Inhibitory Effect of Hwalhyul Composition on Inflammatory Responses in Rheumatoid Arthritis

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This study was performed to examine whether HC is effective in controlling molecular components known to be involved in RA, FLS were used to determine possible regulatory effects of HC on levels of inflammatory cytokines. Major findings are summarized as follows. TNF-α mRNA expression levels in FLS cells which had been repressed by 10, 100 ㎍/㎖ of HC treatment. IL-6 mRNA expression levels in FLS cells which had been repressed by 10, 100 ㎍/㎖ of HC treatment. IL-8 mRNA expression levels in FLS cells which had been repressed by 10, 100 ㎍/㎖ of HC treatment. IL-1β mRNA expression levels in FLS cells which had been repressed by 10, 100 ㎍/㎖ of HC treatment. The present data suggest that FLS which has been activated by IL-1 and TNF-α co-treatment decreased production of inflammatory cytokines then, HC treatment by repressed the production of these molecules.

Key words : Hwalhyul composition, Rheumatoid arthritis

Introduction

Rheumatoid arthritis(RA) is a systemic inflammatory disorder that mainly affects the diarthroidal joint attacking the joints[1]. This disease can occur at any age, but common at ages of 40-70 years, with a female to male ratio of 2.5 to 1[2]. The clinical features include symptoms of joint swelling, pain, weakness, fever, weight loss, articular abnormalities. Also, the formation of locally invasive synovial tissue, pannus is a characteristic feature of RA and involved in the joint erosions seen in RA[3].

In the oriental medicine, clinical treatment for RA involves the use of reagents eliminating Han(寒: means cold) and Sup(濕: means humid) factors and facilitating blood circulation. Acupuncture and herbal medication have also been frequently used together for RA therapy[4].

Here in the present study, a herbal prescription Hwalhyulbokhapbang (活血複合方 : every abbreviation from now on HC) was examined whether it has any attenuating effects on RA. Determination of levels of inflammatory cytokines and gene expression in RA showed significant changes by HC administration.

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Materials and Methods

1. Materials

1) Animals

DBA/1J mice (6 weeks old) obtained from Charles River (Japan), were used in the present study. The animals were maintained in a conventional environment at 12 hr of day light (200~300 Lux) and 12 hr of dark condition at 22±2℃. The animals were supplied with food pellets and water and adjusted for at least 2 weeks before the experiment.

2) Drugs

The HC used in this study was purchased from Daejeon University oriental medicine hospital and the composition of a pack is as follows.

Table 1. Prescription of Hwalhyulbokhapbang (HC)

Scientific name	Amount(g)
Spatholobi Caulis (Gyehyuldeung)	8
Paeonia Radix Rubra (Jeokjakyak)	6
Corydalidis Tuber (Hyunhosaek)	6
Siegesbeckiae Herba (Heuichum)	8
Total amount	28

2. Methods

1) HC extraction

Four packs of dried HC were dissolved in 2 liter of distilled water, extracted for 3 hr. After filtration using the rotary evaporator, the purified powder 13.2 g was obtained by using the freeze dryer, and kept at -84℃ until use. The powder

obtained was diluted with proper concentration before use.

2) Human fibroblast cells(hFCs) culture

The human skin tissues were dissected, washed three times with cold PBS and cut into small pieces. The tissues in 15 mℓ conical tube were centrifuged at 1,400 rpm for 5 min. After adding DMEM containing collagenase A, DNase type Ⅰ, and antibiotics, the dissociated tissue was incubated at 37℃ for 2 hr, and further digested for 30 min in the presence of 0.5% trypsin-0.2% EDTA. Then, the tissue was washed twice with cold PBS by centrufugation at 1,500 rpm, resuspended in DMEM-10% FBS, and cultured for 7 days. The cells were detached from the plate by 0.5% trypsin-0.2% EDTA treatment, and subcultured in DMEM-5% FBS medium for three weeks by transferring every week.

