Effect of glucocorticoid on cytokines TLR9 and TLR7 in peripheral blood for patients with uveitis

HONG-PEI CUI[1], YU-XI PEI[2], GUAN-FENG LI[3] and YAN-RUI LOU[4]

[1]Department of Ophtalmology, Henan Eye Hospital, Henan Eye Institute, Zhengzhou, Henan 450003;
[2]Department of Ophtalmology, Women and Infants Hospital of Zhengzhou, Zhengzhou, Henan 450012;
[3]Department of Ophtalmology, Children's Hospital of Zhengzhou, Zhengzhou, Henan 450053;
[4]Department of Ophtalmology, The Third Affiliated Hospital of Henan University
of Traditional Chinese Medicine, Zhengzhou, Henan 450000, P.R. China

Abstract. The effect of glucocorticoid on cytokines Toll-like receptor (TLR)9 and TLR7 in peripheral blood of patients with uveitis was explored. Forty-six patients with uveitis admitted to our hospital from April 2014 to April 2015 were selected as the research observational group. Thirty-five able-bodied individuals in the same period were selected as the control group. To treat uveitis, the observational group was injected with glucocorticoid (1-2 mg/kg/day) daily, while the control group did not receive any treatment. The quantity of expression of peripheral blood cytokines TLR9 and TLR7 were detected by the methods of fluorescence quantitative PCR, enzyme-linked immunosorbent assay and western blotting. The content of peripheral blood TLR9 and TLR7 (0.21 ± 0.01, 0.19 ± 0.01) decreased significantly (P<0.05) in observational group after glucocorticoid treatment. Compared with data of control group (0.21 ± 0.01, 0.19 ± 0.01), TLR9 and TLR7 content in peripheral blood after glucocorticoid treatment on the patients with uveitis from observation group (0.19 ± 0.01, 0.17 ± 0.01) did not show any significant difference, for correlation between TLR9 and TLR7 in observation group before and after treatment. It was observed that the cytokine content of TLR9 was associated with TLR7 positively (r=0.653, P=0.012). In conclusion, glucocorticoid can improve uveitis by reducing the content of cytokines TLR9 and TLR7 in peripheral blood.

Introduction

Previous findings showed that uveitis, as a common eye disease in clinical practice, mainly occurs in young adults (1). No fundamental solutions have been found to its repeated

Correspondence to: Dr Yu-Xi Pei, Department of Ophtalmology, Women and Infants Hospital of Zhengzhou, 41 Jinshui Road, Zhengzhou, Henan 450012, P.R. China
E-mail: mgw252701@163.com

attacks. Research results of Xu and Lv (2) indicated that uveitis was a type of auto-immunodeficiency disease caused by T lymphocyte which was divided into three main types in clinical practice: Anterior, intermediate and posterior uveitis. The pathogenesis of uveitis is very complex.

In recent years, it has been found that Th1-type immune response played an important role in uveitis attacks. Research results of Trinh *et al* (3) suggested that Toll-like receptors (TLRs) can play a role on the body cell surface as a kind of cell signaling receptor. Thibault *et al* (4) suggested that TLRs played an important role in identifying bacteria, fungi and other exogenous pathogens and triggering body immune reaction signal, and Sun *et al* (5) demonstrated that TLR9, as an important member of TLRs family, was mainly involved in the body for the recognition of invasion exogenous pathogen, whose main mechanism is to activate the body's own immune system by methylated CpG motif in invasion exogenous pathogen. Different to TLR9, the main mechanism of TLR7 is to activate the body's own immune system by the identification of single-stranded RNA virus, promoting the elimination of exogenous pathogen with TLR9 combined action (6), to maintain the stability of the environment within body. Glucocorticoid is also termed 'adrenocortical hormone', which has extensive functions in collectivity, such as biosynthesis and metabolism adjustment of sugar, protein and fat, and obvious anti-inflammatory action found in recent studies as well demonstrating (7) that glucocorticoid could be used for the treatment on auto-immunodeficiency diseases (such as rheumatoid arthritis and systemic lupus), at the same time, it also has a good therapeutic effect for allergic diseases such as urticarial, and hay fever.

In the present study, we provided certain reference significance for the follow-up study of uveitis pathogenesis and the development of specific medicine by conducting glucocorticoid treatment on uveitis and detecting the cytokine content change of TLR9 and TLR7 in peripheral blood to clarify the molecular mechanism of glucocorticoid treatment on uveitis.

