

**Up-regulation of neurotrophic factor-related gene expression in retina with experimental autoimmune uveoretinitis by intravitreal injection of tacrolimus (FK506)**

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Running Title: Up-regulation of neurotrophic factor-related genes in the uveitic eyes receiving intravitreal injection of tacrolimus.

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## **Abstract**

**Aim:** The current study was designed to determine whether intravitreal injection of tacrolimus (FK506) modulates the gene expression of neurotrophic factor-related molecules in the retina from eyes with induced experimental autoimmune uveoretinitis (EAU) in rats.

**Methods:** Rats were immunized with interphotoreceptor retinoid binding protein (IRBP) peptide (R14) and given intravitreal injection of tacrolimus on day 12 after immunization. As control, immunized rats received intravitreal injection of vehicle. On day 15 after immunization, changes in the genetic program associated with neuroprotection and inflammatory responses in the retinas from both groups were determined by DNA microarray analyses and confirmed by RT-PCR analyses.

**Results:** The gene expression of inflammatory responses was markedly reduced in tacrolimus-treated eyes. Genes for molecules associated with neuroprotection (estrogen receptor, erythropoietin receptor, GABA receptor, protein kinase C, glial cell line-derived neurotrophic factor receptor, fibroblast growth factor, and neuropeptide Y receptor) were upregulated in the retinas from tacrolimus-treated eyes.

**Conclusions:** Intravitreal injection of tacrolimus modulated the genes related to neuroprotection in the retina during the ongoing process of EAU. This treatment may be useful for the neuroprotection of retina with severe uveitis as well as for immunosuppression in the uveitic eyes.

## Introduction

Tacrolimus (FK506) is a substance isolated and purified from metabolites of a fungus, *Streptomyces tsukubaensis*.<sup>1</sup> Tacrolimus has been used for the prevention of allograft rejection as an immunosuppressive drug.<sup>2,3</sup> In addition, tacrolimus has been reported to exert a powerful neuroprotective action in experimental stroke and ischemia.<sup>4-7</sup> Furthermore, a previous study has shown that tacrolimus confers neuroprotection on retinal ganglion cells after optic nerve crush.<sup>8</sup>

Experimental autoimmune uveoretinitis (EAU) is an inflammatory disease model that shares many clinical and histopathologic features with human disease such as Behçet's disease.<sup>9-11</sup> In animals, EAU can be induced by immunization with interphoto-receptor retinoid binding protein (IRBP), an eye-specific retinal antigen, or by transfer of the antigen-specific T cells.<sup>12-14</sup> EAU is a Th1 dominant response to the uveitogenic retinal antigen, and uveitogenic effector T cells display a Th1 like cytokine profile.<sup>15,16</sup>

Recently we have demonstrated that intravitreal injection of tacrolimus effectively suppresses ongoing EAU in rats and preserves the retinal structure of eyes with EAU.<sup>17</sup> In the current study, we investigated whether intravitreal injection of tacrolimus modulates gene expression of neurotrophic factor-related molecules in the retinas from eyes with ongoing EAU in rats, using DNA microarrays.

## Materials and Methods

### *Animals*

Six to 8 week-old female Lewis rats, were purchased from Japan CLEA (Tokyo, Japan). All rats were treated in accordance with the ARVO statement for the use of Animals in Ophthalmic and Vision Research. A mixture of ketamine HCl and xylazine was used for anesthesia and administered by intraperitoneal injection.

### *Reagents*

Bovine IRBP peptide (R14),<sup>14</sup> was prepared using synthesizer (Sawady technology, Tokyo, Japan). Complete Freund's adjuvant (CFA) was purchased from Difco Labs (Detroit, MI). Killed Bordetella pertussis suspension was purchased from Sigma Chemical (St. Louis, MO).

### *Induction and Scoring of EAU*

Lewis rats received an injection into one hind footpad of R14 (0.5 µg) in 0.1ml emulsion in CFA,<sup>14</sup> and killed Bordetella pertussis suspension ( $1 \times 10^{10}$  cells) were given intraperitoneally as an additional adjuvant.

Eyes were examined daily after R14 immunization independently by two blinded observers to assess for the onset of inflammation using a slit lamp biomicroscope.<sup>18</sup>

### *Intravitreal injection of tacrolimus*

Tacrolimus (Prograf, Astellas Pharma Inc. , Tokyo, Japan) was dissolved in balanced saline solution (BSS) (BSS plus; Alcon, Fort Worth, TX) at a concentration of 2 mg/ml. Five µl of tacrolimus solution was injected into the vitreous cavity of rats on day 12 after immunization using a 30G needle after paracentesis was performed.