3) Primary culture of fibroblast-like synoviocytes (FLS)

Knee joint tissues surgically dissected out from rheumatoid patients (female, 65 years old) were washed with cold phosphate buffered saline (PBS) and minced into small pieces. The tissues were maintained in serum-free DMEM to remove fat materials and supernatant. Dispase were treated to 10 ㎎ of joint tissue and incubated in joiik's MEM at 37 ℃ for 30 min. The supernatant was transferred to a new tube containing DMEM with 5% fetal bovine serum. Cell suspension was obtained by repeated treatment with dispase three more times. Collected supernatant was contrifugated at 1,200 rpm for 10 min and washed twice with DMEM with 10% FBS. After cell counting, monocytes were isolated by centrifugation under ficoll-hypaque solution and cells adhered to the substrate were cultured in DMEM-15% FBS medium with 5 transfers a week.

4) Stereoscopic microscopy

For stereoscopic microscopy, cell were cultured for 48 hr onto a LAB-Tek tissue culture chamber slide bearing 2 divisions and fixed by the solution containing 2.5% glutaraldehyde, 0.12 M sodium cacodylate buffer (pH 7.3) at 4oC for 30 min. Fixed cells were observed at stereoscopic microscope at × 1000 magnification

5) Real time quantitative RT-PCR

(1) RNA extraction

Cells isolated from the knee joint tissue were plated in 24 well plate with a concentration of 1×10^6 cell per well. One hour after the treatment of HC extract (100 ㎍/mℓ, 10 ㎍/mℓ) or MTX (10 ㎍/mℓ) for 1 hr, hrs IL-1β (10 U/mℓ) and hrs TNF-α (10 ng/mℓ) were added to the cells. Cells were harvested 6 hr later by centrifugation at 2,000 rpm for 5 min. Supernatant was removed and cells were used for RNA isolation using a RNAzolB protocol. Cells were treated with RNAzolB (500 ㎕) to lyse cells and mixed with 50 ㎕ of chloroform ($CHCl_3$) for 15 min. Cell were placed on ice for 15 min, and centrigated at 13,000 rpm. Then, the upper later was taken and mixed with 200 ㎕ of 2-propanol gently on ice for 15 min. After phase separation by centrifugation, the pellets were washed with 80% ethanol. Total RNA exacted were resuspended in 20 ㎕ of diethyl pyrocarbonate (DEPC) water and heat-treated at 75℃ before the use of the first stranded cDNA synthesis.

(2) RT-PCR reaction

Total RNA (3 ㎍) was denatured for 10 min at 75℃. Denatured RNA was mixed with 2.5 ㎕ 10 mM dNPTs mix, 1 ㎕ random sequence hexanucleotides (25 pmole/25 ㎕), 1 ㎕ RNasin (20 U/㎕), 1 ㎕ 100 mM DTT, 4.5 ㎕ 5 × RT buffer (250 mM Tris-Cl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$), 1 ㎕ M-MLV RT (200 U/㎕) and H_2O to make the reaction volume to 20 ㎕. The sample was mixed well and incubated for 60 min for the synthesis of first stranded cDNA and the reaction was stopped by placing for 5 min at 95℃. Synthesized cDNA was then used for PCR.

(3) Real time quantitative RT-PCR

Real time quantitative PCR was carried out by using Applied Biosystems instrument 7,500 Fast Real-Time PCR system. SYBR Green PCR master mix (ABI) was used for measurement of inflammatory cytokine expression, GAPDH was used as an internal control, and primer concentration used was 200 nM. Real-time PCR was performed at the following condition: pre-denaturation for 2 min at 50 ℃, 10 min 94 ℃, 40 cycles, then stop reaction for 0.15 min at 95 ℃ followed by 1 min at 60 ℃.

6) Data analysis

The results are presented as the mean±standard error mean(SEM). Statistical analysis was performed by Student's T-test ($p<0.05$).

