International Immunopharmacology 34 (2016) 71–77

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Interleukin-35 attenuates collagen-induced arthritis through suppression of vascular endothelial growth factor and its receptors

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ARTICLE INFO

Article history:
Received 8 November 2015
Received in revised form 16 February 2016
Accepted 18 February 2016
Available online 26 February 2016

Keywords:
Collagen-induced arthritis
Interleukin-35
Vascular endothelial growth factor
Rheumatoid arthritis

ABSTRACT

Objective: To investigate the effect of interleukin-35 (IL-35) on vascular endothelial growth factor (VEGF) and its receptors, Flt-1 and Flk-1, in a collagen-induced arthritis (CIA) mouse model of rheumatoid arthritis (RA).
Methods: We established a CIA mouse model and injected IL-35 intraperitoneally. The articular index (AI) was measured based on the amount of erythema, swelling, or joint rigidity and synovial histology was measured by hematoxylin and eosin staining (HE staining). The levels of VEGF, Flt-1, Flk-1, and von Willebrand factor (vWF) expression in CIA synovial tissue were determined by immunohistochemistry. The mRNA and protein expression levels of VEGF, Flt-1, Flk-1, TNF-α, and INF-γ were detected by reverse transcription PCR (RT-PCR) and western blots, respectively.
Results: The IL-35 treatment decreased the AI and the synovial histological scores of CIA mice. Immunohistochemistry results revealed that the IL-35 treatment downregulated VEGF, Flt-1, Flk-1, and vWF expression in the CIA mice. RT-PCR results showed that the IL-35-treated mice had lower levels of VEGF, Flt-1, Flk-1, and TNF-α mRNA expression than those of the PBS-treated mice. While there was no significant difference in the level of INF-γ mRNA expression between IL-35-treated and PBS-treated mice. Western blot results showed that the IL-35 treatment downregulated the levels of VEGF, Flt-1, Flk-1, and TNF-α in CIA mice, but the level of INF-γ was not significantly affected.
Conclusion: These findings show that IL-35 may represent a novel therapeutic agent for RA, and the probable mechanisms may rely on inhibiting VEGF and its receptors Flt-1 and Flk-1.

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1. Introduction

RA is a chronic autoimmune disease, which is more common in women. RA is characterized by disabling polyarticular synovitis, and its pathologic properties include synovium hyperplasia, inflammatory cell infiltration, pannus formation, and, finally, cartilage and bone destruction [1]. The collagen-induced arthritis (CIA) mouse model has many similarities with human rheumatoid arthritis (RA). In the CIA mouse model, synovial cells proliferate in a tumor-like manner and cause synovitis. Angiogenesis is highly involved in the disease progression in both the CIA model and in RA [2]. Vascular endothelial growth factor (VEGF), a homologous glycoprotein dimer, promotes angiogenesis in synovial tissues, is a key factor in the pannus formation and plays an important role in injury, repair, inflammation, and RA development [3,4]. The binding of VEGF to Flt-1 and Flk-1, two of its receptors, stimulates the production of VEGF, thus promoting angiogenesis. Flt-1 might contribute to rheumatoid inflammation by triggering production of proinflammatory cytokines [5]. Another study has shown that the angiogenic pathway VEGF/flk-1 might play an important role in the pathogenesis of RA and osteoarthritis [6].

The novel cytokine IL-35 is a heterodimeric protein encoded by the Epstein–Barr virus-induced gene 3 (EBI3) and the p35 gene that is one of the IL-12 subunits [7,8]. EBI3 was first found in the supernatant of B lymphoblastoid cells infected by Epstein–Barr virus [9,10]. Later studies showed that it is mainly expressed in human tonsils and spleen [11]. The high expression levels of EBI3 and p35 in the trophoblast components of human full-term normal placentas suggest that IL-35 plays an important role in immune tolerance [7]. Recent research shows that IL-35 is secreted by both inactive and active T regulatory (Treg) cells [12]. IL-35 plays an anti-inflammatory role by inducing Treg cell production and suppressing T helper type 17 (Th17) cell differentiation in several inflammatory disease models, such as CIA, experimental autoimmune encephalomyelitis (EAE) and inflammatory bowel disease (IBD) models [13–15].