### *DNA microarray hybridization and analysis*

Recent our paper showed that ocular inflammation in the anterior segment was significantly suppressed in the tacrolimus-treated eyes on day 14 and 16 after immunization.<sup>17</sup> However, both tacrolimus- and vehicle-treated eyes showed decreased inflammation in the anterior segment on day 16 after immunization.<sup>17</sup> Therefore, we collected eyes from both groups on day 15. The tacrolimus-treated eyes (3 eyes) and vehicle-treated eyes (3 eyes) were enucleated on day 15 after immunization. Total RNA was extracted from whole retina of enucleated eyes using an Isogen RNA isolation kit (Nippon Gene, Tokyo, Japan). The analysis of RNA quality showed that the 260:280-nm absorbance ratio of RNA samples used in this experiment ranged consistently from 1.8-2.0. The integrity and concentration of total RNA were measured using a bioanalysis unit (Agilent 2100 Bioanalyser; Agilent Technologies, Palo Alto, CA). Fluorescence-labeled antisense RNA was synthesized by direct incorporation of Cy3-UTP or Cy5-UTP (GE Healthcare Bio-Science Corp., Piscataway, NJ) using 2 µg of each RNA sample and an RNA Transcript SureLABEL Core Kit (TaKaRa BIO Inc., Tokyo, Japan). The labeled antisense RNAs were hybridized simultaneously to the microarray chip (FilgenArray Rat 27k, Filgen, Inc., Nagoya, Japan). Array hybridization was performed according to the manufacturer's instructions (Filgen, Inc.). The fluorescence images of hybridized microarrays were obtained with a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA). The Array-Pro Analyzer Ver4.5 (Media Cybernetics, Inc., Silver Spring, MD) was used to determine the signal intensity of each spot and its local background. The net intensity was calculated by subtracting the mean intensity of all pixels within the local background area from the mean

intensity of all pixels within the spot areas. The biases in net intensity were normalized by locally weighted linear regression analysis. Analyzed data were selected using a MicroArray Data Analysis Tool (Filgen, Inc.).

*Real time polymerase chain reaction (PCR)*

First strand cDNAs were synthesized using TaqMan(R) One-step RT-PCR Master Mix Reagents (Applied Biosystems) from total RNA. Levels of estrogen receptor 2 beta and erythropoietin receptor were determined over the time course by real time PCR (TaqMan chemistry with the ABI Prism 7900HT sequence Detection System; Applied Biosystems, Foster City, CA).

TaqManGeneExpressionAssays™ TaqMan™ probes and primer pairs of erythropoietin (assay ID: Rn00690244\_g1), estrogen receptor 2 beta (assay ID: Rn00688791\_m1), and GAPDH (assay ID: Rn01775763\_g1) were obtained from Applied Biosystems.

For relative quantification in real-time PCR experiments, we used the comparative Ct methods (Applied Biosystems). Samples were assayed using thermal cycler conditions consisting of 10 minutes at 48°C and 10 minutes at 95 °C, followed by 60 cycles at 95 °C for 15 seconds and 60 °C for 1 minute.

## RESULTS

### *Comparison of gene expression profiles in tacrolimus-treated eyes and vehicle-treated eyes by DNA microarray analysis*

Slit-lamp examination demonstrated that mean clinical EAU scores on day 12 after immunization were 3.3 in tacrolimus-treated rats and 3.5 in vehicle treated rats. On day 14, mean clinical EAU scores were 0.5 in tacrolimus-treated rats, and 5.7 in vehicle-treated rats, indicating that intravitreal injection of tacrolimus suppressed ongoing EAU. On day 15, eyes were enucleated from both groups and retinas were collected for RNA extraction. The total RNA was applied for DNA microarray analysis.

The signal ratio of each of the 28,800 genes was calculated. Genes with a twofold ratio increase were defined arbitrarily as upregulated in tacrolimus-treated eyes, whereas those with a ratio decrease by half or more were defined as downregulated. Using these criteria, 1828 genes were found to be upregulated, and 1594 genes were found to be downregulated in tacrolimus-treated eyes compared with vehicle-treated eyes.

Summaries of genes differentially expressed between two groups (tacrolimus-treated eyes/vehicle-treated eyes) are shown in Table 1 and 2. The differentially expressed genes are arranged according to two functional categories based on known functions of these genes: 1) inflammation related genes and 2) neuroprotection related genes. As demonstrated in Table 1, the gene expression of IL-1 receptor type 1, inducible nitric oxide synthase 2, IL-6, RANTES (regulated on activation, normal T cell expressed and secreted), CD3 antigen, CCR7 (CC chemokine receptor 7), CXC9 (monokine induced by interferon gamma: MIG), and IL-2 receptor gamma was down regulated in retinas from tacrolimus-treated eyes.

