IL-35 prevents bone loss through promotion of bone formation and angiogenesis in rheumatoid arthritis

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Abstract

Objective

Angiogenesis in bone and osteogenesis appear to be closely linked, suggesting the existence of molecular crosstalk between pro-angiogenic molecules and osteoblasts. The pro-angiogenic molecules vascular endothelial growth factor (VEGF) with its receptors Flt-1, Flk-1 and fibroblast growth factor (FGF)-2 play a crucial role in born formation, an early and critical event in the pathogenesis of rheumatoid arthritis (RA). Interleukin (IL)-35 is demonstrated to be an anti-inflammatory cytokine in RA. However, the mechanisms involved are not fully understood. This study aims to investigate whether IL-35 has an impact on angiogenesis in osteoblasts and its related signalling pathway in RA.

Methods

The effects of IL-35 on osteoblasts proliferation, apoptosis and pro-angiogenic molecules mRNA and protein were examined using osteoblast-like MC3T3E1 cells in vitro. The effects of IL-35 on proliferation and apoptosis were examined using cell counting kit-8 (CCK-8) assay and flow cytometry, respectively. Pro-angiogenic molecules expression were assessed by real time PCR and ELISA, respectively. The signalling pathway between IL-35, bone formation, angiogenesis and signalling pathway was also investigated.

Results

IL-35 promoted osteoblasts proliferation and inhibited apoptosis in a dose-dependent manner in vitro. IL-35 increased basal and TNF-α induced pro-angiogenic molecules expression by osteoblasts. Blocking the Th17/IL-17 signalling pathway with plumbagin inhibited the pro-angiogenic effects of IL-35 in osteoblasts.

Conclusion

These results suggested that IL-35 promotes bone formation and angiogenesis by fostering osteoblasts proliferation, inhibiting apoptosis and upregulating pro-angiogenic molecules through Th17/IL-17 related-signalling pathway. Our findings extend the current understanding of mechanisms modulating bone formation and angiogenesis in RA.

Key words

IL-35, angiogenesis, bone formation, rheumatoid arthritis

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Introduction

Rheumatoid arthritis (RA) is a chronic and autoimmune disease that involved joint inflammation and destruction (1, 2). However, it is not only characterised by inflammatory synovitis, but bone is also implicated. Bone loss takes place at the very beginning following the onset of RA and progressed throughout the entire course of the disease (3, 4). In the bone microenvironment, there is a dynamic balance of resorption and formation, which maintains skeletal homeostasis. The cells responsible for these functions, osteoclasts and osteoblasts, require multiple mediators, including parathyroid hormone, sex steroids, $1,25(OH)_2D_3$ and proinflammatory cytokines (5). Of the latter, tumour necrosis factor (TNF)-α has been shown to play an important role on osteoblasts in the local control of inflammatory bone loss. Several lines of evidence have revealed that TNF-α could increase apoptosis of osteoblasts, indicating a mechanism to induce bone loss (6, 7).

The vascular network provides the necessary oxygen and growth factors for a appropriate bone homeostasis, identifying the roles of osteoclasts, osteoblasts, endothelial cells and angiogenic molecules and their crosstalk in bone is an important event in the pathogenesis of RA with bone loss (8, 9). Evidence suggests that the shortage of oxygen (hypoxia) and the consecutive production of pro-angiogenic molecules such as vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF)-2 have been illustrated to be important in the progress of a regular bone remodelling (10). VEGF have been described to exert functions on osteoblasts by promoting calcium deposits formation and alkaline phosphatase activity in primary human osteoblasts (11).

As a new component of the IL-12 cytokine family, IL-35 has aroused general interest in the field of immune research since it was first reported in 2007. Like other IL-12 family members, IL-35 is a heterodimer consisting of an α-chain p35 subunit and a β-chain EBI-3 subunit (12). It is mainly secreted by regulatory T cells (Tregs), and a few can be secreted by activated B cells, endothelial cells, smooth muscle cells, and monocytes (13, 14). Unlike the pro-inflammatory effects of other IL-12 family cytokines, IL-35 is an anti-inflammatory cytokine which has been explored in many disease (15-18). However, how this interaction works is not fully elucidated. In the present study, we investigated the effect of IL-35 on the expression of pro-angiogenic molecules in osteoblasts. The focus of our study was to clarify the effect of IL-35 on bone formation and angiogenesis in osteoblasts.

