Interleukin-35 inhibits angiogenesis through STAT1 signalling in rheumatoid synoviocytes

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Abstract

Objective

We studied the anti-angiogenic effect of interleukin-35 (IL-35) by investigating its effects on signal transmission through the Janus kinase signal transducer and activator of transcription (JAK-STAT) pathway in fibroblast-like synoviocytes (FLS).

Methods

Using the collagen-induced arthritis (CIA) model of rheumatoid arthritis (RA), we derived and cultured FLS, stimulated FLS with IL-35 at different concentrations and examined the expression levels of mRNA and protein of both vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), endostatin, TNF-α, and IL-6 using reverse transcription polymerase chain reaction (RT-PCR) and immunoblotting. We used Western blotting to study the effects of IL-35 on the function of the JAK-STAT pathway in FLS.

Results

IL-35 treatment inhibited the expression of VEGF, FGF-2, TNF-α and IL-6, and increased the expression of endostatin in FLS. Western blotting showed that IL-35 treatment of CIA-derived FLS resulted in signalling through STAT1, but not through STAT3 or STAT5.

Conclusion

IL-35 signalling through STAT1 and inhibition of the expression of mediators of angiogenesis and inflammation in FLS provide a likely mechanism for anti-angiogenic effects seen in experimental models of RA. Our data suggest that IL-35 and its signalling pathway represent a therapeutic target for the treatment of RA and other angiogenesis-related diseases.

Key words

interleukin-35, rheumatoid arthritis, vascular endothelial growth factor, collagen-induced arthritis, STAT1

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Introduction

Rheumatoid arthritis (RA) is considered a chronic autoimmune disease, with angiogenesis playing a major role in the progression of RA. Angiogenesis is tightly regulated by the balance between pro- and anti-angiogenic mediators. Vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2) are the key factors in the formation and maintenance of pannus tissue. Endostatin is the main anti-angiogenic factor in RA pannus tissue.

IL-35 is a cytokine of the IL-12 family, a heterodimeric protein encoded by the genes IL12A and EBI3. These two genes are encoding the polypeptide chains IL-12α and IL-27β (1, 2). IL-35 has been shown to be secreted by regulatory T cells (Treg) (3) and it has been shown to play an important role in autoimmune and infectious diseases (4). A growing body of research has shown that IL-35 plays an immunosuppressive role by inducing the production of Tregs, inhibiting responder T cell (Tres) responses and increasing IL-10 production in both autoimmune and inflammatory diseases.

In 2007, Niedbala *et al*. first described the effects of IL-35 in collagen-induced arthritis (CIA) (1), showing that IL-35 potently suppresses the development of CIA. In the current study, we investigated the effect of IL-35 on the expression of pro-angiogenic genes and endostatin in CIA-derived fibroblast-like synoviocytes (FLS). IL-35 is a member of the IL-12 family of cytokines and other members of this family are known to signal through the JAK-STAT pathway. Therefore, we hypothesised that the FLS-associated effect of IL-35 on angiogenesis may also be mediated by IL-35 signalling through the JAK-STAT pathway. Therefore, the focus of our study was to elucidate the effect of IL-35 on signal transduction through the JAK-STAT pathway in FLS derived from mice undergoing CIA.

Materials and methods

Induction of CIA and FLS culture

All studies were carried out in agreement with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Male DBA/1 mice, 8 to 10 weeks old, were purchased from Huafukang Biotechnology (Beijing, China). We induced and assessed CIA in mice as previously described (5). After inducing CIA, we peeled off the skin of the knee joint to expose muscle tissue and ligaments. Several pieces of synovial tissue were placed into a tissue culture flask containing high glucose DMEM supplemented with 10% FBS and penicillin/streptomycin, and were cultured at 37°C in humidified air containing 5% CO_2. Analysis of the outgrowing cells for markers of FLS showed that they were vimentin-positive and CD68-negative. All experiments described below were carried out with cultured FLS from the third to sixth generations.

