行政院國家科學委員會專題研究計畫 期中進度報告

細胞素 Fractalkine 在眼球新生管形成及發炎反應功能之研究(2/3)

期中進度報告(精簡版)

計 畫 類 別 ：個別型
計 畫 編 號 ：NSC 95-2314-B-002-058-
執 行 期 間 ：95 年 08 月 01 日至 96 年 07 月 31 日
執 行 單 位 ：國立臺灣大學醫學院眼科

計 畫 主 持 人：楊長豪

處 理 方 式 ：期中報告不提供公開查詢

中 華 民 國 96 年 05 月 31 日

Expression of CX3C Chemokine, Fractalkine, and its Receptor CX3CR1 in Experimental Autoimmune Anterior Uveitis

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

From the [1]Department of Ophthalmology, National Taiwan University Hospital, and the [2]Department of Ophthalmology, Taipei Municipal ZhongXiao Hospital, Taipei, Taiwan

* Correspondence to: Chang-Hao Yang

Department of Ophthalmology, National Taiwan University Hospital

Chung-Shan S. Rd. No.7, Taipei, Taiwan

TEL: 886-2-23123456 ext.3193 Fax: 886-2-23412875

e-mail: chyang@ha.mc.ntu.edu.tw

Abstract.

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 the Lewis rats by injection of melanin-associated antigen into peritoneum and footpad. At defined time points, fractalkine and its receptor CX3CR1 mRNA expressions 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 immunhistochemical staining. In a separate experiment, NF-κB inhibitor, PDTC (200 mg/kg/day) was administrated intraperitoneally daily after immunization. The rats were sacrificed on14th day of immunization. Fractalkine mRNA in iris/ciliary body and fractalkine concentration in aqueous humor after PDTC treatment were determined.

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 (200mg/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 prominently expressed on vascular endothelial 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. 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 fractalkine or NF-κB as a new drug target for uveitis therapy.

Introduction:

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 CX3C chemokine, differs greatly from other chemokines in that it can exit 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's 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. have demonstrated in vivo the presence of fractalkine in normal cultured microvascular endothelial and stromal cells in the iris and retina [27]. 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 cytoplasma 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 dithocarbamate (PDTC) is a potent NF-κB inhibitor that could inhibit NF-κB induction by decreasing I-κB phosphrylation, 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.

Materials and 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 obtained from fresh pigmented bovine eyes. The tissue was gently homogenized with phosphate-buffered saline and filtered through a wire mesh to remove cellular debris and connective tissue. The homogenate was centrifuged at $1.2 \times 10^5 g$ at 4°C for 15 minutes and washed once with phosphate-buffered saline (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 minutes. 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: 1). 0.05 ml MAA was suspended in PBS, emulsified (1:1) in complete Freund's adjuvant (CFA) and injected into left hind footpad. 2). 0.05 ml MAA, emulsified with 1μg purified Bordetella pertussis toxin (PTX), was injected intraperitoneally.

We repeated the following experiments three times on different experimental days.

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 scale ranging from 0 to 4: 0 = normal; 1 = slight iris-vessel dilatation and some anterior chamber cells; 2 = iris hyperemia, with some limitation in pupil dilation, anterior chamber cells, and a slight flare; 3 = a miotic, irregular, hyperaemic, and (sometimes) slightly damaged iris, with a considerable flare and cells (especially with accumulation near the iris); and 4 = a seriously damaged and hyperaemic iris, a miotic pupil often filled with protein, and cloudy gel-like aqueous humor (AqH).

Tissue preparation

Rats were sacrificed on days 3 ($n = 5$), 9 ($n = 5$), 11 ($n = 5$), 14 ($n = 5$), 18 ($n = 5$), and 25 ($n = 5$) after immunization. The eyes were harvested. Both eyes of the experimental animals were used; e.g. 10 eyes from 5 rats were examined at each time point. 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 (Life, Gaithersburg, MD). One microgram of total RNA from each sample was annealed for 5 min at 65°C with 300-ng oligo(dT)(Promega, Madison, WI) and reverse transcribed to cDNA by using 80 U Moloney murine leukemia virus reverse transcriptase (MMLV-RT)(Gibco, Grand Island, NY) per 50 μg reaction for 1 h at 37°C. The reaction was stopped by heating for 5 min at 90°C.

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 10× Taq polymerase buffer, and 1.25 U Taq polymerase (Promega, Madison, WI). Fractalkine primers were: 5'-GAATTCCTGGCGGGTCAGCACCTCGGCATA-3', 5'-AAGCTTTTACAGGGCAGCGGTCTGGTGGT-3' (DNAFax, Taipei, Taiwan); CX3CR1 primers were: 5'-AGCTGCTCAGGACCTCACCAT-3', 5'-GTTGTGGAGGCCCTCATGGCTGAT-3' (DNAFax, Taipei, Taiwan); β-actin primers were: 5'-CTGGAGAAGAGCTATGAGCTG-3', 5'-AATCTCCTTCTGCATCCTGTC-3' (DNAFax, Taipei, Taiwan). Conditions for amplifying fractalkine and CX3CR1 were as follows: denaturation, 1 min at 94°C, and elongation, 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 performing gel electrophoresis on 2% agarose containing ethidium bromide (Sigma, St. Louis, MO) and then analyzed against the 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 AqH

Using a 30-gauge needle, aqueous humor (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 (*n* = 5), 9 (*n* = 5), 11 (*n* = 5), 14 (*n* = 5), 18 (*n* = 5), and 25 (*n* = 5) 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 50μl and used for the tests. Optical density was determined at A_{450} 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 xylenes, 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, Minneapolis, MN) 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.

