Expression of CX3C chemokine, fractalkine, and its receptor CX3CR1 in experimental autoimmune anterior uveitis

I-Mo Fang,1,2 Chang-Pin Lin,1 Chung-May Yang,1 Muh-Shy Chen,1 Chang-Hao Yang1

1Department of Ophthalmology, National Taiwan University Hospital, Taipei, Taiwan; 2Department of Ophthalmology, Taipei Municipal ZhongXiao Hospital, Taipei, Taiwan

Purpose: To demonstrate the expression and location of CX3C chemokine, fractalkine, and its receptor, CX3CR1, in the iris/ciliary body and thus establish their roles in experimental autoimmune anterior uveitis, an animal model of human acute anterior uveitis.

Methods: Uveitis was induced in Lewis rats by injection of melanin associated antigen into the peritoneum and footpad. At defined times, fractalkine and its receptor CX3CR1 mRNA expression in the iris/ciliary body were measured by using a semiquantitative polymerase chain reaction method. Fractalkine in aqueous humor was determined by enzyme linked immunosorbent assay. The cellular sources of fractalkine were determined by immunohistochemical staining. In a separate experiment, NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC; 200 mg/kg/day) was administrated intraperitoneally daily after immunization. The rats were sacrificed on day 14 of immunization. Fractalkine mRNA in iris/ciliary body and fractalkine concentration in aqueous humor were determined after PDTC treatment.

Results: Fractalkine mRNA was found to be upregulated in the iris/ciliary body nine days after immunization, preceding clinical disease onset. CX3CR1 mRNA exhibited peak levels at day 14, coincident with disease onset. Fractalkine in aqueous humor showed an expression profile similar to mRNA expression. PDTC (200 mg/kg) markedly inhibited the expression of fractalkine mRNA in the iris/ciliary body, and fractalkine protein in aqueous humor. Immunohistochemical staining revealed that fractalkine was expressed on vascular endothelial cells and infiltrated inflammatory cells. Treatment with PDTC significantly reduced both the number of leukocyte infiltrations in the iris/ciliary body and fractalkine expression on vascular endothelial cells.

Conclusions: The sequential expression of fractalkine may direct distinct CX3CR1 receptor expressing mononuclear cell subsets to inflammatory sites. Fractalkine expression is modulated, at least in part, through the NF-κB signaling pathway. These findings provide new insight into the molecular mechanisms of acute anterior uveitis and suggest fractalkine or NF-κB as a new drug target for uveitis therapy.

Acute anterior uveitis (AAU) is the most common form of uveitis in humans. It is also a major visual problem because its recurrent nature may result in secondary complications such as cataract, cystoid macular edema, or glaucoma [1]. Experimental autoimmune anterior uveitis (EAAU) is a T-cell mediated autoimmune disease that serves as an animal model for human acute anterior uveitis [2,3]. Immunization of Lewis rat with bovine melanin associated antigen (MAA) and appropriate adjuvants resulted in a disease with many of the clinical and pathologic features of the human disease [4]. EAAU differed particularly from other models because the inflammation remained exclusively anterior without retina and choroid involvement [5,6]. Studies of the molecular mechanisms of EAAU would provide insight into the pathogenesis of human AAU.

Chemokines are small chemoattractant cytokines that induce leukocyte accumulation at inflammatory sites and modulate inflammatory activities via the recruited cells [7,8]. Previous studies have demonstrated that chemokine are implicated in the pathogenesis of many autoimmune diseases, including uveitis [9,10]. According to NH2-terminal cysteine motifs, chemokines can be grouped into four families; C, CC, CXC, and CX3C [11]. Fractalkine, the only member of the CX3C chemokine family, differs greatly from other chemokines in that it can exit the cell either as a soluble form or a membrane bound form [12].