RT-PCR and Western blotting to assess the expression of VEGF, FGF-2, endostatin, TNF-α, and IL-6 in FLS treated with various concentrations of IL-35

After FLS ($1x10^5$) had been cultured in 6-well plates in serum-free DMEM for two hours at 37°C in a 5% CO_2 atmosphere, the following experimental groups were established: CIA FLS (control); CIA FLS plus 25 ng/mL IL-35 (Sigma, St. Louis, USA); CIA FLS plus 50 ng/mL IL-35; CIA FLS plus 100 ng/mL IL-35. FLS were cultured for 48 h at 37°C in a 5% CO_2 atmosphere. Thereafter, total RNA was extracted using the Trizol reagent (Invitrogen, Massachusetts, USA) according to the manufacturer's instructions, and total protein was extracted using 0.5 mL protein pyrolysis buffer, including addition of 0.05 mL protease inhibitor cocktail to each sample. PCR reactions were set up using a RT-PCR kit (Beijing Quanshijin Biotechnology, Beijing, China) using the following PCR conditions: 94°C for 2 min, followed by 30 cycles of melting at 94°C for 30 s, annealing at 65°C for 30 s, and extension at 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';

FGF-2 sense primer: 5'- AACGGCGGCTTCTTCCTGCG-3',

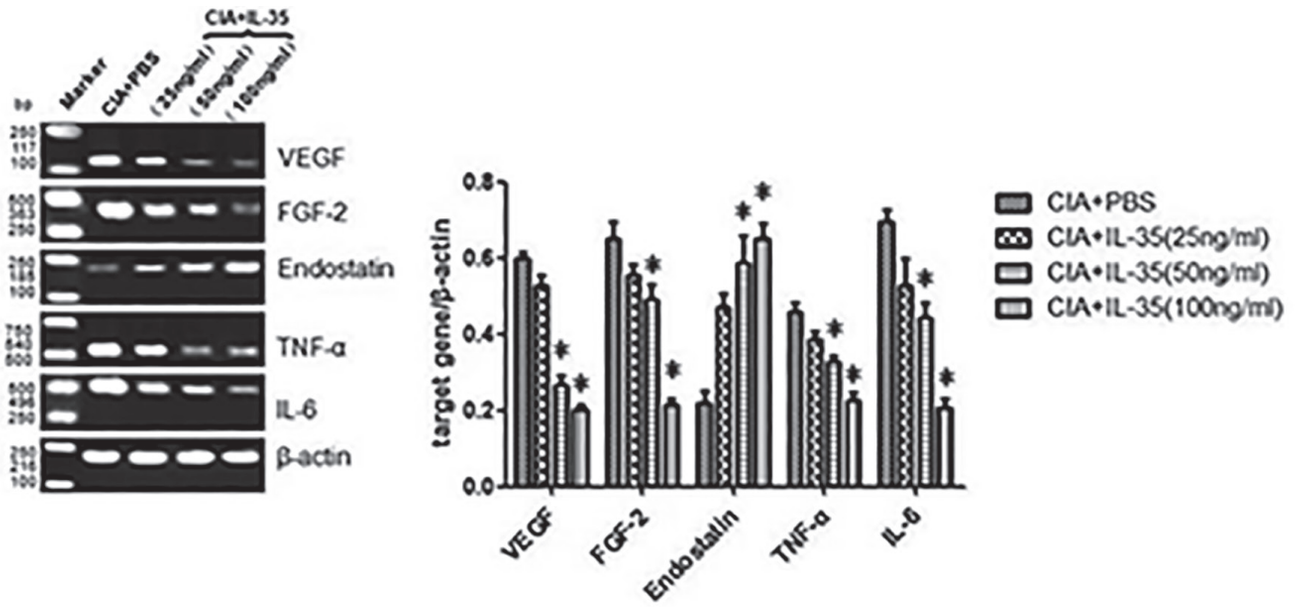

Fig. 1. RT-PCR analysis of IL-35-stimulated FLS. After stimulation with various concentration of IL-35 (25 ng/mL, 50 ng/mL, or 100 ng/mL) for 48 h. RT-PCR assays were performed on samples of IL-35-treated or untreated FLS. The levels of VEGF, FGF-2, endostatin, TNF-α, and IL-6 mRNA expression in FLS derived from CIA mice after various treatments are shown. Data shown represent means ± SEM; n=3; **p*<0.05 compared to FLS controls.