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 intrapertoneal injection of PDTC (200 mg/kg/day) (Sigma, St.Louis, MO) 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, a day with high levels of fractalkine mRNA and protein expression. 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 are expressed as mean ± standard deviation mean (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 time points and between control and PDTC-treated groups were evaluated by one-way analysis of variance followed by Bonferroni multiple comparison test, as appropriate. Values of $p < 0.05$ were considered as significant. The Mann-Whitney U and Kruskal-Wallis test were used to examine differences between the clinical scores of control and experimental groups. When this test was used, $p<0.05$ was considered significant.

Results:

Clinical scores of EAAU

Clinical signs for EAAU appeared on day 14 post-immunization. The disease reached its peak on day 19, with a clinical score of 2.60 ± 0.35. Then, recovery started. 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 level duration the evolution of clinical disease. At the recovery stage of disease, fractalkine mRNA expression also declined in parallel with the subsidence of clinical disease (Fig. 1A).

CX3CR 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 control level on day 18. (Fig. 1B)

Fractalkine levels in aqueous humor

In aqueous humor (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. High concentration of fractalkine protein in AH was detected from day 11 to day 18, correlated 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. (Fig. 2)

Quantification of leukocytes in Aqueous humor

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 aqueous humor were closely correlated to the expression of fractalkine protein in aqueous humor. (Fig.3)

Effects of NF-κB inhibitor PDTC on fractalkine mRNA in the iris/ciliary body and fractalkine protein in aqueous humor

After conducting a preliminary dose-ranging study (data not shown), we use 200mg/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 post-immunization (0.62 ± 0.16 vs. 1.79 ± 0.15, P=0.01, Fig 4C).

PDTC-treated eyes resulted in a reduction in fractalkine mRNA in the iris/ciliary body (P=0.001). (Fig. 4A)

The fractalkine protein concentration in aqueous humor 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). (Fig. 4B)

Immunohistochemical staining of fractalkine

By immunohistochemistry, stronger fractalkine expression was observed in vascular endothelial cells in the ciliary process and in the infiltrating cells widespread in the iris/ciliary process and posterior chamber of rats with EAAU (Fig.5A-C), compared with those of normal rats (Fig. 5E). Treatment with PDTC (200mg/kg/day) led to the reduction of the leukocyte infiltrations and fractalkine expression in the iris/ciliary processes (Fig. 5D). After omission of primary antibodies, control sections

showed only background staining (Fig. 5F).

For the immunochemical stain of retina, rats with EAAU were found to have higher fractalkine staining in the nerve fiber layer and the outer plexiform layer and fainter in the nuclear layers (Fig 6A). Normal rats showed similar staining pattern. There is no difference of the staining intensity between the normal and EAAU rats (Fig. 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 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 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 leukocytes 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 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 level 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 expressions on these cells makes them attractive and leads to them being firmly entrapped by fractalkine-secreting cells, 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 [40-43]. In this study, by immunohistochemistry, we observed that vascular endothelial cells of the iris/ciliary body and infiltrating mononuclear cells are the cellular sources of fractalkine in rat model of acute anterior uveitis. 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 fractalkine accessibly contact leukocytes in 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 immunuhistochemical 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 [44,45]. Brand et al. proposed that, through the activation of NF-κB, fractalkine may intersect with other inflammatory mediators to coordinate the expression of genes whose products have key roles in mediation of immune responses [46]. 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 molcecular mechanisms of acute anterior uveitis and suggest that fractalkine or NF-κB may be a drug target for therapeutic applications.

Reference:

1. Nussenblatt RB, Palestine AG. Uveitis: Fundamentals and Clinical Practice. Chicago: Year Book Medical Publishers; 1989.

