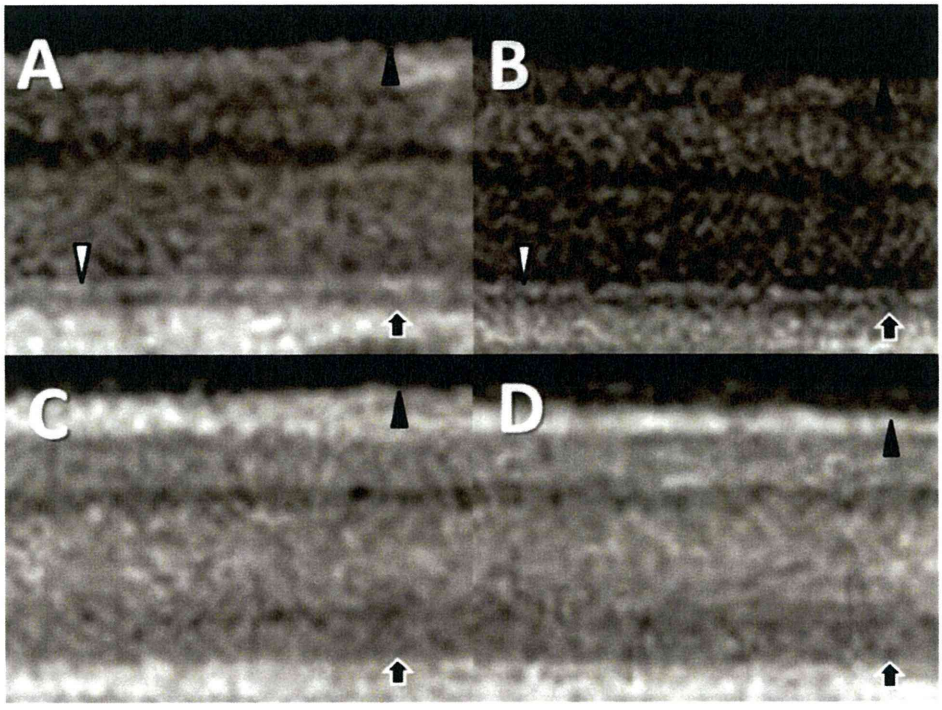

Figure 5 Optical coherence tomography (OCT) images corresponding to figure 3. (A, B) OCT images of the retina 3 h after the *N*-methyl-*N*-nitrosourea injection (figure 3A, B). The intersection of the inner and outer photoreceptor segments (IS/OS) on the OCT images can be observed (white arrowhead), and the arrow and black arrowhead show the retinal pigment epithelium line and internal limiting membrane line, respectively. (C, D) OCT images of the retina 6 h after the *N*-methyl-*N*-nitrosourea injection (figure 3C, D). The IS/OS on the OCT images is not depicted. The arrow and black arrowhead show the retinal pigment epithelium line and internal limiting membrane line, respectively.

observations (figures 4B, 5A,B). In OCT images 6 h after injection, the OS was preserved, but the IS/OS was not observed (figures 4C, 5C,D). In OCT images 24 h after injection, the IS and OS were disorganised, and the IS/OS was not depicted (figure 4D). In OCT images 1 week after injection, the photoreceptor cells disappeared, and the IS/OS were not depicted. These results also corresponded to the observed histopathology.

DISCUSSION

In our earlier study, we hypothesised that when the IS/OS is not seen in OCT images, the IS structure is probably destroyed, or the IS and OS may have disappeared entirely.[14] However, in this study, we discovered that IS disorganisation results in a lack of IS/OS depiction on OCT images, despite relative preservation of the OS. These findings suggest that the IS might play a key role in the observation of the IS/OS on the OCT images. A certain structure in the IS (eg, the basal body of the connecting cilia or mitochondria) might be the origin of the IS/OS on OCT images. It is suggested that disruption of the outer segments will certainly be associated with significant visual disability, but the ultimate prognosis is still unknown. Lin *et al* reported photoreceptor remodelling in mice.[17] Therefore, OS remodelling in humans probably occurs as well.

A possible counterargument is that the structural change between the IS and OS seems to be a good explanation of the origin of IS/OS and that the OS discs probably reflect the light well. Near-infrared scattering signals have been extensively investigated in photoreceptors in a number of studies.[18 19] These works suggested that cell swelling and shrinkage and membrane depolarisation resulting from ionic fluxes likely cause the observed optical changes. Bizheva *et al* hypothesised that photoreceptors hyperpolarise during light stimulation, which may result in temporary modulation of the optical reflectivity of the cell membrane. However, scattering changes observed in that study were relatively slow and corresponded more to the time course of the ion shifts involved in electroretinogram generation than to the rapid hyperpolarisation of the photoreceptor membrane evoked by light stimuli.[20] In addition, they observed two different scattering signals, one at the OS level and the other at the IS level.[20] Whereas the OS is packed with thousands of densely stacked discs that contain the photosensitive agent rhodopsin and the G protein that mediates phototransduction, the IS ellipsoids of the photoreceptor consist of mitochondria that are tightly packed in parallel.[21] Altered metabolic rates during light stimulation could cause changes in the mitochondrial refractive index, which in turn may result in IS reflectivity changes.[20] Therefore, the IS might be where temporary modulation of the optical reflectivity of the cell membrane occurs, even though many studies have hypothesised that the OS is the IS/OS origin on OCT images.

Srinivasan *et al* reported that the short focal length of the eye increases aberration, which degrades OCT signal and image quality. For this reason, imaging of an eye of a small animal was performed with a contact lens made of a flat microscope cover slip and hydroxypropyl methylcellulose in the normal young adult C57BL6 mouse and Long–Evans rat.[8] However, in this study, normal commercial Cirrus HD-OCT was used without a contact lens because the contact lens did not work well. In addition, the aberration of the rat lens and cornea may decrease OCT image quality. Therefore, the shadow caused by the intermediate line and the external limiting membrane was not depicted on the OCT images. However, IS/OS status was demonstrated in the controls, and so estimation of the presence or absence of IS/OS on the OCT images could be proven. We need to consider that OCT findings correspond to optical reflection alterations and that biological change might influence the optical response.

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Correlation between high-resolution optical coherence tomography (OCT) images and histopathology in an *N*-methyl-*N*-nitrosourea –induced retinal degeneration rat model

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Review Article

Analysis of the Pathogenesis of Experimental Autoimmune Optic Neuritis

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Optic neuritis associated with multiple sclerosis has a strong association with organ-specific autoimmune disease. The goal of our research is to establish an optimal organ-specific animal model to elucidate the pathogenetic mechanisms of the disease and to develop therapeutic strategies using the model. This paper is divided into five sections: (1) clinical picture of optic neuritis associated with multiple sclerosis, (2) elucidation of pathogenesis using animal models with inflammation in optic nerve and spinal cord, (3) clinical relevance of concurrent encephalomyelitis in optic neuritis model, (4) retinal damage in a concurrent multiple sclerosis and optic neuritis model, and (5) development of novel therapies using mouse optic neuritis model. Advanced therapies using biologicals have succeeded to control intractable optic neuritis in animal models. This may ultimately lead to prevention of vision loss within a short period from acute onset of optic neuritis in human. By conducting research flexibly, ready to switch from the bench to the bedside and from the bedside to the bench as the opportunity arises, this strategy may help to guide the research of optic neuritis in the right direction.

1. Introduction

When ocular inflammation involves other connecting intraocular or extraocular tissues, the name of the disease changes depending on the site of inflammation. For example, the diagnosis of the disease may vary from conjunctivitis, scleritis, uveitis (intraocular inflammation), chorioretinitis, to optic neuritis. Ocular inflammation usually has an infectious or autoimmune etiology. When the cause is autoimmunity, inflammation usually involves specific organs or tissues. Our group has taken a keen interest on the study of optic neuritis associated with multiple sclerosis, which is an intractable organ-specific inflammatory disease unresponsive to conventional treatments and consequently has a poor prognosis. Optic neuritis associated with multiple sclerosis has strong association with organ-specific autoimmune disease. Our research, which aims to establish an optimal organ-specific animal model to elucidate the pathogenesis of the disease and to develop therapeutic strategies using animal model, will contribute to the understanding of the pathophysiology of intractable optic neuritis in humans and ultimately to ameliorate the disease in the future.

Optic neuritis associated with multiple sclerosis may manifest in two forms: opticospinal multiple sclerosis (OSMS) which is accompanied by cerebral lesion associated with multiple sclerosis and neuromyelitis optica (NMO) which is usually not accompanied with cerebral lesion. No conclusion has been reached on whether the two forms are in fact the same entity. The definition of NMO is the presence of optic neuritis together with spinal cord lesion extending over three or more vertebral segments, but not necessarily cerebral lesion. The presence of antibodies against aquaporin 4 (AQP4) is the most important factor associated with the pathophysiology of NMO, and it has been closed up recently [1, 2].

Anti-AQP4 antibody has been shown to react with AQP4 expressed on astrocytes, inducing complement fixation reaction and leading to cell death [2]. If we consider NMO as one type of autoimmune disease, B cells that are responsible for antibody production and T cells that regulate cytokines which promote antibody production should play important roles in the development of NMO. Although the pathogenesis of multiple sclerosis remains incompletely understood, some antigens of cerebrospinal origin, such as

myelin basic protein (MBP), have been proposed to cause inflammation in the brain and spinal cord via some autoimmune mechanisms. At cellular level, immunity is acquired through the following pathway: antigen-antigen presenting cell-T cell-B cell. This may provide a framework to explain the clinical picture of multiple sclerosis, an autoimmune disease. Among these major cellular transmission routes, (1) the type of antigen, (2) the surface molecules (such as HLA) expressed on antigen-presenting cells including macrophages and dendritic cells, and (3) the type of lymphocytes including T cells and B cells will decide the site of inflammation and the size of the lesion. Each of these will be discussed as follows. (1) Concerning the types of protein antigen, the candidates include MBP, myelin oligodendrocyte glycoprotein (MOG), and the myelin-specific proteolipid protein (PLP). Among these proteins, MOG antigen has a high possibility of causing optic neuritis. MOG has been shown to be present abundantly inside the optic nerve, and inflammatory cells presumably react to the MOG antigen of the optic nerve to cause tissue damage [3]. In contrast, MBP is present abundantly inside the brain and spinal cord, and it has been suggested that optic neuritis rarely develops in the presence of MBP-induced encephalomyelitis. Therefore, the site of lesion is determined depending on which antigen is targeted by the immune system. (2) As for the involvement of antigen-presenting cells, classic multiple sclerosis has been associated with the expression of HLA-DR2 on antigen-presenting cells. In addition, the state of adhesion molecules expressed on antigen-presenting cells is a factor that attenuates inflammation. (3) Regarding the involvement of T cells and B cells, an increase in Th17-type T cells producing IFN-γ and IL-17, as well as an accumulation of B cells that are precursors of the antibody-producing plasma cells have been suggested in optic neuritis associated with multiple sclerosis. In severe NMO cases in which the above-mentioned anti-AQP4 antibody was detected, apart from the autoimmune mechanisms, the antibody *per se* also causes direct cell damage (astroglia in this case), which may account for the higher incidence of severe damage compared to OS-MS. From the immunological point of view, it may be possible to classify anti-AQP4 antibody-positive NMO within the category of OS-MS, with the anti-AQP4 antibody-negative OS-MS classified as a mild type with little antibody-induced tissue damage. The feasibility of this classification can only be verified in a large scale of clinical study regarding NMO.

