Inhibitory Effects of *Gamimahaenggamsuk-tang* on RA-related Inflammatory Responses in Cultured Fibroblast-like Synoviocytes

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Gamimahaenggamsuk-tang (GMHGST) is used for treatment of inflammatory diseases including rheumatoid arthritis (RA). Here, regulatory activity of GMHGST on RA-mediated inflammatory responses was investigated in cultured human fibroblast-like synoviocytes (FLS). Levels of mRNAs encoding for inflammatory cytokines such as IL-1 β, IL-6 and IL-8 and NOS-II enzyme, which had been induced by TNF-α and IL-1β cotreatment, were decreased to the similar levels as those in cells treated with anti-inflammatory agent MTX. mRNA expressions of matrix metalloproteinase-3 (MMP-3) and tissue inhibitor of metalloproteinases (TIMPs) as well as intercellular adhesion molecule (ICAM) were also downregulated by increasing doses of GMHGST in activated FLS. Moreover, GMHGST appeared to protect cells by decreasing NO levels, and inhibited cell proliferation which had been induced by inflammatory stimulation by TNF-α and IL-1. These results suggest that GMHGST is effective as an inhibitory agent for regulating inflammatory responses in activated FLS.

Key words : *Gamimahaenggamsuk-tang*(GMHGST), RT-PCR, Rheumatoid arthritis, fibroblast-like synoviocyte, inflammatory cytokine, nitric oxide

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disorder that inflict significant morbidity on approximately 1% of the population showing women thee times more than men. Joint destruction and extra-articular inflammation result in a long-term reduction in independence and therefore up to 50% of patients are no longer in full-time employment after 10 years of their illness[30]. Furthermore, population studies have suggested that the standardized mortality ratio of RA patients is up to 5.5 times that of the general population[18].

RA is a complex, multi-system disease, and multiple cell types are known to contribute to the pathogenic picture in rheumatoid synovium (pannus), and many studies implicate the T cell as a primary mobile of RA. Also, accumulating evidence suggests that fibroblast-like synoviocytes (FLS, also called synovial fibroblasts) play crucial roles in both joint damage and the propagation of inflammation[15]. In normal joint homeostasis, FLS function in secreting high levels of long-chain, polymeric hyaluronan into joint cavity, which has both lubricating and immunomodulatory properties. Then in RA patients, FLS are hyperproliferative[17], and several in vitro studies suggest that FLS in RA divide more rapidly than cells from normal or osteoarthritic joints. Several growth factors that drive FLS mitosis in vitro are overexpressed in the rheumatoid joint[23]. Platelet-derived growth factor (PDGF), and basic fibroblast growth factor (bFGF) are overexpressed by FLS[20]. Transforming growth factor-β (TGF-β) can drive FLS proliferation[3], and activins, members of the TGF-β superfamily, which are overexpressed in RA synovium, induce RA FLS proliferation[8,28]. Finally, the presence of type C retroviral particles in synovial fluid suggests the potential involvement of a virus in the development of a semi-transformed phenotype[36].

FLS mediate inflammation and autoimmunity through a wide range of mechanisms. FLS respond to, and themselves produce, inflammatory mediators including IL-1, 4, 6, 8, 10, 12, 13, 17, 18, 21, TNF-α, TGF-β, IFN-γ, VIP, iNOS, and, via upregulation of cyclooxygenase-2 (COX-2), prostaglandin

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E_2[9,11,14,24,25,35,]. Many of these pro-inflammatory cytokines are known to be overexpressed in RA patients, especially in those with long-term disease. Since they promote activation of T cells, neutrophils, and macrophages, their neutralization hold promise for therapeutic targeting in RA[19]. FLS are thus important drivers of RA inflammation.

The use of complementary and alternative medical therapies has been increasing worldwide among patients with rheumatologic diseases. Forty-seven percent of older adults with osteoarthritis use complementary medicine. Non-prescribed medications now increase, while massage therapy and chiropractic services make up the remainder in western as well as oriental societies[32]. Within the category of alternative medicine, herbal medications are commonly used for rheumatic conditions. Several of these medications are easily available and claim a long history of safety and efficacy. In the present study, we investigated potential attenuating effects of Gamimahaenggamsuk-tang (GMHGST) on RA-related inflammatory responses in cultured FLS cells. GMHGST is the mahaenggamsuk-tang (MHGST) fortified with several herbal drugs (see Table 1). MHGST is commonly used for the treatment respiratory diseases such as asthma and several inflammatory diseases. By activating FLS with co-treatment of TNF-α and IL-1β, we examined regulatory effects of GMHGST on the production of inflammatory cytokines, metalloproteinase and its inhibitor protein, adhesion molecule, and nitric oxide (NO). In most of the cases, we found significant decreases in inflammatory cytokine mRNA levels, decreased production of NO, and decreased cell proliferation.