Materials and methods

Chemicals and reagents

IL-35 (mouse) was from Sigma Company (USA). Fetal bovine serum (FBS) was purchased from Clark Bioscience Company (USA). α-Modified minimal essential medium (α-MEM) was from Gibco (USA), the Cell Counting Kit was purchased from Dojindo Molecular Technologies (Japan), Annexin V-FITC/PI apoptosis kit was from Beyotime biotechnology (China), and Plumbagin was from R&D systems (USA). Real time-PCR kits, TRizol reagents, were obtained from Takara Biotechnology Company (China), VEGF ELSIA kit was from Boster Biological Technology Co. Ltd (China).

Cell culture

MC3T3E1 were cultured in α-MEM, supplemented with 10%FBS, 100U/ml penicillin and 100μg/ ml streptomycin at 37°C in 5%CO_2 humidified atmosphere. Cells were passaged (third to seven generations) used for the *in vitro* experimental procedures. Recombinant IL-35 or TNF-α was dissolved in 0.1% bovine serum.

CCK-8 assay to evaluate MC3T3E1 cells proliferation

MC3T3E1 proliferation was evaluated using the CCK-8 kit. MC3T3E1 in a density of 3×10^3cells/ml was seeded into 96-well plates and then was incubated at 37°C in 5%CO_2 atmosphere. With and without induction of TNF-α (20 ng/ml), the medium was replaced with fresh medium containing different concentrations of IL-35 (0, 25, 50 and 100 ng/ml). The 10μl CCK-8 solution was added to each well and then was in-

cubated for 4h. The absorbance for the solution in each well was read at 450 nm in a microtitre plate reader.

Flow cytometry to evaluate MC3T3E1 cells apoptosis

Flow cytometry was used to detect MC3T3E1 frequency of apoptosis. 5×10^4 cells/well were seeded into 6-well plates. After incubation with IL-35 (0, 25, 50 and 100ng/ml) for 48h without or with induction of TNF-α (20ng/ml), cells were harvested by trypsin digestion, followed by centrifugation at 1000rmp for 5min. For apoptosis analysis purpose, Annexin V-FITC/PI apoptosis kit was used according to the manufacturer's instructions. Cells were resuspended gently in 195μl binding buffer and incubated with 5μl Annexin V-FITC. Then cells were incubated with 10μl PI solution for 15min in the dark at room temperature.

Real time-PCR to assess the expression of proangiogenic molecules mRNA

After incubation with IL-35 (0, 25, 50 and 100ng/ml) for 48h without or with induction of TNF-α, total RNA was isolated from MC3T3E1 using Trizol reagent according to the manufacturer's protocol. PCR reactions were conducted using a real time-PCR kit using the following PCR conditions: 94°C for 2min, 30 cycles as 94°C for 30s, 65°C for 30s, 72°C for 30s and finally 72°C for 2min. The primer sequences used for real time-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';
FGF-2 sense primer: 5'-AACGGCGGCTTCTTCCTGCG-3';
anti-sense primer: 5'-TCAGCTCTTAGCAGAGATTGG-3';

The relative expression of VEGF, Flt-1, Flk-1 and FGF-2 were normalised with a β-actin internal control.

Immunoassay to assess VEGF levels in cell culture supernatants

Subsequently, we quantified VEGF protein levels in cell supernatants using enzyme linked immunosorbent assay (ELISA) kit. MC3T3E1 was treated with IL-35 at different concentrations (0, 25, 50 and 100ng/ml) for 48h without or with induction of TNF-α. Cell supernatants were collected to detect VEGF protein levels. The absorbance was measured at 450nm using a microplate reader.

Angiogenic analysis after blockage with Plumbagin

After pretreatment with 1uM Plumbagin for 24h at 70–90% confluence, the cells were treated with IL-35 at different concentrations (0, 25, 50 and 100ng/ml) for 48h without or with induction of TNF-α. The experimental procedures for real time-PCR and ELISA on pro-angiogenic molecules expression were conducted as previously described.

Statistical analysis

Analyses were performed using SPSS 17.0. The figures were created using GraphPad Prism 6. One-way ANOVA was performed to analyse differences among different groups. Probability values less than 0.05 were considered as statistically significant.