2. Elucidation of Pathogenetic Mechanisms Using Animal Models with Inflammations in Optic Nerve and Spinal Cord

As discussed above, immunological mechanisms are considered to play a role in optic neuritis associated with multiple sclerosis. However, this hypothesis can only be proven when one demonstrates the development of specific lesion by adjuvant immunizing the host with the causative protein antigen. So far, research analyzing the optic nerve lesion using animal models of multiple sclerosis has not been conducted actively. In the 1990s when experimental autoimmune encephalomyelitis (EAE), which is an animal model of multiple sclerosis, was being developed, a study reported that immunizing mice with the central nervous system- (CNS-) specific PLP resulted in the onset of optic neuritis on day 17 after immunization [4]. Histopathological examination of the optic nerve showed infiltration of polymorphonuclear cells and monocytes [4], resembling the findings of optic neuritis associated with multiple sclerosis in human. Likewise, another CNS protein MOG, when used to immunize Brown Norway (BN) rats, also caused optic neuritis within 3 weeks in 90% of the animals, in addition to encephalomyelitis [5]. These studies provide clear evidence that in certain strains of rodents, optic neuritis develops as a result of immunization with antigens derived from neuron. However, even in rats and mice, the MHC types (haplotypes) in which optic neuritis can develop are probably restricted, a phenomenon probably similar to the increased susceptibility of HLA-DR2-positive human to multiple sclerosis.

In the mouse model that develops both encephalomyelitis and optic neuritis, the onset of encephalomyelitis is usually observed 13 days on average after adjuvant-immunization with the MOG35-55 peptide. However, when T cells obtained from these mice are injected intraperitoneally (adoptive immunization) to normal mice, optic neuritis also develops in these mice [6]. This finding indicates that the T cells possessing the pathogenetic factor of optic neuritis are important in the development of autoimmune optic neuritis. As an evidence that these MOG-specific T cells are an important pathogenetic factor, transgenic mice possessing T cells with MOG35-55-specific T cell receptor (TCR) developed optic neuritis spontaneously, with a clinical course resembling that of multiple sclerosis in humans, which is an interesting observation [7]. Furthermore, when these MOG-specific TCR transgenic mice were crossed with MOG-specific Ig heavy-chain (closely associated with B cells) knock-in mice, the incidence of spontaneous optic neuritis increased markedly [8]. As a possible mechanism, Bettelli et al. [8] proposed that IL-17- and IFN-γ-producing $CD4^+$ T cells are involved in the development of optic neuritis in mice. The IL-17-secreting Th17 cells possess unique properties different from those of Th1 cells that are closely associated with the development of EAE and experimental autoimmune uveoretinitis (an animal model of human uveitis), and those of Th2 cells that are involved in allergic diseases. IL-17 has been considered as an important cytokine for the development of autoimmune diseases. Taken together, the following hypothesis may be proposed. Through the action of the newly discovered Th17 mechanism, but not Th1 or Th2, the T cells, IgH-producing B cells, and antigen that causes CNS lesion interact to produce the pathophysiology of mouse encephalomyelitis and optic neuritis.

Oligodendrocyte-specific protein- (OSP-) induced EAE is a relatively new model of multiple sclerosis, and similar findings have been reported by other institutes. OSP is a protein present in the myelin sheath of the CNS. Mice immunized with OSP develop optic neuritis together with encephalomyelitis [9]. Study has shown that OSP55-80 and

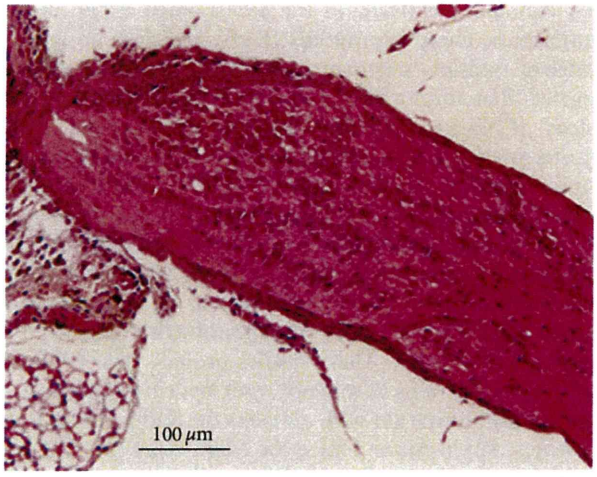

FIGURE 1: Histopathological findings of the optic nerve from a mouse adjuvant immunized with MOG peptide. Marked cell infiltration is observed in the optic nerve.

OSP179-207 derived from the OSP molecule are epitopes recognized by $CD4^+$ T cells that mediate lesion formation. In addition, the antibodies induced by OSP were predominantly directed against OSP22-46 [9]. These findings suggest that T cells and B cells collaborate in an antigen-specific manner to give rise to the clinicopathological manifestations of this model.

From these studies, the pathogenesis of multiple sclerosis and the associated optic neuritis become gradually unraveled. However, the incidence of optic neuritis in mouse models has stayed at 60% to 70%, even when gene transfer technology is used. In order to be used in therapeutic experiments in the future, a higher incidence of optic neuritis of the animal model has to be achieved. By mixing dimethyl sulfoxide (DMSO) during immunization with MOG peptide, we have successfully increased the incidence of optic neuritis from 80% to 90% in mice (report in preparation). The histopathological picture of optic neuritis obtained using this animal model is shown in Figure 1, and the Luxol myelin sheath staining of optic neuritis is shown in Figure 2. In the section stained by Luxol fast blue for myelin sheath, unstained regions indicating destruction of the myelin sheath were consistent with areas of cell infiltration. In an *in vitro* experiment, spleen cells collected from a mouse that developed optic neuritis were cultured, and the cytokines in the culture supernatant were assayed. IL-17 level was high, and IFN-γ was slightly elevated in the supernatant. On the other hand, secretion of IL-10, an inhibitory cytokine, was not remarkable. These results confirm previous cytokine data of mouse optic neuritis model and probably predict the results of analysis of optic neuritis in human.

3. Clinical Relevance of Concurrent Encephalomyelitis in Optic Neuritis Model

When experimental autoimmune optic neuritis (EAON) is produced, EAE occurs at the same time. EAE is known to be a model of human multiple sclerosis. Regarding the relationship between EAE and multiple sclerosis, although the two share many common features histopathologically, they also differ in many aspects which remain unclarified. When we try to analyze EAON, the interpretation of EAE, which has been extensively studied, is important.

In the EAE animal model, MOG protein is considered to be the major causative antigen. In humans, approximately 50% of multiple sclerosis patients are positive for anti-MOG antibody, while an equal number of patients are negative. Recently, a tetramer radioimmunoassay has been developed, which is more sensitive than the conventional technique such as ELISA and flow cytometric assays. This radioimmunoassay is capable of detecting conformational differences in MOG protein and allows discrimination between native MOG (or folded MOG) and denatured MOG (or unfolded MOG). Human study using this method has demonstrated that antibodies from adult-onset multiple sclerosis do not bind native MOG while antibodies from children with acute disseminated encephalomyelitis (ADEM) react with native MOG. On the other hand, adult-onset multiple sclerosis cases react immunologically with denatured MOG [10]. In animal study using this method, antibodies from EAE do not bind native MOG [10].

It remains unknown whether native MOG or denatured MOG is the main target protein in autoimmune optic neuritis or NMO. If one can establish that the antibodies from optic neuritis-related diseases recognize a configuration of the MOG protein different from that recognized by antibodies from general multiple sclerosis (e.g., antibodies of patients with ADEM that recognize native MOG), this will provide evidence that optic neuritis and multiple sclerosis are independent diseases. Further analyses are expected to generate interesting new insights.

4. Retinal Damage in a Concurrent Multiple Sclerosis and Optic Neuritis Animal Model

In an animal model of multiple sclerosis, retinal ganglion cells are also damaged, although this finding has not been proven in humans. Meyer et al. [11] reported that encephalomyelitis developed in rats after immunization with MOG, inflammation occurred in the optic nerve axons, and apoptosis of retinal ganglion cells was induced. These results are very interesting, because it is generally believed that peripheral nerves without a myelin sheath are not attacked in multiple sclerosis. In fact, peripheral nerve fibers in the retina are not directly damaged in patients with multiple sclerosis. Likewise, a study using MOG-specific TCR transgenic mice showed that optic neuritis occurred spontaneously and retinal ganglion cells were gradually damaged starting from day 12, with TUNEL-positive apoptotic cells increasing to 83% on day 16 [12]. Therefore, in animal models of multiple sclerosis, retinal ganglion cells may be concurrently damaged, and the involvement of IL-1β and glial cell line-derived neurotrophic factor in neurodegeneration has attracted interest [13]. These molecules are probably related to neuroprotection. Future studies using optical coherence

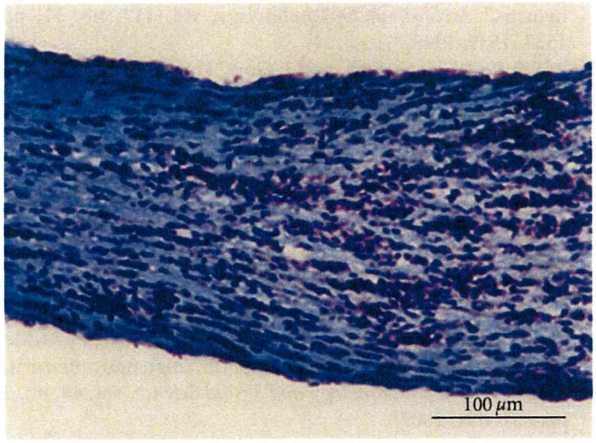

FIGURE 2: Luxol fast blue staining for myelin sheath in the optic nerve of a mouse adjuvant immunized with MOG peptide. Regions of weak staining of the myelin sheath corresponds to regions of cell infiltration in the optic nerve.

tomography (OCT) to analyze the perioptic nerve retina in multiple sclerosis patients may provide proof of retinal ganglion cell damage in humans.