Materials and Methods

1. Materials

1) Animals

DBA/1J mice (6 week old) obtained from Charles River (Japan), were used in the present study. The animals were housed in a conventional system at 12 hr of day light (200 ~ 300 Lux) and 12 hr of dark condition in 22±2℃. The animals were fed with food pellets [composition: crude protein (22.1%), crude lipid (8.0%), crude fibers (5.0%), crude minerals (8.0%), calcium (0.6%), and phosphorus (0.4%); Samyang Co, Inc. Korea] and water ad libitum. Animals were adjusted to the environment at least 2 weeks before the experiment.

2) Drugs

The Gamimahaenggamsuk-tang (GMHGST) used in this study was purchased from Daejeon University Oriental medicinal hospital and the composition of a seal is as follows.

3) Reagents and apparatus

Diethyl pyrocarbonate (DEPC), methotrexate (MTX), trypsin-0.2% EDTA, 3,4,5-dimethylthiazol-2,5-carboxymethoxyphenyl-2,4-sulfophenyl-2H-tetrazolim (MTS), 2,7,-dichloro-dihydrofluore scindiacettate (DCFH-DA), chloroform, RPMI-1640 medium, trichloroacetic acid, isopropanol, ethidium bromide, Dulbecco's phosphate buffered saline (D-PBS), formaldehyde, polyacrylamide, magnesium chloride ($MgCl_2$) were all purchased from Sigma (St. Louis, USA). Taq polymerase and deoxynucleotide triphosphates (dNTPs) were from Takara (Japan), and reverse transcriptase and ribonuclease inhibitor were from Promega (Madison, USA). RNAzolB was purchased from Tel-Test (Friendswood, USA), fetal bovine serum (FBS) from Hyclone (Logan, USA), ribonuclease from Pharmingen (Torreyana, USA), rIL-6 from R&D system (Minneapolis, USA), and 3H-thymidine from Amersham (Buckinghamshire, UK). Other chemicals used in the present study were the highest quality available. The instruments used in this study are listed below with manufacturers in parenthesis. Real time quantitative RT-PCR (Applied Biosystems, USA), flow cytometer (Becton Dickinson, USA), spectrophotometer (Shimazue. Japan), centrifuge (Centrikon, Sigma), bio-freezer (Sanyo, Japan), Primus 96 thermocycler system (MWG Biotech., Germany), turbo thermal-cyclerTM (Bioneer Co., Korea), ice maker (Vision, Korea), CO_2 incubator (Forma scientific Co., USA), clean bench (Vision Co., Korea), rotary vacuum evaporator (Büchi 461, Switzerland), autoclave (Hirayama, Japan), imager system (Kodak, U.S.A), plate shaker (Lab-Line, USA), ELISA leader (molecular devices, USA), homogenizer(OMNI, USA).

Table 1. Prescription of *Gamimahaenggamsuk-tang* (GMHGST)

Korean Name	Herbal nomenclatures	Amount (g)
Euyin (의이인)	*Coisis semen*	20
Sanyak (산 약)	*Dioscoreae rhizoma*	4
Haengin (행 인)	*Armeniacae amarum semen*	4
Gobon (고 본)	*Ligustici rhizoma*	4
Mahwang (마 황)	*Ephedrae herba*	4
Nabokja (나복자)	*Raphani semen*	4
Gamcho (감초)	*Glycyrrhizae radix*	4
Maikmundong (맥문동)	*Liriopis tuber*	4
Ganghwal (강 활)	*Notopterygii rhizoma*	2
Gilgyung (길 경)	*Platycodi radix*	2
Jigak (지 각)	*Aurantii fructus*	2
Changchul (창 출)	*Atractylodis rhizoma*	2
Omija (오미자)	*Schizandrae frunctus*	2
Total amount		58

