IL-2</td>
<td>GATCTAGCCAGCGAGAACCT</td>
<td>CTCTGGGACATCTCCGCA</td>
</tr>
<tr>
<td>TNF-α</td>
<td>CTACGGAAAGATCTCAATTGCG</td>
<td>GGAGCTCTCAAAATCTCGCA</td>
</tr>
<tr>
<td>IL-4</td>
<td>AAGGAGCTACGGGACTCATAT</td>
<td>CTGCTGGAGTCACATC</td>
</tr>
<tr>
<td>IL-10</td>
<td>GAAGAGCTCAATTGCGACA</td>
<td>AATCTCCAAATCTCGCA</td>
</tr>
</tbody>
</table>

ELISA detection for the evaluation of Th1 and Th2 cytokine expression in serum

Serum Th1 and Th2 cytokines IL-2, TNF-α, IL-4, and IL-10 expression levels were detected by ELISA according to the manufacturer protocol. The plate was read at 450 nm to measure the absorbance value (OD value). The sample concentration was calculated according to the OD value and standard curve.

Statistical analysis

All statistical analyses were performed using the SPSS16.0 software (Chicago, IL, USA). Numerical data are reported as means ± SD. Differences between multiple groups were analyzed by one-way ANOVA. Linear regression analysis was used to calculate correlation. P < 0.05 was considered as statistically significant.

RESULTS

Blood glucose levels

We measured FBG, P2hG, and HbA1c levels in each group and found that the T2DM and DR groups had higher FBG, P2hG, and HbA1c levels compared to the normal group (P < 0.05). The difference between the T2DM and DR groups was not significant (P > 0.05) (Table 2). Therefore, blood glucose changes are not for predicting the induction of T2DM with DR.

| Table 2. Blood glucose index comparison in each group. |
|---------------------------------|------------------|-----------------|
|                                  | Control          | T2DM group      | DR group        |
| FBG (mM)                        | 5.42 ± 1.29      | 8.97 ± 2.18*    | 8.92 ± 2.03*    |
| P2hG (mM)                       | 9.35 ± 3.62      | 16.27 ± 3.31*   | 17.14 ± 4.11*   |
| HbA1c (%)                       | 4.45 ± 1.51      | 12.07 ± 2.26*   | 11.99 ± 3.12*   |

*P < 0.05, compared with normal control.

mRNA expression of Th1 cytokines IL-2 and TNF-α

Real-time PCR was used to determine mRNA expression of Th1 cytokines IL-2 and TNF-α in each group. Compared with the normal group, IL-2 and TNF-α mRNA levels increased in both T2DM and DR groups (P < 0.05). Expression levels in the DR group were higher than in the T2DM group (P < 0.05) (Figure 1).
mRNA expression of Th2 cytokines *IL-4* and *IL-10*

Real-time PCR was used to determine mRNA expression of Th2 cytokines *IL-4* and *IL-10* in each group. Different from Th1 cytokines, *IL-4* and *IL-10* mRNA expression in T2DM group was not significantly different from the normal group (P > 0.05) (Figure 2). However, *IL-4* and *IL-10* mRNA level was lower in the DR group compared to the normal control and T2DM groups (P < 0.05) (Figure 2).

Figure 1. *IL-2* and *TNF-α* mRNA expression level in each group. A. *IL-2*; B. *TNF-α*. *P* < 0.05, compared with normal control. #P < 0.05, compared with T2DM group.

Figure 2. *IL-4* and *IL-10* mRNA expression level in each group. A. *IL-4*; B. *IL-10*. *P* < 0.05, compared with normal control. #P < 0.05, compared with T2DM group.

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Expression of Th1 cytokines IL-2 and TNF-α in serum

ELISA was performed to measure serum protein levels of Th1 cytokines IL-2 and TNF-α. Similar to mRNA expression, IL-2 and TNF-α protein expression in the serum of patients from the T2DM and DR groups was higher than that of subjects in the normal group (P < 0.05). Expression levels in the DR group were higher than in the T2DM group (P < 0.05) (Figure 3).

