



Short communication

Triptolide inhibits CCR5 expressed in synovial tissue of rat adjuvant-induced arthritis

Wang Yifan^{1,2}, Wei Dengming¹, Lai Zheng^{1,2}, Le Yanping¹, Shen Junkan³¹Department of Pathology, School of Medicine, Ningbo University, 315211 Ningbo, China²Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Graduate School of the Chinese Academy of Sciences, 200031 Shanghai, China³Department of Pathology, the Third Hospital of Ningbo, 315020 Ningbo, China**Correspondence:** Wei Dengming, e-mail: weidengming@hotmail.com.

Abstract:

Triptolide has been clinically used to treat patients with rheumatoid arthritis, in which chemokine receptors play an important role in immune and inflammatory responses. To investigate the effect of triptolide on CCR5, we used complete Freund's adjuvant to produce adjuvant-induced arthritis (AIA) in rats. Our data show that both CCR5 mRNA and protein levels in synovial tissue of rats with AIA are significantly higher than those in normal rats. Triptolide can significantly inhibit rat AIA-induced overexpression of CCR5 at both mRNA and protein levels. These results may contribute to better understanding of the therapeutic effects of triptolide in rheumatoid arthritis.

Key words:

triptolide, CCR5, adjuvant induced arthritis, rheumatoid arthritis

Abbreviations: AIA – adjuvant-induced arthritis, CIA – collagen-induced arthritis, RA – rheumatoid arthritis, SF – synovial fluid, ST – synovial tissue, TWHF – *Tripterygium wilfordii* Hook. F

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory condition that affects multiple joints and results in the accumulation of leukocytes within the synovial tissue (ST) and synovial fluid (SF). The pathogenesis of RA is still largely unknown but leukocytes and their prod-

ucts play an important role in the development of inflammation, joint destruction and pain [16]. The attraction of leukocytes into the joints is controlled by chemokines. Chemokine activity is dependent on the presence of an interaction with chemokine receptors on the leukocyte surface. Indeed, chemokines and their receptors are involved together in the development and perpetuation of inflammation [3]. Chemokine receptors have a regulatory effect on the maturation and traffic of leukocytes, and they are implicated in several disease states, including RA [10]. Of particular interest regarding therapeutic intervention in RA is CCR5, the receptor for RANTES/CCL5, MIP-1 α /CCL3, and MIP-1 β /CCL4. CCR5 showed strong expression on ST fibroblasts and ST T lymphocytes in RA [15].

CCR5 has also been shown in several studies to be present on mononuclear cells in SF from patients with RA [4, 9].

Tripterygium wilfordii Hook. F (TWHF), a perennial vine-like member of the *Celastraceae* plant family, has been reported to show efficacy in the treatment of RA, psoriatic arthritis, and systemic lupus erythematosus. Triptolide, a highly oxygenated diterpenoid triepoxide, as an active component in TWHF, has been identified to possess potent anti-inflammatory and immunosuppressive activities in patients and animals with a variety of inflammatory and autoimmune diseases, including RA [5, 11]. However, no data about the effect of triptolide on CCRs are available to date. The present study was designed to explore the effect of triptolide on the expression of CCR5 in ST of rat adjuvant-induced arthritis (AIA), which is a widely used experimental model for studying the pathogenesis of human RA.

Materials and Methods

Animals

Female adult Sprague-Dawley rats (200 ± 20 g) were used throughout the study. Animals were kept in cages (four animals per cage) at a room with controlled temperature (23 ± 2°C) and on a 12-h light-dark cycle. Water and food were given *ad libitum*. The local animal ethics committee has approved all experimental procedures described below.