5. Development of Novel Therapies Using Mouse Optic Neuritis Model

Recently, progress in the elucidation of the pathogenesis of intractable optic neuritis has reached a level that allows application of the research findings to treatment clinically. Obviously animal models are indispensable for the research and development of therapeutic modalities. Gene therapy has been attempted using animal model of optic neuritis. Guy et al. [14, 15] injected an adenovirus vector containing the human gene for catalase (possessing antioxidant activity) over the optic nerve head of mice with EAE and succeeded to suppress optic neuritis together with encephalomyelitis in these mice. Furthermore, by injecting an adenovirus vector containing both catalase and extracellular superoxide dismutase (ECSOD) genes (dual gene therapy) into the eye of EAE models, demyelination was attenuated [16, 17]. Moreover, gene therapy using another antioxidant gene, superoxide dismutase 2 (SOD2), also suppressed mitochondrial oxidative stress and ameliorated the optic neuritis and encephalomyelitis in a mouse model [18].

Our group is currently conducting trials to suppress optic neuritis in mice using an approach entirely different from those reported hitherto. It is known that a specific immunoregulatory mechanism termed anterior chamber-associated immune deviation (ACAID) is present in the eye to maintain immune privilege in the intraocular environment [19]. This unique immunoregulatory mechanism in the anterior chamber maintains humoral immunity including serum antibody production, but suppresses cell-mediated immunity represented by intradermal reaction. Immunosuppressive substances such as TGF-ß and neuropeptides present in the anterior chamber play important roles in the ACAID phenomena. Inside the anterior chamber which is an immune privilege site, cells including macrophages are stimulated by antigen in the presence of immunoregulatory substances, and subsequently come into contact with T cells; these T cells migrate out of the eye and are transformed into regulatory T cells. We have examined the possibility of suppressing optic neuritis using the ACAID immunoregulatory system [20], and we found that the incidence was reduced to 70% in the group that received injection of MOG peptide antigen into the anterior chamber while 100% of mice in the positive control group developed optic neuritis. When the severity of optic neuritis was compared by histopathological scores, the anterior chamber injected group which was markedly reduced compared to the positive control group. These results prove that the ACAID immunoregulation is effective in controlling optic neuritis. The salient point about this immunoregulatory system is that disease development is mediated by the host's own immunoregulatory cells. We thus prepared immunoregulatory cells by transferring the calcitonin gene-related peptide (CGRP, known as a neuropeptide) gene into dendritic cells isolated from a mouse. Intravenous injection of the gene-transferred cells into the same mouse ameliorated optic neuritis that developed in the EAE model (report under preparation). This experimental study indicates the possibility to ameliorate optic neuritis by autologous cell therapy.

6. Conclusion

Research to elucidate the pathogenetic mechanism of intractable optic neuritis has shown great progress in recent years, both clinically and also in experimental models. Some of these study results have begun to reach a level permitting their application to analyses of the pathophysiology of human optic neuritis and development of treatment clinically. If cell-mediated therapy, gene therapy, and other advanced therapies using biologicals succeed to control intractable optic neuritis in animal models, this may ultimately lead to prevention of vision loss of optic neuritis in humans. By conducting research flexibly, ready to switch from the bench to the bedside and from the bedside to the bench as the opportunity arises, this strategy may help to guide the research of optic neuritis in the right direction.

Abbreviations

EAON: Experimental autoimmune optic neuritis
MS: Multiple sclerosis
NMO: Neuromyelitis optica
AQP4: Aquaporin-4
MOG: Myelin oligodendrocyte glycoprotein.

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Effect of Infliximab on Gene Expression Profiling in Behçet's Disease

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Purpose. Recent studies have demonstrated that a new anti-tumor necrosis factor (TNF)-α antibody, infliximab, is effective in controlling ocular inflammatory attacks in Behçet's disease. In this study, the effect of infliximab on gene expression patterns in peripheral blood mononuclear cells of Behçet's disease patients was investigated before and after initiation of infliximab treatment.

Methods. A human whole-genome microarray of 54,359 genes was used to analyze mRNA expression profiles of peripheral blood mononuclear cells obtained from four patients (three women, one man, 21-64 years at age) at baseline and at 22 weeks after initiation of infliximab. Quantitative polymerase chain reaction (PCR) analysis was performed for selected up- or downregulated genes, to confirm the microarray results.

Results. Anti-TNF-α therapy reduced the frequency of ocular episodes in three of four patients. Among inflammatory cytokine-related genes, TNF blockade reduced expression of interleukin (IL)-1 receptor type 2, interferon-γ receptors, IL6, IL6 receptor, gp130, and IL17 receptors. Furthermore, gene expression of Toll-like receptor 2 (*TLR2*), receptor for mycobacterial glycolipid (C-type lectin domain family 4, member E: *CLEC4E*), and complexin 2 (*CPLX2*) was downregulated in all patients.

Conclusions. Several up- or downregulated genes identified in this study may be candidates for further investigation in identifying the molecular mechanism of infliximab in the treatment of Behçet's disease with refractory uveoretinitis. (*Invest Ophthalmol Vis Sci.* 2011;52:7681-7686) DOI:10.1167/iovs.11-7999

Behçet's disease is a systemic occlusive vasculitis, resulting in the four major clinical manifestations of (1) recurrent and chronic intraocular inflammation, (2) recurrent aphthous ulcers of the mouth, (3) genital ulcers, and (4) skin lesions that may include erythema nodosum, acneiform lesions, and cutaneous hypersensitivity thrombophlebitis.[1,2] Behçet's disease is characterized by unilateral or bilateral acute episodes of iridocyclitis with or without hypopyon, and/or panuveitis.[3-5] Although the etiology is unknown, both genetic and environmental factors play a role in the pathogenesis of this disease.[2,6] Behçet's disease is particularly common in the Far East and the Mediterranean basin, and is frequently noted between the 30th- and 45th-degree latitudes in Asian and European populations, corresponding to the Old Silk Road.[7] In addition, it has been reported that activation of both innate and adaptive immune responses to infection with streptococci or heat shock proteins (HSPs) may be involved in the development of Behçet's disease.[6,8,9]

Until very recently, the treatment of Behçet's disease was based on a strategy of inducing immunosuppression in the patient, by attacking the pathways of the immune system in a broad and rather nonspecific manner using drugs such as corticosteroids and cyclosporine. Several reports in the literature have described new anti-inflammatory biological agents such as infliximab, an anti-tumor necrosis factor (TNF)-α antibody, as having good efficacy in the treatment of Behçet's disease. Infliximab was approved by the Japanese national health insurance system in January of 2007 for the indication of Behçet's disease with refractory uveoretinitis.[10-12] Although blockade of TNF-α using infliximab reduces ocular attacks in Behçet's disease, the mechanism of action remains to be determined. In the present study, to investigate how infliximab treatment affects gene expression in patients with Behçet's disease, we used DNA microarray technology to analyze mRNA expression profiles in peripheral blood samples from Behçet's disease patients with refractory uveitis before and after initiation of treatment with infliximab. The present study is a first attempt at understanding the molecular mechanisms involved in the effect of infliximab in Behçet's disease through genome-wide gene expression profiling.

Methods

Patients

Four patients (three women, one man, 21-64 years of age) were recruited for the study. To be considered for treatment with infliximab, patients had to be refractory to therapy with at least one immunosuppressive medication and/or corticosteroids or intolerant of such therapy. The diagnosis of Behçet's disease was made based on criteria of the Behçet's Disease Research Committee of Japan.[13] Before starting infliximab, all patients underwent a complete rheumatological examination, tuberculin protein purified derivative (PPD) skin testing, and chest radiography. If patients had a positive PPD skin test, oral isoniazid was concomitantly administered for tuberculosis prophylaxis. All patients received infliximab (Remicade: Mitsubishi Tanabe Pharma, Osaka, Japan) infusions at a dose of 5 mg/kg at 0, 2, and 6 weeks and every 8 weeks thereafter. Three of four patients were receiving cyclosporine (2.0-2.5 mg/kg/d), and two patients were receiving prednisolone (5.0 mg/d) at the time of initiation of infliximab therapy. This study was conducted in accordance with the tenets of the Declaration of Helsinki, and was approved by the Kyorin University Hospital Research Ethics Committee. All patients provided written informed consent at the time of enrollment in the study.

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Study Procedures and Evaluations

Blood samples for mRNA profiling were obtained immediately before the first intravenous infusion of infliximab and at 22 weeks, just before the fifth infusion. Visual acuity measurements (using Landolt C charts) and slit lamp and fundus examinations were performed at every visit. Ocular attacks were defined as the sudden onset of inflammation in the anterior segment, vitreous body, or fundus, as confirmed by ophthalmic examination.[14] The number of ocular attacks before and after initiation of infliximab treatment was converted to frequency per 6-month period. Retinal vasculitis was evaluated by fluorescein angiography at baseline and at 6 months after initiation of infliximab treatment. Degree of retinal vasculitis was evaluated based on fluorescein dye leakage from retinal vessels (periphery, posterior pole, and optic disc) and was scored from 0 to 3 (0, absence of vascular leakage; 1, mild vascular leakage; 2, moderate vascular leakage; and 3, severe vascular leakage) as previously described.[15] Two ophthalmologists performed the assessment in a masked fashion, and the mean score was calculated.