Fractalkine is expressed in various organs, including the skin, brain, and kidneys [13-15], and interacts with its unique receptor, CX3CR1, which is expressed on monocytes, natural killer cells, and some T cells [16]. Fractalkine and CX3CR1 represent a novel type of leukocyte trafficking molecule that regulates both adhesive and chemotactic functions [17]. The membrane bound fractalkine is able to mediate firm adhesion of CX3CR1 expressing leukocytes without requiring selectin mediated rolling of activation of integrins [18,19]. Furthermore, fractalkine is released from the cell surface by means of proteolytic cleavage as soluble fractalkine, which has potent chemoattractant activity for CX3CR1+ leukocytes and thus activates the leukocytes [20,21]. Because CX3CR1 is preferentially expressed on Th1 cells, with respect to Th2 cells, cell infiltration through fractalkine-CX3CR1 interaction especially promotes Th1 responses [22]. Numerous studies have demonstrated that fractalkine-CX3CR1 interaction contributes to the development of various inflammatory diseases including...
rheumatoid arthritis, psoriasis, and glomerulonephritis [23-26]. In the eye, Silverman et al. [27] have demonstrated in vivo the presence of fractalkine in normal cultured microvascular endothelial, and stromal cells in iris and retina. However, the expression of fractalkine in ocular inflammatory diseases has remained unknown.

The nuclear factor kappa B (NF-κB) is a transcriptional factor that can regulate the expression of pro-inflammatory genes, including cytokines, adhesion molecules, and chemokines [28,29]. In the cytoplasm of inactive cells, NF-κB exists as a complex bound to its inhibitor, known as inhibitory protein κB (I-κB) [30]. Many stimuli trigger the phosphorylation of I-κB, resulting in the dissociation of the NF-κB-IκB complex. This allows NF-κB to migrate to the nucleus, where it binds to specific promoter sequences of DNA and thereby initiates gene transcription. Pyrrolidine dithiocarbamate (PDTC) is a potent NF-κB inhibitor that could inhibit NF-κB induction by decreasing I-κB phosphorylation, thus preventing the dissociation of the NF-κB-IκB complex [31,32]. Previous in vitro studies have demonstrated that NF-κB mediates cytokine induced fractalkine activation [33]. However, there are no data available concerning the role of NF-κB in fractalkine activation in vivo in ocular inflammatory diseases.

The purpose of this study was to investigate the expression of fractalkine and its receptor, CX3CR1, in the iris/ciliary body in EAAU. Additionally, we examined the effect of the NF-κB inhibitor PDTC on fractalkine gene and protein expression in vivo to establish the role of NF-κB in the activation of fractalkine expression of EAAU.

METHODS

Antigen and Induction of EAAU: MAA was prepared by modifying the method originally described by Broekhuyse et al. [3-6]. The iris and ciliary body were carefully removed from fresh pigmented bovine eyes. The tissue was gently homogenized with phosphate buffered saline (PBS) and filtered through a wire mesh to remove cellular debris and connective tissue. The homogenate was centrifuged at 1.2x10^5 g at 4 °C for 15 min and washed once with PBS at pH 7.4. The resulting pellet was suspended in 2% sodium dodecyl sulfate (SDS; Bio-Rad, Richmond, CA) and incubated at 70 °C for 10 min. After centrifugation, the pellet was washed three times with water. The insoluble antigen was dried and stored at -20 °C.

Lewis rats, 6-8 weeks old and weighing 125-160 g, were used for the experiment. All animals were treated in accordance with the ARVO statement for the Use of Animals in Ophthalmic and Vision Research. To induce EAAU, the rats were given two separate injections simultaneously. The first injection was 0.05 ml MAA, suspended in PBS, emulsified (1:1) in complete Freund’s adjuvant (CFA; Sigma, St. Louis, MO) and injected into the left hind footpad and the second injection was 0.05 ml MAA, emulsified with 1 μg purified Bordetella pertussis toxin (PTX; List, Campbell, CA), and injected intraperitoneally. The experiments were repeated three times on different experimental days.