Figure 3. IL-2 and TNF-α expression level in serum in each group. A. IL-2; B. TNF-α. *P < 0.05, compared with normal control. **P < 0.05, compared with T2DM group.

Expression of Th2 cytokines IL-4 and IL-10 in serum

ELISA was performed to measure serum protein levels of Th2 cytokines IL-4 and IL-10. Similar to mRNA expression, IL-4 and IL-10 expression in the serum of patients from T2DM group was similar to the subjects in the normal group (P > 0.05). Expression levels in the DR group were lower than in the T2DM group (P < 0.05) (Figure 4).

Correlation analysis between Th1 and Th2 cytokines in the serum of DR patients

We analyzed the correlation between serum Th1 and Th2 cytokines in DR patients. IL-2 and TNF-α are negatively correlated with IL-4 and IL-10, respectively (P < 0.05) (Table 3).
Th1/Th2 imbalance in diabetic retinopathy

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DISCUSSION

DR is a common DM microvascular complication. As a chronic inflammatory disease, DR can change retinal microvascular permeability, increase leukocyte adhesion, and elevate inflammation-related factors expression. DR can lead to DM microvascular progressive damage and decrease the patient’s quality of life (Tamadon et al., 2015). About a third of DM patients will have DR complications characterized as diabetic macular edema or proliferative diabetic retinopathy, DM patients with the duration for 20 to 25 years may have DR in different degrees (Hao et al., 2015), ranging from serious visual impairment to even blindness. Thus, clarifying the DR mechanism and providing the relevant theoretical basis for DR clinical treatment and prevention is important.

In T1DM, T cell subgroup Th1 mediates the immune response involved in the pathogenesis, while Th2 cells play a protective role by secreting cytokines (Ma et al., 2015). However, the importance of Th1/Th2 balance changes in T2DM patients and its impact on DR complications is unknown. We analyzed Th1/Th2 balance by detecting cytokine secretion. We found that IL-2 and TNF-α mRNA levels in T2DM and DR groups were higher than in

![Figure 4. IL-4 and IL-10 expression level in serum in each group. A. IL-4; B. IL-10. *P < 0.05, compared with normal control. ‘P < 0.05, compared with T2DM group.]

| Table 3. Correlation analysis of serum Th1 cytokines and Th2 cytokines in DR patients. |
|---------------------------------|-----------------|-----------------|
|                                | IL-4            | IL-10           |
| IL-2                           | -0.726          | -0.641          |
| TNF-α                          | -0.572          | -0.718          |

| DISCUSSION |

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the normal group, and levels were higher in the DR group compared with the T2DM group. *IL-4* and *IL-10* levels were significantly lower in the DR group than in the normal and T2DM groups, but were similar between the normal and T2DM groups. Correlation analysis of Th1/Th2 cytokines in the DR group showed that *IL-2* and *TNF-α* are negatively correlated with *IL-4* and *IL-10*, respectively. These results suggest that *IL-2* and *TNF-α* can activate an inflammatory response, facilitating leukocyte adhesion and immune cell maturation and migration. This leads to inflammation, causes retinal ganglion cell damage, and aggravates DR occurrence and development (Håkansson et al., 2015). Secretion of *IL-2* and *TNF-α* by Th1 cells can promote cell apoptosis, leading to adhesion molecule expression and the acceleration of reactive T cell infiltration in the pancreas (Song et al., 2015b). By secreting *IL-4* and *IL-10*, Th2 cells can suppress immune damage caused by Th1 and stimulate B cell differentiation to plasma cells. The plasma cells can secrete antibody and generate memory B cells to enhance the humoral immune response (Coomes et al., 2015). In DR, increase in *IL-2* and *TNF-α* expression and the decrease of *IL-4* and *IL-10* secretion, leading to Th1/Th2 imbalance and DR pathological progress.

Identifying immune treatments that target Th1/Th2 balance may improve the immune response of DR patients and may aid in DR prevention and treatment.

**Conflicts of interest**

The authors declare no conflict of interest.

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