Honokiol possesses potential anti-inflammatory effects on rheumatoid arthritis and GM-CSF can be a target for its treatment

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Abstract: Objective: To observe the anti-inflammatory effects of honokiol in primary cultures of peripheral blood mononuclear cells of rheumatoid arthritis patients, the pro-inflammatory cytokines and potential targets were investigated. Methods: The levels of GM-CSF, IL-1β, TNF-α and IL-8 were determined by ELISA assay. The genes and proteins expression were analyzed by real-time PCR and Western blotting respectively. Results: The serum IL-1β, TNF-α and GM-CSF levels were 1.76-, 2.16- and 3.57-fold increased in patients with RA as compared to those of control group. Honokiol inhibited the expression levels of IL-1β, TNF-α, GM-CSF and IL-8 in PBMCs with a dose-dependent manner. Measurements obtained from supernatants were positively correlated between TNF-α and IL-1β, moreover, similar results found TNF-α levels positively correlated with GM-CSF and IL-8 activity in the supernatants of PBMCs isolated from RA patients. Furthermore, the mRNA and protein expression of IL-1β, GM-CSF and IL-8 were up-regulated when the PBMCs exposure to TNF-α, however, honokiol treatment significantly reversed the expression of IL-1β, TNF-α and GM-CSF in response to TNF-α with a dose-dependent manner. Conclusions: This study demonstrates that honokiol could possess potential anti-inflammatory effects and inhibits TNF-α-induced IL-1β, GM-CSF and IL-8 production in PBMCs from rheumatoid arthritis patients.

Keywords: Rheumatoid arthritis, TNF-α, IL-1β, GM-CSF, IL-8

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease with chronic inflammatory of peripheral joints and abnormal immune responses, about 1% of the world population who have suffered from this progressive joint destruction disease [1, 2]. The pathogenesis of RA is thought that the interaction of environmental factors, genetic and immunoinflammatory responses contributes to the progression of RA [3]. In addition to the deposition of immune complexes (IC) of autoantigens and their antibodies, however, the interaction between rheumatoid factor and pro-inflammatory cytokines play a vital roles in the pathophysiology of RA [4]. Active pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) or IL-6 are involved in the synovial inflammation of RA [5, 6]. Macrophages are the major cells involved in the pathogenesis of inflammatory arthritis. These cells are abundant in the inflamed synovial tissue and their number in the synovial sublining layer is correlated with disease activity and response to treatment [7, 8]. RA macrophages directly contribute to the degradation of articular cartilage and subchondral bone [9]. Furthermore, the activation of RA monocyte-macrophages is not locally restricted at the synovial level, but involves also those of the peripheral circulation [10, 11].

Previous studies have shown that macrophage colony-stimulating factor (M-CSF) or granulocyte-macrophage colony-stimulating factor (GM-CSF), main factors of monocytes/macrophages survival, are expressed in the synovial fluid and membrane of RA patients [12, 13]. In vivo, M-CSF loss-of function is protected against antigen induced arthritis [10]. Fur-
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In the recent studies, GM-CSF has been shown to exacerbate arthritic disease in animals, meanwhile, lacking functional GM-CSF is protected from collagen-induced arthritis in mice [11, 12]. Intriguingly, synovial CD4+ T-cell-derived GM-CSF supports the differentiation of an inflammatory dendritic cell population in RA [14]. These studies demonstrate that M-CSF and GM-CSF can exacerbate the inflammatory response and support the role of monocytes/macrophages in the pathogenesis of inflammatory arthritis. However, the pro-inflammatory and immunomodulatory effects of GM-CSF appear to depend on the dose and the presence of other relevant cytokines in the context of an immune response [15]. A recent study reveals that pro-inflammatory cytokines (IL-1β and TNF-α) promote monocyte viability via GM-CSF [16]. The results suggest that there could be interaction between GM-CSF and IL-1β, as well as TNF-α.