Subjects and methods

Subjects. Forty-six patients (25 males and 21 females) with uveitis admitted to the Women and Infants Hospital of

Zhengzhou (Henan, China) from April, 2014 to April, 2015 were selected as the research observational group. Their age averaged 32.4±8.64 years. Thirty-five able-bodied individuals, including 18 males and 17 females with an average age of 34.1±9.72 years were enrolled at the same period as members of the control group. There was no statistical difference between the observation and control groups regarding age and gender (χ^2=0.318, t=0.523, P>0.05). The diagnosis of patients with uveitis in observational group refers to the International Uveitis Research Organization Diagnosis and Classification Criteria (8). Exclusion criteria: the patients with other eye diseases, such as keratitis, cataracts, diabetic retinopathy and so on, were excluded. The able-bodied individuals in the control group were healthy and did not take any antibiotics, glucocorticoid hormone and other related drugs in the recent 3 months that could affect the experiment.

Methods. Blood (5 ml) was extracted from the patients vein in early stage of uveitis attack and after glucocorticoid treatment for 1-2 months, respectively. Blood samples were stored in heparin anticoagulant tube and placed within liquid nitrogen and used for the follow-up extraction of RNA and proteins.

Fluorogenic quantitative PCR
RNA extraction. RNA extraction plan was conducted according to the Takara kit instructions and improved to some extent (9).

　Fluorogenic quantitative PCR. Kit of fluorogenic quantitative PCR applied was from Applied Biosystems (Beijing, China). Two-step method was used to detect different gene expression quantity. Detailed plan was preceded according to the instruction and improved to some extent, and the primers was combined by Invitrogen-Life Technologies (Carlsbad, CA, USA), the primer sequences are shown in Table I.

Enzyme-linked immunosorbent assay (ELISA). In the experiment, the genetic expression quantity was detected by double antibody sandwich method, and the detailed method is as follows:

　i) Coat: In this study, we made a proper dilution of antibody protein by PBS buffer solution of pH 9.0, among which, the concentration was about 1-10 μg/m. Then, 0.1 ml was added into a 96-well plate with overnight process under 4˚C, the liquid in the 96-well plate was discarded the next day, the plate was cleaned 5 times by scrubbing solution, 2 min/time.

　ii) Sample adding: 0.1 ml processed serum sample was added into above 96-well plates, keeping static for 1 h under 37˚C, and cleaned 5 times by scrubbing in buffer solution, 2 min/time (conducted with blank well, negative control and positive control).

　iii) Second antibody: 0.1 ml new second antibody (Roche Diagnostics, Basel, Switzerland) was added to a 96-well plate after cleaning for 0.5-1.2 h under 37˚C, and cleaned 5 times by scrubbing solution, 2 min/time.

　iv) Chromogenic substrate: 0.1 ml new chromogenic substrate (Roche Diagnostics) was added to a 96-well plate after cleaning, which was incubated for 30 min under 37˚C.

　v) Stop solution: At the appropriate time to finish, 0.005 ml 0.2 M vitriol stop solution was added to the above-mentioned 96-well plates.

Table I. Fluorogenic quantitative PCR primer.

Genes	Primer sequences
TLR9	F: 5'-CTGCCTTCCTACCCTGTGAG-3'
	R: 5'-AGTTGCCGTCCATGAATACG-3'
TLR7	F: 5'-CTGGAGGTATTCCCACGAAC-3'
	R: 5'-AAACAGTAGGGACGGCTGTG-3'
GAPDH	F: 5'-TCATGGGTGTGAACCATGAGAA-3'
	R: 5'-GGCAGGACTGTGGTCATGAG-3'

TLR, Toll-like receptor.

　vi) Indication; qualitative detection: The above-mentioned 96-well plate was placed on plain paper to observe the shade of color, that is to say, the deeper color, the stronger the positive level, meaning the higher protein content of TLR9/TLR7, negative control without any color; quantitative detection: 96-well plate was placed on ELISA to conduct quantitative detection, with a wavelength of 450 nm. Zero set was carried out by blank well, in the experimental results, OD value greater than negative control value was determined to be positive.

Western blotting detection. In the present study, we used the combined protein of animal cell protein extraction kit from Roche Diagnostics (according to the instructions) with some improvements. Then antibody dilution was carried out according to the instruction provided by Roche Diagnostics, the final dilution ratio was 1:4,000, and the correlation operation referred to molecular clone handbook.