Angiogenesis is a key process in the development of RA. IL-35 was shown to attenuate the severity of RA [16], however the function of IL-35 towards the process of angiogenesis of RA has not been studied. In this study, we firstly demonstrated that IL-35 could inhibit

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http://dx.doi.org/10.1016/j.intimp.2016.02.018
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angiogenesis of CIA mice as well as downregulate the expression of VEGF and its receptors. Furthermore, the severity of synovitis in CIA mice was ameliorated by IL-35 treatment.

2. Materials and methods

2.1. Animals and reagents

With the help from the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health, this study was performed congruously. And the Committee on the Ethics of Animals Experiments of China Medical University approved this protocol. Sodium pentobarbital anesthesia was the necessary condition of the performance of all surgery and the study also reduced suffering at full stretch.

Male, 8–10-week-old DBA/1 mice were bought from Huafukang Biotechnology (Beijing, China). They were fed and cared for by the staff of the Liaoning University of Traditional Chinese Medicine. IL-35, complete Freund's adjuvant (CFA), incomplete Freund's adjuvant (IFA), and collagen II (CII) were bought from Sigma (St. Louis, USA). Rabbit anti-mouse VEGF, Flt-1, Flk-1, TNF-α, beta-actin and horseradish peroxidase conjugated goat anti-rabbit antibodies were purchased from Abcam (Cambridge, UK). Rabbit anti-mouse vWF antibody, goat anti-mouse IFN-γ and Trizol were purchased from Proteintech Group (Chicago, USA), R&D systems, Inc (Minneapolis, US) and Invitrogen (Massachusetts, USA), respectively. Reverse transcription polymerase chain reaction (RT-PCR) kits, a DNA marker, and a protein marker were purchased from Beijing Quanshijin Biotechnology (Beijing, China). Primers were synthesized by Beijing Sanboyuanzhi Biotechnology (Beijing, China). Immunocytochemistry (SP) kits and color development kits were purchased from Beijing Boaosen Biotechnology (Beijing, China).

2.2. Induction of CIA and assessment of arthritis

The CIA mouse model was established according to previously published protocols [17]. Briefly, in a sterile procedure, CII was dissolved thoroughly with 0.1 mol/l glacial acetic acid. Then, CII (2 mg/ml) was dissolved thoroughly with an equal volume of CFA, and 200 μl was injected at the end of each mouse's tail. After 21 days, the mice received 2 mg/ml CII + IFA in the same manner as described above. CIA was assessed based on previously published methods [17]. The experimental model was considered to be successfully established in mice with an articular index (AI) score > 4 and a volume of unilateral lateral malleolus >1.6. The AI was evaluated on a scale of 0–3 based on the amount of erythema, swelling, or joint rigidity, and giving a maximum score of 12 per mouse as previously described [17]. From day 24, the mice were injected intraperitoneally (i.p.) with IL-35 (2 μg) or PBS daily for 10 days. After 40 days, ankle joints and plantar, decalcified in 10% ethylenediaminetetraacetic acid, were made into 5-μm-thick paraffin sections and then stained with hematoxylin and eosin (HE). We observed inflammatory cell infiltration, pannus formation, and bone destruction of CIA mice joints for histological assessments of arthritis lesions [17]. Scales of 1–5 were scored as follows. 1 = normal joint tissue; 2 = a small amount of inflammatory cell infiltration in the joint tissue with little or no pannus formation, no decalcification, and no bone erosion; 3 = a moderate amount of inflammatory cell infiltration in the joint tissue with distinct pannus formation, mild decalcification, and mild bone destruction; 4 = a large amount of inflammatory cell infiltration in the joint tissue with a large amount of pannus formation, moderate decalcification, and moderate bone destruction; 5 = a large amount of inflammatory cell infiltration in the joint tissue with a large amount of decalcification and bone destruction. Scores were combined to evaluate the severity of CIA mice joints, and thus the maximum score for each mouse was 20 points.

