J Vet Clin 26(6) : 520-523 (2009)

Journal of Veterinary Clinics
www.ksvc.or.kr

Quantitative Differences in mRNA Expression of Toll-like Receptor (TLR)-2, -4, and -9 in Normal Equine Eyes and Eyes with Equine Recurrent Uveitis

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(Accepted : December 09, 2009)

Abstract : The purpose of this study was to evaluate the quantitative differences in mRNA expression of TLR-2, -4, and -9 in normal equine eyes and eyes with equine recurrent uveitis (ERU). Normal equine eyes (n = 6) and eyes with naturally-occurring ERU (n = 6) were collected. Real time PCR assay was performed to compare mRNA expression of TLR-2, -4, and -9 between normal and ERU eyes. A significant up-regulation of TLR-2 and -9 mRNA in the ciliary body and TLR-2 mRNA in the iris was found in eyes with ERU compared to the mRNA levels in these same tissues of normal equine eyes. There were no remarkable differences observed in TLR-4 mRNA expression between normal eyes and eyes with ERU. The current data suggest the potential involvement of TLR-2 and -9 in the pathogenesis of ERU. However, further study is required to determine the role of TLRs in ERU.

Key words **:** Toll-like receptors, mRNA, recurrent uveitis, immune-mediated, horse.

Introduction

Equine recurrent uveitis (ERU) is a leading cause of blindness in horses and is characterized by recurrent bouts of active ocular inflammation followed by variable-length periods of quiescence (13). ERU is known to have multiple causes and to be related to infectious organisms, especially *Leptospira* spp., but it is widely considered to be an immune-mediated disease (8,12,14,25). Recent studies have demonstrated that the initial episode of uveitis may be caused by microorganisms, such as *Leptospira,* and immunogenic potential of fragments of these infectious organisms may play an important role in development of immune-mediated disease in the equine eye (20,22). However, the persistence of organisms in the eye is not likely the cause of ERU (15). Although it is unclear how these potentially immunogenic fragments are involved in initiation and recurrence of uveitis, increasing evidence, such as seen in arthritis and lung disease, suggests the role of toll-like receptors (TLRs) (3,5).

TLRs are type I trans-membrane microbial pathogen-associated receptors that recognize various microbial components and mediate adaptive immunity through the activation of the NF-κB pathway (3,4,18). TLRs also respond to certain cellular remnants of inflammation, such as necrotic cells, fibrinogen and heat-shock protein and may function as a feedback mechanism in the regulation of inflammation. Dysregulation of the expression of TLRs and this feedback regulation of the receptors are thought to be instrumental in the initiation and recurrence of immune-mediated diseases such as ERU (2,6, 19,24). Specifically, TLR-2, -4 and -9 have been found to induce autoimmune inflammation by reacting to self-antigen DNA (TLR-9) or inflammation-altered cellular debris (TLR-2 and -4) (1,3,6,19,21,24). Therefore, identification of the specific TLRs related to autoimmune inflammation in ERU and their regulation will further elucidate the pathogenesis of autoimmune ocular inflammation, with the potential of identifying specific targets for therapeutic intervention.

The purpose of this study was to compare mRNA expression of TLR-2, -4, and -9 in the uveal tract and retina in normal equine eyes and eyes with ERU.

Materials and Methods

Animals

The use of animals in this study adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and was reviewed and monitored by the North Carolina State University Institutional Animal Care and Use Committee. As normal samples, adult horses with normal ophthalmic examination and complete vaccination and deworming schedules, which were donated to the North Carolina State University, were used. Horses with spontaneous ERU were used in this study if they had the following: greater than a 3 month history of recurrent uveitis, documented episodes of recurrent ocular inflammation, clinical signs consistent with ERU (i.e., aqueous flare, hypopyon, corpora nigra atrophy, iris hyperpigmentation and fibrosis, cataract, vitreal cellu-

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lar infiltrate, retinal degeneration), lack of corneal inflammatory disease, and a complete evaluation by a veterinary ophthalmologist (15).

Collection of Samples

Normal eyes and eyes with ERU were collected from horses immediately after euthanasia. Spleen and lymph node tissues were collected to use as positive controls (26). Directly following collection, ciliary body, iris, and choroid/retina were harvested and obtained samples were stabilized using RNA later® (Qiagen, Valencia, CA), then transferred to -80°C according to the manufacturer's instructions. Spleen and lymph node samples were stored similarly.