<table>
<thead>
<tr>
<th>Name</th>
<th>Primer alignment</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fractalkine</td>
<td>Forward</td>
<td>GAATTCTTGCGGCTAGCTGCCTTCGTCCATT</td>
</tr>
<tr>
<td>Reverse</td>
<td>AGCTGCTCAGGACCTCACCAT</td>
<td></td>
</tr>
<tr>
<td>CX3CR1</td>
<td>Forward</td>
<td>AGGCTGCAGGCCTCACCAT</td>
</tr>
<tr>
<td>Reverse</td>
<td>GTGTTGAGGCCCTCATGGCTGAT</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward</td>
<td>CTGGAAAGGAAGCTCTATGAGCTG</td>
</tr>
<tr>
<td>Reverse</td>
<td>AATCTCCTTCTGAGCTCCCTGC</td>
<td></td>
</tr>
</tbody>
</table>

Primer sequences for fractalkine, CX3CR1, and β-actin are listed. All of the primers were prepared by DNAFax (Taipei, Taiwan).
Clinical activity scoring: The rats were clinically observed with slitlamp biomicroscopy on a daily basis. Two different observers looked for clinical signs of ocular inflammation in a masked manner. Disease severity was assessed with a score from 0 to 4 (0: normal; 1: slight dilatation of iris vessels and some anterior chamber cells; 2: iris hyperemia, some limitation in pupil dilatation, anterior chamber cells, and slight flare; 3: miotic, irregular, hyperemic, and (sometimes) slightly damaged iris with considerable flare and cells [especially with accumulation near the iris]; 4: seriously damaged and hyperemic iris, miotic pupil often filled with protein, and cloudy gel-like aqueous humor [AqH]).

Tissue preparation: Rats were sacrificed on day 3, 9, 11, 14, 18, and 25 (n=5 for each group) after immunization. The eyes were quickly dissected, and the iris and ciliary body were isolated from the remaining ocular tissue using an operating microscope.

Preparation of RNA and cDNA: Total RNA was extracted from the iris/ciliary body with Trizol reagent (Invitrogen, Carlsbad, CA). Total RNA (1 µg) from each sample was annealed for 5 min at 65 °C and then cooled to 4 °C with 300 ng oligo(dT), obtained from Promega (Madison, WI) and reverse transcribed to cDNA with 0.4% trypan-blue solution, and the numbers of different types of leukocytes were counted using phase contrast microscopy.

Polymerase chain reaction: The amplification was performed with a thermocycler (MJ Research, Waltham, MA). The 50 µl reaction mixture consisted of 5 µl cDNA, 1 µl of sense and antisense primer, 200 µM of each deoxynucleotide, 5 µl 10X Taq polymerase buffer, and 1.25 U Taq polymerase (Promega). Primer sequences for fractalkine, CX3CR1, and β-actin are listed in Table 1. All of the primers were prepared by DNAFax (Taipei, Taiwan). Conditions for amplifying fractalkine and CX3CR1 were as follows: denaturation for 1 min at 94 °C and elongation for 3 min at 72 °C. The annealing temperature for fractalkine and CX3CR1 was 62 °C. At the end of amplification, the reaction mixture was heated for 10 min at 72 °C and then cooled to 4 °C. A 10 µl sample of each polymerase chain reaction (PCR) product was separated by gel electrophoresis on 2% agarose containing ethidium bromide (Sigma, St. Louis, MO) and then analyzed against DNA molecular length markers under ultraviolet light. The intensity of the products was analyzed using an image analyzer (Digital 1D Science; Eastman Kodak, Rochester, NY), and the amount of PCR-amplifiable material in each reverse transcribed sample was standardized against the amount of a housekeeping gene, rat β-actin.

Quantification of leukocytes in aqueous humor: Using a 30 gauge needle, AqH was collected from the eyes immediately after the animal was sacrificed. The AqH was pooled in silicon treated microcentrifuge tubes (Fisher Scientific, Pittsburgh, PA). A volume of 2 µl AqH from one rat was stained with 0.4% trypan-blue solution, and the numbers of different types of leukocytes were counted using phase contrast microscopy.

Quantification of fractalkine in AqH: The levels of fractalkine in the AqH obtained from rats with EAAU were quantitated on days 3, 9, 11, 14, 18, and 25 (n=5 for each group) after immunization using a sandwich enzyme linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. The
ELISA assay was repeated twice. The sample was diluted up to 50 µl and used for testing. Optical density was determined at A450 with a microplate reader (Bio-Rad, Richmond, CA). Using recombinant standards supplied by the manufacturer, fractalkine concentration was determined from standard curves.