2. Chan CC, Hikita N, Dastgheib K, Whitcup SM, Gery I, Nussenblatt RB. Experimental melanin-protein induced uveitis in the Lewis rat: Immunopathogenic process. Ophthalmology 1994;101:1275-80.
3. Broekhuyse RM, Kuhlman ED, Winkens HJ. Experimental autoimmune anterior uveitis (EAAU), a new form of experimental uveitis. I. Induction by a detergent-insoluble, intrinsic protein fraction of the retinal pigment epithelium. Exp Eye Res. 1991;52: 465-74.
4. Broekhuyse RM, Kuhman ED, Winkens HJ. Experimental autoimmune anterior uveitis (EAAU). II Dose-dependent induction and adoptive transfer using a melanin-bound antigen of the retina pigment epithelium. Exp Eye Res.1992;55:401-11.
5. Broekhuyse RM, Kuhman ED. Experimental autoimmune anterior uveitis: the preparation of uveitogenic ocular melanin. Invest Ophthalmol Vis Sci. 1993;34:698-700.
6. Bora NS, Kim MC, Kabeer NH. Experimental autoimmune anterior uveitis (EAAU): induction with melanin associated antigen from the iris and ciliary body. Invest Ophthalmol Vis Sci. 1995;36:1056-66.
7. Verma MJ, Lloyd A, Rager H. Chemokines in acute anterior uveitis. Curr eye Res. 1997;16:1202-12088.
8. Baggiolini M. Chemokines and leukocyte traffic. Nature. 1998;392:565-568.
9. Fang IM, Yang CH, Lin CP, Yang CM, Chen MS. Expression of chemokine and receptors in Lewis rats with experimental autoimmune anterior uveitis. Exp Eye

Res. 2004;78:1043-1055.

10. Admaus G, Manczak M, Machnicki M. Expression of CC chemokines and their receptors in the eye in autoimmune anterior uveitis associated with EAE. Invest Ophthalmol. Vis. Sci. 2001;42:2894-2903.
11. Lindley I, Westwich J, Kunkel S. Nomenclature announcement: the chemokines. Immunol Today. 1993;14:24.
12. Bazan JF, Bacon KB, Hardeiman G. Fractalkine: a new class of membrane-bound chemokine with a CX3C motif. Nature.1997; 385:640-644.
13. Harrison JK, Jiang Y, Chen S, Xia Y, Maciejewski D, McNamara RK, Streit WJ, Salafranca MN, Adhikari S, Thompson DA, Botti P, Bacon KB, Feng L. Role for neuronally derived fractalkine in mediating interactions between neurons and CX3CR1-expressing microglia. Proc Natl Acad Sci USA. 1998;95:10896-10901.
14. Papadopoulos EJ, Fitzhugh DJ, Tkacayk C, Gillfillan AM, Sassetti C, Metcalfe DD, Hwang ST. Mast cells migrate, but do not degranulate, in response to fractalkine, a membrane-bound chemokine expressed constitutively in diverse cells of the skin. Eur J Immunol 2000;30;2355-2361.
15. Chakravorty SJ, Cockwell P, Girdlestone J, Brooks CJ, Savage COS. Fractalkine expression on human renal tubular epithelial cells: potential role in mononuclear cell adhesion. Clin Exp Immunol. 2002;129:150-159.
16. Imai T, Hieshima K, Haskell C, Baba M, Nagira M, Nishimura M, Kakizaki M, Takagi S, Nomiyama H, Schall TJ, Yoshie O. Identification and molecular characterization of fractalkine receptor CX3CR1 which mediates both leukocyte migration and adhesion. Cell. 1997;91;521-530.
17. Al-Aoukaty A, Rolstad B, Giaid A, Maghazachi AA. MIP-3α, MIP-3β and fractalkine induce the locomotion and the mobilization of intracellular calcium, and activate the heterotrimeric G-proteins in human natural killer cells.

Immunology. 1998;95:618-624.

18. Fong AM, Robinson LA, Steeber DA, Tedder TF, Yoshie O, Imai T, Patel DD. Fractalkine and CX3CR1 mediate a novel mechanism of leukocyte capture, firm adhesion, and activation under physiologic flow. J Exp Med. 1998;188:1413-1419.
19. Umehara H, Bloom E, Steeber DA, Domae N, Imai T. Fractalkine and vascular injury. Trends Immunol 2001;22:602-607.
20. Yoneda,O, Imai T, Goda S, Inoue H, Yamauchi A, Okazaki T. Imai H, Yoshie O, Bloom ET, Domae N, Umehara H. Fractalkine-mediated endothelial cell injury by NK cells. J Immunol. 2000;164:4055-4062.
21. Foussat A, Coulomb-L'Hermine A, Gosling J, Krzysiek R, Durand-Gasselin I, Schall T, Balian A, Richard Y, Galanaud P, Emilie D. Fractalkine receptor expression by T lymphocyte subpopulations and in vivo production of fractalkine in human. Eur J Immunol. 2000;30:87-97.
22. Fraticelli P, Sironi M, Bianchi G, D'Ambrosio D, Albanesi C, Stoppacciaro A, Chieppa M, Allavena P, Ruco L, Girolomoni G, Sinigaglia F, Vecchi A, Mantovani A. Fractalkine (CX3CL1) as an amplification circuit of polarized Th1 responses. J Clin Invest. 2001;107:1173-1181.
23. Volin MV, Woods JM, Amin MA, Connors MA, Harlow LA, Koch AE. Fractalkine: a novel angiogenic chemokine in rheumatoid arthritis. Am J Pathol. 2001:159:1521-1530.
24. Raychaudhuri SP, Jiang WY, Farber EM. Cellular localization of fractalkine at sites of inflammation: antigen-presenting cells in psoriasis express high levels of fractalkine. Br J Dermatol. 2001;144:1105-1113.
25. Cockwell P, Chakravorty SJ, Girdlestone J, Savage COS. Fractalkine expression in human renal inflammation. J Pathol. 2002; 196:85-90.
26. Ruth JH, Volin MV, Haines GK III. Fractalkine, a novel chemokine in rheumatoid

arthritis and in rat adjuvant-induced arthritis. Arthritis Rheum. 2001;44: 1568-1581.