Drug treatment

After being anesthetized, rats were immunized subcutaneously at the palmar surface of the right hind paw on day 0 with 0.1 ml of 10 mg/ml *Mycobacterium tuberculosis* in incomplete Freund's adjuvant for the induction of arthritis, while rats in normal group were injected with a single dose of 0.1 ml of physiological saline. Triptolide was dissolved in physiological saline containing 4% 1,2-propylene glycol and applied intraperitoneally once a day. Two treatment groups received an injection of 0.1 mg/kg or 0.2 mg/kg of triptolide on day 14 after arthritis induction and maintained until day 20. Two control groups received an

equal amount of 4% 1,2-propylene glycol saline solution.

Immunohistochemistry

On day 21, the animals were sacrificed for immunohistochemical analysis. Right hind ankle joints were fixed in 10% buffered formalin, followed by treatment with 8% nitric acid solution for decalcification, dehydrated, cleared and embedded in paraffin. Immunostaining was performed on ST sections (4 µm), using SABC kits (Boster, China) and 3,3'-diaminobenzidine (DAB) as a chromogen, followed by counterstaining with hematoxylin. Sections were incubated overnight at 4°C with primary antibody, a rabbit anti-rat CCR5 polyclonal antibody (1:150) (Boster, China), while PBS was used as a negative control.

After immunohistochemical staining, all sections were examined by using a LOGENE-I PAS9000 computer-assisted color video image analysis system (LOGENE, China). Measurement of the integrated optical density (IOD, proportional to the total amount of protein staining) was made by an observer who was unaware of the order of sections.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from ST of knee joints was extracted using Trizol reagent (BBI, Canada) according to the manufacturer's instructions. First strand cDNA was synthesized using AMV RTase XL reverse transcriptase (Takara, Japan) at 46°C for 45 min. For PCR, 2 µl of each cDNA sample was amplified by using Ex Taq polymerase (Takara, Japan). The primer sequences for CCR5 were: sense, 5'-CAC CCT GTT TCG CTG TAG GAA TG-3'; and antisense, 5'-GCA GTG TGT CAT CCC AAG AGT CTC-3'. Those for GAPDH were: sense, 5'-TGA TGA CAT CAA GAA GGT GGT GAA G-3'; and antisense, 5'-TCC TTG GAG GCC ATG TAG GCC AT-3'. The sizes of amplified products were 214 bp for CCR5 and 240 bp for GAPDH. Amplification conditions were denaturation at 94°C for 1 min, annealing for 1 min at 62°C for CCR5 and 60°C for GAPDH, and extension at 72°C for 1 min for 30 cycles. Amplification products (2.5 µl each) were electrophoretically separated on 1.5% agarose gels and stained with ethidium bromide and analyzed by using a Gel Doc 2000 analysis system (Bio-RAD, USA).

Statistical analysis

The experimental results were expressed as the mean \pm SD of n rats per group. SPSS was used for data analysis. Statistical analysis was evaluated by Dunnett's t -test, with the level of significance chosen at $p < 0.05$.

Results

Immunohistochemical study of CCR5 in synovial tissue

CCR5 could be detected in ST in all groups. Normal rat ST exhibited weak constitutive expression of

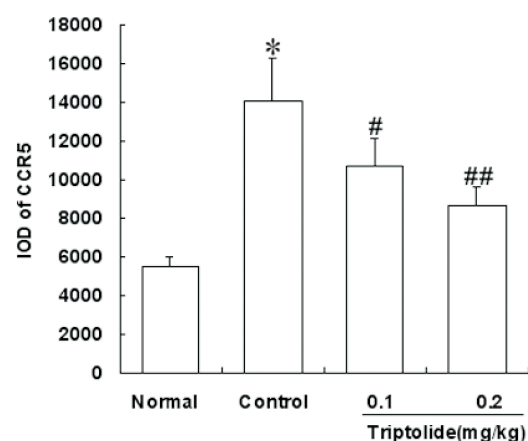
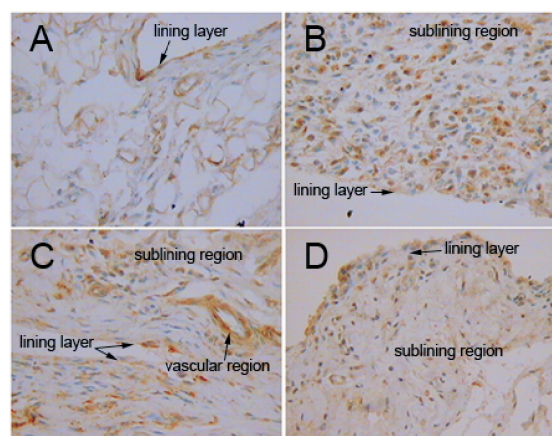