Honokiol, a small molecular weight natural product isolated and purified from the Magnolia officinalis, has been shown to possess potent anti-oxidation [17] and anti-inflammatory [18]. Functional studies reveal that honokiol can prevent cartilage matrix degradation in human osteoarthritis chondrocytes [19] and inhibit the progression of collagen-induced arthritis [20]. Further research shows that honokiol as an effective inhibitor can prevent tumor necrosis factor-α-induced up-regulation of inflammatory cytokine and chemokine production in human synovial fibroblasts [21]. There has been no study on the effect of honokiol on human peripheral blood mononuclear cells-derived macrophages, major cells involved in the pathogenesis of inflammatory RA. Therefore, we explored the effects of honokiol in primary cultures of human monocyte-derived macrophages, isolated from whole blood of patients with RA, in terms of proinflammatory cytokines (IL-1β, IL-8 and TNF-α).

Materials and methods

Serum samples and cell culture

Human blood samples were obtained with written informed consent from Department of Immunology and Rheumatology, Affiliated Hospital of Weifang Medical University. The study was approved by the Ethics Committee of the Department of Immunology and Rheumatology, Affiliated Hospital of Weifang Medical University. 14 serum samples of RA patients and 13 cases of healthy control were collected between May 2014 and Jan. 2015.

The peripheral blood mononuclear cells (PBMCs) were separated from erythrocytes by density centrifugation in the blood samples of RA patients and healthy control, and maintained in RPMI-1640 (Invitrogen, USA) supplemented with 10% FBS (Invitrogen, USA) at 37°C in a humidified incubator (Thermo, USA), 5% CO₂, 95% air atmosphere. The medium was replenished two days.

Quantitative real-time PCR

The PBMCs RNA extraction was performed according to the Trizol manufacturer’s protocol (Invitrogen, Carlsbad, CA, USA). Synthesis of cDNAs was performed by reverse transcription reactions with 1 μg of total RNA using moloney murine leukemia virus reverse transcriptase (Promega, Switzerland) with oligo dT (15) primers (Fermentas) as described by the manufacturer. Real-time PCR was carried out with the Applied Biosystems 7300 Real-Time PCR System. A total of 20 μL reactions were prepared with the TaqMan Universal PCR Master Mix. The Ct (cycle threshold fluorescence values) was automatically given by SDS 2.1 software (Applied Biosystems), and the relative expression levels of VEGF mRNA were calculated using the 2ΔΔCt method. GAPDH as an internal control was used to normalize the data to determine the relative expression of the target genes. PCR with the following primers: IL-1β, Forward 5’-ATGCCATTGTGTAATGTCCT-3’ and Reverse 5’-GTCCGGTCCACAGTCGG-3’; GM-CSF, Forward 5’-ATGCGAATCTGTGCTGTGCT-3’ and Reverse 5’-CTGCCGTCCCTAGCTGTGGG-3’; IL-8, Forward 5’-CTGCGATCATGTGCATGTGCT-3’ and Reverse 5’-GTCCGAGTCCTCATTGGG-3’; GAPDH, Forward 5’-ACAGGGAGGTGATAGCATT-3’ and Reverse 5’-GACCAAAGCCCTTCATACTTC-3’.

Western blotting

The PBMCs were homogenized and extracted in NP-40 buffer, followed by 5-10 min boiling and centrifugation to obtain the supernatant. Samples containing 50 μg of protein were separated on 10% SDS-PAGE gel, transferred to PVDF Transfer Membrane (Millipore). After sat-
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Table 1. Physiological and biochemical parameters of patients with rheumatoid arthritis and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>Age (Years)</th>
<th>Sex ratio (M/F)</th>
<th>CRP (mg/L)</th>
<th>ESR (mm/h)</th>
<th>IL-1β (pg/mL)</th>
<th>TNF-α (pg/mL)</th>
<th>GM-CSF (pg/mL)</th>
</tr>
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<tbody>
<tr>
<td>Control (n = 13)</td>
<td>56 ± 14</td>
<td>1.06</td>
<td>3.7 ± 1.4</td>
<td>12.8 ± 4.6</td>
<td>325 ± 30</td>
<td>113 ± 27</td>
<td>35 ± 9.4</td>
</tr>
<tr>
<td>RA (n = 14)</td>
<td>58 ± 13</td>
<td>0.53</td>
<td>14.8 ± 9.2</td>
<td>27.4 ± 14.2</td>
<td>572 ± 126</td>
<td>244 ± 56</td>
<td>125 ± 30.7</td>
</tr>
<tr>
<td><em>P value</em></td>
<td></td>
<td></td>
<td>0.49</td>
<td>0.27</td>
<td>0.001</td>
<td>0.001</td>
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CRP, C-reactive protein; ESR, Erythrocyte sedimentation rate; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; GM-CSF, granulocyte-macrophage colony-stimulating factor.