Results

IL-35 promoted basal and TNF-α induced cell proliferation of MC3T3E1 cells

To identify the effect of IL-35 on TNF-α induced MC3T3E1 cell growth, we tested the cell viability rate using CCK-8 assay. TNF-α induced MC3T3E1 cells were exposed to IL-35 at different concentration. As shown in Fig. 1, the results of CCK-8 assay showed that IL-35 improved the proliferation of MC3T3E1 cells and TNF-α induced MC3T3E1 cells dose dependently ($p<0.05$).

IL-35 inhibited basal and TNF-α induced cell apoptosis of MC3T3E1 cells

We next aimed to examine the agiogenic effect of VEGF secreted by osteoblasts in response to IL-35 *in vitro*. As shown in Fig. 2, after co-culturing the MC3T3E1 with IL-35, the apoptotic rate was decreased in a dose dependent manner as compared to the control group cultured under normal conditions, suggesting that IL-35 suppressed MC3T3E1 cells apoptosis.

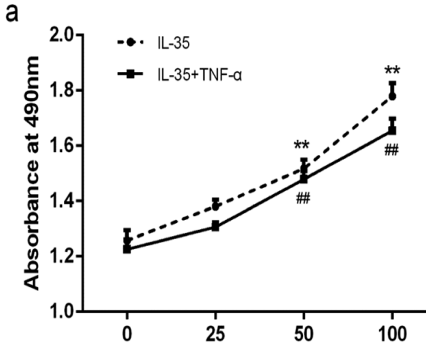

Fig. 1. The effects of IL-35 at different concentration (0, 25, 50, 100ng/ml) on the proliferation of MC3T3E1 cells without and with induction of TNF-α (20ng/ml). Mean ± SD, n=6. $^{**}p<0.01$ vs. IL-35(0ng/ml)/TNF-α(-); $^{\#\#}p<0.01$ vs. IL-35(0ng/ml) /TNF-α(+).

IL-35 inhibited basal and TNF-α induced angiogenesis molecules mRNA expression in MC3T3E1 cells

Following the treatment with IL-35, VEGF with its receptors Flt-1 and Flk-1 and FGF-2 mRNA expression were further analysed by real-time PCR. The results of real time-PCR showed IL-35 dose-dependently elevated the VEGF, Flt-1 and VEGF mRNA expression. After induction of TNF-α, VEGF, Flt-1 and FGF-2 mRNA expression were significantly increased by IL-35 in a dose dependently manner (Fig. 3). However, both basal and TNF-α induced Flk-1 mRNA were not detected using real-time PCR (data not shown).

IL-35 increased basal and TNF-α induced VEGF protein levels in MC3T3E1 cells supernatant

The quantitative analysis of VEGF protein levels in the MC3T1E1 cells supernatant was conducted by using VEGF Immunoassay kit according to manufacturer's protocol. In present study, we found that IL-35 at 100 ng/ml significantly increased VEGF protein levels by MC3T3E1 cells supernatant after 48 h incubation.