Immunohistochemistry: Formalin fixed, paraffin embedded tissue sections were placed on slides, deparaffinized in xylene, and rehydrated through graded ethanol into PBS. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol. Then the sections were treated with 5% normal rat serum and incubated overnight with goat anti-rat fractalkine immunoglobulin G (IgG, 150 nm/ml; R&D Systems) at 4 °C. Thereafter, a biotinylated horse secondary antibody against goat IgG and an avidin-biotinylated peroxidase complex (Santa Cruz Biotechnology, Santa Cruz, CA) were used with 3,3’-diaminobenzidine as a peroxidase substrate. Sections were counterstained with hematoxylin, dehydrated, and mounted. Goat IgG (R&D Systems) were used as primary antibody in the sections of negative control.

Treatment with NF-κB inhibitor PDTC: To examine whether NF-κB is involved in regulating fractalkine gene activation, rats were randomly divided into two groups of ten. One group received intraperitoneal injections of PDTC (200 mg/kg/day; Sigma) daily after MAA induction. The other group received intraperitoneal injection of PBS daily after MAA induction as a control. The rats were sacrificed on day 14 after immunization. Fractalkine mRNA in the iris/ciliary body and fractalkine concentration in aqueous humor after PDTC treatment were determined.

Statistical analysis: All values in the figures and the text were expressed as mean ± standard deviation (SD). With respect to the effects of PDTC on clinical scores, the figure shown is representative of three experiments performed on three different experimental days. Differences between the amounts of the cytokine mRNA and protein concentrations at different times and between control and PDTC treated groups were evaluated by one way analysis of variance followed by the Bonferroni multiple comparison test, as appropriate. Values of p < 0.05 were considered significant. The Mann-Whitney U and Kruskal-Wallis test were used to examine differences be-

Figure 4. Effect of PDTC on ocular inflammation and fractalkine expression in EAAU. Effect of the NF-κB inhibitor, pyrrolidine dithiocarbamate (PDTC), on fractalkine mRNA expression in the iris/ciliary body (A), fractalkine protein concentration in aqueous humor (B) and clinical score (C) of Lewis rats immunized with melanin-associated antigen. The rats were treated with PDTC after immunization. They were sacrificed on day 14. Total RNA and aqueous humor was extracted. Groups shown are the rats immunized with MAA and treated with PDTC (200 mg/kg) every day (“PDTC-treated”), rats immunized with MAA (“MAA”), and rats without MAA immunization (“Normal”). The intensity of mRNA was analyzed using an image analyzer and the relative intensity was determined by comparison of its intensity with that of β-actin. Data are presented as the mean±SD in ten rats. The asterisk indicates a p<0.05, when compared to the normal control.
tween the clinical scores of control and experimental groups. When these tests were used, a p<0.05 was considered significant.

RESULTS
Clinical scores of EAAU: Clinical signs for EAAU appeared on day 14 after immunization. The disease reached its peak on day 19, with a clinical score of 2.60±0.35, after which recovery began. Recovery was complete by around day 30.

Iris and ciliary body fractalkine and CX3CR1 mRNA expression: Fractalkine mRNA expression started increasing on day 9 and reached its peak on day 11, preceding onset of clinical disease. It remained at relatively high expression levels during the evolution of clinical disease. At the recovery stage of disease, fractalkine mRNA expression also declined in parallel with the subsidence of clinical disease (Figure 1A).

CX3CR1 mRNA expression (the receptor for fractalkine) was upregulated on day 11, coincident with the peak of fractalkine mRNA expression. The maximal expression of CX3CR1 mRNA was on day 14, concurrent with disease onset. It returned abruptly to a control level on day 18 (Figure 1B).

Fractalkine levels in AqH: In AH, the increase in fractalkine protein started on day 9, prior to clinical disease onset. It reached its peak on day 14, concurrent with disease onset. A high concentration of fractalkine protein in AH was detected from day 11 to day 18, correlating with the evolution stage of clinical disease. With the abrupt decline in the concentration of fractalkine from day 18, the severity of clinical disease also reduced (Figure 2).

Quantification of leukocytes in AqH: Leukocytes, mainly lymphocytes and monocytes, were found in the anterior chamber of rats with EAAU from day 11, coincident with onset of clinical disease. The increase and decrease of leukocytes number in AqH were closely correlated to the expression of fractalkine protein in AqH (Figure 3).

