Effect of iguratimod and methotrexate on RANKL and OPG expression in serum and IL-1β-induced fibroblast-like synoviocytes from patients with rheumatoid arthritis

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Abstract: The receptor activator of nuclear factor κB ligand (RANKL)/receptor activator of nuclear factor κB (RANK)/osteoprotegerin (OPG) system plays a key role in rheumatoid arthritis (RA)-associated bone erosion. The upregulation of the RANKL/OPG ratio promotes bone erosion. The objective of this study is to explore the effects of iguratimod, a small-molecule disease-modifying antirheumatic drug (DMARD), alone or in combination with methotrexate (MTX), on RANKL and OPG expression in RA. We performed an enzyme-linked immunosorbent assay (ELISA) to investigate the modulatory effects of iguratimod, MTX, or their combination on serum RANKL and OPG levels of patients with RA before and after treatment for 12 and 24 weeks. Furthermore, fibroblast-like synoviocytes (FLS) from patients with RA were interleukin (IL)-1β-stimulated and then treated with different concentrations of iguratimod, MTX, or both, and RANKL and OPG expressions were investigated by using ELISA, quantitative real-time polymerase chain reaction (qPCR) and western blot analysis. We found that RANKL levels and the RANKL/OPG ratio significantly decreased in both serum and IL-1β-induced RA FLS after treatment. Moreover, combination therapy with iguratimod and MTX showed an even stronger inhibition than each drug alone did. Our results suggest that iguratimod and MTX, especially in combination, efficaciously protected against bone erosion by suppressing the production of RANKL.

Key words: RANKL, OPG, iguratimod, methotrexate, fibroblast-like synoviocytes, serum, rheumatoid arthritis, interleukin-1β.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by progressive synovitis and joint destruction (1). Bone destruction seriously affects the normal shape and function of joints and eventually leads to joint deformities and ankylosis. Bone erosion in patients with RA occurs rather rapidly, and a number of osteoclast differentiation and function regulators have been implicated. Three important implicated regulatory molecules are the receptor activator of nuclear factor κB (RANK), receptor activator of nuclear factor κB ligand (RANKL), and osteoprotegerin (OPG) (2-4). RANKL and the macrophage colony-stimulating factor (M-CSF) are essential factors that stimulate the growth and differentiation of osteoclasts (5). RANKL binds with RANK, which is expressed in osteoclast and preosteoclast precursors and this interaction activates processes such as preosteoclast recruitment and osteoclast activation (6). On the other hand, OPG, a decoy receptor for RANKL, is a protective factor against bone loss by combining with RANKL to interfere with the interaction between RANKL and RANK (4, 6). The balance of the RANKL/OPG system affects the process of bone erosion and remodeling. Serum ratio of soluble RANKL/OPG (sRANKL/OPG) has been suggested to be a marker of bone damage in patients with RA (7).

Iguratimod (T-614) is a novel disease-modifying antirheumatic drug (DMARD) that has been widely used to treat patients with RA in Japan and China. It is a small molecule with immunomodulatory and anti-inflammatory effects (8, 9). For bone metabolism, previous studies have shown that iguratimod greatly suppressed bone loss. It promoted osteoblast differentiation and strongly inhibited osteoclast differentiation (10, 11). The production of RANKL in collagen-induced arthritis (CIA) mouse joint tissues decreased significantly after treatment with iguratimod, as demonstrated in an immunohistochemical assay (12). In addition, iguratimod suppressed RANKL production in IL-6 and soluble IL-6 receptor (sIL-6r)-induced fibroblast-like synoviocytes (FLS) (13). However, the effects of iguratimod and methotrexate (MTX) on serum RANKL and OPG levels and their expression in IL-1β-induced FLS from patients with RA are still unclear.

In the present study, we investigated the effects of iguratimod and MTX, alone or in combination, on RANKL and OPG levels. Serum levels of RANKL and OPG were analyzed in patients with RA before and after treatment with iguratimod, MTX, or their combination for the first time. Furthermore, we investigated the effects of iguratimod and MTX on the ratio of RANKL and OPG expression in IL-1β-induced FLS from patients with RA.