Fig. 1. Immunohistochemical study of CCR5 expression in rat synovial tissue. Upper panel: Immunostaining of CCR5 in synovial tissue (original magnification, $\times 400$). Photomicrographs were taken from normal rats (A), control AIA rats (B), 0.1 mg/kg triptolide-treated (C) and 0.2 mg/kg triptolide-treated AIA rats (D). Lower panel: Quantitative analysis was processed by using LOGENE-I PAS9000 image analysis system. Values are expressed as the mean \pm SD ($n = 7$). * $p < 0.01$ compared with normal group. # $p < 0.05$ compared with control AIA group. ## $p < 0.01$ compared with control AIA group

CCR5. The synovial lining layer and synovial sublining region were diffusely immunoreactive for CCR5. CCR5 was also localized in the vascular regions (Fig. 1). Slides were negative when the primary antibody was omitted (data not shown). In general, CCR5 was up-regulated at the protein level on ST cells in AIA rats and significantly decreased in both triptolide treatment groups (Fig. 1).

Expression of CCR5 mRNA in synovial tissue

To strengthen the results from immunohistochemical staining, we next performed RT-PCR to examine CCR5 gene expression in ST. CCR5 mRNA was expressed in ST of normal rats, but was up-regulated in ST of AIA rats compared with that of normal rats. Triptolide exhibited an inhibitory effect on CCR5 mRNA expression, while this effect was most evident after the dose of 0.2 mg/kg of triptolide (Fig. 2).

Discussion

In this study, we induced AIA and harvested rat joints after injection of triptolide or vehicle. CCR5 was analyzed by immunohistochemistry at protein level and RT-PCR at mRNA level. To our knowledge, this is the first study to demonstrate that triptolide can inhibit

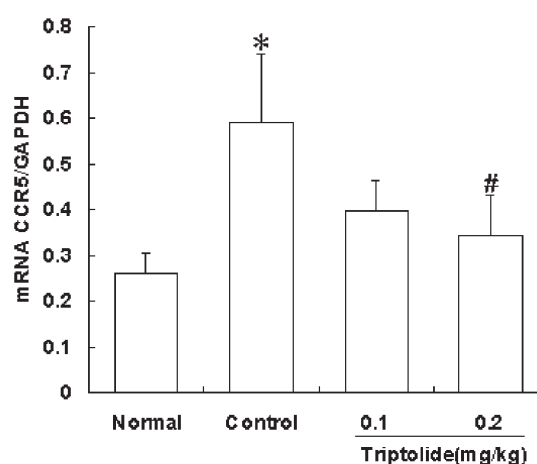


Fig. 2. Effect of treatment with triptolide on the expression of CCR5 mRNA in rat synovial tissue. The result was analyzed by Gel Doc 2000 image analysis system. Values are expressed as the means \pm SD ($n = 7$). * $p < 0.01$ compared with normal group. # $p < 0.05$ compared with control AIA group

the expression of CCR5. We also confirmed previous reports that the expression of CCR5 was up-regulated in ST of AIA rats [2, 13].