27. Silverman MD, Zamora DO, Pan Y, Texeira PV, Baek SH, Planck SR, Rosenbaum JT. Constitutive and inflammatory mediator-regulated fractalkine expression in human ocular tissues and cultured cells. Invest Ophthalmol Vis Sci. 2003;44:1608-1615.
28. Tak PP, Firestein GS. NF-κB: a key role in inflammatory diseases. J Clin Invest. 2001;107:7-11.
29. Chen F, Castranova V, Shi X. New insights into the role of nuclear factor-kappa B, a ubiquitous transcription factor in the initiation of diseases. Clin Chem. 1999;45:7-17.
30. Baeuerle PA, Henkel T. Function and activation of NF- κB in the immune system. Annu. Rev. Immunol. 12:141-179, 1994.
31. Liu SF, Ye X, Malik AB. Pyrrolidine dithiocarbamate prevents I-kB degradation and reduces microvascular injury induced by lipopolysaccharide in multiple organs. Mo. Pharmacol. 55: 658-667, 1999.
32. Schreck R, Meier B, Mannel DN, Droge W, Baeuerle PA.. Dithiocarbamates as potent inhibitors of nuclear factor KB activation in intact cells. J. Exp.Med. 175:1181-1194, 1992.
33. Garcia GE, Xia Y, Chen S, Wang Y, Ye RD, Harrison JK, Bacon KB, Zewes H-G, Feng L. NF-κB-dependent fractalkine induction in rat aortic endothelial cells stimulated by IL-1β, TNF-α and LPS. J Leukoc Biol. 2000;67: 577-584.
34. Pan Y, Lloyd C, Zhou H, Dolich S, Deeds J, Gonzalo JA, Vath J, Gosselin M, Ma J, Dussault B, Woolf E, Alperin G, Culpepper J, Gutierrez-Ramos JC, Gearing D. Neurotactin, a membrane anchored chemokine upregulated in brain inflammation. Nature. 1997;387:611-617.

35. Kim MC, Kabeer NH, Tandhasetti MT, Kaplan HJ, Bora NS. Immunohisto-chemical studies on melanin associated antigen (MAA) induced experimental autoimmune anterior uveitis (EAAU). Curr Eye Res.1995;14:703-10.
36. Springer TA. Traffic signals for lymphocyte recruitment and leukocyte emigration: the multistep paradigm. Cell. 1994;76:301.
37. Chapman GA, Moores KE, Gohil J, Berkhout TA, Patel L, Green P, Macphee CH, Stewart BR. The role of fractalkine in the recruitment of monocytes to the endothelium. Eur J Pharmacol. 2000;392:189-195.
38. Nanki T, Imai T, Nagasaka K, Urasaki Y, Nonomura Y, Taniguchi K, Hayashida K, Hasegawa J, Yoshie O, Miyasaka N. Migration of CX3CR1-Positive T cells producing type 1 cytokines and cytotoxic molecules into the synovium of patients with rheumatoid arthritis. Arthritis Rheum. 2002;46:2878-2883.
39. Blaschke S, Koziolek M, Schwarz A, Benohr P, Middel P, Schwarz G, Hummel K-M, Muller G.. A. Proinflammatory role of fractalkine in rheumatoid arthritis. J Rheumatol. 2003;30:1918-27.
40. Umehara H, Imai T. The role of fractalkine in leukocyte adhesion and migration, and vascular injury. Drug News Perspect. 2001;14:460-464.
41. Ludwig A, Berkhout T, Moores K, Groot P, Chapman G. Fractalkine is expressed by smooth muscle cells in response to IFN-gamma and TNF-alpha and is modulated by metalloproteinase activity. J immunol. 2002;168:604-12.
42. Papadopoulos EJ, Sassetti C, Sacki H, Yamada N, Kawamura T, Fitzhugh DJ, Saraf MA, Schall T, Blauvelt A, Rosen SD, Hwang ST. CX3CL1, a CX3C chemokine, is expressed by dendritic cells and is upregulated upon dendirtic cell maturation. Eur J Immunol 1999; 29:2551-2559.
43. Harrison JK, Jiang Y, Chen S, Xia Y, Maciejewski D, McNamara RK, Streit WJ, Salafranca MN, Adhikari S, Thompson DA, Botti P, Bacon KB, Feng. Role for

neuronally derived CX3CL1 in mediating interactions between neurons and CX3CR1-expressing microglia. Proc. Natl. Acad Sci USA 1998;95:10896-10901.