Figure 5. Immunohistochemical staining for fractalkine expression in the iris/ciliary body from Lewis rats. Rats with experimental autoimmune anterior uveitis showed stronger fractalkine expression in vascular endothelial cells (arrows) in the ciliary body (CB; A, B) and in the infiltrating cells (arrows) in the iris/CB and posterior chamber (A, C), compared with those of normal rats (E). Treatment with PDTC (200 mg/kg/day) contributed to reduced mononuclear cell infiltration and fractalkine expression in the iris/CB (D). The negative control (F) was obtained using goat IgG as primary antibody. A ciliary process (CP) is also labeled. The original magnifications were 200x in A, D, E and 400x in B, C.
Effects of NF-κB inhibitor PDTC on fractalkine mRNA in the iris/ciliary body and fractalkine protein in AqH: After conducting a preliminary dose ranging study (data not shown), we used 200 mg/kg/day PDTC for the experiments. Rats treated with PDTC demonstrated a significant reduction in clinical severity than those treated with PBS on day 14 after immunization (0.62±0.16 compared to 1.79±0.15, p=0.01, Figure 4A). PDTC treated eyes resulted in a reduction in fractalkine mRNA in the iris/ciliary body (p=0.001; Figure 4B). The fractalkine protein concentration in AqH was 720±110 pg/ml in rats 14 days after MAA induction, and treatment with PDTC reduced the protein concentration to 280±70 pg/ml (p=0.001; Figure 4C).

**Immunohistochemical staining of fractalkine:** By immunohistochemistry, stronger fractalkine expression was observed in vascular endothelial cells in the ciliary body and in the infiltrating cells in the iris/ciliary body and posterior chamber of rats with EAAU (Figure 5A-C), compared with those of normal rats (Figure 5E). Treatment with PDTC (200 mg/kg/day) led to a reduction not only in the number of leukocyte infiltrations, but also fractalkine expression in the iris/ciliary body (Figure 5D). Control sections showed only background staining (Figure 5F). For the immunohistochemical stain of retina, rats with EAAU were found to have fractalkine staining in the nerve fiber layer and the outer plexiform layer and faint in the nuclear layers (Figure 6A). Normal rats showed a similar staining pattern. There was no difference in the staining intensity between normal and EAAU rats (Figure 6B).

**DISCUSSION**

Fractalkine has been shown to play a role in a variety of pathological conditions related to inflammation in the brain, kidney, skin, and joints [23-26,34]. In this study, we broadened the scope and demonstrated an upregulation of fractalkine before and during the evolution of an experimental model of uveitis in eyes. Additionally, the expression of fractalkine was associated with the upregulation of its receptor, CX3CR1, indicating that fractalkine and CX3CR1 interaction may participate in the pathogenesis of the rat model of uveitis. Furthermore, the NF-κB inhibitor PDTC effectively reduced the expression of fractalkine, suggesting that the regulation of fractalkine in EAAU involves, at least in part, a NF-κB dependent mechanism. This is the first study to identify the expression of fractalkine and CX3CR1 in ocular inflammatory disease.

Large leukocyte infiltration in the iris/ciliary body, especially by monocytes and T lymphocytes, is the pathological hallmark of EAAU [35]. To recruit certain types of leukocytes to the site of inflammation, multi-step processes including establishment of a chemattractant gradient and firm attachment of leukocytes to and migration across the vascular endothelial cells should be properly regulated [36,37]. Previous studies have demonstrated that fractalkine could act as a chemoattractive cytokine to attract monocytes and lymphocytes, and adhesion molecules to promote transendothelial migration of leukocytes in a variety of inflammatory diseases [38,39]. In this study, we found that fractalkine was upregulated prior to the infiltration of monocytes and lymphocytes, and prior to onset of clinical disease. Given the mechanistic parallels between various inflammations elsewhere in the body, we...
could reasonably postulate that early expression of fractalkine may represent an important level of control in directing migration and adhesion of the trafficking monocytes and T cells into inflammatory sites, the iris and the ciliary body.