The migration of leucocytes from the bloodstream into the inflammation sites is a dynamic process consisting of multiple steps involving adhesion molecules and chemotactic factors. Chemokines regulate the traffic of leucocytes by inducing cell motility and by activating adhesion molecules. Chemokine receptors allow leucocytes to sense chemokine gradients, thereby directing these cells into tissue compartments. As a result, the interaction of chemokines and chemokine receptors plays an important role in regulating leucocyte traffic within the immune system. Since chemokines and their receptors have been suggested to be potential targets in the therapy of RA [1], a better characterization of the functional relevance of the CCR5 receptor to cell migration in response to synovial chemokines is warranted. Indeed, it has recently been reported that accumulation of CCR5-positive T lymphocytes is seen in the inflamed synovium and in the SF of RA, and seems to be of importance in the recruitment of Th1 lymphocytes into the joint [8, 15]. CCR5 expression was also significantly elevated on synovial lining cells, macrophages, and endothelial cells in ST of AIA rats [2, 13]. An array-based analysis of not-yet-inflamed joints in mice with autoimmune arthritis also identified CCR5 as one of the most highly up-regulated genes [7]. The interaction of CCR5 with its ligands is crucial for the development and severity of RA, on the basis of the recent demonstration that RA activity correlates with the polymorphism of CCR5 [20].

Murine experimental arthritis can be partially ameliorated by blocking CCR5 using selective receptor antagonists. A recent study showed that the collagen-induced arthritis (CIA) phenotype in CCR5-deficient mice was similar to that in wild-type mice [12]. A CCR5 nonpeptide antagonist, when used preventively, inhibited mouse CIA and migration of leukocytes to the joints [19]. Blocking of CCR5 with Met-RANTES in rat AIA resulted in reduced joint destruction [14]. Furthermore, in a nonhuman primate model of RA, treatment with a CCR5 antagonist resulted in clinical improvement of the disease [17]. Taken together, these findings indicate that CCR5 targeting is an interesting potential therapeutic approach.

We have reported that treatment with triptolide from day 14 could effectively reduce the edema of rat hind paw [18]. It also has been reported that triptolide

can inhibit the expression of MIP-1 α , MIP-1 β and RANTES, the ligands of CCR5, at both mRNA and protein levels [6, 18]. Our results demonstrate that triptolide not only inhibits the production of the ligands of CCR5, but also CCR5 itself. The inhibition of CCR5 expression in ST may be explained by down-regulation of the receptor or decreased migration of cells expressing the receptor towards the sites of inflammation, or a combination of both. This inhibition may also contribute to the therapeutic effects of triptolide in RA.

Acknowledgment:

This study was supported by the Ningbo Agriculture and Society Development Project (No. 2006C100059).

References:

1. Cascieri MA, Springer MS: The chemokine/chemokine-receptor family: potential and progress for therapeutic intervention. *Curr Opin Chem Biol*, 2000, 4, 420–427.
2. Haas CS, Martinez RJ, Attia N, Haines GK 3rd, Campbell PL, Koch AE: Chemokine receptor expression in rat adjuvant-induced arthritis. *Arthritis Rheum*, 2005, 52, 3718–3730.
3. Haringman JJ, Kraan MC, Smeets TJM, Zwinderman KH, Tak PP: Chemokine blockade and chronic inflammatory disease: proof of concept in patients with rheumatoid arthritis. *Ann Rheum Dis*, 2003, 62, 715–721.
4. Katschke KJ Jr, Rottman JB, Ruth JH, Qin S, Wu L, LaRosa G, Ponath P et al.: Differential expression of chemokine receptors on peripheral blood, synovial fluid, and synovial tissue monocytes/macrophages in rheumatoid arthritis. *Arthritis Rheum*, 2001, 44, 1022–1032.
5. Lipsky PE, Tao XL: A potential new treatment for rheumatoid arthritis: thunder god vine. *Semin Arthritis Rheum*, 1997, 26, 713–723.
6. Liu Q, Chen T, Chen G, Li N, Wang J, Ma P, Cao X: Immunosuppressant triptolide inhibits dendritic cell-mediated chemoattraction of neutrophils and T cells through inhibiting Stat3 phosphorylation and NF-kappaB activation. *Biochem Biophys Res Commun*, 2006, 345, 1122–1130.
7. Li Z, Jiang H, Xie W, Zhang Z, Smrcka AV, Wu D: Roles of PLC-beta2 and -beta3 and PI3Kgamma in chemoattractant-mediated signal transduction. *Science*, 2000, 287, 1046–1049.
8. Loetscher P, Uguccioni M, Bordoli L, Baggiolini M, Moser B, Chizzolini C, Dayer JM: CCR5 is characteristic of Th1 lymphocytes. *Nature*, 1998, 391, 344–345.
9. Mack M, Bruhl H, Gruber R, Jaeger C, Cihak J, Eiter V, Plachy J et al.: Predominance of mononuclear cells expressing the chemokine receptor CCR5 in synovial effu-