2. Methods

1) GMHGST extraction

Three seals of dried GMHGST were dissolved in 2000 ml distilled water, and extracted in a heating bath for 3 hr. The filtrates were then condensed by using the rotary evaporator (Büchi B-480, Switzerland), and freeze-dried by using the drier (Freezer drier; EYELA FDU-540, Japan). The powder obtained was kept at -84℃ until use, and diluted with distilled water at 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 1400 rpm for 5 min. After adding collagenase A (5 ㎎/㎖ in DMEM; Boehringer Mannheim, USA), DNase I (0.15 ㎎/㎖, Sigma, USA), and antibiotics (penicillin 104 U/㎖, streptomycin 10 ㎎/㎖, amphotericin B 25 ㎍/㎖), the dissociated tissue was incubated at 37 ℃ for 2 hr, and further incubated for 30 min in the presence of 0.5% trypsin/0.2% EDTA. The tissue was then washed twice with cold PBS, centrifuged at 1500 rpm to remove the supernatant, resuspended in DMEM-10% FBS, and cultured for 7 days. The cells were dissociated from the plate by 0.5% trypsin/0. 2% EDTA treatment, and cultured in 96 well plate with a cell concentration of 10^5 cells/㎖ in DMEM-5% FBS culture medium.

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

Knee joint tissues 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 washed with serum-free DMEM and fat debris and culture medium was removed. Dispase (1-1.5 mg/㎖, grade 11, Boehringer Mannheim) were treated to 10 mg of joint tissue and incubated in jolik's MEM at 37℃ for 30 min. The supernatant was transferred to a new tube containing DMEM with 5% FBS. Cell suspension was obtained by repeated treatment with dispase three more times. Collected supernatant was centrifuged at 1200 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 FLS adhered to the substrate were cultured in DMEM containing 15% FBS medium with 5 transfers a week.

4) Stereoscopic microscopy

For microscopy, cells were cultured for 48 hr in a LAB-Tek tissue culture chamber slide (Nunc Inc., USA) and fixed by the solution containing 2.5% glutaraldehyde, 0.12 M sodium cacodylate buffer (pH 7.3) at 4℃ for 30 min. Fixed cells were observed under the stereoscopic microscope (Nikon, Japan) at 1000x magnification.

5) Measurement of cytotoxicity

The cytotoxicity was measured by modified SRB method. After incubation in 5% CO_2 incubator at 37℃ for 1 hr, cells were dissociated by trypsin-EDTA treatment, The culture medium was removed and the cells (2.0×10^4 well in 96 well plate) were incubated at 37℃, 5% CO_2. Cells were treated with 1000 ㎍/㎖, 500 ㎍/㎖, 250 ㎍/㎖, 125 ㎍/㎖, 62.5 ㎍/㎖, 32 ㎍/㎖, 16 ㎍/㎖, and 1.6 ㎍/㎖ of GMHGST for 48 hr. Then, cells were washed with PBS twice, treated with 50 ㎕ of 50% trichloroacetic acid (TCA) for 1 hr at 4℃, washed with distilled water five times, and air dried. Cells were stained with SRB solution (0.4%/1% acetic acid; 100 ㎕/well) for 30 min at room temperature, and washed with 0.1% acetic acid for 4-5 times. Cells were air dried, and solublized in 10 mM Tris base (100 ㎕/well). Cells in the plate were dispersed in the culture medium by using a plate shaker for 5 min and used for the measurement of optical density at 540 nm using ELISA reader (Molecular Devices, USA).

6) Real Time Quantitative RT-PCR

(1) RNA extraction

Cells isolated from the knee joint tissue were plated in a 24 well culture dish with 1×10^6 cells per well, and treated with GMHGST extract (100, 10 ㎍/㎖) or MTX (10 ㎍/㎖). One hour later, recombinant IL-1β (10 U/㎖) and TNF-α (10 ng/㎖) were added to cells. Cells were harvested 6 hr later by centrifugation at 2000 rpm for 5 min. Supernatant was removed and the cells were subject to RNA extraction using RNAzolB protocols. 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 centrifuged at 13000 rpm. Then, the upper layer 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 was resuspended in 20 ㎕ of 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℃. The denatured RNA was mixed with 2.5 ㎕ 10 mM dNPT 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 bring up the reaction 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 7500 Fast Real-Time PCR system. The primers used in the present experiment are listed in the Table below.