44. Chen YM, Tu CJ, Hung KY, Wu KD, Tsai TJ, Hsieh BS. Inhibition by pentoxifylline of TNF-α-stimulated fractalkine production in vascular smooth muscle cells: evidence for mediation by NF-κB down-regulation. Br. J. Pharmacol. 2003:138;950-958.

45. Chandrasekar B, Mummidi S, Perla R.P., Bysani S, Dulin NO, Liu F, Melby PC. Fractalkine stimulated by nuclear factor κB-dependent inflammation signals induces aortic smooth muscle cell proliferation through an autocrine pathway. Biochem J. 2003;373:547-558.

46. Brand S, Sakaguchi T, Gu X, Colgan SP, Reinecher H-C. Fractalkine-mediated signals regulate cell-survival and immune-modulatory responses in intestinal epithelial cells. Gastroenterology 2002;122:166-177.

Legends:

Figure 1

Expression of fractalkine (A) and CX3CR1(B) mRNA in the iris/ciliary body from Lewis rats at different time points during the course of EAAU.

The control represents the normal rats that have not been immunized. Bar charts represent the relative intensity of mRNA of fractalkine. Line charts represent the clinical scores of the disease. 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 five rats. * $p < 0.05$, when compared to normal control.

Figure 2

The concentration of fractalkine in aqueous humor from Lewis rats at different time points during the course of EAAU were determined by ELISA.

The control represents the normal rats that have not been immunized. Bar charts represent the concentrations of fractalkine in aqueous humor. Line charts represent the clinical scores of the disease. Data are presented as the mean ±SD in five rats. * $p < 0.05$, when compared to normal control.

Figure 3

Correlations of fractalkine concentration with numbers of different subsets of leukocytes in aqueous humor from Lewis rats at different time points during the course of EAAU.

The control represents the normal rats that have not been immunized. Bar charts represent number of different types of leukocyte in aqueous humor. Line charts represent the concentration of fractalkine. Data are presented as the mean ±SD in five

rats. * $p < 0.05$, when compared to normal control.

Figure 4

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. Columns marked as “Normal” represent rats without immunization with MAA, while “MAA” represents rats immunized with MAA. “PDTC-treated” represents rats immunized with MAA and treated with PDTC (200mg/kg) every day. 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. Significant differences: *$P<0.05$

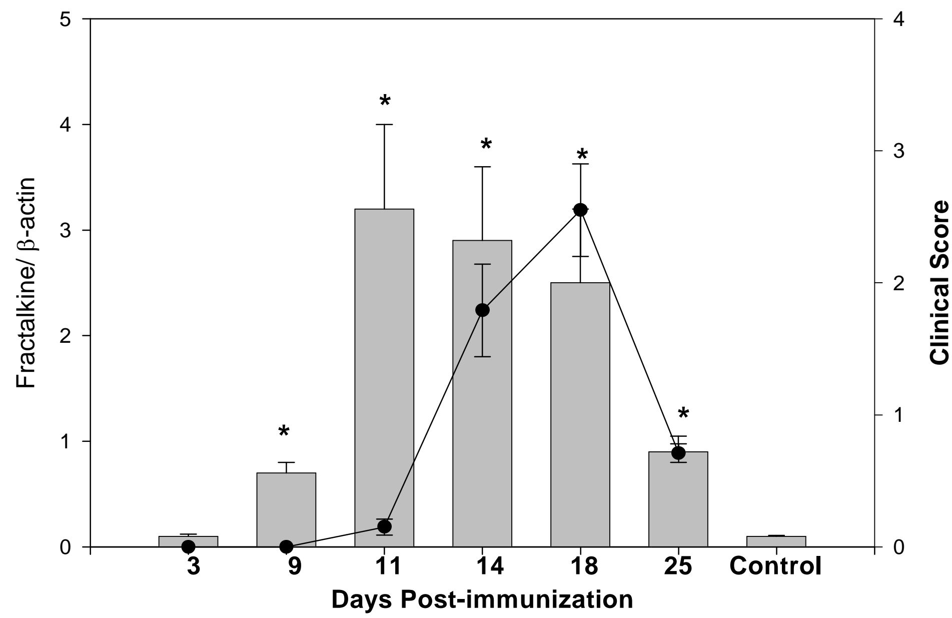
Figure 5

Immunohistochemical staining for fractalkine expression in the iris/ciliary body from Lewis rats. Rats with experimental autoimmune anterior uveitis were found to have stronger fractalkine expression in vascular endothelial cells in the ciliary process (CP) and in the infiltrating cells widespread in the iris/ciliary process and posterior chamber (A), compared with those of normal rats (E) (X200). At higher magnification (X400), significant fractalkine expression was detected in many subsets of vascular endothelial cells (arrow) (B) and infiltrating mononuclear cells (arrow) in the posterior chamber (C). Treatment with PDTC (200mg/kg/day) contributed to reduced mononuclear cells infiltrations and fractalkine expression in the iris/ciliary process (D)

(X200). Faint constitutive fractalkine staining was found in the iris/ciliary process of normal rats (E) (X200). Control sections showed only background staining after omission of primary antibodies (F).