Materials and Methods

Patients

A total of 93 patients with established RA who were treated at the Department of Rheumatology and Immunology of China-Japan Union Hospital of Jilin University in China (January 2014 to February 2015) were en-
rolled in this study. All patients were diagnosed with RA according to the 1987 American College of Rheumatology (ACR) criteria and presented with active forms of the disease. They were divided into 3 groups based on their medication and treated orally as follows: group A (n=28, iguratimod, 25 mg, twice daily), group B (n=33, MTX, 15 mg, once a week), and group C (n=32, iguratimod, 25 mg, twice daily, and MTX, 15 mg, once a week). No other DMARDs (including biological DMARDs) were involved in the treatment. The medication of the patients also contained a demand-based intake of the non-steroidal anti-inflammatory drugs (NSAIDs, ibuprofen or meloxicam in 25 patients in group A, 29 in patients in group B and 30 patients in group C). The patient clinical characteristics were examined while serum samples were collected at 0, 12, and 24 weeks and were stored at -80°C until the analysis. The characteristics of the patients with RA at baseline (week 0) are summarized in Table 1. Synovial tissues were obtained from 3 patients with RA when they underwent total knee replacement surgeries in the Department of Orthopedics at our hospital. The study was conducted in accordance with the Helsinki Declaration and was approved by the Ethics Committee of China-Japan Union Hospital.

**FLS culture and treatment**

The synovial tissue mentioned above was minced into small pieces and treated with 4 mg/mL of collagenase (Sigma-Aldrich, St. Louis, MO, USA) for 2 h at 37°C in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Carlsbad, CA, USA). The digested tissue was filtered through a 70-μm cell strainer, and the cell suspension was centrifuged at 1500 rpm for 6 min. The cell pellet was washed twice with DMEM, suspended and dispensed into culture flasks containing DMEM supplemented with 10% fetal bovine serum (FBS, TBD, Tianjin, China), 100 U/mL penicillin, and 100 μg/mL streptomycin in a humidified 5% CO₂ atmosphere at 37°C. After overnight culturing, the non-adherent cells were removed, and the adherent cells, fibroblast-like synoviocytes, were used for the experiments when they were between the passage 4 and 8.

The FLS were plated at a density of 5×10⁴/mL in 60-mm dishes at 37°C in a humidified atmosphere of 5% CO₂. After overnight culturing, the FLS were treated with IL-1β (10 ng/mL, Peprotech, NJ, USA), and at the same time, MTX (100 nM), iguratimod with different concentrations (5, 10, and 20 μg/mL, Simcere Pharmaceutical, Nanjing, China) or MTX (100 nM) combined with iguratimod (20 μg/mL) were added into the culture system. The cells were then cultured for 48 h. Igaratimod was dissolved in dimethyl sulfoxide (DMSO), the final concentration of which was less than 0.1% to ensure it did not affect cell viability. The cell pellets and culture supernatants were collected for further experiments.

**Serum sRANKL and OPG analysis**

All serum samples were centrifuged and stored at -80°C without freeze/thaw cycles until they were analyzed. The serum total soluble RANKL and OPG concentrations were measured by using an enzyme-linked immunosorbent assay (ELISA) kit for sRANKL and OPG (Biovendor, Brno, Czech Republic) according to the manufacturer’s instructions. The OD value was measured using a microplate reader at sampling and reference wavelengths of 450 and 630 nm, respectively. Then, we calculated the concentration of sRANKL and OPG from the constructed standard curve. Each serum sample was tested in triplicate wells.

**FLS RANKL and OPG analysis**

**ELISA**

All the culture supernatants mentioned above were centrifuged and stored at -80°C without freeze/thaw cycles until they were analyzed. The OPG production in the supernatants was determined using the Human OPG ELISA kit (BOSTER-BIO, Wuhan, China). The OD value was measured using a microplate reader at sampling and reference wavelengths of 450 and 630 nm, respectively. Then, we calculated the concentration of OPG from the constructed standard curve.

**Quantitative real-time polymerase chain reaction (qPCR)**

The total RNA was extracted from the FLS using TRIzol reagent (Life Technologies, USA) according to the manufacturer’s instructions. Then, first-strand complementary DNA (cDNA) was synthesized using the Transcript One-Step gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China), and the quantitative real-time polymerase chain reaction (qPCR) was performed with RANKL, OPG and glycerose.