Table 2. The primer sequences for the genes used in the present study

Target gene	Primers	Sequences
human IL-1β	Forward	5' tccagggacaggatatggag 3'
	Reverse	5' tctttcaacaGMHGSTcaggacag 3'
human IL-6	Forward	5'GMHGSTccccacacagacaGMHGSTcactc 3'
	Reverse	5'tGMHGSTctctttGMHGSTtGMHGSTtttcacaca 3'
human IL-8	Forward	5'GMHGSTtgGMHGSTGMHGSTtgGMHGSTtctcttg3'
	Reverse	5' tggggtggaaaggtttggagtat 3'
G3PDH	Forward	5'tGMHGSTGMHGSTtctagaaaaacctGMHGSTcaa 3'
	Reverse	5'GMHGSTcccagGMHGSTtcaaaggtg 3'
human NOS-II	Forward	5' acaaGMHGSTctacccctccagat 3'
	Reverse	5' ctcctttgttacGMHGSTcttcca 3'
human MMP-3	Forward	5'cGMHGSTagaaatGMHGSTagaagttcc 3'
	Reverse	5' gatGMHGSTcaggaaaggttctga 3'
human TIMP-1	Forward	5'agtgGMHGSTactcattGMHGSTttgtg 3'
	Reverse	5'GMHGSTaggattcagGMHGSTtatctgg 3'
human ICAM-1	Forward	5' aggttgaaccccacagtcac 3'
	Reverse	5' gtgtctcctgGMHGSTtctggttc 3'

GMHGST green PCR master mix (ABI) was used for the measurement of inflammatory cytokine expression, and human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control. The primer concentration used was 200 nM. Real-time PCR was performed by following condition: predenaturation for 2 min at 50℃, 10 min 94℃, 40 cycle progression, then stop reaction for 0.15 min at 95℃ followed by 1 min treatment at 60℃. Quantitative PCR (RQ) data were analyzed by following formula. $y=x(1+e)n$. Here, x=starting quantity, y=yield, n=number of cycles, and e=efficiency

7) Measurement of inhibition of FLS proliferation

FLS were cultured in a 96 well plate with 2 × 10^6 cells per well and treated with GMHGST extract (100 ㎍/㎖, 10 ㎍/㎖, 1 ㎍/㎖) and recombinant IL-6 for 72 hr at 37℃. Then, the cells were treated with 50 ㎕ of MTS solution, cultured for 4 hr by shaking on a plate shaker (Lab-Line, USA). The absorbance was measured at 490 nm using a ELISA reader (Molecular devise, USA).

8) Determination of NO production

Griess solution was prepared immediately before the experiment by mixing solution A (0.2 % naphthylethylene diamine dihydrochloride in water) and solution B (2 % sulfonylamide in 5 % H_3PO_4) with 1:1 ratio. FLS after recombinant IL-6 (R&D system) treatment were plated in a 24 well plate with 5×10^5 cells per culture dish and incubated in the presence of GMHGST (100 ㎍/㎖, 10 ㎍/㎖, 1 ㎍/㎖) and IL-6 at 37℃for 48 hr. After the culture, 100 ㎕ of supernatant from each well was mixed with 100 ㎕ of Griess solution, and the optical density at 540 nm was measured.

Results

1. Determination of cytotoxicity of GMHGST treatment in human fibroblast cells

Observation of cultured human fibroblast cells (hFC) and human FLS under bright field microscope showed morphological features characteristic to individual cell types (Fig. 1A). To determine possible toxicity of GMHGST treatment, the viability of GMHGST treated-hFCs was determined by a modified SRB method. Values of cell viability were gradually decreased as increasing GMHGST concentration. Yet, consistently significant levels of toxicity were not observed until the GMHGST doses were higher than 125 ㎍/㎖ (Fig 1B). Thus in the current study, the effects of GMHGST administration on inflammatory responses in FLS were examined in a range of 1-100 ㎍/㎖ of GMHGST concentration at which no significant cytotoxicity was detected.