anti-sense primer: 5'- TCAGCTCT-TAGCAGAGATTGG-3';
endostatin sense primer: 5'-AAGGAC-GAGGTGCTATCT-3',
anti-sense primer: 5'-TCACAG-TAACTCTCCATCAG-3';
TNF-α sense primer: 5'-ATGAG-CACGGAAAGCATGATCC-3',
anti-sense primer: 5'-AGGGCAA-GGCTCTTGATGGCAG-3';
IL-6 sense primer: 5'-GAGAG-CATTGGAAGTTGGGG-3',
anti-sense primer: 5'-CTTCCAGC-CAGTTGCCTTCT-3';
β-actin sense primer: 5'-CCAGAG-CAAGAGAGGTATCC-3',
anti-sense primer: 5'-GGGGTGTT-GAAGGTCTCAAA-3'.

RT-PCR was used to establish the expression of VEGF, FGF-2, endostatin, TNF-α, and IL-6 mRNA in each treatment group of FLS. Western blotting was used to assess the protein expression in the same groups. Rabbit anti-mouse VEGF (Lot: GR169425-1), TNF-α (Lot: GR117041-10) and IL-6 (Lot:GR254783-3) antibodies were purchased from Abcam (Cambridge, UK). Rabbit anti-mouse FGF-2 (Lot: B4901) antibody was purchased from ImmunoWay (Newark, USA). Rabbit anti-mouse endostatin (Lot: AE060912) antibody was purchased from BIOSS (Beijing, China).

Western blotting to assess the JAK/STAT signalling pathway in IL-35-stimulated FLS

FLS were cultured as described above. We examined the effect of IL-35 on the STAT1, STAT3 and STAT5 signalling pathways in CIA-derived FLS. To examine the effects of IL-35 on the STAT1 pathway, we used the following experimental groups: CIA FLS (control); CIA FLS plus 50 ng/mL: IL-35; CIA FLS plus 50 ng/mL IL-35 plus fludarabine (STAT1 inhibitor). For the study of the effect of IL-35 on the STAT3 pathway, we used the following experimental groups: CIA FLS (control); CIA FLS plus 50 ng/mL IL-35; CIA FLS plus 50 ng/mL IL-35 plus stattic (STAT3 inhibitor). For the study of the effect of IL-35 on the STAT5 pathway we used the following experimental groups: CIA FLS (control); CIA FLS plus 50 ng/mL IL-35; CIA FLS plus 50 ng/mL IL-35 plus IQDMA (STAT5 inhibitor). The FLS to be treated with inhibitors of STAT1, 3, or 5 were pre-treated with each of the inhibitors for two hours before stimulation with IL-35. All cells were then stimulated with 50 ng/mL of IL-35 (FLS plus IL-35 and FLS plus IL-35 plus inhibitor groups) for 30 min. Thereafter, immunoblotting was used to assess the presence of phosphorylated STAT proteins in FLS after treatment. Rabbit anti-mouse p-STAT1 (Lot: GR95628-3), p-STAT3 (Lot: GR57259-6), and p-STAT5 (Lot: GR16174-6) antibodies, STAT1 inhibitor fludarabine (Lot: APN-12341-1-1), STAT3 inhibitor stattic (Lot: APN-12243-1-1) and STAT5 inhibitor IQDMA (Lot: APN-12535-2-7) were purchased from Abcam (Cambridge, UK).

Statistical analysis

Data were analysed using SPSS 16.0 software. The levels of mRNA and protein expression in FLS were all expressed as mean ± SEM. One-way ANOVA was used for the analyses of differences among groups, and $p<0.05$ was considered statistically significant.