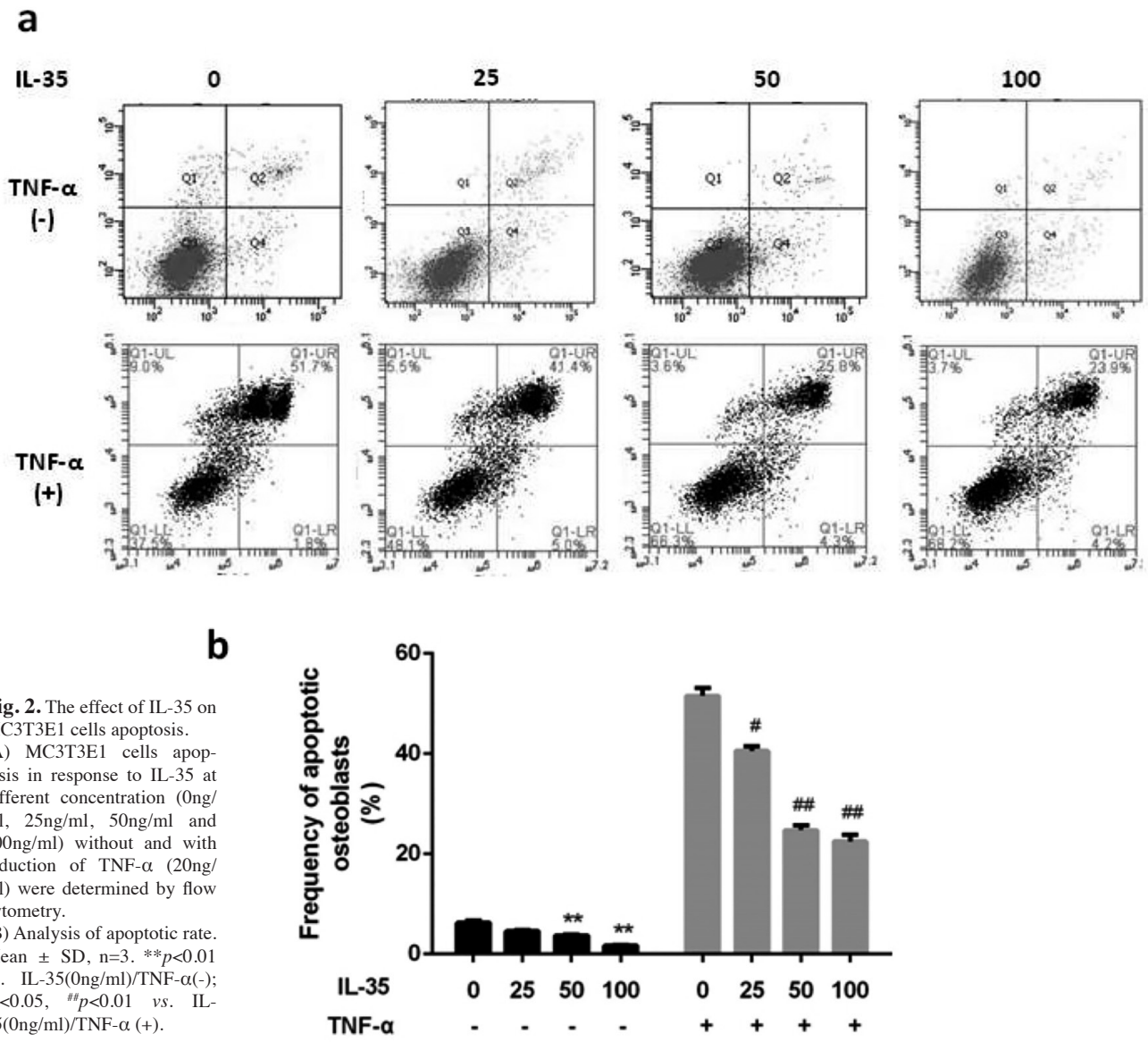

Fig. 2. The effect of IL-35 on MC3T3E1 cells apoptosis.
(**A**) MC3T3E1 cells apoptosis in response to IL-35 at different concentration (0ng/ml, 25ng/ml, 50ng/ml and 100ng/ml) without and with induction of TNF-α (20ng/ml) were determined by flow cytometry.
(**B**) Analysis of apoptotic rate. Mean ± SD, n=3. $^{**}p<0.01$ *vs*. IL-35(0ng/ml)/TNF-α(-); $^{\#}p<0.05$, $^{\#\#}p<0.01$ *vs*. IL-35(0ng/ml)/TNF-α (+).

IL-35 foster angiogenesis through Th17/IL-17 related signalling pathway in MC3T3E1 cells

To confirm the involvement of the pathway in the angiogenic effects of IL-35, we tested the effects of exogenous plumbagin (IL-17A Inhibitor) which can block the Th17/IL-17 related pathway. After blocking with plumbagin (1uM) for 18h, VEGF, Flt-1 and FGF-2 mRNA levels in MC3T3E1 cells were decreased in response to IL-35 at different concentrations (0ng/ml, 25ng/ml, 50ng/ml and 100ng/ml). These results demonstrated plumbagin inhibited the pro-angiogenic effects of IL-35 in osteoblasts, confirming that IL-35 exerts its pro-angiogenic effects through Th17/IL-17 related signalling pathway.