Results

1. Effects on TNF-α mRNA expression

Relative levels of TNF-α mRNA were 0.1201 in cells with no rIL-1/TNF-α treatment. In cells with MTX treatment, mRNA level was decreased to 0.574 compared with control group (CT). HC treatment inhibited TNF-α mRNA levels in a dose-dependent manner (Fig. 1).

2. Effects on IL-6 mRNA expression

Relative levels of IL-6 mRNA in wild type (WT) to the rIL-1/TNF-α-treated control were 0.115. In cells with MTX treatment, mRNA level was decreased to 0.415 compared to the control group (CT), HC treatment decreased IL-6 mRNA levels in a dose-dependent manner (Fig. 2).

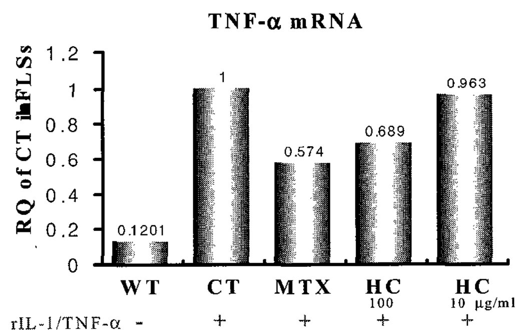

Fig. 1. Inhibitory effects of HC on TNF-α mRNA expression determined by quantitative real-time PCR in human fibroblast-like synoviocytes.

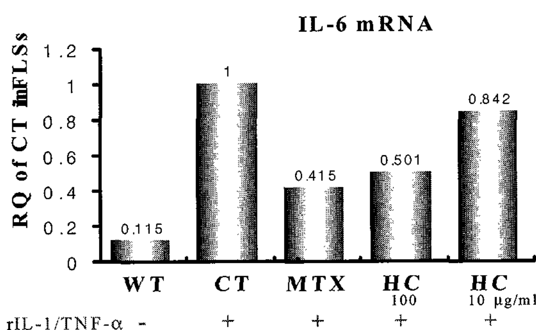

Fig. 2. Inhibitory effects of HC on IL-6 mRNA quantitative real-time PCR in human fibroblast-like synoviocytes.

3. Effect on IL-8 mRNA expression

Relative levels of IL-8 mRNA in cells with no rIL-1/TNF-α were 0.187 relative to rIL-1/TNF-α treated control (CT) group. In cells with MTX treatment in the presence of rIL-1/TNF-α, mRNA level was increased to 0.347. HC treatment decreased IL-8 mRNA levels to 0.461 and 0.429 with 10 μg/mℓ and 100 μg/mℓ of HC respectively(Fig. 3).

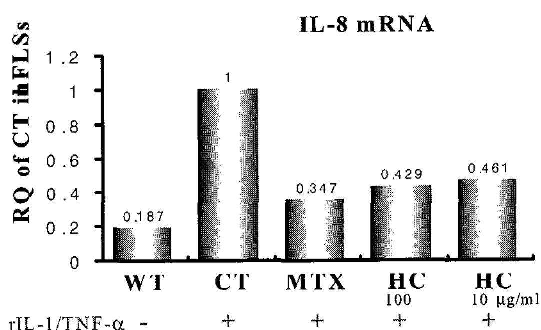

Fig. 3. Inhibitory effects of HC on IL-8 mRNA quantitative real-time PCR in human fibroblast-like synoviocytes.

4. Effects on IL-1β mRNA expression

Relative levels of IL-1β mRNA compared to the control (CT) were 0.223 in cells with no rIL-1/TNF-α. mRNA levels were 0.117 in cells treated with MTX and were 0.684 and 0.898 with 100 and 10 μg/mℓ of HC respectively (Fig. 4).