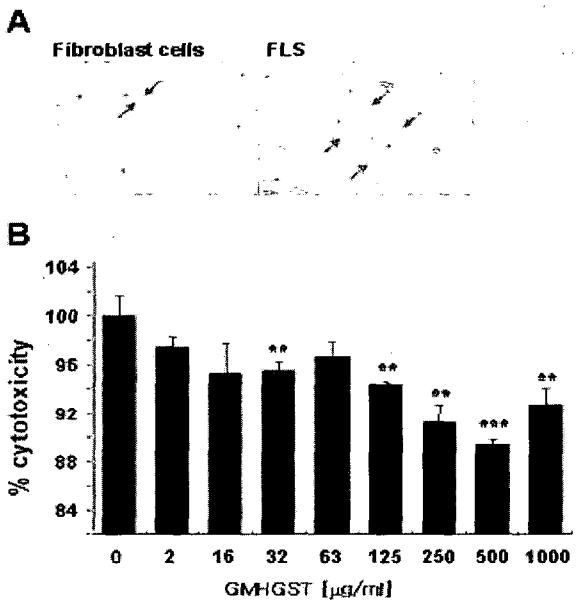

Fig. 1. (A) Human lung fibroblast cells and FLS were cultured, and cell morphologies were photographed under bright field microscope. Human fibroblast cells and FLS showed a large surface area (marked by arrows) indicating healthy states. (B) Human lung fibroblast cells were pretreated with various concentrations of GMHGST, and% toxicity relative to the cells treated with 0 ㎍/㎖ GMHGST was determined. The bars denote mean ± S.E.M. (N=6). (Student's t-test, *p<0.05, **p<0.01, ***p<0.001).

2. Effects of GMHGST on the production of inflammatory cytokines in FLS

Possible anti-inflammatory activity of GMHGST was examined in cultured FLS which had been activated by cotreatment of IL-1β and TNF-α. As shown in Fig. 2A-C, activated FLS (labeled 'CT' in the Fig.) showed several fold increases in mRNA synthesis encoding IL-1β, IL-6 and IL-8 compared with untreated control (labeled "WT" in the Fig.).

Addition of anti-inflammatory agent MTX to the IL-1β/TNF-α -stimulated cells decreased mRNA synthesis levels to 11 - 42%, suggesting clear suppression of the production of inflammatory cytokines by MTX. Treatment of GMHGST at 10 ㎍/㎖ did not decrease mRNA levels, and then, the synthesis of IL-1β, IL-6 and IL-8 mRNA was all effectively inhibited by 100 ㎍/㎖ of GMHGST treatment. It was particularly noted that 100 ㎍/㎖ of GMHGST treatment was most effective for decreasing IL-8 mRNA levels similar to those in MTX-treated cells.

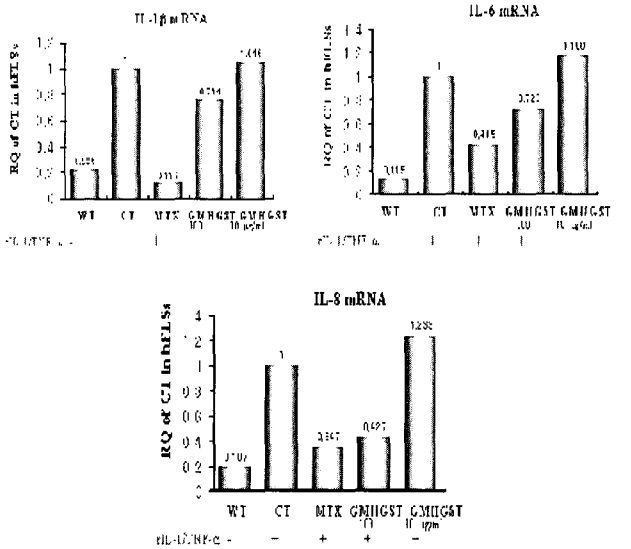

Fig. 2. Inhibitory effects of GMHGST on IL-1β, IL-6, and IL-8 mRNA expression levels in FLS. Human FLS were stimulated with IL-1β (10 U/ml) and TNF-α (100 ng/ml), and further stimulated for 6 hr with MTX (positive control) or GMHGST(100 ㎍/ml and 10 ㎍/ml). The amount of GMHGST green was measured at the end of each cycle. The cycle number at which the emission intensity of the sample rises above the baseline is referred as to the RQ and is proportional to the target concentration. WT: mRNA from cells with no treatment. CT: control cells with IL-1β/TNF-α, but no GMHGST, treatment

3. Effects of GMHGST treatment in FLS on MMP-3 and TIMP mRNA

It is known that increased synthesis of metalloproteinase-1 and tissue inhibitor of metalloproteinase (TIMP) is associated with the tissue destruction such as cartilage in RA patients[10]. Thus, MMP and TIMP mRNA expression in response to GMHGST treatment was investigated by real time PCR. MMP-3 and TIMP mRNA levels in cells teated with both IL-1β and TNF-α were increased 2.9 and 8.3 folds in GMHGST-treated group compared with non-treated group respectively(Fig. 3A, B). GMHGST treatment at 100 ㎍/㎖ decreased MMP-3 and TIMP mRNA levels which were similar to those in MTX-treated cells. However, 10 ㎍/㎖ of GMHGST was not effective for the inhibition of MMP-3 mRNA synthesis and was only slightly effective for TIMP mRNA regulation. Together, these data suggest that GMHGST at 100 ㎍/㎖ can inhibit the synthesis of MMP-3 and TIMP mRNA synthesis in activated FLS.