- sions of patients with different forms of arthritis. *Arthritis Rheum*, 1999, 42, 981–988.
10. Murdoch C, Finn A: Chemokine receptors and their role in inflammation and infectious diseases. *Blood*, 2000, 95, 3032–3043.
 11. Qiu D, Kao PN: Immunosuppressive and anti-inflammatory mechanisms of triptolide, the principal active diterpenoid from the Chinese medicinal herb *Tripterygium wilfordii* Hook F. *Drugs R D*, 2003, 4, 1–18.
 12. Quinones MP, Ahuja SK, Jimenez F, Schaefer J, Garavito E, Rao A, Chenux G et al.: Experimental arthritis in CC chemokine receptor 2-null mice closely mimics severe human rheumatoid arthritis. *J Clin Invest*, 2004, 113, 856–866.
 13. Shahrara S, Amin MA, Woods JM, Haines GK, Koch AE: Chemokine receptor expression and *in vivo* signaling pathways in the joints of rats with adjuvant-induced arthritis. *Arthritis Rheum*, 2003, 48, 3568–3583.
 14. Shahrara S, Proudfoot AE, Woods JM, Ruth JH, Amin MA, Park CC, Haas CS et al.: Amelioration of rat adjuvant-induced arthritis by Met-RANTES. *Arthritis Rheum*, 2005, 52, 1907–1919.
 15. Suzuki N, Nakajima A, Yoshino S, Matsushima K, Yagita H, Okumura K: Selective accumulation of CCR5⁺ T lymphocytes into inflamed joints of rheumatoid arthritis. *Int Immunol*, 1999, 11, 553–559.
 16. Świerkot J, Szechiński J: Methotrexate in rheumatoid arthritis. *Pharmacol Rep*, 2006, 58, 473–492.
 17. Vierboom MP, Zavodny PJ, Chou CC, Tagat JR, Pugliese-Sivo C, Strizki J, Steensma RW et al.: Inhibition of the development of collagen-induced arthritis in rhesus monkeys by a small molecular weight antagonist of CCR5. *Arthritis Rheum*, 2005, 52, 627–636.
 18. Wang Y, Wei D, Lai Z, Le Y: Triptolide inhibits CC chemokines expressed in rat adjuvant-induced arthritis. *Int Immunopharmacol*, 2006, 6, 1825–1832.
 19. Yang YF, Mukai T, Gao P, Yamaguchi N, Ono S, Iwaki H, Obika S et al.: A non-peptide CCR5 antagonist inhibits collagen-induced arthritis by modulating T cell migration without affecting anti-collagen T cell responses. *Eur J Immunol*, 2002, 32, 2124–2132.
 20. Zapico I, Coto E, Rodriguez A, Alvarez C, Torre JC, Alvarez V: CCR5 (chemokine receptor-5) DNA-polymorphism influences the severity of rheumatoid arthritis. *Genes Immun*, 2000, 4, 288–289.

Received:

February 27, 2007; in revised form: November 12, 2007.