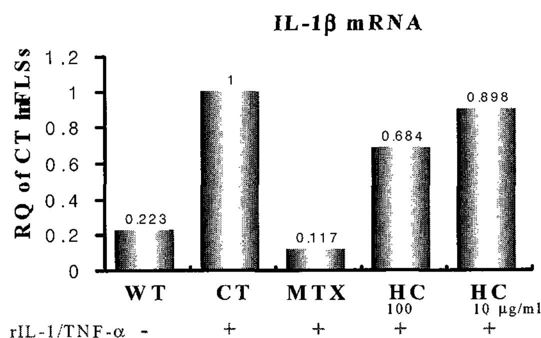

Fig. 4. Inhibitory effects of HC on IL-1β mRNA quantitative real-time PCR in human fibroblast-like synoviocytes.

Discussion

Pathophysiological studies of inflammatory synovitis in RA revealed overall pathogenesis and related molecular factors[5,6]. Results of work in animals have suggested a central role for TNF-α and IL-1β in the process of synovitis and joint destruction. TNF-α and IL-1β seem to function synergistically in inducing effector function, and are both present in large quantities in affected synovial fluid and synovial tissue[7,8].

Besides IL-1 and TNF-α, other IL family proteins are functionally involved in inflammatory responses in RA. For instance, a potential role of IL-6 in RA was demonstrated by treating antibody of IL-6 receptor[9].

In the present study, we investigated possible effects of HC on regulation of RA in cultured human FLS, known to show produce several inflammatory cytokines in responses to external, stressful inflammatory stimulation[10,11]. We first identified that HC treatment in a concentration range of 1 - 250 μg/mℓ did not have any cytotoxic effects on cultured cells. Then, the stimulation of cells with combined treatment of IL-1 and TGF induced several inflammatory cytokines. Our data on the induction levels of IL-1 and TNF-α by real time RT-PCR showed large increases in FLS and then strong inhibition by MTX treatment. MTX was originally developed as a folate antagonist for the treatment of cancer, and has been used in the therapy of rheumatoid arthritis, although its mechanism of action in the therapy of rheumatoid arthritis remains less clear[12,13]. In our study, MTX treatment to IL-1/TNF-α stimulated cells efficiently decreased IL-1 and TNF, and thus used as a control monitoring the RA effect.

HC extract is a mixed prescription composing of 4 different herbal drugs. We found that HC treatment in FLS activated by TNF/IL-1 decreased levels of inflammatory cytokines TNF-α, IL-6, and IL-8. As mentioned above, these

cytokines are strongly induced in activated FLS, and thus it is presumed that HC may attenuate inflammatory responses in RA tissues[14,15].

The present data showed that HC treatment regulated several species of mRNA expression in activated FLS cells. In most of cases, induction of mRNA expression involves transcriptional activation of target gene by recruiting transcription factors. Since HC treatment induced multiple mRNA expression, several different kinds of transcription factors might be activated to induce target gene expression. Another possibility is the regulation at the post-transcriptional levels by increasing the stability of pre-existing mRNA.

The present study showed that the treatment of HC extract decreased the production of several inflammatory cytokines which were upregulated in human FLS cells by IL-1 β and TNF-α co-treatment. the present data implicate that HC may be clinically relevant for the treatment of RA by modulating molecular factors.

Conclusion

To examine whether HC is effective in controlling molecular components known to be involved in RA, FLS were used to determine possible regulatory effects of HC on levels of inflammatory cytokines. Major findings are summarized as follows. TNF-α mRNA expression levels in FLS cells which had been repressed by 10, 100 ㎍/㎖ of HC treatment. IL-6 mRNA expression levels in FLS cells which had been repressed by 10, 100 ㎍/㎖ of HC treatment. IL-8 mRNA expression levels in FLS cells which had been repressed by 10, 100 ㎍/㎖ of HC treatment. IL-1β mRNA expression levels in FLS cells which had been repressed by 10, 100 ㎍/㎖ of HC treatment. The present data suggest that FLS which has been activated by IL-1 and TNF-α co-treatment decreased production of inflammatory cytokines then, HC treatment by repressed the production of these molecules.

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