Real-Time PCR assay

Total RNA was isolated from the spleen (n = 6), lymph node (n = 6), and ciliary body, iris, and choroid/retina of normal equine eyes (n = 6) and eyes with ERU (n = 6) using a RNeasy kit (Qiagen) with Qiashredder homogenization and on-column DNAse digestion following manufacturer's instructions. First-strand cDNA synthesis was performed from 1 μg RNA using a Superscript II kit (Invitrogen, Carlsbad, CA), 2 mM random hexamers (Applied Biosystems, Foster City, CA), and 10 mM dNTPs (Applied Biosystems). Real-time PCR was performed in a Bio-Rad iCycler using MyiQ Optical System Software version 2.0 (Bio-Rad, Hercules, CA). Reaction mixtures had a final volume of 25 μl including SYBR® Green Mastermix (Applied Biosystems), PCR-grade water, 200 nM reverse and forward primers, and cDNA samples. The primers used are listed in Table 1. Reaction conditions involved initial denaturation at 95°C for 2 minutes, followed by denaturation at 95°C for 15 seconds, annealing at 57.1°C (TLR-4) or 58.9°C (TLR-2) or 63°C (TLR-9) for 60 seconds, and elongation at 72°C and 0.5°C temperature increments (90 repeats). For each cDNA sample, triplicate reactions were performed on each plate for detection of the target genes. A non-template control was included to evaluate DNA contamination. The results were analyzed by the comparative threshold cycle (C_T) method and normalized by GAPDH.

Statistical analysis

Differences in mRNA and protein expression levels between the normal and ERU tissues were analyzed by use of Student's *t*-test. Differences were considered significant at $p < 0.05$. (JMP v.5.1, SAS Institute, Cary, NC).

Results

Spleen and lymph node samples showed significantly higher expression of TLR-2, -4, and -9 compared to ocular samples ($p < 0.01$). A 4 to 6-fold elevation of TLR-2 mRNA and 6 to 12-fold elevation of TLR-9 mRNA in the iris, ciliary body, and choroid/retina was found in eyes with ERU compared to the mRNA levels in these same tissues of normal equine eyes (Fig 1). In TLR-2 expression, there was a significant difference between normal and ERU ciliary body and iris ($p < 0.05$). In TLR-9 expression, there was a significant difference between normal and ERU ciliary body ($p < 0.05$).

Table 1. Primer sequences used for amplification of equine TLR-2,-4, and -9, and GAPDH

Gene	Accession No.	Primer sequence (5'-3')	Annealing Temp (°C)	Product Length (bp)
TLR-2	AY429602	F: ACGGCAGCTGTGAAAAGTCT	58.9	213
		R: CCTGAACCAGGAGGACGATA		
TLR-4	AY005808	F: TCTGGAGACGACTCAGGAAAGC	57.1	136
		R: GCAAGAAGCACCTCAGGAGTTT		
TLR-9	DQ390541	F: ACGGAACAACCTGGTAACAGTCCA	60	155
		R: TGTTATGGGACAAGTCCAGCACCGT		
GAPDH	AF097178	F: AAGTGGATATTGTCGCCATCAAT	60	88
		R: AACTTGCCATGGGTGGAATC		

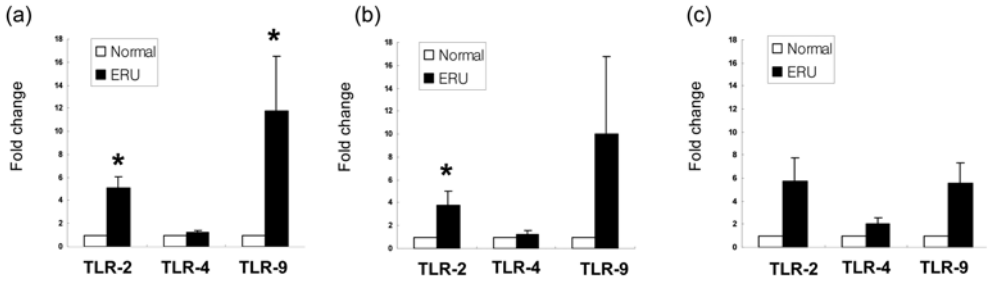

Fig 1. mRNA expressions of TLR-2, -4, and -9 in the ciliary body (a), iris (b), and choroid/retina (c) in normal equine eyes (n = 6) and eyes with ERU (n = 6). TLR-2 and -9 mRNA expression was significantly up-regulated in the ciliary body from eyes with ERU ($p < 0.05$). TLR-2 mRNA expression was significantly up-regulated in the iris of eyes with ERU ($p < 0.05$).

However, there was no significant difference in TLR-4 expression between normal eyes and eyes with ERU although a 2-fold elevation was noted in the choroid/retina of ERU eyes.