Data processing. Software SPSS 20.0 (Chicago, IL, USA) was used for statistical analysis. Data were presented as mean ± standard deviation. The comparison of multi-sample mean adopted one-way analysis of variance. The comparison between two groups was made using t-test and pairwise comparison within group employed q-test to check. P<0.05 was considered statistically significant.

Results

Detection of cytokine TLR9 and TLR7 mRNA content in peripheral blood of patients from observational group before and after treatment. From the comparison between the relative quantity of expression of cytokines TLR9 and TLR7 mRNA in peripheral blood of patients from the observational group before and after treatment, and those of patients from the control group, we determined that the relative expression of the quantity of cytokines TLR9 and TLR7 mRNA in peripheral blood after glucocorticoid treatment decreased significantly (P<0.05) (Fig. 1).

　The relative expression of the quantity of cytokines TLR9 and TLR7 mRNA in peripheral blood for the patients with uveitis after glucocorticoid treatment had no significant difference with those of control group. This suggested that glucocorticoid could reduce the expression of cytokines TLR9 and TLR7 mRNA in peripheral blood in the patients suffering
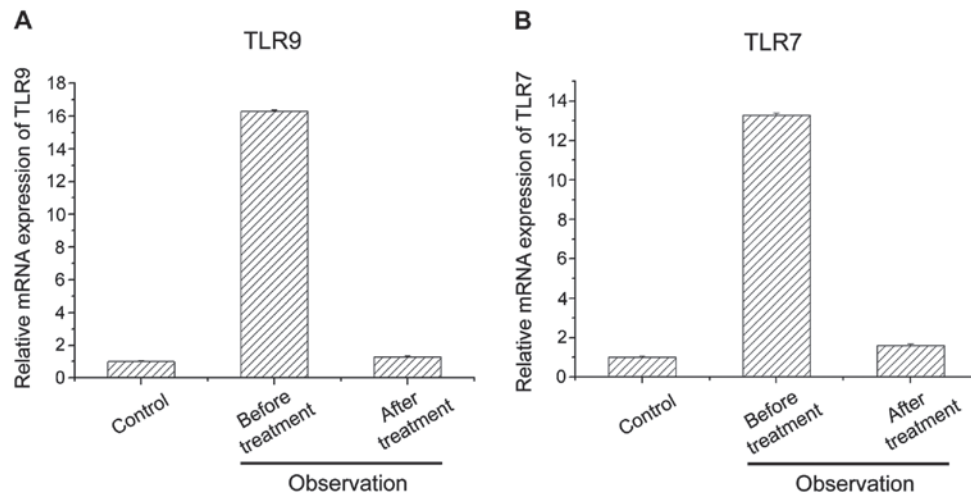
Figure 1. Detection of cytokine (A) Toll-like receptor (TLR)9 and (B) TLR7 mRNA content in peripheral blood of patients from the observational group before and after treatment.

Table II. Detection of TLR9 and TLR7 protein content (ng/l) in peripheral blood fromthe observation group patients after glucocorticoid treatment.

Groups	Cases	TLR9 cytokine	TLR7 cytokine
Observation			
Before treatment	46	0.48 ± 0.03^{a}	0.38 ± 0.02^{a}
After treatment	46	$0.21\pm0.01^{a,b}$	$0.19\pm0.01^{a,b}$
Control	35	0.19 ± 0.01	0.17 ± 0.01

[a]Compared with control, $P<0.05$. [b]Compared with before treatment, $P<0.05$. TLR, Toll-like receptor.
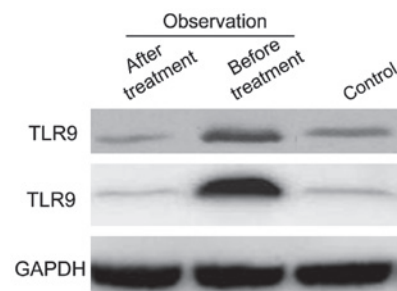

Figure 2. Detection of cytokine Toll-like receptor (TLR)9 and TLR7 protein expression quantity of the observational and control group patients by western blotting.

uveitis. This reduction may be associated with glucocorticoid treatment on uveitis.