Preparation of RNA, DNA Hybridization, and Analysis

Peripheral blood mononuclear cells (PBMCs) were prepared from heparinized blood samples by centrifugation using density gradients (Lymphoprep; Axis-Shield, Oslo, Norway). Total RNA was first extracted from PBMCs (Isogen RNA isolation kit; Nippon Gene, Tokyo, Japan) and purified (RNeasy Mini Kit; Qiagen, Tokyo, Japan). Total RNA was analyzed (2100 Bioanalyzer; Agilent Technologies, Santa Clara, CA) and UV spectrophotometry was used to check quality. Total RNA (1 μg) was taken, and biotin-labeled cRNA was synthesized (MessageAmp II-Biotin Enhanced Kit; Ambion, Austin, TX). The biotin-labeled cRNA was fragmented and hybridized (CodeLink Human Whole Genome Bioarray; Applied Microarrays, Tempe, AZ) for 18 hours (300 rpm shaker) at 37°C. The hybridized slides were washed and incubated with streptavidin-Alexa Fluor 647 (GE Health Care Bio-Science; Piscataway, NJ) for 30 minutes at 25°C, to label the cRNA, and washed again. The slides were scanned with a laser-based detection system (GenePix 4000B; Molecular Devices, Sunnyvale, CA).

Data Processing

Scanned image files were analyzed with the microarray-associated software (CodeLink Expression Analysis 5.0 software; Applied Microarrays). The net intensity was calculated by subtracting the median intensity of all pixels within the local background area from the mean intensity of all pixels within the spot areas. The net intensity of each spot was normalized by quantile with a microarray data-analysis program (Microarray Data Analysis Tool, ver. 3.2; Filgen, Nagoya, Japan), and gene expression data of test sample and control sample were compared.

Real-Time Polymerase Chain Reaction

First-strand cDNAs were synthesized (TaqMan One-step RT-PCR Master Mix Reagents; Applied Biosystems [ABI], Foster City, CA) from total RNA. Real-time PCR quantification (qPCR) was performed (TaqMan Gene Expression Assay; 7500 real-time PCR System; ABI). Probes and primer pairs (TaqMan; ABI) of *CLEC4E* (assay ID: Hs00907314_m1), *ETS2* (assay ID: Hs01036305_m1), *IL1R2* (assay ID: Hs01030384_m1), *KIAA0101* (assay ID: Hs00207134_m1), *TLR2* (assay ID: Hs01872448_s1) and *GAPDH* (assay ID: Hs99999905_m1) were obtained from ABI. The assays were performed in 20 μL of reaction volume. The conditions of one-step reverse transcriptase (RT)-PCR were as follows: 30 minutes at 48°C, and 10 minutes at 95°C, followed by 60 cycles of 15 seconds at 95°C and 1 minute at 60°C. All reactions were run in duplicate. The threshold cycle (Ct) was defined as the fractional cycle number at which the fluorescence passes the fixed threshold. Target gene expression levels were quantified using GAPDH as endogenous control. The calibration curve was obtained using fivefold serial dilutions of total RNA from each sample.

Data Analysis

Enrichment analysis was performed to identify significant pathways/categories using pathway data from the National Center for Biotechnology Information (NCBI, Bethesda, MD, http://www.ncbi.nlm.nih.gov/sites/entrez?db=biosystems). Software used for the analysis included Gene Ontology (GO) and the data analysis tool (Microarray Data Analysis Tool; Filgen), and pathways that satisfied a z-score > 0 and $P < 0.01$ were considered to be significant.[16] Clustering analysis was performed using MeV version 4.6.1 (http://www.tm4.org/mev). P values were calculated by Fisher's exact test.

Results

Patient Characteristics and Response to Infliximab Treatment

Table 1 provides demographic and clinical response information for the four Behçet's disease patients with refractory uveitis at baseline and at 6 months of infliximab therapy. The mean duration of Behçet's disease associated uveitis was 56 months. Other manifestations in these patients included oral aphthous lesions (four patients), skin lesions (two patients), and genital ulcers (one patient). Three of the four patients were receiving cyclosporine at the time of initiating infliximab therapy, and three of these patients were still receiving cyclosporine (at a lower dose) at 6 months. Two of four patients were receiving oral corticosteroids in addition to cyclosporine at the initiation of infliximab therapy; the corticosteroids were continued through 6 months in two patients. As shown in Table 1, although ocular attacks were observed in three of four patients before infliximab treatment, all patients had no recurrence during the first 6 months on infliximab. The ocular attacks in patients 1 and 3 involved inflammation in the fundus (posterior pole), whereas the ocular attacks in patient 4 involved the anterior segment. Patient 2 experienced no ocular attacks per se during the 6-month period before initiating infliximab treatment; however, this patient had a history of

Table 1. Characteristics of Enrolled Patients at Baseline and 22 Weeks after Initiation of Infliximab Treatment

						Number of Ocular Attacks First		Fluorescein Leakage Score First	
	Sex	Age (y)	Duration (mo)	CyA (mg/d)	PSL (mg/d)	Baseline*	6 Months	Baseline	6 Months
1	F	50	69	100	5	2	0	3.5	2.0
2	M	34	40	—	—	0	0	13	12.0
3	F	21	14	150	—	2	0	6	2.5
4	F	64	105	150	5	4	0	5.5	5.0

CyA, cyclosporine; PSL, prednisolone.

* The number of ocular attacks before and after initiation of infliximab treatment was converted to frequency per 6-month period.

ocular attacks in the fundus prior to that period. In addition, the retinal vascular leakage score by fluorescein angiography was reduced in all patients at 6 months of infliximab treatment compared to the baseline (Table 1). No adverse effects were observed in any patient during the study period.

Analysis of Inflammatory Cytokine and Chemokine-Related Genes Differentially Expressed before and after Initiation of Infliximab Treatment

Of 54,359 in the microarray, a 2.0-fold or higher alteration in the expression ratio between baseline and 22 weeks after starting infliximab was observed for 138 genes in patient 1, 188 genes in patient 2, 271 genes in patients 3, and 1717 genes in patient 4. In contrast, a twofold or greater decrease in the expression ratio was observed for 267 genes in patient 1, 456 genes in patient 2, 356 genes in patient 3, and 456 genes in patient 4. We attempted to identify hierarchical clustering specific to the four patients; however, no characteristic clustering group was found due to the small sample size (data not shown). Genes related to inflammatory cytokines including interleukin (IL), interferon (IFN)-γ, TNF, and chemokines that were up- or downregulated compared to the baseline (greater than 2.0-fold change) were analyzed in each patient. As shown in Table 2, several cytokine receptors such as *IL1R2*, *IL2RG*, *IFNGR1/2*, *IL6R/IL6ST* (*GP130*), and *IL17R A/E* were downregulated in at least two patients. In addition, the IL1 receptor associated kinase 3 (*IRAK3*) and signal transducer and activator of transcription 6 (*STAT6*) involved in signal transduction of macrophages and T cells were downregulated with infliximab treatment. On the other hand, *IL8*, *IL12A/23A*, and *IL32* were upregulated in two patients. As listed in Table 3, several chemokine or chemokine receptor genes were down- or upregulated in each patient, although there were differences between patients. In particular, *CX3CR1* was downregulated in three patients; however, this same gene was found to be overexpressed in one patient.

Analysis of Genes Up- or Downregulated in All Four Patients

Next, we determined what genes were up- or downregulated (by at least 1.5-fold) in all four patients with infliximab treatment. As shown in Table 4, *CLEC4E* (C-type lectin domain family 4, member E: NM_014358), *CPLX2* (complexin 2: NM_001008220), UI-H-DF1-auj-n-04-0-UI.s1 NCI_CGAP_DF1: BM991706), *ETS2* (v-ets erythroblastosis virus E26 oncogene homolog 2: NM_005239), *TLR2* (BC032464), *PLXNC1* (plexin C1: AI290473), *LHFPL2* (lipoma HMGIC fusion partner-like 2: NM_005779), and *TPCN2* (two pore segment channel 2: NM_139075) were downregulated in all four patients. In contrast, only one gene *KIAA0101* (NM_014736), was upregulated in all four patients.

qPCR Validation of Down- or Upregulated Gene Expression

To confirm some of the data obtained by cDNA microarray, we also used real-time PCR to analyze mRNA expression. Figure 1 shows the real-time PCR data for the downregulated genes *IL1R2*, *TLR2*, *CLEC4E*, and *ETS2*, and for the upregulated gene *KIAA0101*. The gene expression of *IL1R2*, *TLR2*, and *CLEC4E* was reduced in all four patients compared with baseline, whereas the expression of *ETS2* was reduced in three patients. As for *KIAA0101*, gene expression was elevated in all four patients compared to baseline.

Discussion

Recent reports have demonstrated that infliximab is effective in suppressing ocular attacks in Behçet's disease,[10–12] but details of its molecular mechanism of action are still unclear. To our knowledge, this study is the first to use DNA microarray technology to investigate gene expression profiles in PBMCs from patients with Behçet's disease before and after initiation of infliximab treatment. Our data revealed the effect of TNF blockade on active ocular inflammation in Behçet's disease to be associated with changes in gene expression related to specific inflammatory responses, including those of cytokine and chemokines and of innate immune responses.