4. Inhibition of NO production in activated FLS by GMHGST

Increased NO production can contribute the progression of inflammatory responses in several cell types. Thus, inhibition of NO production may be critical for controlling inflammation. In FLS stimulated with IL-1/TNF-α, both NOS-II mRNA and NO levels were largely elevated compared with non-teated control(Fig. 4). MTX then inhibited both NOS and NO levels. GMHGST treatment decreased NOS-II mRNA levels in a dose-dependent manner, showing lower NOS-II mRNA than MTX-treated cells at 100 ㎍/㎖(Fig. 4A). Decreases in NO levels were significantly lower than IL-1/TNF-α-control group though the value was higher than MTX-treated group(Fig. 4B).

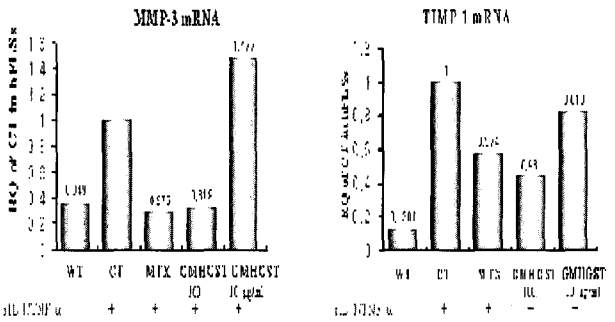

Fig. 3. Inhibitory effects of GMHGST on MMP-3 and TIMP mRNA expression levels in FLS. Human FLS were stimulated with IL-1β (10 U/ml) and TNF-α (100 ng/ml), and further stimulated for 6 hr with MTX (positive control) or GMHGST(100 ㎍/ml and 10 ㎍/ml). The amount of GMHGST green was measured at the end of each cycle. The cycle number at which the emission intensity of the sample rises above the baseline is referred as to the RQ and is proportional to the target concentration. WT: mRNA from cells with no treatment. CT: control cells with IL-1β/TNF-α, but no GMHGST, treatment .

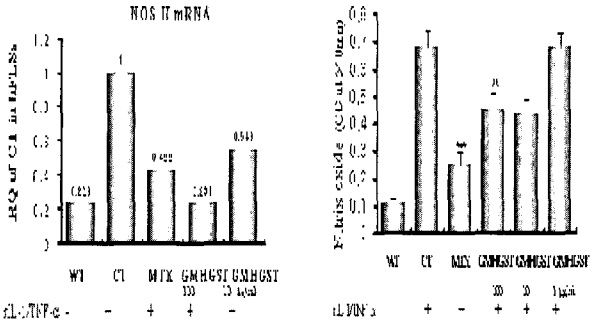

Fig. 4. Inhibitory effects of GMHGST on NOS and NO levels in stimulated FLS. (A) Human FLS were stimulated with IL-1β (10 U/ml) and TNF-α (100 ng/ml), and further stimulated for 6 hr with MTX (positive control) or GMHGST (100 ㎍/ml and 10 ㎍/ml). NOS-II mRNA levels were determined by RT-PCR. (B) NO production in FLS. Twenty hours after the culture in the presence of absence of GMHGST, the culture supernatant was collected and NO concentration was determined. The bar graphs denote mean ± S.E.M. (N=6). (Student's t-test, *p<0.05, **p<0.01, ***p<0.001). CT: control cells with IL-1β/TNF-α, but no GMHGST, treatment .

5. Effects of GMHGST treatment in FLS on ICAM mRNA synthesis

Previous studies have shown increased infiltration of intercellular adhesion molecule (ICAM) in the synovial tissue

(ST) from patients with RA[37]. Real-time measurement of ICAM mRNA synthesis in FLS activated by IL-1/TNF-α co-treatment showed about three fold increase compared with untreated cells. MTX treatment decreased mRNA levels to 50%. GMHGST treatment also decreased ICAM mRNA levels. As shown in Fig. 5, ICAM mRNA levels in cells treated with 100 μg/mℓ of GMHGST were lower than those treated with MTX, indicating that GMHGST were effective for downregulating ICAM mRNA synthesis in IL-1/TNF-α stimulated cells.