Results

Effect of IL-35 on expression of VEGF, FGF-2, endostatin, TNF-α, and IL-6 in FLS

We first explored the expression levels of VEGF, FGF-2, endostatin, TNF-α and IL-6 in the IL-35-treated (25 ng/mL, 50 ng/mL and 100 ng/mL) FLS of CIA mice. After stimulating FLS with various concentrations of IL-35 for 48 h, RT-PCR and Western blot results show that VEGF, FGF-2, TNF-α, and IL-6 expression levels in the IL-35-treated (50 ng/mL and 100 ng/mL) FLS were lower than those in the FLS controls

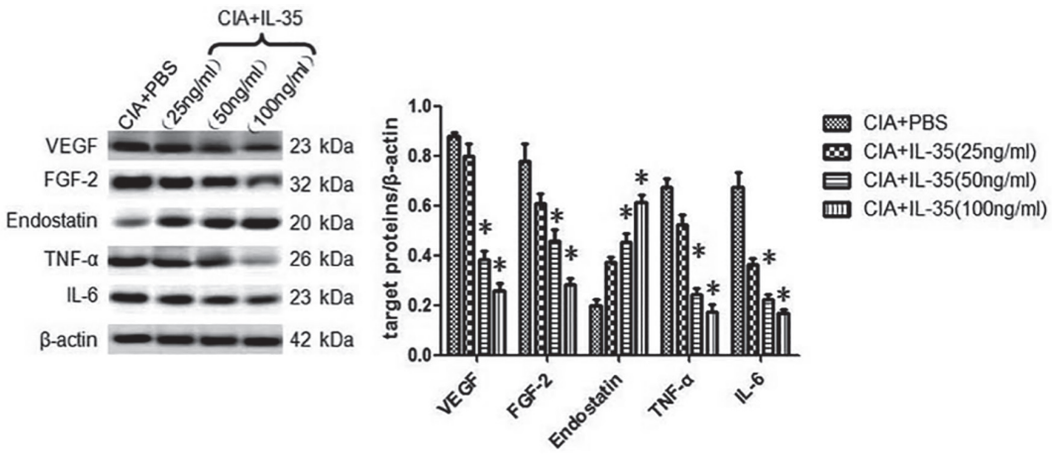

Fig. 2. Western blot analysis of IL-35-stimulated FLS. After stimulation with various concentrations of IL-35 (25 ng/mL, 50 ng/mL, 100 ng/mL) for 48h, Western blot assays were performed on samples of IL-35-treated or untreated FLS. The levels of VEGF, FGF-2, endostatin, TNF-α, and IL-6 protein expression in FLS derived from CIA mice after various treatments are shown.
Data shown represent means ± SEM; n=3; *p<0.05 compared to FLS controls.