Microarray and qPCR analysis showed that gene expression of *TLR2* was downregulated in all four patients after starting infliximab. This finding is in line with a recent report that infliximab reduced *TLR2* and *TLR4* expression in monocytes from patients with spondyloarthropathy.[17] Furthermore, since

Table 2. Significantly Down- or Upregulated Cytokine-Related Genes in Behçet's Disease Patients after Initiation of Infliximab Treatment

Patient 1			Patient 2			Patient 3			Patient 4		
Gene Symbol	GenBank ID	Ratio	Gene Symbol	GenBank ID	Ratio	Gene Symbol	GenBank ID	Ratio	Gene Symbol	GenBank ID	Ratio
Downregulated Genes											
IRAK3	AI652770	0.346	*IL6*	NM_000600	0.049	*IRAK3*	AI652770	0.159	*IL1R2*	NM_004633	0.285
IL6R	XI2830	0.499	*IL1RN*	NM_173843	0.056	*IL17RE*	H18179	0.478	*IL6ST*	NM_002184	0.375
IL1R2	NM_004633	0.345	*IL8RB*	NM_001557	0.257				*IFGR1*	NM_000416	0.422
IFNGR1	NM_000416	0.468	*IRAK3*	AI652770	0.294				*IL13RA1*	U81379	0.430
NFIL3	NM_005384	0.484	*STAT6*	NM_003153	0.399				*IL2RG*	BC071710	0.435
			IL7R	NM_002185	0.426				*IL17RA*	NM_014339	0.481
			IFNGR2	NM_005534	0.434				*IL17C*	NM_013278	0.490
			TNF	NM_000594	0.466				*STAT6*	BF808288	0.493
			IL23A	NM_16584	0.477						
			IL2RG	NM_000206	0.477						
Upregulated Genes											
IL7R	NM_002185	2.719	None			*IL8*	NM_000584	2.948	*IL8*	NM_000584	4.139
IL32	NM_001012631	2.227				*IL12A*	NM_000882	2.091	*IL32*	NM_001012631	3.967
						IL12RB2	RO1220	2.088	*IL4R*	AW449273	2.896
						IL22RA2	NM_181310	2.034	*IL23A*	X00437	2.635
									IL2RG	NM_000206	2.015

Relative ratios between baseline and 22 weeks after starting infliximab were calculated for each patient.

TABLE 3. Significantly Down- or Upregulated Chemokine-Related Genes in Behçet's Disease Patients after Initiation of Infliximab Treatment

Patient 1			Patient 2			Patient 3			Patient 4		
Gene Symbol	GenBank ID	Ratio	Gene Symbol	GenBank ID	Ratio	Gene Symbol	GenBank ID	Ratio	Gene Symbol	GenBank ID	Ratio
Downregulated Genes											
None			*CCL3*	NM_002983	0.033	*CX3CR1*	NM_001337	0.184	*CCR2*	NM_000648	0.147
			CX3CR1	U28934	0.036	*CCR5*	AA287788	0.359	*CX3CR1*	NM_001337	0.330
			CXCL2	BF509029	0.041	*CXCL5*	NM_002994	0.409			
			CCL7	NM_006273	0.049	*CX3CR1*	U28934	0.419			
			CCL2	BQ188762	0.134	*CCR3*	NM_178329	0.439			
			CXCL3	NM_002090	0.140	*CCR6*	NM_031409	0.468			
			CCR3	NM_178329	0.283	*CCL3*	NM_002983	0.497			
			CCR1	NM_001295	0.361						
			CX3CR1	NM_001337	0.429						
			CCR4	NM_005508	0.451						
			CXCR6	NM_006564	0.483						
			CXCL10	NM_001565	0.494						
Upregulated Genes											
CX3CR1	NM_001337	4.756	None			*CCL7*	NM_006273	2.582	*CCL3*	NM_002983	3.390
CCR5	AA287788	2.495				*CXCL2*	BF509029	2.132	*CCR7*	BF508279	2.446
									CCL4	NM_002984	2.322
									CCL7	NM_006273	2.008

Relative ratios between baseline and 22 weeks after starting infliximab were calculated for each patient.

stimulation with inflammatory cytokines such as TNF-α, IFN-γ, and IL-6 has been demonstrated to elevate the expression of *TLR2* and *TLR4*,[18,19] it is likely that the downregulation of *TLR2* that we observed in this study was due to an indirect effect through infliximab-associated changes in cytokine expression.

The present microarray analysis revealed that the expression of eight genes was reduced in all four patients. In addition, we confirmed that the gene expression of *CLEC4E* was downregulated in all four patients by qPCR. CLEC4E, also known as Mincle, is expressed in macrophages and is induced after exposure to various stimuli.[20] Ishikawa et al.[21] have demonstrated that CLEC4E is a pivotal receptor for the mycobacterial cord factor, which is the most abundant glycolipid in the mycobacterial cell wall. TNF-blockers have been shown to increase the risk of reactivation of latent tuberculosis in patients.[22,23] Although TNF blockade has been reported to interfere with innate and adaptive immune responses to mycobacterium tuberculosis in several ways,[24] it is possible that downregulation of *CLEC4E* increases the risk of tuberculosis disease in Behçet's disease patients treated with TNF blocker. Furthermore, it has been found that *CLEC4E* also recognizes a nuclear protein released by dead or dying cells and that it mediates the recruitment of neutrophils.[25] Given that increased neutrophil chemotaxis has been suspected of playing a role in Behçet's disease,[26,27] we speculate that infliximab

TABLE 4. Down- or Upregulated Genes in All Behçet's Disease Patients after Initiation of Infliximab Treatment

Gene Symbol	Gene Name	GenBank ID	Mean Ratio	GO Biological Process
Downregulated Genes				
CLEC4E	C-type lectin domain family 4, member E	NM_014358	0.350	Immune response (GO:0006955)
CPLX2	Complexin 2	NM_001008220	0.352	Neurotransmitter transport (GO:0006346) Vesicle docking during exocytosis (GO:0006904) Mast cell degranulation (GO:0043303)
N/A	UI-H-DFI-auj-n-04-0-UI.s1 NCI_CGAP_DF1	BM991706	0.379	Unknown
ETS2	v-ets erythroblastosis virus E26 oncogene homolog 2	NM_005239	0.408	Skeletal system development (GO:0001501) Positive regulation of transcription (GO:0045893)
TLR2	Toll-like receptor 2	BC032464	0.459	Innate immune response (GO:0045087) Inflammatory response (GO:0006954)
PLXNC1	Plexin	A1290473	0.468	Cell adhesion (GO:0007155) Signal transduction (GO:0007165) Multicellular organismal development (GO:0007275)
LHFPL2	lipoma HMGIC fusion partner-like 2	NM_005779	0.497	Unknown
TPCN2	Two pore segment channel 2	NM_139075	0.579	Ion transport (GO:0006811) Calcium ion transport (GO:0006816)
Upregulated Genes				
KIAA0101	KIAA0101	NM_014736	1.843	Unknown

Mean ratios were calculated based on relative ratios between baseline and 22 weeks after starting infliximab for each patient ($n = 4$). N/A, not available.

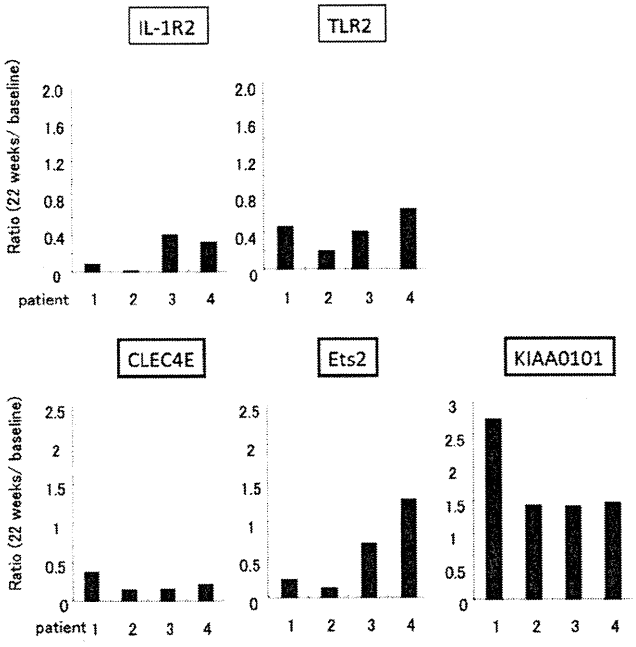

FIGURE 1. qPCR validation of down- or upregulated gene expression. Results of real time PCR data for the downregulated genes *IL1R2*, *TLR2*, *CLEC4E*, and *ETS2*, and for the upregulated gene *KIAA0101* are shown for each patient. Relative ratios between baseline and 22 weeks after starting infliximab were calculated.

therapy reduces the expression of *CLEC4E*, resulting in the inhibition of recruitment of neutrophils to inflammatory sites.

The present study revealed that infliximab treatment downregulated the expression of *CPLX2* in all four patients. Complexins (CPLXs) are soluble proteins that regulate the activity of soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complexes necessary for vesicle fusion.[28] Recently, Remmers et al.[29] identified a genetic association in the promoter region of *CPLX1* in European but not in Japanese Behçet's disease patients. Although it remains unknown how downregulation of *CPLX2* is involved in the suppressive effect of infliximab in Behçet's disease, regulation of exocytosis by CPLXs may be associated with potential pathogenic mechanisms in this disorder.

Recent studies have demonstrated that IFN-γ and IL-17/IL-23 production are elevated in Behçet's disease patients with active uveitis.[30,31] Therefore, we suspected that TNF blockade reduced the expression of these genes in PBMCs. However, IFN-γ gene expression was not downregulated in Behçet's disease patients treated with infliximab compared with baseline. In contrast, we found that T-cell receptor (TCR) signaling pathways, in particular phosphorylation of CD3/TCR zeta chains and translocation of ZAP-70 (ζ-chain [TCR] associated protein kinase 70 kDa) to immunologic synapse, were significantly upregulated in two patients by pathway analysis (data not shown). Interestingly, it has been reported that persistent expression of TNF in vitro and in vivo impairs the T-cell immune response, and that exposure of T cells in vitro to TNF downregulates expression of CD3ζ.[32] Furthermore, TNF blockade has been shown to elevate IFN-γ production in PBMCs from RA patients, suggesting that anti-TNF therapy reverses T-cell immune reactivity in vivo.[30,33,34] In addition, recent reports have shown that with infliximab treatment, regulatory T-cell (Treg)–mediated suppression was restored to levels found in healthy individuals[35] and that infliximab elevated the frequency of Tregs in PBMCs from patients with Behçet's disease.[36] All the evidence taken together indicates that TNF blockade may not only suppress innate immune responses but also restore the function of circulating T cells in Behçet's disease.