Discussion

RA is a chronic systemic disorder in which angiogenesis can foster the infiltration of inflammatory cells into the joints leading to synovial hyperplasia and progressive bone destruction (19). The bone destruction is caused by a decrease of bone formation by osteoblasts. It is well known TNF-α plays a significant role in bone loss related to RA. Therefore, we established a microenvironment with TNF-α which mimics inflammatory induced osteoblasts of RA *in vitro*. Furthermore, there is evidence indicating that angiogenesis and bone formation are intrinsically coupled (20). It has been reported that vascular endothelial growth factor (VEGF) is one of the most influential regulators of angiogenesis and it is essential for bone formation. In these processes VEGF plays a dual role, working both on endothelial cells to facilitate their migration and proliferation, and motivating osteogenesis through regulating osteogenic growth factors (10). VEGF isoforms bind to two receptors, VEGF receptor-1 (VEGFR-1), also named fms-like tyrosine kinase (Flt-1) (21), and VEGF receptor-2 (VEGFR-2), named Flk-1 in mouse (22). In the present study, we investigated the effect of IL-35 on the expression of VEGF in MC3T3E1 cells, a cell line derived from the mouse cranium and possessed the characteristics of osteoblasts. Besides the angiogenic effects of VEGF on endothelial cells, VEGF exerts actions on bone cells,

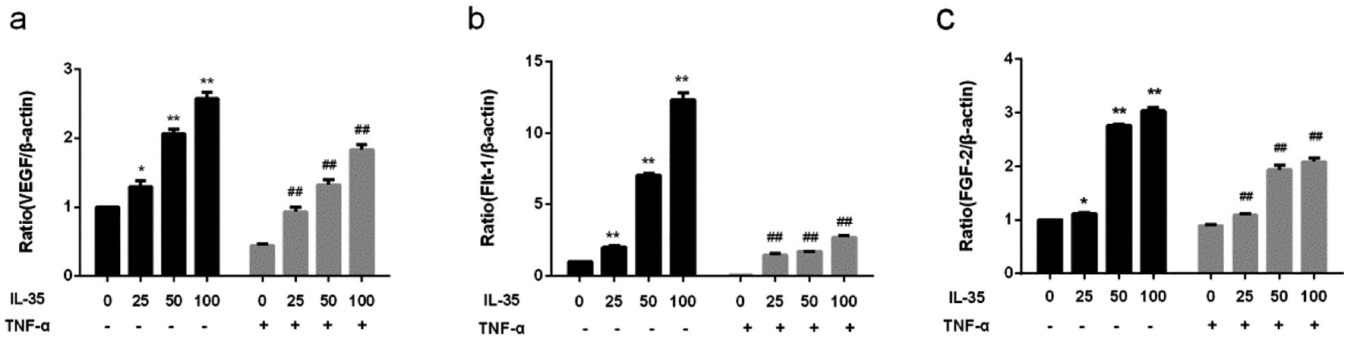

Fig. 3. VEGF mRNA expression in MC3T3E1 cells. VEGF, Flt-1 and FGF-2 mRNA expression in MC3T3E1 cells in response to IL-35 at different concentrations (0ng/ml, 25ng/ml, 50ng/ml and 100ng/ml). **P<0.01vs.IL-35(0ng/ml)/TNF-α(-); #P<0.05,##P<0.01vs.IL-35(0ng/ml)/ TNF-α(+).

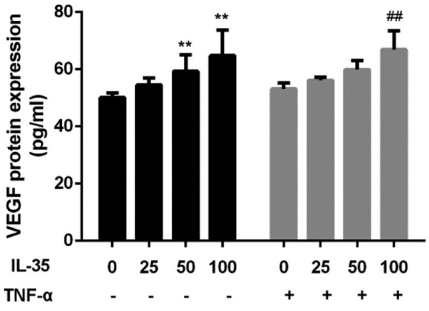

Fig. 4. VEGF protein levels in MC3T3E1 cells. VEGF protein levels in MC3T3E1 cells in response to IL-35 at different concentrations (0ng/ml, 25ng/ml, 50ng/ml and 100ng/ml). **p<0.01vs.IL-35(0ng/ml)/TNF-α(-); #p<0.05,##p<0.01vs.IL-35(0ng/ml)/TNF-α(+).

such as osteoblasts and osteoclasts (23). It was also demonstrated that Flk-1 was expressed on osteoblasts and VEGF has been shown to upregulate the expression of Flk-1 in M3CT3E1 osteoblastic cells and subsequently enhance osteoblasts activity (24). This indicates that, on one hand, VEGF enhances angiogenesis through interaction with its receptors on endothelial cells indirectly trigerring osteogenesis; on the other hand, VEGF interacts with its receptor on osteoblastic cells, directly contributing to bone formation. FGF-2, which is a member of the FGF polypeptide family, is expressed in osteoblasts and stored in the extracellular matrix (25). It is not only a strong pro-angiogenic molecular, acting as a factor of the positive effects of angiogensis but also play an important role in bone formation (26, 27). The obtained results showed that IL-35 could promote the proliferation of osteoblasts and inhibited the apoptosis of osteoblasts. Furthermore, IL-35 dose-dependently increased the expression of VEGF, Flt-1 and FGF-2 in cultured osteoblasts. These findings suggests IL-35 as a crucial positive mediator of vascular growth in bone formation, which may be a potential therapeutic agent for the treatment of RA, especially with bone loss.