In this study, CX3CR1 mRNA showed a particular expression pattern; increasing expression at disease initiation, but abruptly dropping to control levels at maximal clinical disease. CX3CR1 is reported to express mostly on the surface of monocytes, T lymphocytes, and natural killer cells, the main infiltrating cells in EAAU [16]. Therefore, our observation that CX3CR1 mRNA was upregulated during disease initiation probably reflects the ongoing infiltrations of these inflammatory cells. However, it is still possible that increased CX3CR1 density in these leukocytes accounts for this upregulation. It is now becoming evident that, under the influence of cytokines, chemokine receptors on leukocytes could change dynamically to modulate chemokine function. The increase in CX3CR1 expression on these cells make them attractive to fractalkine-secreting cells, leading to them being firmly entrapped, and subsequently promotes their migration into inflammatory tissues. Similarly, the decreases in CX3CR1 mRNA expression at the peak of disease make these cells unresponsive to fractalkine. As a result the cells are recruited less to the inflammatory sites, marking the initiation of disease resolution.

Previous studies have reported that endothelial cells, smooth muscle cells, dendritic cells, neurons, and macrophages are capable of producing fractalkine [13,40-42]. Silverman et al. [27] have shown a widespread expression of fractalkine in microvascular endothelial cells and stromal cells in iris and retina. In this study, by immunohistochemistry, we also demonstrated a constitutive expression of fractalkine in iris/ciliary body in normal rats. However, compared with that of normal rats, stronger fractalkine staining could be detected in vascular endothelial cells in ciliary process and in infiltrated cells widespread in the iris/ciliary process and posterior chamber of rats with EAAU. In retina, rats with EAAU were found to have fractalkine staining in the nerve fiber layer and the outer plexiform layer and faint in the nuclear layers. Normal rats showed a similar staining pattern. There was no difference in the staining intensity between the normal and EAAU rats. This finding further confirmed that the inflammations in EAAU are confined to iris/ciliary body without retina and choroid involvement. Fractalkine expressed on inflamed endothelium could play a dual role, as a selective capturing molecule for circulating CX3CR1 cytotoxic effectors and as a subsequent co-signaling molecule promoting their migration into tissues via downstream chemokines. Only in vascular endothelial cells could fractalkines contact leukocytes in the systemic circulation to act as an adhesion molecule in the membrane-bound form, and build a concentration gradient between local tissue and systemic circulation to act as a chemoattractant cytokine in soluble form.

The present study revealed that the blockade of NF-κB activation by an NF-κB inhibitor effectively suppressed fractalkine mRNA and protein expression. In addition, by immunohistochemical staining, we demonstrated that treatment with an NF-κB inhibitor resulted in attenuated fractalkine expression in vascular endothelia. These results suggest that NF-κB plays a role in regulating fractalkine gene expression. Our findings are in agreement with previous in vivo studies, which have showed that the TNF-α-stimulated fractalkine production by rat aortic endothelial cells and vascular smooth muscle cells is NF-κB dependent [43,44]. Brand et al. [45] proposed that, through the activation of NF-κB, fractalkine may interact with other inflammatory mediators to coordinate the expression of genes whose products have key roles in mediation of immune responses. Indeed, we have previously demonstrated that TNF-α was upregulated in parallel with disease progression in EAAU. However, further study is needed to clarify the issue of whether NF-κB-dependent fractalkine activation in EAAU is induced by TNF-α.

In summary, we demonstrated expression of fractalkine during experimental autoimmune anterior uveitis. The sequential expression of fractalkine may direct distinct CX3CR1 receptor-expressing mononuclear cell subsets to inflammatory sites. Furthermore, vascular endothelial cells are the main sources of fractalkine expression. Fractalkine expression is modulated, at least in part, through the NF-κB signaling pathway. These findings provide new insight into the molecular mechanisms of acute anterior uveitis and suggest that fractalkine or NF-κB may be a drug target for therapeutic applications.

ACKNOWLEDGEMENTS

This study was supported by research grant NSC-93-2314-13-002-093 from the National Science Council, Republic of China and the Department of Health, Taipei City Government. We are grateful to Mr. Ghi-Hsin Fong for preparing the manuscript.

REFERENCES

41. Ludwig A, Berkhout T, Moores K, Groot P, Chapman G. Fractalkine is expressed by smooth muscle cells in response to...