Some limitations of this study should be considered. First, since the number of patients in the present study is very small, more samples are needed to confirm the data obtained in this study. Second, as shown in the Results section, the number of genes up- and downregulated by infliximab varied greatly between our patients, all of whom responded well to infliximab. This variation suggests that inflammatory gene responses to anti-TNF therapy are not always the result of the suppressive effect of infliximab on ocular inflammation in Behçet's disease. Further study is needed to examine whether differences in gene expression profiles influence the clinical response to infliximab and/or the development of side effects. Third, since concomitant use of cyclosporine in three of four patients and corticosteroids in two of four patients was maintained throughout the 6-month period after the infliximab therapy started, the effect of these drugs on gene profiles in our patients must also be considered. Fourth, given that more women than men were included in this study, it is possible that the sex of the subject may have influenced the gene expression profiles we obtained with infliximab treatment. A greater number of patients, particularly male patients, must be examined to explore this possibility. Fifth, one of the patients was a woman of postmenopausal age (patient 4), and a larger number of upregulated genes (over 1500 genes) was observed in this patient with infliximab treatment compared with the other three patients. It has been reported that menopause may alter gene expression profiles of circulating monocytes,[37] and therefore the effect of menopause on our data also should taken into consideration.

In conclusion, the present study demonstrated the potential of using gene expression profiling analysis to understand the molecular mechanism of the TNF blocker, infliximab, on refractory uveoretinitis in Behçet's disease. Several up- or downregulated genes identified in this study may be candidates for further investigations in identifying molecular mechanism of infliximab in the treatment of Behçet's disease with refractory uveoretinitis. Our study is only a first attempt in this direction, but we believe that it lays a basis for further research.

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Decreased ocular inflammatory attacks and background retinal and disc vascular leakage in patients with Behçet's disease on infliximab therapy

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ABSTRACT

Aim To evaluate the efficacy of infliximab treatment in patients with refractory uveoretinitis associated with Behçet's disease.

Methods Clinical records of 14 patients were retrospectively reviewed. Patients received infliximab infusions (5 mg/kg) at weeks 0, 2 and 6, and every 8 weeks thereafter. The main outcome measures were frequency of clinically observable ocular inflammatory attacks, background retinal and disc vascular leakage as assessed by fluorescein angiography during periods of clinical quiescence, visual acuity and adverse effects.

Results The median follow-up after initiating infliximab therapy was 19 months (range 12–29 months). At 12 months, eight of 14 patients (57%) had experienced no inflammatory attacks, and the frequency of attacks was significantly reduced when compared with the 6-month period just prior to infliximab use. Background retinal and disc vascular leakage assessed at 12 months improved in 11 of 14 patients (79%). Visual acuity improved or remained unchanged at 12 months in 26 of 28 eyes (93%). Infliximab therapy was terminated in two patients owing to infusion reactions. However, no serious adverse effects were observed.

Conclusion Infliximab over the first year of treatment appeared effective in reducing ocular inflammatory attacks, as well as background retinal and disc vascular leakage, in patients with refractory uveoretinitis associated with Behçet's disease.

INTRODUCTION

Behçet's disease is a multisystem inflammatory disorder with a chronic and relapsing course.[1–3] The four major clinical manifestations are intraocular inflammation, recurrent oral aphthous ulcers, genital ulcers and skin lesions.[1–3] Patients with ocular involvement usually have unilateral or bilateral acute episodes of anterior uveitis with or without hypopyon, posterior uveitis and/or panuveitis.[1–3] The disease eventually affects both eyes in the majority of cases, and is believed to be particularly severe in young men.[4] The frequency and severity of ocular attacks determine the extent of damage to intraocular structures and ultimately visual prognosis.[5 6] However, continuous background retinal and disc vascular leakage is well documented, even during periods of clinical quiescence,[7] and such 'background inflammation' may also contribute to slow destruction of ocular tissues and decline in vision. To our knowledge, the effect of treatment on such retinal and disc vascular leakage in Behçet's disease has never been reported.

Until recently, the treatment of Behçet's disease was based on a strategy of inducing immunosuppression using drugs such as corticosteroids and ciclosporin, thereby inhibiting the pathways of the immune system in a broad and rather non-specific manner.[8] New agents such as infliximab, an anti-tumour necrosis factor α antibody, have demonstrated their effectiveness in the treatment of rheumatoid arthritis and Crohn's disease.[9] Recently, open-label clinical trials in Japan and Turkey have shown infliximab to be effective also in Behçet's disease in terms of reducing ocular inflammatory attacks, although no analysis of background retinal and disc vascular leakage was carried out in these studies.[10 11]

The overall aim of this retrospective study was to evaluate efficacy and safety of infliximab in ocular Behçet's disease over the first year of treatment. Efficacy was assessed, not only by the number of clear clinically observable ocular inflammatory attacks but also by the degree of background retinal and disc vascular leakage as observed by fluorescein angiography.

METHODS

Patients

Medical records were reviewed of 14 patients with Behçet's disease followed in the Ocular Inflammation Service at the Kyorin Eye Center, Kyorin University Hospital (Tokyo, Japan) who received infliximab therapy for at least 6 months between January 2007 and December 2009. Eight patients met the International Study Group criteria for Behçet's disease, while six patients were diagnosed based on the criteria of the Behçet's Disease Research Committee of Japan.[12 13] As per Japanese Ministry of Health, Labour, and Welfare (MHLW) guidelines concerning the indication for infliximab therapy, all 14 patients had ocular inflammation refractory to treatment with at least one immunosuppressive drug and/or corticosteroids, or were judged to be intolerant to such therapy owing to adverse effects.

Treatment protocol

Before starting infliximab, all patients underwent a complete internal medical examination, tuberculin protein purified derivative (PPD) skin testing and chest x-ray examination. Patients found to be PPD-positive received tuberculosis prophylaxis consisting of 400 mg/day of isoniazid for 9 months. The treatment protocol as per MHLW guidelines involved intravenous infusions of infliximab at a dose of 5 mg/kg at weeks 0, 2 and 6, and every

8 weeks thereafter. In patients receiving ciclosporin at the time of initiating infliximab therapy, the ciclosporin was tapered down to a lower dose and then maintained in an attempt to inhibit the development of human antichimera antibodies.[14 15] All other immunosuppressive agents were tapered, with the goal of eventual discontinuation.

Main outcome measures

The main outcome measures were the number of clinically observable ocular inflammatory attacks, background retinal and disc vascular leakage as assessed by fluorescein angiography during periods of clinical quiescence, visual acuity and adverse effects.

Anterior chamber cells and flare were graded as per the SUN Working Group.[16] Ocular inflammatory attacks were defined as the sudden onset of cells and/or flare in the anterior chamber or vitreous, or cellular infiltrates and/or haemorrhage in the retina, as assessed clinically by slit-lamp biomicroscopy and funduscopy. The number of ocular attacks before and after initiating infliximab treatment was converted to frequency per 6-month period. To assess the degree of background retinal and disc vascular leakage, fluorescein angiography (FA) was performed prior to initiation of infliximab therapy (baseline) and at 12 months, at times of clinical quiescence defined as the absence of signs of an ocular inflammatory attack. FA was also performed at 24 months in five patients. The extent of fluorescein leakage was graded on a scale of 0 to 3 (0=none, 1=mild, 2=moderate, 3=severe) for the peripheral retina, the macula and the optic disc. Two ophthalmologists (HK and AO) performed the FA grading in a masked fashion. The mean scores were calculated for the peripheral retina, macula and optic disc separately, with the total vascular leakage score representing the sum of these three mean scores.

Best-corrected visual acuities were measured at each clinic visit. Adverse events including infusion reactions (occurring during and up until 2 h after conclusion of the infliximab infusion) were recorded. This study was conducted in accordance with the tenets of the Declaration of Helsinki and was deemed by the Kyorin University Hospital Research Ethics Committee not to require institutional review board approval.

Statistical analysis

Statistical analysis was performed using the Wilcoxon signed-rank test. A p value of <0.05 was considered to indicate statistical significance. All results were expressed as mean±SE.

RESULTS

The demographic characteristics of the 14 Behçet's disease patients who received infliximab are summarised in table 1. Twelve patients were men, and two were women, with ages ranging from 13 to 68 years (mean 38 years). The median duration of intraocular inflammation associated with Behçet's disease prior to starting infliximab therapy was 30 months. Manifestations or history of oral aphthous ulcers, skin lesions and genital ulcers were noted in 14, six and four patients, respectively. One patient had also been diagnosed as having neurological manifestations of Behçet's disease. Twelve of the 14 patients were receiving ciclosporin at the time of initiating infliximab therapy, and the ciclosporin was tapered down in nine patients. At 12 months on infliximab, seven patients were receiving a ciclosporin dose of 100 mg/day or less. Six of the 14 patients were receiving oral corticosteroids in addition to ciclosporin at the initiation of infliximab therapy, and the corticosteroid was tapered to a dose of 10 mg/day or less in five of six patients and discontinued in two patients. Two patients were receiving methotrexate in addition to ciclosporin and corticosteroids at the initiation of infliximab therapy, and the methotrexate was continued at a dose of 8 mg/wk in one patient for neurological manitfestations and discontinued in the other patient.

Table 1 Baseline patient demographics and clinical manifestations

No of patients	14
Gender	
Male	12
Female	2
Age (years)	
Mean	38
Range	13–68
Duration of ocular disease (months)	
Median	30
Range	13–105
Systemic manifestations	
Oral aphthous ulcers	14
Skin lesions	6
Genital ulcers	4
Therapy at baseline	
Ciclosporin	12
Prednisolone	6
Methotrexate	2

The median follow-up after initiating infliximab therapy was 19 months (range 12–29 months). Eight of 14 patients (57%) had no ocular inflammatory attacks over the first 12 months. Two of five patients examined after 24 months on infliximab also had no clinically observable ocular attacks. As shown in figure 1A, the mean frequency of ocular attacks before infliximab treatment was significantly reduced during the first 6 months of infliximab administration. However, over subsequent 6-month periods, this mean frequency of ocular attacks gradually rose. Of note, infliximab treatment was terminated owing to severe infusion reactions in two patients at 6 and 12 months of initiating therapy. As shown in figure 1B, when these two patients were excluded from the data, the mean numbers of ocular attacks per 6-month period were significantly reduced at 6, 12 and 18 months after initiating therapy. Concomitant drugs were reduced or discontinued in 10 of the 12 patients who were able to continue infliximab therapy.