Detection of cytokine TLR9 and TLR7 protein expression quantity of observation group and control group by ELISA. From the results of ELISA detection of the quantity of expression of protein TLR9 and TLR7 in peripheral blood from observational group before and after treatment and those of control group, we can see that the TLR9 and TLR7 content in peripheral blood of patients from observational group before treatment was 0.48 ± 0.03 and 0.38 ± 0.02 ng/l and the TLR9 and TLR7 content in peripheral blood of patients from observation group after glucocorticoid treatment was 0.21 ± 0.01 and 0.19 ± 0.01 ng/l (Table II). The comparison between them has significant deference ($P<0.05$). As well, compared with control group, the TLR9 and TLR7 content in peripheral blood of patients from observation group before treatment was significantly higher ($P<0.05$) than the TLR9 and TLR7 protein content of control group patients. This indicated that glucocorticoid could reduce protein content of cytokines TLR9 and TLR7 in peripheral blood for patients with uveitis. The related articles showed that TLR9 and TLR7 content in peripheral blood had a positive correlation with uveitis disease, which may relate to the therapeutic mechanism of glucocorticoid on uveitis (10,11).

Detection of cytokine TLR9 and TLR7 protein expression quantity of the observation and control groups by western blotting. The quantity of cytokine TLR9 and TLR7 protein expression of observational group before and after treatment in peripheral blood of observational group after glucocorticoid treatment decreased insignificantly, whereas obviously compared with cytokines TLR9 and TLR7 protein content in peripheral blood of control group, which was accordant with ELISA result and suggested that glucocorticoid could improve uveitis by reducing the protein content of cytokines TLR9 and TLR7 in peripheral blood (Fig. 2).

Correlation detection of TLR9 and TLR7 content in peripheral blood before and after glucocorticoid treatment. Through the detection of TLR9 and TLR7 content in peripheral blood before and after glucocorticoid treatment by SPSS 20.0, we found that for the patients with uveitis, the content of TLR9 and TLR7 in peripheral blood before and after treatment showed a positive correlation ($r=0.653$, $P=0.012$). This showed that TLR9 and TLR7 content in peripheral blood had a positive correlation with uveitis disease and glucocorticoid could improve uveitis by reducing cytokines TLR9 and TLR7 content in peripheral blood. At the same time, due to the correlation between TLR9 and TLR7, both can be used as a new detection and recovery index for uveitis disease.

Discussion

Uveitis, is a kind of autoimmune disease whose pathogenesis is not clear, so that there is no specific effect medicines for its treatment (12). The results of Yu and Liu showed that uveitis, as a type of auto-immunodeficiency disease, was caused by the deficiency of immune response mechanism that involved T lymphocytes (12). Sun *et al* suggested that TLRs, as a kind of natural immune receptor protein of human body, played an important role of signal transduction in the body's immune response (13). For example, the expression of TLR can be detected in macrophages, B lymphocytes, monocytes and T lymphocyte, which explained that TLRs widely participated in collective immune response mechanism (14). Additionally, TLRs could selectively identify the echogenic microbes and virus that entered the body by their special spatial structure and triggered the response mechanism of body's immune system to echogenic pathogenic microorganism by other related signal transduction pathways (15). Therefore, TLRs play a significant role in identifying exogenous pathogenic factors and starting body's immune response mechanism to clear pathogens. As a type of autoimmune deficiency disease, the main cause of uveitis is that the immune system starts mechanism for their own cells and tissues incorrectly, leading to immune deficiency diseases. Thus, as an important part of the body's own immune response mechanism, the distribution and dysfunction of TLRs also can result in the generation and deterioration of immunodeficiency diseases (16). Studies have shown that the main expression of TLR9 and TLR7 was in the endoplasmic reticulum, TLR7 often participated in CpGDNA of TLR9 mediation as an auxiliary receptor, with the function of immune cell activation (17). Prinz *et al* (18) found that the quantity of expression of TLR9 and TLR7 mRNA had positive correlation with the state of this illness by the detection of TLR9 and TLR7 mRNA expression in experimental autoimmunity encephalomyelitis, which illustrated that TLR9 and TLR7 are involved in the process of experimental autoimmunity encephalomyelitis attack (19).

As a kind of autoimmunity deficiency disease, the main pathogenesis of uveitis is also caused by body's autoimmunity system deficiency (20,21). In the present study, we selected glucocorticoid that has good effect on uveitis treatment. The results showed that the content of TLR9 and TLR7 in peripheral blood in the patients with uveitis decreased significantly compared with the data before treatment, and the content of TLR9 and TLR7 in peripheral blood was close to the content of normal people. This suggested that glucocorticoids could reduce the content of TLR9 and TLR7 in the body and the response of body's immune system to external environment to reach our aim of cure. However, the present study did not investigate the way by which glucocorticoid acts on TLR9 and TLR7 to decrease their content. This is crucial to future studies that are to be conducted.

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