Figure 1. Effect of honokiol on pro-inflammatory cytokines production in PBMCs. PBMCs were treated with honokiol (0, 5, 10 or 20 μM) for 48 h, the levels of TNF-α (A), IL-1β (B), GM-CSF (C) and IL-8 (D) were determined by ELISA assay. Values are expressed as mean ± SD, n = 5 in each group. *P < 0.05, **P < 0.01, ***P < 0.001 versus control group.

Statistical analysis

The data from these experiments were reported as mean ± standard errors of mean (SEM) for each group. All statistical analyses were performed by using PRISM version 5.0 (GraphPad). Inter-group differences were analyzed by one-way ANOVA, and followed by Tukey's multiple comparison test as a post test.
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Physiological and biochemical parameters of patients with rheumatoid arthritis and healthy controls

To investigate the biochemical parameters of inflammation, we results shown that CRP and ESR were significantly higher in patients with RA than that of the healthy control (Table 1). The serum IL-1β, TNF-α and GM-CSF levels were 1.76-, 2.16- and 3.57-fold increased in patients with RA as compared to those of control group (Table 1).

Effect of honokiol on pro-inflammatory cytokines production in PBMCs

IL-1β, TNF-α, GM-CSF and IL-8 levels were detected in supernatants of the pathological cells after treatment with honokiol or untreated cells (controls). As shown in Figure 1, honokiol inhibited the expression levels of IL-1β, TNF-α, GM-CSF and IL-8 in PBMCs with a dose-dependent manner (Figure 1A-D). These observations suggested that IL-1β, TNF-α, GM-CSF and IL-8 plays an important role in the pathological courses of RA, and honokiol played an anti-inflammatory role and might have beneficial effects in preventing and treating RA.

TNF-α levels positively correlated with IL-1β, GM-CSF and IL-8 activity in PBMCs isolated from RA patients

To test whether there was a relationship between TNF-α and IL-1β levels, as well as GM-CSF and IL-8, the levels of TNF-α, IL-1β level, GM-CSF and IL-8 were measured in the cell culture supernatants by ELISA assay. As shown in Figure 2, measurements obtained from supernatants were positively correlated between TNF-α and IL-1β (r = 0.823, Figure 2A). Moreover, similar results found TNF-α levels positively correlated with GM-CSF and IL-8 activity in the supernatants of PBMCs isolated from RA patients (Figure 2B and 2C).

Honokiol inhibits TNF-α-induced IL-1β, GM-CSF and IL-8 production

IL-1β and IL-8 are critical proinflammatory cytokines in pathogenesis of RA. To explore the effect of TNF-α on PBMCs isolated from RA.

Figure 2. TNF-α levels positively correlated with IL-1β (A), GM-CSF (B) and IL-8 (C) activity in PBMCs isolated from RA patients. Values are expressed as mean ± SD, n = 14 in each group.
patients, the expression of IL-1β, GM-CSF and IL-8 were evaluated after exposure to TNF-α for 24 h. In our study, we shown that the mRNA and protein expression of IL-1β increased when the PBMCs exposed to TNF-α (Figure 3A-C). However, honokiol treatment significantly reversed the expression of IL-1β in response to TNF-α with a dose-dependent manner. Moreover, similar results found that honokiol could inhibit the mRNA and protein expression of IL-8, however, the inhibitory effect of honokiol on IL-8 was independent of the drug concentration when the PBMCs exposed to TNF-α (Figure 4A-C). Furthermore, the mRNA and pro-

Figure 3. Honokiol inhibited TNF-α-induced IL-1β production. mRNA (A) and protein (B) expression were measured by real-time PCR and western blotting respectively. Densitometric quantification for western blotting (C). Values are expressed as mean ± SD, n = 4 in each group. *P < 0.05 versus control group; #P < 0.05, ##P < 0.01 versus TNF-α-treated group.