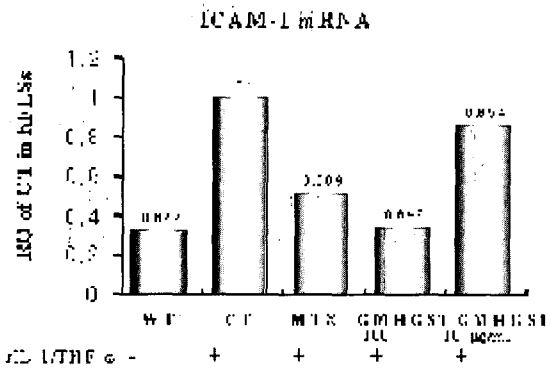

Fig. 5. Inhibitory effects of GMHGST on ICAM mRNA levels in stimulated FLS. Human FLS were stimulated with IL-1β (10 U/ml) and TNF-α (100 ng/ml), and further stimulated for 6 hr with MTX (positive control) or GMHGST (100 μg/ml and 10 μg/ml). Then, ICAM mRNA levels were determined by RT-PCR. CT: control cells with IL-1β/TNF-α, but no GMHGST, treatment.

6. Effects of GMHGST on FLS proliferation

The proliferation of FLS showed more than three fold increase by IL-1/TNF-α stimulation above untreated cells(Fig. 6). MTX inhibited cell proliferation of activated FLS. Then the treatment of GMHGST into activated cells decreased cell numbers in a dose-dependent manner. Cell counts at 100 μg/mℓ of GMHGST were significantly lower than untreated control cells(Fig. 6). These results suggest that GMHGST had an inhibitory effect on FLS proliferation related to the inflammatory responses in RA tissue.

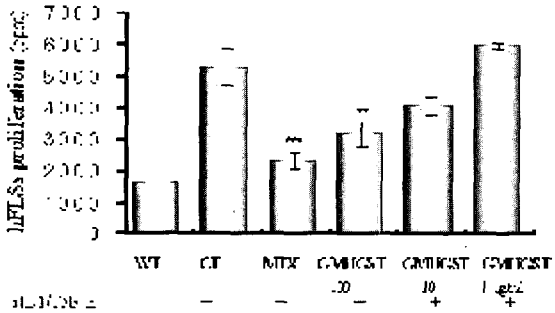

Fig. 6. Inhibitory effect of GMHGST on FLS proliferation. Human FLS were stimulated with IL-1β (10 U/ml) and TNF-α (100 ng/ml), and further treated with MTX (10 mg/ml) or GMHGST(100 μg/ml and 10 μg/ml) for 72 h. After ^{3}H-thymidine incorporation reaction and harvest, cell proliferation was assayed. The bars in the graph represent the mean ± S.E.M.(N=4; Student's t-test, *p<0.05, **p<0.01, ***p<0.001).

Discussion

RA is a chronic inflammatory disease leading to substantial disability and health care expenditures[40]. In RA, synovial proliferation of joints leads to erosions and joint-space narrowing in up to two thirds of patients within the first 2 to 3 years of disease onset[26,31]. Recommended clinical practice has been rapidly changed toward more aggressive initial treatment with disease-modifying anti-rheumatic drugs (DMARDs)[4,39].

The pathogenesis of RA involves numerous factors involving genetic, environmental, and immunologic influences[19]. Several case studies showed that disease concordance was higher in monozygotic twins than dizygotic twins[1,34]. Mechanistic studies showed that multiple cell types including T cell and FLS contribute to the pathogenesis in rheumatoid synovium. Recent evidences further suggest that fibroblast-like synovial cells (FLS) play an important role in both joint damage and the propagation of inflammation while the participation of T cells in the pathogenesis accounts for part of the mononuclear infiltrate in the synovial sublining. Normal FLS function involves the control of synovial fluid volume and normal inflammatory responses. Also, FLS appear to regulate routine leukocyte trafficking, via their capacity to interact with leukocytes. In pathological situation, FLS are appreciated to be responsible for cartilage destruction and also derive both inflammation and autoimmunity. Thus, in the current study, we examined GMHGST-mediated regulation of inflammatory cytokines in FLS activated by TNF-α and IL-1β co-treatment.