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**Table 1. Baseline clinical characteristics of patients.**

<table>
<thead>
<tr>
<th>Patient (n)</th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>p-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Women n (%)</td>
<td>24 (85.7)</td>
<td>29 (87.9)</td>
<td>28 (87.5)</td>
<td>1.000</td>
</tr>
<tr>
<td>Age (years)</td>
<td>47.32±8.90</td>
<td>49.64±8.01</td>
<td>51.75±7.13</td>
<td>0.108</td>
</tr>
<tr>
<td>RA duration (months)</td>
<td>11.34±5.96</td>
<td>13.40±5.43</td>
<td>14.00±5.84</td>
<td>0.186</td>
</tr>
<tr>
<td>TJC (n)</td>
<td>17.36±6.06</td>
<td>16.88±5.69</td>
<td>19.25±6.02</td>
<td>0.243</td>
</tr>
<tr>
<td>SJC (n)</td>
<td>11.57±5.31</td>
<td>11.18±4.75</td>
<td>13.25±5.48</td>
<td>0.242</td>
</tr>
<tr>
<td>ESR (mm/1^st^ h)</td>
<td>39.07±20.88</td>
<td>35.03±22.79</td>
<td>40.47±20.70</td>
<td>0.574</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>27.42±38.18</td>
<td>35.82±61.46</td>
<td>42.25±43.36</td>
<td>0.689</td>
</tr>
<tr>
<td>DAS28 (ESR)</td>
<td>6.79±1.18</td>
<td>6.72±1.37</td>
<td>6.80±1.39</td>
<td>0.324</td>
</tr>
<tr>
<td>DAS28 (CRP)</td>
<td>6.26±1.09</td>
<td>5.95±1.15</td>
<td>6.56±1.17</td>
<td>0.296</td>
</tr>
<tr>
<td>HAQ</td>
<td>1.08±0.59</td>
<td>0.87±0.67</td>
<td>1.28±0.75</td>
<td>0.212</td>
</tr>
</tbody>
</table>

TJC, tender joint count; SJC, swollen joint count; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein, DAS28: Disease Activity Score in 28 joints; HAQ, Health Assessment Questionnaire. There were no statistically significant differences in clinical characteristics between the treatment groups. Values are mean±standard deviation (SD) unless otherwise indicated.
aldehyde 3-phosphate dehydrogenase (GAPDH) using the TransStart Tip Green qPCR SuperMix (TransGen Biotech, Beijing, China). The primer sequences are provided in Table 2. The reaction systems were incubated at 50°C for 2 min, and then 95°C for 10 min for an initial holding, followed by 94°C (5 s) and 60°C (34 s) for 40 cycles. The data were collected and quantitatively analyzed using an ABI 7500 sequence detection system (Applied Biosystems, USA). Then, the gene expression level was calculated using the threshold cycle (Ct) value for amplification of the human RANKL and OPG with the human GAPDH as a reference gene. The relative gene expression was determined by using the 2^−ΔΔCt method and the experiment was performed thrice in triplicate.

**Western blot analysis**

The FLS were harvested and washed twice with phosphate-buffered saline (PBS), and whole cell lysates were prepared using a radioimmunoprecipitation assay (RIPA) buffer (BOSTER-BIO, Wuhan, China) and protease inhibitor cocktail (Biotool, USA). After incubation for 2 h at 4°C, the lysed cells were centrifuged to remove the debris at 12,000 rpm for 30 min at 4°C, and the supernatants were collected. The protein concentrations were measured using a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China) according to the manufacturer’s instructions. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using a mini-protein system (Bio-Rad, USA). The protein samples (40 μg) were boiled for 5 min with the SDS-PAGE loading buffer (CWBio, Beijing, China), loaded onto 10% SDS gels, and after the electrophoresis, the proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). The membranes were subsequently blocked with 5% nonfat dried milk in Tris-buffered saline plus Tween (TBST) for 2 h at room temperature, and then it was incubated overnight with mouse monoclonal antibodies against human RANKL (mab6263, R&D, MN, USA), OPG (ab11994, Abcam, UK), or β-actin (#3700, Cell Signaling Technology, USA) at 4°C. Then, the membranes were washed and incubated with peroxidase-conjugated AffiniPure goat anti-mouse IgG (ZSGB-BIO, Beijing, China) diluted in TBST (1:3,000) for 1 h at room temperature. After washing the membranes, the protein bands were detected by using the SuperSignal West Pico kit (Thermo Scientific, USA). The experiment was carried out thrice in triplicate.

**Statistical analysis**

The statistical analysis was performed using the statistical package for the social sciences (SPSS) version 19.0. The results were presented as the mean-standard deviation (SD) unless otherwise indicated. Firstly, we performed a one-sample Kolmogorov–Smirnov test to determine if the variables could be described as normal or non-normal distributions, and then the Fisher’s exact test was used to describe the categorical variables. Then, we performed a repeated-measures analysis of variance (ANOVA) to analyze the serum evaluation data of patients with RA before and after treatment. Furthermore, to describe the difference between the treatment groups, the ANOVA was used for the independent normally distributed variables while the Kruskal-Wallis test was used for the non-normal ones. Then, the data of the FLS experiments were assessed to determine the difference between the groups using an ANOVA while p-values<0.05 were considered significant.