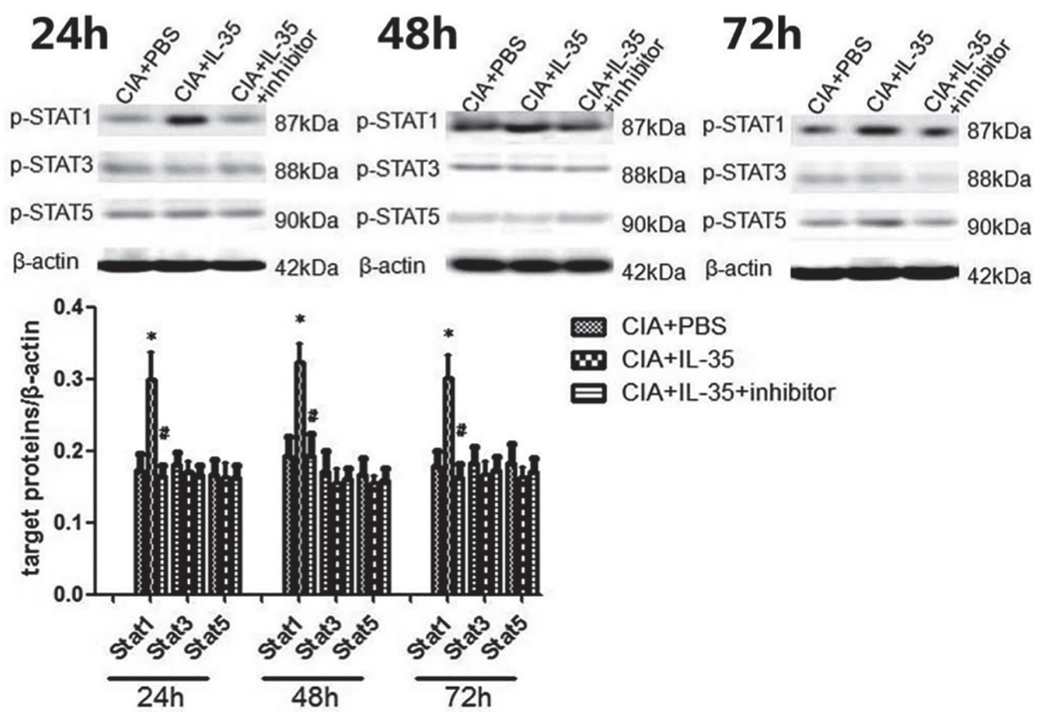

Fig. 3. IL-35 activates STAT1 in FLS derived from CIA mice. FLS were pretreated with fludarabine (STAT1 inhibitor), stattic (STAT3 inhibitor), or IQDMA (STAT5 inhibitor) for two hours. Cells were then stimulated with IL-35 at 50 ng/mL for 30 min. Western blotting was carried out using antibodies against the phosphorylated forms of STAT1, STAT3 and STAT5 after treatment for 24, 48 or 72h.
Data represent means ± SEM; n=3; *p<0.05 compared to control FLS. #p<0.05 compared to FLS treated with IL-35.

(p<0.05). The levels of VEGF, FGF-2, TNF-α, and IL-6 expression in FLS treated with 25 ng/mL IL-35 and FLS controls were not significantly different (p>0.05). Endostatin expression levels in the IL-35-treated (50 ng/mL and 100 ng/mL) FLS were higher than those in the FLS controls (p<0.05). The levels of endostatin in FLS treated with 25 ng/mL IL-35 and FLS controls were not significantly different (p>0.05). (Fig. 1-2).

Effect of IL-35 on the JAK1/STAT1 signalling pathway in FLS

Western blotting was used to assess the effect of IL-35 on the STAT1 pathway. p-STAT1 levels in the FLS plus IL-35 (50 ng/mL) group were higher than those in the control FLS group and in the FLS plus IL-35 plus fludarabine group after treatment for 24, 48 and 72h (p<0.05). The p-STAT1 levels in the FLS plus IL-35 plus fludarabine groups and in the control FLS group were not significantly different from one another after 24, 48 and 72h of treatment (p>0.05).

IL-35 treatment on the STAT3 and STAT5 pathways showed that the FLS plus IL-35 (50 ng/mL) group and the control FLS group did not exhibit statistically significant differences in the levels of p-STAT3 and p-STAT5 after 24, 48 or 72h (p>0.05). The p-STAT3 levels in the FLS plus IL-35 plus stattic group and in the control FLS group were not significantly different after 24, 48 or 72h (p>0.05). The p-STAT5 levels in the FLS plus IL-35 plus IQDMA group and in the control FLS group were not significantly different after treatment for 24, 48 or 72h (p>0.05). Taken together, the results show that IL-35-treated CIA-derived FLS transfer signalling through STAT1 but not through STAT3 or STAT5 (Fig. 3).