Discussion

Numerous studies have shown that TLRs are expressed in a variety of tissues and cells in the eye and play an important role in ocular defense against microbial infection (4). This preliminary study demonstrated the mRNA expression of TLR-2, -4, and -9 by iris, ciliary body, and choroid/retina in normal eyes and eyes with ERU. A significant up-regulation of TLR-2 and -9 mRNA in the ciliary body and TLR-2 mRNA in the iris was observed in the eyes with ERU.

ERU develops after primary uveitis, when the blood-ocular barrier is disrupted, allowing CD4+ T-cells to enter and remain in the eye (14). Recurrent bouts of inflammation develop as a consequence of new antigenic detection when CD4+ T-cells are up-regulated, resulting in inter- or intra-molecular epitope spreading (7). Recent findings have indicated that subsequent episodes of ERU are associated with immune response to various ocular retinal autoantigens, such as S-antigen, interphotoreceptor retinoid binding protein (IRBP), and cellular retinaldehyde-binding protein (CRALBP) (9-11). These studies have demonstrated that the pathogenesis of recurrent uveitis in horses is autoimmune in origin; however, the exact mechanism of initiation and recurrence of the inflammatory episodes is still unknown. TLR-2, -4 and -9 have been found to induce autoimmune inflammation (1,3,6,19,21, 24). TLR-9, currently known as an immune sensor for DNA, also recognizes self-DNA, which is derived from tissues damaged by inflammation through NF-κB pathway activation. This suggests that TLR-9 plays an important role in the initiation and regulation of inflammation in autoimmune inflammation. Previous research has indicated that inflammation-altered cellular debris activates TLR-2 and -4. TLR-2, especially, has been shown to mediate the inflammation in leptospiral disease (12,13). In addition, leptospiral infection has been associated with ERU in horses although persistence of this organism does not directly cause recurrent disease (15). Therefore, the increased mRNA expression of TLR-9 in ERU ocular tissues in this study may suggest the regulatory role of TLR-9 in the recurrence of inflammation in ERU. Furthermore, up-regulated TLR-2 expression in ERU ocular tissues noted here may support the theory that leptospiral infection or inflammatory debris are factors in the development of ERU.

TLR inhibitors have recently been developed and shown to be effective in various inflammatory disorders caused by activation of TLRs (17,23). The clinical application of TLR inhibitors appears to be promising especially for microbial inflammation, such as bacterial sepsis, and autoimmune diseases, such as systemic lupus erythematosus (SLE) (17). In addition, subconjunctival injection of TLR-9 small interfering RNA (siRNA) reduced inflammation in mice with *Pseudomonas aeruginosa* keratitis (16). Therefore, use of TLR inhibitors or knockdown of specific TLRs, which follows identification of the specific TLRs related to autoimmune inflammation in ERU and their regulation, may serve as a potent and specific therapy for patients with ERU.

In conclusion, the current data implicate the potential involvement of TLR-2 and -9 in the pathogenesis of ERU. However further studies to localize TLRs to specific cells in the eye and to investigate their inhibition are required to determine the role of TLRs in ERU.

Acknowledgement

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD)" (KRF-2006- E00020).

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말의 정상안과 재발성 포도막염이 있는 안구에서의 Toll-like Receptor-2, -4, -9 발현 비교

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요 약 : 본 연구는 말의 정상안과 재발성 포도막염이 있는 안구에서의 TLR-2, -4, -9 mRNA 발현의 정량적 차이를 비교하기 위해 수행되었다. 정상 및 재발성 포도막염이 있는 말 각 6두 에서 안구를 적출하여 모양체, 홍채, 망막 및 맥락막을 수집하였다. Real-time PCR assay 통해 정상안과 재발성 포도막염이 있는 안구에서의 TLR-2, -4, -9의 mRNA 발현 차이를 정량적으로 비교하였다. 말의 재발성 포도막염 시에는 모양체, 홍채에서 정상인 경우에 비해 4-12 배의 TLR-2 와 TLR-9 mRNA 발현증가를 보였으며, 맥락막 및 망막에서는 2-6 배의 TLR-2, -4, -9 mRNA 발현 증가를 보였다. 본 연구 결과는 Toll-like receptor 2, -4, -9이 말의 재발성 포도막염의 병리기전에 영향을 미치고 있음을 시사한다. 하지만 재발성 포도막염 시의 Toll-like receptor 2, -4, -9의 구체적 역할을 밝히기 위해서는 다양한 후속 연구가 요구된다.

주요어 : Toll 유사 수용체, mRNA, 재발성 포도막염, 면역 매개성, 말.