Six patients had ocular attacks during the first 12 months after initiating infliximab therapy (figure 2). Patients 4 and 5 developed ocular attacks at various time points after the last infliximab infusion, although these were the two patients mentioned above who experienced infusion reactions leading to discontinuation of infliximab treatment. Thereafter, patient 4 moved to a different city, where he was reportedly restarted on infliximab. In patient 5, the dose of concomitant ciclosporin was increased with moderate control of ocular recurrences.

The remaining four patients who had ocular attacks experienced them at weeks 7 to 8 after the last infliximab infusion. These consisted of asymptomatic small cellular infiltrates in the peripheral retina with no decrease in visual acuity in patients 2, 6 and 11, for which additional treatments were not performed. In patient 7, mildly symptomatic anterior chamber cells were noted at week 8, for which topical corticosteroids were prescribed and quickly tapered. The interval between infliximab infusions was not shortened in these four patients.

One patient, a 38-year-old man, was also diagnosed as having neuro-Behçet's disease based on symptoms of leg weakness and

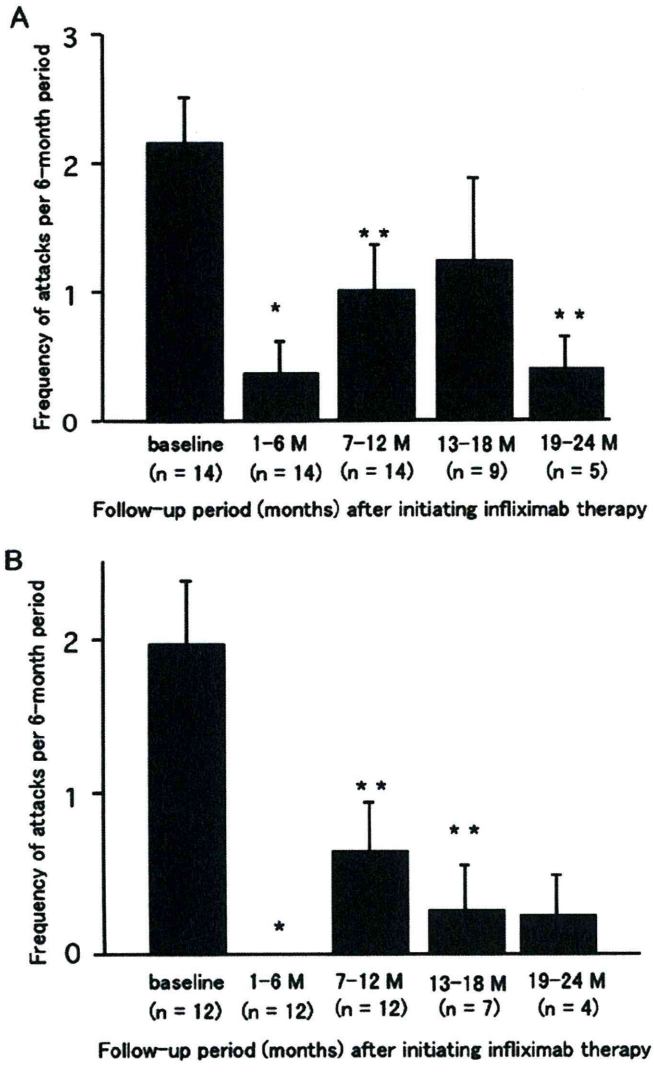

Figure 1 Frequency of ocular inflammatory attacks before and after initiation of infliximab treatment. (A) Number of ocular inflammatory attacks before and after initiation of infliximab treatment, converted to the frequency per 6-month period, with the mean±SE shown for each period. (B) Mean number of ocular inflammatory attacks (±SE) before and after initiation of infliximab treatment, shown after excluding data from two patients in whom infliximab therapy was terminated due to severe infusion reactions. A statistical analysis was performed using the Wilcoxon signed-rank test. *$p<0.01$ versus baseline. **$p<0.05$ versus baseline.

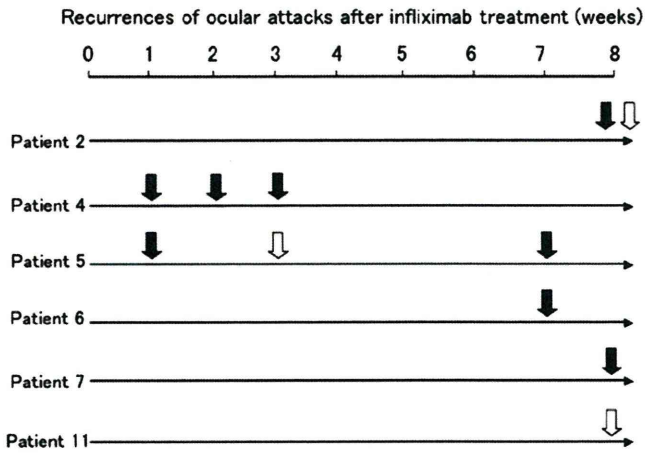

Figure 2 Timing of recurrence of ocular attacks from the last infliximab infusion. The interval between the last infusion of infliximab and recurrence of ocular attacks was analysed. Six patients had recurrence of ocular attacks during 12 months. In four patients (patients 2, 6, 7 and 11), recurrence occurred 7 or 8 weeks after the last infliximab infusion. Patients 4 and 5 developed recurrence at various time points. Arrows indicate the occurrence of ocular attacks. Open arrows indicate bilateral ocular attacks. The numbers shown in the figure indicate the exact time point after infliximab treatment in the 8-week interval between infliximab infusions.

urinary retention, with corresponding parenchymal abnormalities on brain MRI and cerebrospinal fluid pleocytosis. This patient had no neurological or ocular inflammatory recurrences over the first 24 months of infliximab.

Figure 3A shows the mean grade of retinal vascular leakage as assessed by FA at baseline before initiating infliximab therapy and at 12 months of therapy. There was a significant decrease in the total vascular leakage score with infliximab treatment ($p=0.0338$). The score was reduced for peripheral retina, macula and optic disc, although the difference was statistically significant only for peripheral retina. As shown in figure 3B, the total vascular leakage score on infliximab decreased in 11 of 14 patients (79%). The two patients in whom infliximab therapy had been terminated owing to severe infusion reactions (patients 4 and 5) represented two of the three patients in whom no improvement in total vascular leakage score was noted. Figure 4 shows representative FA images (patient 2), showing decreased peripheral retina and disc vascular leakage at 21 months after initiation of infliximab therapy.

As shown in table 2, the percentage of patients with a best-corrected visual acuity (BCVA) of 1.0 or better rose from 46% at baseline to 61% at 12 months. Three eyes (10%) with a BCVA of 0.1 or less at baseline experienced no improvement in visual acuity, although these eyes had optic disc atrophy and/or macular atrophy prior to the initiation of infliximab therapy. The BCVAs before and after initiating infliximab treatment are shown in figure 5. The BCVA was maintained or improved in 26 of 28 eyes (92.8%) at 12 months, and in eight of 10 eyes (80%) at 24 months. The two eyes that had a decrease in BCVA at 12 months were from patient 4, who discontinued infliximab therapy (at 6 months) owing to severe infusion reactions; this patient subsequently moved to another city after 22 months of follow-up. A further two eyes had a decrease in BCVA at 24 months; these belonged to patient 5, who also had to discontinue infliximab therapy (at 12 months) owing to severe infusion reactions.

With regard to adverse effects, five patients experienced symptoms consistent with the common cold; all these patients recovered without specific treatment. One patient had suspected bacterial pharyngitis that responded to antibiotic therapy.

Four patients experienced infliximab-related infusion reactions consisting of rash, itchiness and/or respiratory distress. These infusion reactions occurred at the fifth infusion in two patients, the eighth infusion in one patient, and the 15th infusion in one patient. All four of these patients had been receiving concomitant ciclosporin. After the initial infusion reaction, subsequent infusions were performed with preadministration of intravenous hydrocortisone (100 mg), and the infusion reactions abated in two patients. However, infusion reactions continued in the remaining two patients with increasing severity, and infliximab therapy was discontinued as mentioned previously at 6 and 12 months. The dose of concomitant ciclosporin was

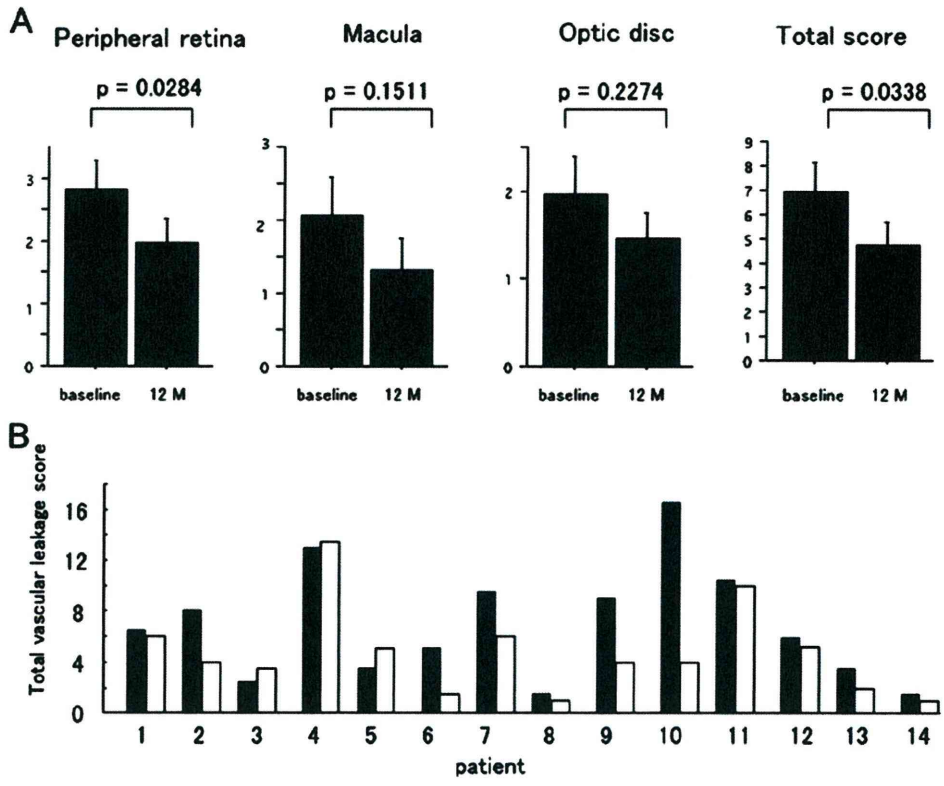

Figure 3 Improvement in retinal vasculitis after infliximab infusions. (A) Fluorescein angiography (FA), performed at times of clinical quiescence prior to initiation of infliximab treatment (baseline) and at 12 months to assess the degree of vascular leakage. The extent of fluorescein leakage was graded on a scale of 0 to 3 (0=none, 1=mild, 2=moderate, 3=severe) for the peripheral retina, the macula and the optic disc. The data represent means±SEM within each group. A statistical analysis was performed using the Wilcoxon signed-rank test. (B) Total vascular leakage score, calculated for each individual patient, with the black bar indicating the score at baseline and the white bar indicating the score at 12 months.

subsequently increased to 250 mg/d in patient 5 in whom infliximab therapy was terminated at 12 months; no increase in ocular inflammatory attacks has been observed in this patient over a 24-month period since stopping infliximab.