Discussion

The main pathology of RA includes hyperplasia of synovium, which is accompanied by angiogenesis (6, 7). Abundantly present mediators such as VEGF, FGF-2, EGF and TNF-α have pro-angiogenic activity, while endostatin can be considered an anti-angiogenic factor in

RA (8). The main pro-angiogenic mediator VEGF does not only induce vascular endothelial cell proliferation, but also increases vascular permeability and promotes cell migration, thereby playing a central role in the regulation of angiogenesis. VEGF-A, upon binding to the VEGF receptors (VEGFR) VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1), induces dimerisation and transphosphorylation of the VEGFRs resulting in receptor activation and stimulation of angiogenesis in RA. Accumulating evidence suggests that TNF-α and IL-6 may also promote angiogenesis in RA by increasing the expression of VEGF-A (9). However, TNF-α and IL-6 are also important inflammatory cytokines (9, 10) which can also promote the production of other inflammatory cytokines. Recent studies have shown that in adjuvant arthritis, endostatin expression was associated with down-regulation of VEGF expression and suppression of TNF-α production (11). After stimulation of CIA-derived FLS with various concentrations of IL-35 *in vitro*, our study showed that IL-35 significantly decreases the expression of VEGF, FGF-2, TNF-α and IL-6. Furthermore, the expression of endostatin was enhanced, accounting for a mechanism by which IL-35 may relieve the severity of RA. Our data indicate that IL-35 might act, at least partially, through up-regulation of the anti-angiogenic factor endostatin, which may lead to reduction of VEGF. The observed down-regulation of pro-angiogenic and pro-inflammatory cytokines by IL-35 may also involve an indirect action mediated by endostatin. In RA angiogenesis is associated with the action of pro-inflammatory cytokines. Therefore, the anti-angiogenic effect of IL-35 on FLS is likely to involve both the inhibition of the expression of VEGF and of pro-inflammatory cytokines, and the induction of the expression of endostatin, employing both direct actions of IL-35 and indirect actions mediated by, *e.g.* endostatin.

Recent studies have shown that in RA, the cytokine IL-35, which belongs to the IL-12 family of cytokines, mainly induced expansion of Treg cells and inhibited the differentiation of Th17 cells (1). In addition, it has also been shown that IL-35 directly promotes inhibition of Tres responses in RA by Treg cells (12). We have shown previously that IL-35 decreased bone destruction by synovial tissue in the CIA model of RA (13), and here we demonstrate that IL-35 inhibits angiogenesis in CIA-derived FLS. Both mechanisms may contribute to the inhibition of RA by IL-35. In the current study we addressed the effect of IL-35 on the JAK-STAT signal transduction pathway in CIA-derived FLS. The identity of the IL-35 receptor is currently still under investigation. Collison *et al*. (14) reported that the IL-35 receptor is different from the receptors shared by other IL-12 family members. They suggested that the IL-35 receptor may be a heterodimer of gp130 and IL-12R or a homodimer of gp130 or of IL-12R β2. The study further showed that IL-35 signalled through gp130 and IL-12R β2 and signal transduction was conveyed through STAT1 and STAT4. The study also suggested that IL-35 may also utilise different receptors and different STAT signalling pathways, as suggested by the variable affects seen in different diseases and on different cell types (15). The signalling molecules of the JAK/STAT pathway are vital for the regulation of RA. We studied the significance of STAT1, STAT3 and STAT5 in CIA as an experimental model for RA. Our results show that IL-35 increases levels of p-STAT1 in FLS, while fludarabine blocked this effect. In contrast, IL-35 had no effect on the levels of p-STAT3 and p-STAT5 after 24, 48 and 72h of treatment. Therefore we concluded that IL-35 transmits signals exclusively through STAT1 in CIA-derived FLS. IL-35 may act in a similar way in RA, resulting in blockade of pro-angiogenic and pro-inflammatory signals by inhibiting the expression of both VEGF and FGF-2 and, importantly, also by inhibiting the expression of TNF-α and IL-6.

In conclusion, our study shows that IL-35 inhibits the expression of pro-angiogenic and pro-inflammatory mediators in CIA-derived FLS through effects on STAT1 signalling, suggesting that IL-35 and its signalling pathway may represent therapeutic targets in human RA and other angiogenesis-related conditions.

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