On the other hand, as shown in Table 2, the expression of neuroprotection-related genes such as estrogen receptor 2 beta, erythropoietin receptor, GABA receptor, protein kinase C, glial cell line-derived neurotrophic factor receptor, fibroblast growth factor, and neuropeptide Y receptor was elevated in the retinas from tacrolimus-treated eyes.<sup>19-30</sup>

Table 1. Differential expression of inflammation related genes in retinas derived from uveitic eyes receiving intravitreal injection of tacrolimus

Common Name	GenBank Accession No.	Ratio
IL-1 receptor type 1	M95578	0.01
Nitric oxide synthase 2, inducible	U03699	0.21
IL-6	M26744	0.23
C-C ligand 5 (RANTES)	-	0.31
CD3 antigen, gamma polypeptide	BF560704	0.32
CCR7	BC089762	0.35
CXC ligand 9 (MIG)	BC087594	0.36
IL-2 receptor gamma	BC079343	0.49

IL: interleukin, RANTES: regulated on activation, normal T cell expressed and secreted, CCR: CC chemokine receptor, MIG: monokine induced by interferon gamma.

Table 2. Differential expression of neurotrophic factor related genes in retinas derived from uveitic eyes receiving intravitreal injection of tacrolimus

Common Name	GenBank Accession No.	Ratio
Estrogen receptor 2 beta	U57439	4.51
Erythropoietin receptor	BC089810	3.31
Similar to Bcl-X, short	-	2.78
GABA A receptor, gamma 1	X57514	2.72
Protein kinase C, alpha	-	2.28
GDNF receptor family receptor alpha 4	AJ294476	2.25
Fibroblast growth factor 8	-	2.17
Neuropeptide Y receptor Y1	BC089981	2.15
Protein Kinase C, beta 1	X04440	2.08

GDNF: glial cell line derived neurotrophic factor receptor.

#### ***Confirmation of differential expressed genes***

To confirm that observed differences in DNA microarray analysis correlated with differences in steady levels of the corresponding mRNAs, the expression patterns of selected genes were determined by real time PCR. Levels of selected genes relative to that of a housekeeping gene (GAPDH) were compared between tacrolimus-treated eyes and vehicle-treated eyes. This method confirmed the differential expression of each of the selected genes in the expected manner (Figure 1). mRNA expression of estrogen receptor 2 beta and erythropoietin receptor was elevated in retinas from tacrolimus-treated eyes.

## DISCUSSION

We recently demonstrated that intravitreal injection of tacrolimus is capable of suppressing the ongoing process of autoimmune uveoretinitis in the eye and can preserve the retinal structure of the uveitic eye.<sup>17</sup> Tacrolimus has been shown to exert profound neuroprotective and neuroregenerative effects in vivo and in vitro.<sup>4,5,7,31</sup> However, the underlying molecular pathways of neuroprotection are not fully understood. We used DNA microarray technology to define genes that are related to neuroprotection in the retinas from tacrolimus treated eyes with EAU. We obtained evidences that intravitreal injection of tacrolimus upregulated the gene expression of neuroprotection-related molecules as well as decreased the expression of inflammatory responses related genes. These data support the notion that increased expression of neuroprotection-related genes by intravitreal injection of tacrolimus may play a potential role in retinal protection of the eyes with ongoing ocular inflammation, as well as in immune regulation.

Microarray analysis showed that gene expression of estrogen receptor 2 was upregulated in the retinas derived from tacrolimus-treated eyes. In addition, we also observed that protein kinase C gene expression was increased in tacrolimus-treated eyes. Recent reports have demonstrated a neuroprotective effect of estrogen.<sup>19-21</sup> Furthermore, Cordey et al. have shown that estrogen-induced neuroprotection of neurons depends on activation of protein kinase C.<sup>26,27</sup> These reports, together with the results of the microarray study, suggest that estrogen receptor 2 and protein kinase C genes upregulation by intravitreal injection of tacrolimus may be a important factor involved in the preservation of sensory retina with ongoing EAU.

Erythropoietin receptor was induced in the tacrolimus treated retina. So far, a wide variety of experimental studies have shown that both erythropoietin and the erythropoietin receptor are functionally expressed in the nervous system and that this cytokine exerts a remarkable neuroprotection.<sup>22-24</sup> In the field of ophthalmology, it has been reported that erythropoietin has a potential role as therapeutic molecule against apoptotic neuronal cell death in the context of glaucoma or neurodegenerative diseases.<sup>32,33</sup> Taken together, the upregulation of erythropoietin receptor induced by tacrolimus delivered into vitreous cavity may play a part in the neuroprotection of sensory retina in the uveitic eyes.

In conclusion, the observation based on this DNA microarray experiment suggests that upregulation of neurotrophic factor-related gene expression and downregulation of inflammation related genes may be important mechanisms by which intravitreal injection of tacrolimus not only prevent ocular inflammation but also facilitates preservation of retinal architecture in the eyes with uveoretinitis.

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**Competing interests**

There is no conflict of interest related to this manuscript.

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**Figure legend**

Figure 1. Real time PCR assay of relative mRNA levels of the estrogen receptor 2 beta and erythropoietin receptor in retina from tacrolimus-treated eyes and vehicle-treated eyes. GAPDH served as the reference gene. Relative expression of each gene was determined from the Ct. Changes in expression (x-fold) of selected upregulated genes in retina from tacrolimus-treated eyes compared with those from vehicle treated eyes.

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Figure 1