Niedbala *et al*. showed that IL-35 restrained differentiation of Th17 cells and subsequently downregulated production of IL-17 (12). Considering this hallmark of IL-35, this could be a convincing explanation that IL-35 may regulate angiogenesis and bone formation through Th17/IL-17-related signalling pathways. Plumbagin is a organic bicyclic naphthoquinone extracted from roots of the natural remedial plant Plumbago zeylanica. The plumbagin was known for its strong biological activities such as anti-inflammatory, antitumour, and anti-bacterial activities (28, 29). Several studies reported that plumbagin suppressed NF-κB signalling pathway (30, 31) and the pro-inflammatory cytokines triggered by NF-κB activation in the *in vivo* experimental models of osteoporosis (32). Lately, many studies appraised the medicative implication of plumbagin and pinpointed its potential use in treating RA, depression, and diabetes (33-35). Furthermore, plumbagin was discovered to have anti-cancer features to treating prostate cancer, pancreatic cancer, cervical cancer and brain cancer (36-39). Therefore, the present study is designed to examine whether IL-35 have an impact on angiogenesis in MC3T3E1 cells via Th17/IL-17-related signalling pathways. In current study, we demonstrated that IL-35 induced-VEGF mRNA

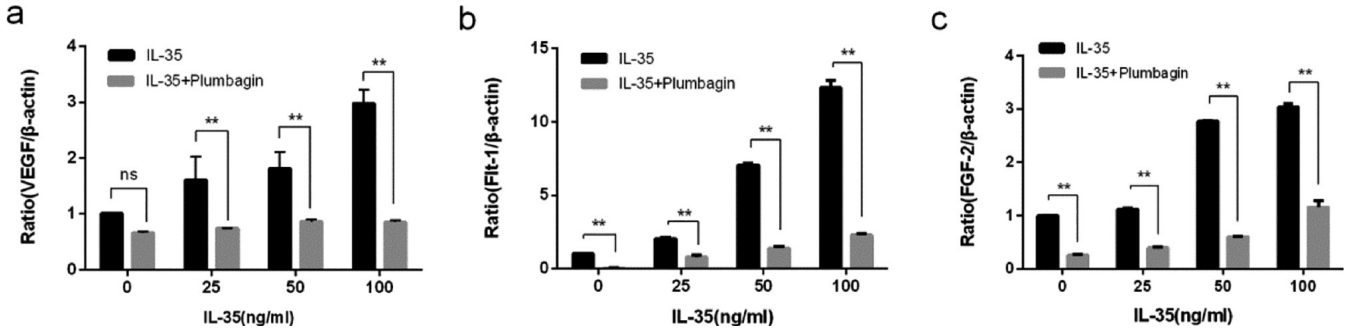

Fig. 5. VEGF, Flt-1 and FGF-2 mRNA levels in MC3T3E1 cells were determined by real time-PCR. Without or with blockage of plumbagin, basal VEGF, Flt-1 and FGF-2 mRNA levels in MC3T3E1 cells in response to IL-35 at different concentrations (0ng/ml, 25ng/ml, 50ng/ml and 100ng/ml). *p<0.05, **p<0.01, ns: no significant.

expression was decreased after blocking production of IL-17 in MC3T3E1 cells, and VEGF protein levels was decreased accordingly. Hence, the regulation of IL-35 on angiogenesis and bone formation may be mediated by Th17/IL-17-related signalling pathways.

Conclusions

RA angiogenesis and bone formation are coupled and are in crosstalk with each other. Our findings in this work suggested that IL-35 might prolong bone loss progression in RA through promoting bone formation and angiogenesis regulated by Th17/IL-17 related signalling pathway. Focusing on the only one player may not be adequate to impact RA progression. Additionally, these findings establish a molecular crosstalk coupling osteogenesis and angiogenesis, which may confirm considerable value for the development of future RA clinical applications.

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