**Results**

**Serum sRANKL and OPG levels before and after iguratimod and MTX treatment**

The ELISA was performed to evaluate the serum levels of sRANKL and OPG in patients with RA before and after treatment with iguratimod, MTX, or a combination of both. The results revealed that the serum sRANKL decreased significantly after treatment. Compared with the baseline, the sRANKL levels decreased significantly 12 or 24 weeks after treatment regardless of the therapy (respectively, p<0.01), but only treatment with both iguratimod and MTX showed a significant reduction in serum sRANKL at 24 weeks compared with values at 12 weeks (p<0.01, Figure 1a). However, OPG was insensitive to treatment, and all groups showed similar values before and after treatment (all p>0.05, Figure 1b). Similar results to those obtained with sRANKL were observed when we analyzed the ratio of sRANKL/OPG (respectively, p=0.01, Figure 1c).

To further determine whether there were significant differences between the three groups, we analyzed the ratio of sRANKL, OPG, and sRANKL/OPG at 12 and 24 weeks after treatment to baseline values (ratio of 12 weeks/baseline and 24 weeks/baseline for sRANKL, OPG, and sRANKL/OPG, Figure 1d and 1e). We found no significant difference 12 weeks after treatment between the groups (respectively, p>0.05); however, the 24 weeks/baseline ratio of sRANKL differed significantly between group B and C (p<0.05) while that of sRANKL/OPG differed significantly between group A and C, and group B and C (p<0.01).

**Effects of iguratimod and MTX on IL-1β-induced RANKL and OPG expression in FLS**

**Effects of iguratimod and MTX on the expression of RANKL and OPG mRNA in IL-1β-induced FLS**

The qPCR was performed to evaluate the mRNA expression of RANKL and OPG in 48-h IL-1β-stimulated FLS from patients with RA. The expression of both RANKL and OPG mRNA increased after stimulation while treatment with iguratimod and MTX alone or in combination had no significant effects on OPG mRNA
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expression (Figure 2a). The MTX, which was the positive control, reduced RANKL mRNA expression and iguratimod inhibited the effects of the IL-1β-induced expression of RANKL mRNA (respectively, \( p<0.01 \)). The effects of iguratimod were similar to those of MTX and tended to increase along with the concentration. In addition, the combination of the both medicines showed a higher reduction in RANKL mRNA expression than either of the drugs did alone (respectively, \( p<0.05 \), Figure 2b). Furthermore, we observed that the synovial RANKL mRNA/OPG mRNA ratio was also decreased after treatment regardless of the therapy, and the effects of the combination treatment were stronger than that of MTX only \( (p<0.05) \). However, there was no significant difference between the iguratimod and combination treatments (Figure 2c).

**Effects of iguratimod and MTX on the expression of OPG in the culture supernatants of IL-1β-induced FLS**

We performed an ELISA to evaluate the OPG levels in the cell culture supernatants, and the results revealed they were increased following IL-1β stimulation of the FLS from patients with RA for 48 h \( (p<0.01) \). Furthermore, iguratimod or MTX treatment both slightly decreased the level, but the results were not significant \( (p>0.05) \). Unfortunately, we were unable to accurately measure the RANKL levels in the supernatants.
Effects of iguratimod and MTX on the expression of RANKL and OPG protein in IL-1β-induced FLS

To further evaluate the expression of RANKL and OPG protein, we performed western blot analysis in FLS from patients with RA. There was an increase in intracellular OPG expression following stimulation with IL-1β for 48 h (p<0.01). In addition, iguratimod and MTX tended to further induce the OPG expression, but the results were not significant (p>0.05, Figure 4a, and 4b, respectively). Furthermore, both MTX and iguratimod (10 and 20 μg/mL) inhibited the increase in RANKL induced by IL-1β (p<0.05); however, at 5 μg/mL, iguratimod tended to suppress RANKL expression, but there was no significant difference. Moreover, the effects of iguratimod were concentration-dependent, and the 20 μg/mL concentration showed significantly greater suppression than 5 μg/mL and 10 μg/mL did (respectively, p<0.01 and p<0.05). In addition, the combination treatment with both iguratimod and MTX was more efficient in suppressing RANKL expression than either drug was alone (p<0.05, Figure 4c and 4d), which was consistent with RANKL mRNA expression. Then, we analyzed the ratio of RANKL/OPG protein and obtained similar results to those obtained with RANKL (Figure 4e).