Figure 4. Honokiol inhibited TNF-α-induced IL-8 production. mRNA (A) and protein (B) expression were measured by real-time PCR and western blotting respectively. Densitometric quantification for western blotting (C). Values are expressed as mean ± SD, n = 4 in each group. *P < 0.05 versus control group; #P < 0.05 versus TNF-α-treated group.
tein expression of GM-CSF significantly increased when the PBMCs exposed to TNF-α. Intriguingly, honokiol treatment significantly reversed the expression of GM-CSF in response to TNF-α with a dose-dependent manner (Figure 5A-C).

Discussion

Honokiol has been demonstrated to possess anti-inflammatory activities in rat aortic smooth muscle cells [22] and murine macrophages [23]. In human synovial fibroblasts, TNF-α-induced expression of these inflammatory factors, such as monocyte chemotactic protein-1 (MCP-1) and macrophage inflammatory protein-1α (MIP-1α), is inhibited in a dose-dependent manner by pre-treatment with honokiol [21]. However, the role of honokiol in RA and its contribution to resistance against inflammation has so far remained unclear. In this study, the anti-inflammatory effect of honokiol and its underlying mechanisms in PBMCs treated with TNF-α were explored.

Our results indicated that serum IL-1β, TNF-α and GM-CSF levels were increased in RA patients compared to HC. We observed a positive correlation between TNF-α levels and IL-1β, as well as GM-CSF and IL-8, in PBMCs of RA patients. Moreover, the mRNA and protein expression of IL-1β, GM-CSF and IL-8 were up-regulated when the PBMCs exposure to TNF-α, however, honokiol treatment significantly reversed the expression of IL-1β, TNF-α and GM-CSF in response to TNF-α with a dose-dependent manner. Altogether, our results indicated that active pro-inflammatory cytokines TNF-α, IL-1β, GM-CSF and IL-8 played an important role in the progression of RA, and honokiol could possess potential anti-inflammatory effects and inhibited TNF-α-induced IL-1β, GM-CSF and IL-8 production in PBMCs from rheumatoid arthritis patients. Anti-inflammatory drugs are a vital element of RA therapeutic strategies. Conventional anti-inflammatory drugs, such as methotrexate, leflunomide and glucocorticoids, show significantly more side effects in the treatment of RA, which include serious infections, expensive and inducible malignant tumors [2, 24]. However, more effective, safe and affordable drugs are needed to be exploited to the treatment of RA. Many natural compounds exhibit anti-inflammatory properties and have the potential for treating inflammatory disorders. In human rheumatoid arthritis fibroblast-like synoviocytes, resveratrol inhibits TNF-α-induced IL-1β, MMP-3 production via modulation of PI3 kinase/Akt pathway [2]. Moreover, genkwa flos flavonoids have an antioxidant effects on Freund’s adjuvant-induced rheumatoid arthritis in rats [25]. Moreover, vitamin D down-regulates proinflam-
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Inflammatory mediators in monocyte-derived macrophages, and RA cells appear more sensitive than normal cells [11]. In our study, we found that honokiol was a potential inhibitor of TNF-α-induced expression of inflammatory factors in PBMCs, which held promise as a potential anti-inflammatory drug.

In this study, we identified that GM-CSF as one of the growth factor involved in TNF-α-induced inflammatory effect in PBMCs from RA patients. Interestingly, this effect is observed using conditioned media from TNF-α and IL-1β pre-stimulated synovial fibroblasts form RA patients [16]. These results were in line with previous studies that show the importance of TNF-α in the regulation of growth factor expression in PBMCs, and it confirmed that GM-CSF could be an interesting target in the treatment of RA.

In conclusion, the preliminary data presented in this report demonstrated for the anti-inflammatory activity of honokiol under these experimental conditions and pointed out its inhibitor potential in peripheral RA PBMCs mediators, including the proinflammatory cytokines and growth factor.

Disclosure of conflict of interest

None.

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References

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[17] Zhao C and Liu ZQ. Comparison of antioxidant abilities of magnolol and honokiol to scavenge radicals and to protect DNA. Biochimie 2011; 93: 1755-1760.