Figure 6

Immunohistochemical staining for fractalkine expression in retina from Lewis rats. Rats with experimental autoimmune anterior uveitis were found to have higher fractalkine staining in the nerve fiber layer, the outer plexiform layer and fainter in the nuclear layers (A), which were identical to the fractalkine expression patterns of normal rats with nearly equal intensity (B). Control sections showed only background staining after omission of primary antibodies (C). Original magnification: (X400).



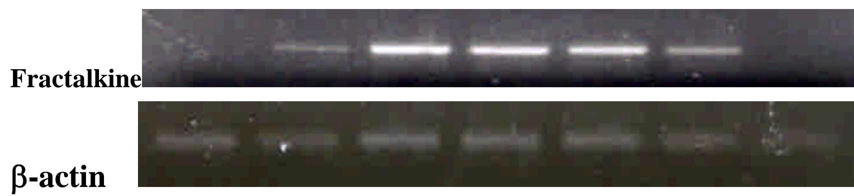


Fig 1A

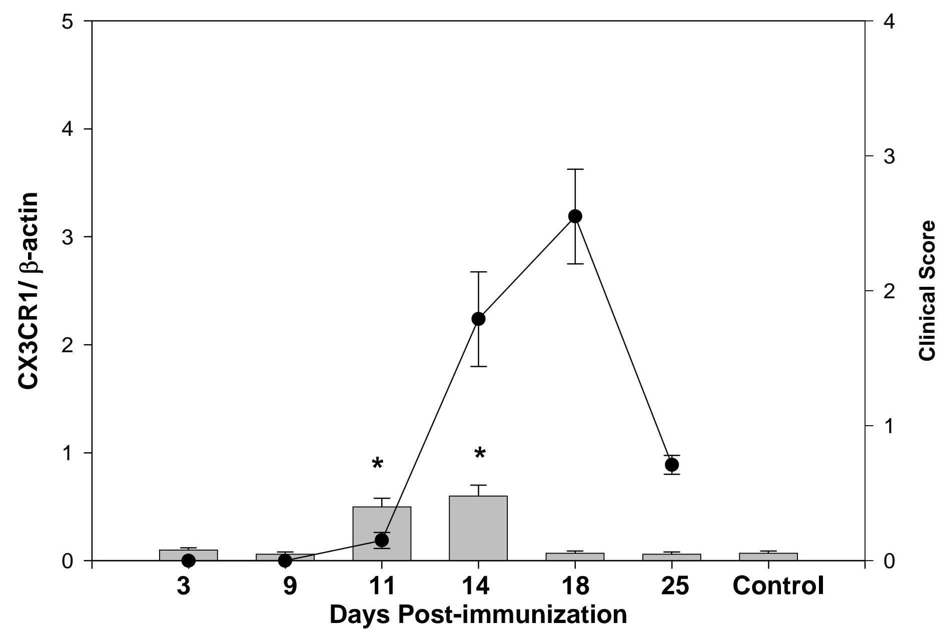


Fig. 1B

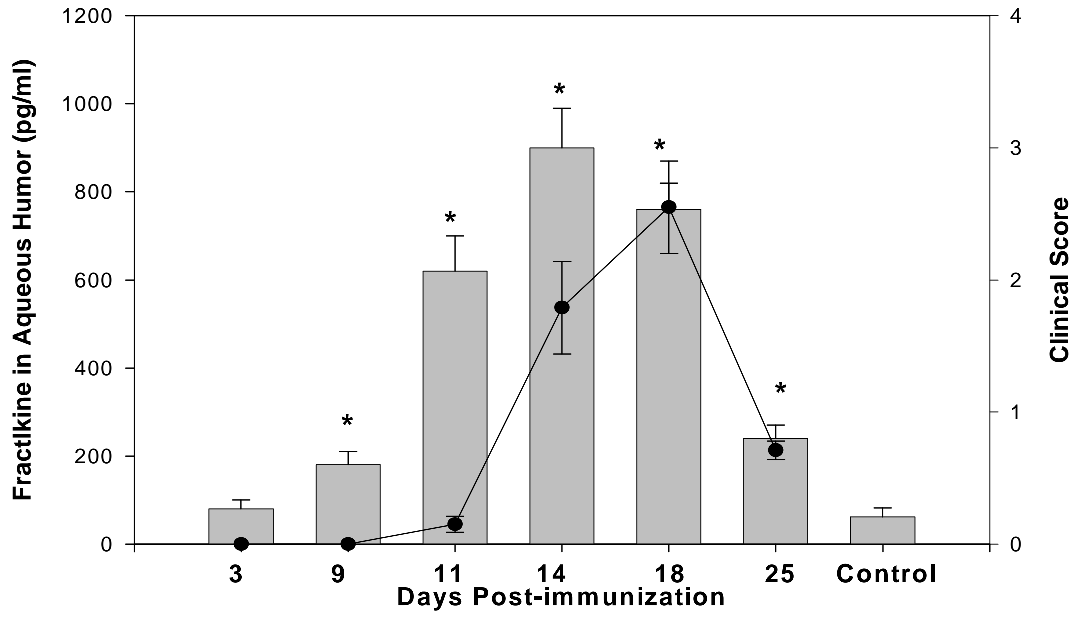


Fig. 2

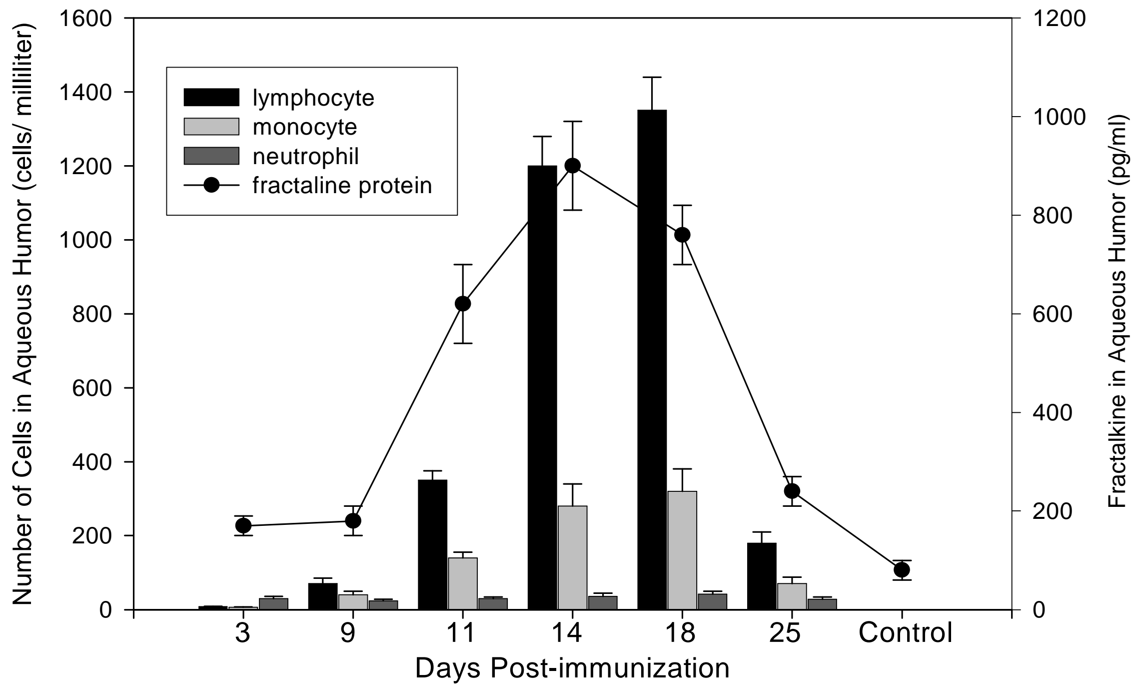


Fig. 3

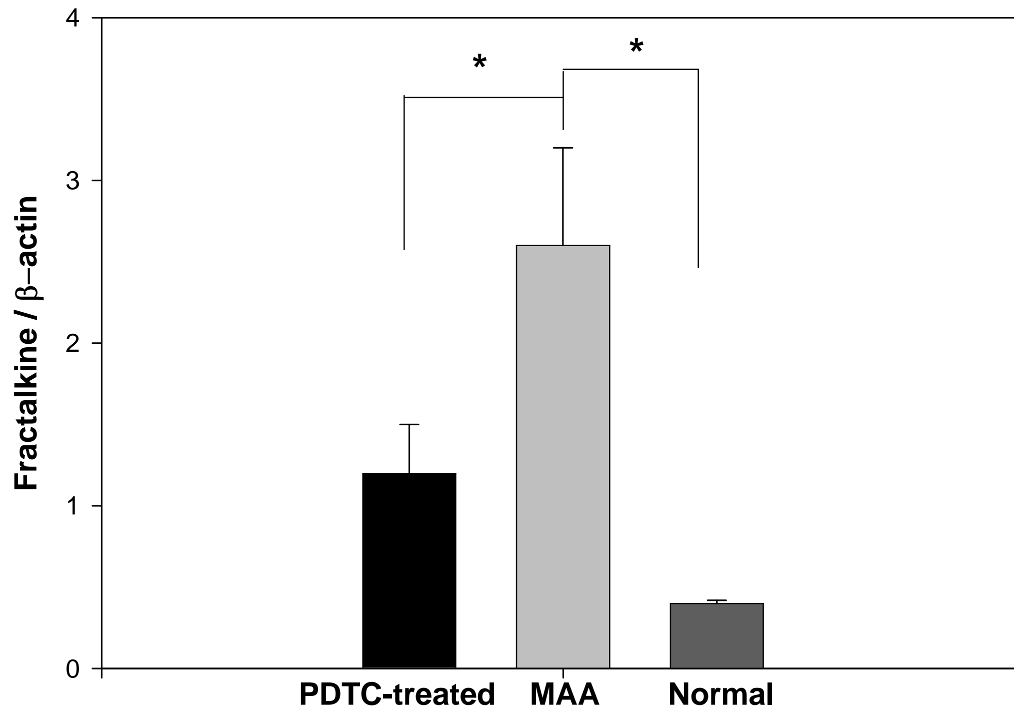


Fig. 4A

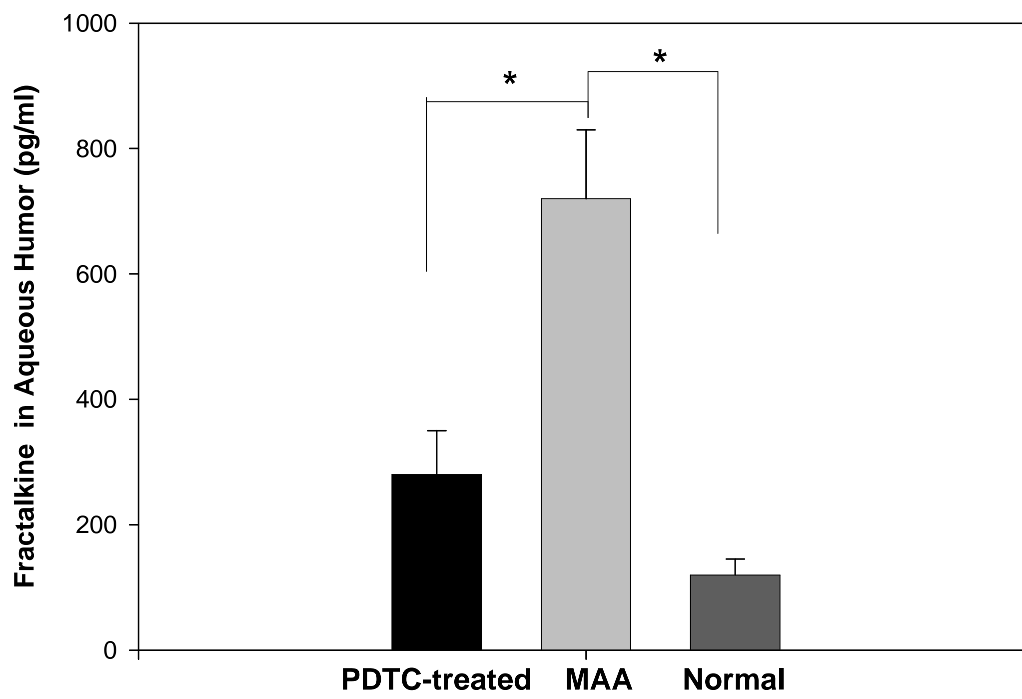


Fig. 4B

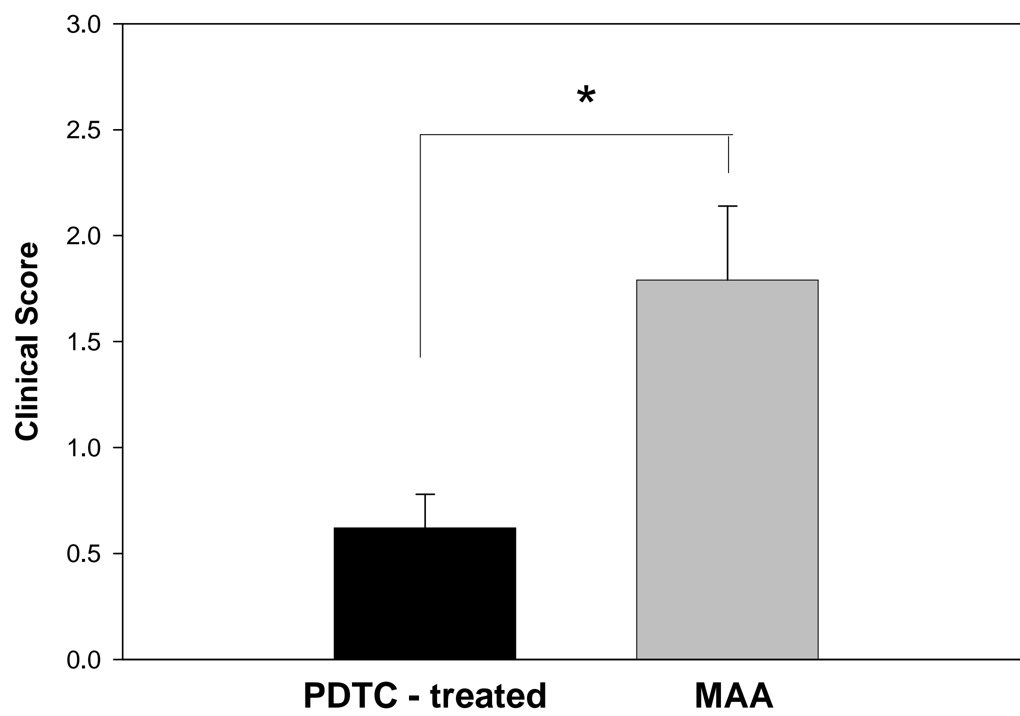


Fig. 4C