2.3. Immunohistochemistry to assess the levels of VEGF, Flt-1, Flk-1, and vWF expression

Using the immunohistochemistry SP method, we tested the levels of VEGF, Flt-1, Flk-1, and vWF expression from untreated normal mice, PBS-treated and IL-35-treated CIA mice on the 40th day of the model. Synovial tissue paraffin sections were prepared with a 5-μm thickness. Subsequently, dimethylbenzene dewax was applied to the sections and then eluted with an ethanol gradient. Finally, antigen retrieval was achieved by the addition of compound protease, and the endogenous peroxidase activity was quenched by incubation with 3% H_2O_2. Primary antibodies were diluted at 1:500. Each paraffin section was treated with 100 μl of the diluted primary antibodies and incubated at 4 °C overnight. Then, biotinylated goat anti-rabbit IgG was added, and the sections were incubated at 37 °C for 20 min, followed by the addition of streptavidin-horseradish peroxidase and an additional incubation at 37 °C for 20 min. Diaminobenzidine was used as a chromogen. The sections were also counterstained with Mayer hematoxylin. Positive cell membranes and cytoplasms were stained as tan and brown. Images were collected with a Leica Q550CW, and the average optical density (AOD) of each section were used in the statistical analyses of the relative expression levels of VEGF, vWF, Flt-1, and Flk-1 in synovial tissues.

2.4. RT-PCR to assess the VEGF, Flt-1, Flk-1, TNF-α, and INF-γ mRNA expression in synovial tissue

Cryopreserved synovial tissue (100 mg) was prepared and ground up in liquid nitrogen. Total RNA was extracted using Trizol Reagent according to the manufacturer's instructions. Reverse transcription was performed in 20 μl using 1 μg total RNA and random hexamers (TaKaRa Biotechnology, Ltd., Dalian, China), and subsequently subjected to PCR reactions with a RT-PCR kit using the following PCR conditions: 94 °C for 2 min, 30 cycles at 94 °C for 30s, 65 °C for 30 s, 72 °C for 30 s and finally 72 °C for 2 min. The primer sequences used for RT-PCR were as follows:

VEGF sense primer: 5′-GCCAGAAAATCACTGTGAGCCTTGT-3′,
anti-sense primer: 5′-AGCTGCCTCGCCTTGCAACG-3′;
Flt-1 sense primer: 5′-GCTCGAGCGTGCCGCGT-3′,
anti-sense primer:5′-TCCGTGGTGGCGGTGCAGTT-3′;
Flk-1 sense primer: 5′-TACCGGGAAACTGACTTGGCCT-3′,
anti-sense primer: 5′-TCAGTTCTTGCTGTACAATTTA-3′;
TNF-α sense primer: 5′-ATGAGCACGGAAAGCATGATCC-3′,
anti-sense primer: 5′-AGGGCAAGGCTCTTG ATGGCAG-3′;
INF-γ sense primer: 5′-TTTGAGGTCAACAACCCACA-3′,
anti-sense primer: 5′-CGCAATCACAGTCTTGGCTA-3′;
β-actin sense primer: 5′-CCAGAGCAAGAGAGGTATCC-3′and
anti-sense primer: 5′-GGGGTGTTGAAGGTCTCAAA-3′.

The PCR-amplified products were subjected to electrophoresis in a 1.5% agarose gel. β-actin was used as an internal standard and the results were analyzed by a gel imaging and analysis system (4200SF, Tanon, Shanghai, China). Then, the integral optical density (IOD) of the target gene band and the reference gene band were detected by 4200SF. The ratio of the target gene band IOD to the reference gene band IOD was used to statistically analyze the differences in VEGF, Flt-1, Flk-1, TNF-α, and INF-γ mRNA expression.

2.5. Western blot to assess the VEGF, VEGF, Flt-1, Flk-1, TNF-α, and INF-γ protein expression

Cryopreserved synovial tissue (100 mg) was prepared, and 1 ml of protein pyrolysis buffer was added. Minced tissue homogenates were kept in an ice bath for 10 min, and then centrifuged for 15 min at 14,000 rpm. The supernatants were collected for use in determining the total protein concentrations of the cells, which were measured by the Coomassie blue staining method. Proteins were loaded at 30 μg

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