Discussion

We sought to investigate the effects of iguratimod and MTX on the RANKL/RANK/OPG system, and elucidated the potential underlying mechanism of the iguratimod-mediated bone protection, to further support and modulate its use in clinical practice. To achieve this, we investigated the effects of iguratimod, alone or combined with MTX, on serum RANKL and OPG levels in patients with RA, and the IL-1β-induced expression of these proteins in FLS from patients with RA. Our results greatly contributed to clarifying the mechanism of the bone protectant effects of iguratimod.

The RANKL/RANK/OPG system is one of the most important regulators of bone metabolism. RANKL is produced by osteoblasts, FLS, T cells, and other cells in the form of a membrane-bound or soluble protein (4, 14). It induces osteoclast activation and osteoclast survival, and its expression can be increased by cytokines including tumor necrosis factor (TNF)-α, IL-6, IL-17, and IL-1β (5). OPG is a secretory protein that is produced by osteoblasts, stromal cells, and other cells, and it binds with RANKL to interrupt the RANKL/RANK interaction, thereby blocking RANKL-mediated responses such as preosteoclast recruitment, osteoclast activation, and osteoclast survival (4-6). The balance of RANKL and OPG regulates bone metabolism. Previous studies showed that serum sRANKL levels and sRANKL/OPG were significantly higher in patients with RA than in healthy controls and decreased after treatment with DMARDs or biological agents and, therefore, sRANKL/OPG was suggested as a marker of bone damage in patients with RA (7, 15-17). Igratimod is a small molecule that has proven efficacious in the clinical treatment of patients with RA. Furthermore, clinical trials show that compared with the placebo group, the ACR response rate is significantly higher in iguratimod-treated groups, and it significantly reduces the blood concentrations of CRP, RF, IgG, IgM, and IgA with an efficacy that is comparable to that of salazosulfapyridine (18-22). In addition, iguratimod decreases the serum levels of proinflammatory cytokines and sup-
presses matrix metalloproteinases (MMP) production in cultured human synovial cells (23, 24).

To the best of our knowledge, our study is the first to show that treatment with iguratimod, MTX, or a combination of both significantly decreased the serum sRANKL level and the ratio of sRANKL/OPG. Although there was no significant difference between the groups 12 weeks posttreatment, the combination therapy showed a greater effect on sRANKL/OPG ratio than single drug treatment did 24 weeks posttreatment. In addition, only the combination therapy showed a significant reduction in serum sRANKL and the ratio of sRANKL/OPG at 24 weeks compared with values at 12 weeks. This observation indicated that iguratimod or MTX alone likely achieved their ceiling effects while the combination did not and, therefore, exhibited stronger effects. This indicates that iguratimod likely regulated the RANKL/RANK/OPG system, and was even more efficient when used in combination with MTX.

Then, we evaluated the effects of iguratimod, MTX, or their combination on FLS from patients with RA, which are target cells for iguratimod. A previous study reported that IL-6/sIL-6r or IL-1β could induce RANKL expression in vitro while TNF-α could not (25). We chose IL-1β (10 ng/mL) rather than TNF-α to induce RANKL expression in FLS while MTX (100 nM) was used as the positive control because it has been shown to suppress RANKL expression and RANKL/OPG (26). We chose concentrations of iguratimod (5, 10, and 20 μg/mL) that have been proven to efficiently inhibit inflammation without affecting the growth of FLS (13, 24). We used qPCR, ELISA, and western blot analyses to investigate mRNA and protein expressions of RANKL and OPG, and all 3 methods yielded comparable results. As expected, the expressions of RANKL and OPG were significantly induced by IL-1β while the RANKL/OPG ratio was increased. Iguratimod inhibited the expression of RANKL but not OPG, which decreased the RANKL/OPG ratio and this was consistent with the results of a previous report (13). Moreover, the RANKL suppression effect of iguratimod was concentration-dependent. Furthermore, we demonstrated for the first time that the combination of iguratimod and MTX suppressed RANKL and reduced the RANKL/OPG ratio more significantly than either drug did alone.

In summary, we demonstrated that iguratimod and MTX reduced the serum sRANKL levels and sRANKL/OPG ratio, as well as inhibited the RANKL expression and RANKL/OPG in FLS from patients with RA. This implies that iguratimod may potentially protect against bone destruction and, therefore, may prevent joint deformities and ankylosis through regulating the RANKL/RANK/OPG system. Finally, further studies are required to fully elucidate the signal pathways that mediate these effects.

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