DISCUSSION

In our hands, infliximab therapy significantly reduced the frequency of ocular inflammatory attacks in 12 of 14 Behçet's disease patients, with eight patients experiencing no attacks

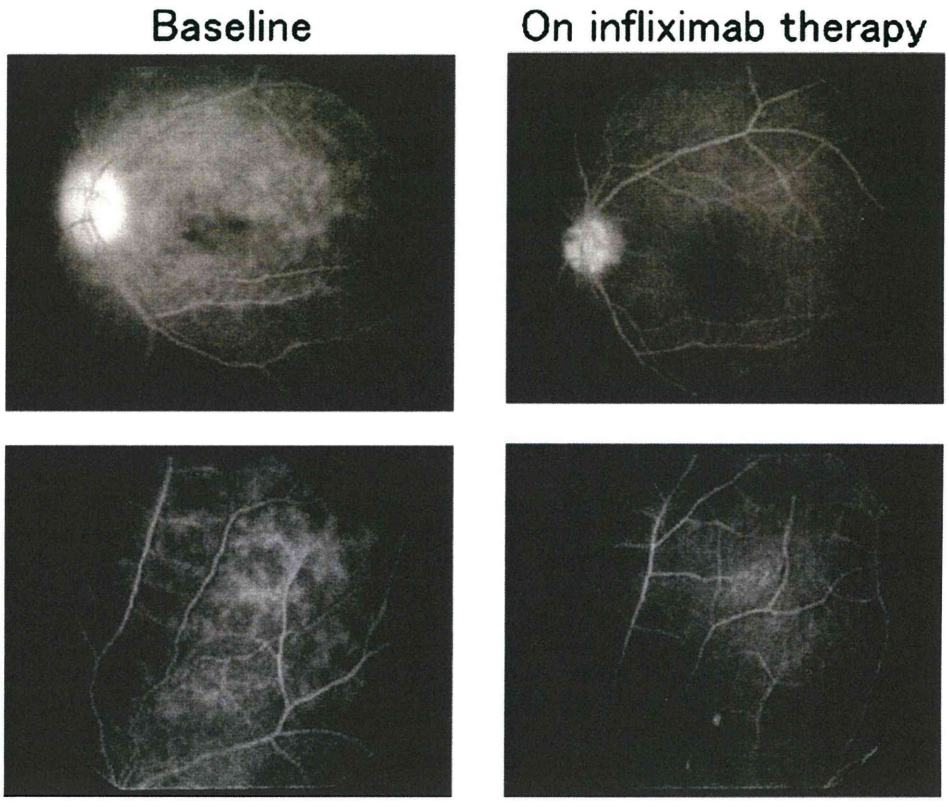

Figure 4 Fluorescein angiographic images of a representative Behçet's disease patient at baseline and on infliximab therapy. Fluorescein angiographic images of the left eye of patient 2 at baseline and at 21 months after initiation of infliximab treatment during times of clinical quiescence. Fluorescein leakage from vessels in the macula, the peripheral retina and the optic disc were observed before infliximab treatment (left upper and lower images). This fluorescein leakage showed an improvement on infliximab therapy (right upper and lower images).

Table 2 Best-corrected visual acuities in Behçet's disease patients treated with infliximab (number of eyes)

	Baseline N=28 eyes	12 months N=28 eyes	24 months N=10 eyes
≥1.0 (%)	13 (46.4)	17 (60.7)	5 (50.0)
0.5–0.9 (%)	8 (28.6)	4 (14.3)	2 (20.0)
0.2–0.4 (%)	4 (14.3)	4 (14.3)	1 (10.0)
≤0.1 (%)	3 (10.7)	3 (10.7)	2 (20.0)

whatsoever over the first 12 months of therapy. Furthermore, on infliximab, we were able to reduce the concomitant immunosuppressive regimen in 10 of 12 patients. These results suggest that in Behçet's disease patients who are treated continually with infliximab therapy, ocular inflammatory attacks are markedly suppressed with successful tapering or discontinuation of immunosuppressive drugs in the majority of patients. These results are in line with previous reports, indicating that infliximab is effective for suppressing ocular attacks in Behçet's disease.[10 11 17 18] In addition, the frequency of ocular inflammatory attacks was found not to rise with time in patients who were able to continue successfully with infliximab therapy (figure 1B).

The ocular attacks while on infliximab we observed were mild and occurred just prior to the next infliximab infusion in most cases, similar to the experience of other clinicians.[19] The serum infliximab concentration in these patients may have dropped to lower than a certain threshold necessary for control of ocular disease.[20] However, in our study, two patients developed recurrences at various time points regardless of their infusion schedule. In these patients, ocular recurrence may have been related to inadequate infliximab dosing owing to the development of infusion reactions.

We also investigated whether infliximab therapy would affect the degree of background retinal and optic disc vascular leakage as assessed by fluorescein angiography at times of clinical quiescence. It is well known that retinal and optic disc atrophy are major causes of morbidity in Behçet's disease,[21 22] and we suspect that damage to these intraocular structures occurs not only because of acute inflammatory attacks, but also because of chronic smoldering inflammation as evidenced by background vascular leakage. In our study, we found that infliximab significantly reduced vascular leakage in the peripheral retina at 12 months of therapy. In addition, vascular leakage in the macula and at the optic disc also improved, although this was not statistically significant. Suppression of such chronic vascular leakage, whether via infliximab therapy or other treatments, would be expected to aid in the preservation of tissue function and therefore ultimately visual function. Furthermore, vascular leakage on FA may serve as an important surrogate marker for the degree of inflammatory control for Behçet's disease patients on various drug regimens.

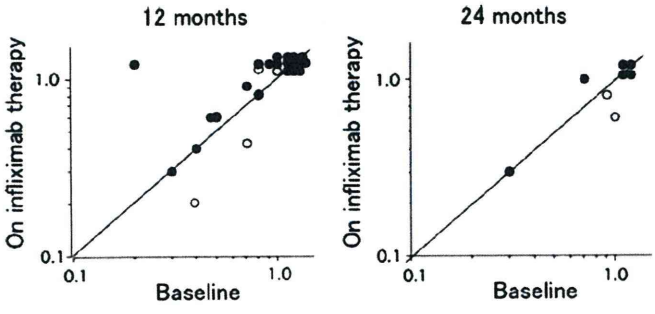

Figure 5 Visual acuities at baseline and on infliximab therapy. Best-corrected visual acuities of all study eyes are shown at baseline and at 12 months (n=14) and 24 months (n=5) after initiation of infliximab administration. The closed circles represent the 12 patients who received infliximab treatment for over 1 year. The open circles represent the two patients who discontinued infliximab treatment due to infusion reactions at 6 and 12 months, respectively.

In the present study, the percentage of eyes with a BCVA of 1.0 or better increased from 46% at baseline to 61% at 12 months after initiating infliximab therapy. In contrast, three eyes (10%) with a BCVA of 0.1 or less at baseline experienced no improvement in visual acuity owing to prior optic disc and/or macular atrophy. Previous studies have shown that the major reasons for poor visual outcomes in Behçet's disease are macular atrophy, optic disc atrophy and chorioretinal atrophy.[5 6] Moreover, a relationship between the number of ocular inflammatory attacks and poor visual prognosis has been documented.[5 6] Taken together, this strongly suggests that the initiation of infliximab therapy early on during the uveitis disease course would be beneficial in maintaining good visual function in patients with Behçet's disease.

Serious adverse effects were not observed in the present study. Notably, although six of 14 patients had positive PPD test results and received isoniazid prophylaxis, no patients developed tuberculosis during the follow-up period. Consistent with our findings, previous studies have also reported that infliximab treatment did not result in PPD-positive patients developing tuberculosis if proper prophylaxis was used.[11] However, in a preliminary open label study of infliximab in Behçet's disease in Japan, Ohno and colleagues reported one case of miliary tuberculosis among 13 patients.[10]

Some patients on infliximab have been known to develop antibodies against the drug (human antichimera antibodies), and it has been speculated that the presence of these antibodies may be associated with a greater risk of infusion reactions.[14] Furthermore, it has been reported that the development of anti-infliximab antibodies is associated with low circulating drug levels.[23] In patients with rheumatoid arthritis and Crohn's disease, the use of infliximab with concomitant methotrexate has been shown to reduce the incidence of infusion reactions.[14 15] Given such data, in our study we elected to continue concomitant ciclosporin albeit at lower doses, with the hope of reducing the immunogenicity of infliximab treatment. However, four of 14 patients developed infusion reactions, and the infliximab therapy was eventually halted in two of these patients. Anti-infliximab antibodies were not measured, and therefore any relation between infusion reactions and the development of antidrug antibodies cannot be determined. Furthermore, the dose of concomitant ciclosporin may have been inadequate to suppress the development of antibodies. Finally, it is possible that methotrexate, as commonly administered in rheumatoid arthritis, may be more effective than ciclosporin in suppressing antibody development.

The limitations of this study are that the data were examined retrospectively, all patients were from a single institution, and the number of the patients was small. Furthermore, several years of follow-up would be necessary to evaluate the full scope of the efficacy and safety of using infliximab in Behçet's disease. Even so, our results suggest strong efficacy for infliximab in suppressing not only ocular inflammatory attacks but also background retinal and optic disc vascular leakage, with the BCVA improving or being maintained in 93% of eyes at 12 months.

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