First, we found that treatment of FLS with both TNF-α and IL-1β significantly elevated levels of IL-1β, IL-6, and IL-8 mRNAs known to be actively involved in inflammatory responses in RA. These data are consistent with previous findings. Overproduction of IL-6 and IL-8 is known to play a crucial role in the pathology of RA. They are responsible for the activation of auto-reactive T cells and the appearance of auto-reactive antibodies, including rheumatoid factors. In particular, IL-6 is involved in the bone destruction and osteoporosis observed in the disease and further increased in both the synovial fluid and the serum of patients[6,27,38]. Studies further suggest that IL-6, and IL-8 correlate with disease activity[22,27]. Similarly, studies using experimental animals have suggested a central role for IL-1β in the process of synovitis and joint destruction. Addition of IL-1β into experimental models of arthritis induces and exacerbates synovitis.

Previous studies implicated that anti-proliferating effects of MTX positively correlated with decreases inflammatory responses, which all together, are associated with RA therapy[7].

Treatment of MTX in FLS activated by IL-1β and TNF-α co-treatment significantly decreased inflammatory cytokine levels, rendering it to use as a positive control for GMHGST examination. Although the effects of GMHGST treatment on regulation of inflammatory cytokines were variable among cytokines, increasing dosages of GMHGST clearly decreased cytokines levels.

We further found that GMHGST treatment in stimulated FLS decreased TIMP and MMP-3 mRNA expression levels, which were strongly induced by IL-1β and TNF-α co-treatment. Matrix metalloproteinases (MMPs) are a family of zinc-containing enzymes involved in the degradation and remodeling of extracellular matrix proteins. Under normal physiological conditions, the activities of these enzymes are well-regulated by endogenous tissue inhibitor of metalloproteinases (TIMPs). Chronic stimulation of MMP activities due to an imbalance in the levels of MMPs and TIMPs has been implicated in the pathogenesis of a variety of diseases such as cancer, osteoarthritis, and rheumatoid arthritis. MMPs contribute to joint destruction in part by directly enhancing proteolytic degradation of the ECM of the cartilage and bone[2,16]. GMHGST treatment in activated FLS effectively decreased levels of TIMP and MMP-3 mRNA, indicating that proteolytic destruction in RA might be down-regulated by GMHGST.

In addition to regulatory function of GMHGST for inflammatory mediator molecules, GMHGST was shown to lower NO production in activated FLS. Overproduction of reactive oxygen species (ROS) is detrimental to cells, and the main ROS produced by cells (e.g., chondrocytes) significantly affected in RA are nitric oxide (NO) and superoxide anion that generate derivative radicals. NO is synthesized by NO synthase (NOS) enzymes. Of the three NOS isoforms, the inducible form (NOS II) is regulated at the gene level by a variety of stimulations (; growth factors, cytokines and endotoxins). Many drugs used for the treatment of RA are believed to mediate their therapeutic actions by multiple mechanism, and one of them is a reduction of oxidant damage at site of inflammation[5]. Our data also showed that stimulation of FLS with TNF-α and IL-1βsignificantly elevated NOS 2 mRNA expression as well as NO, whose production was then significantly inhibited by GMHGST, confirming beneficial effects of antioxidant on RA treatment. These data further suggest that GMHGST might include a certain molecular components exerting as antioxidant activity. In this regards, it would be interesting to notice that rasanyana drugs including the plants Allium sativum, Centella asiatica, Ocimum sanctum, Vitis vinifera and Zingiber officinale have been implicated to have antioxidation function[33].

Taken together, our data strongly suggest that oriental medicinal drug GMHGST might have a protective effect on inflammatory FLS. In many cases, induction of mRNA expression involves transcriptional activation of target gene by recruiting transcription factors. Since GMHGST treatment induced multiple mRNA expression, several different kinds of transcription factors might be activated to induce target gene expression. Another possibility is regulation at the post-transcriptional levels by increasing the stability of preexisting mRNA. Previous studies have shown that protein factors induced in the cytoplasm contribute to mRNA stability by interacting with 3′ untranslated region of target mRNA. In any case, GMHGST appears to regulate levels of mRNA synthesis important for the regulation of RA progression.

GMHGST has been clinically used in the oriental medicine for the treatment of RA[12]. Our examination of GMHGST in stimulated FLS strongly implicate possible positive actions of GMHGST. Yet, it should be cautious in interpreting the current data because the efficacy of the GMHGST may be due to synergistic action of multiple compounds or a balance between synergist and antagonist actions. Identification of active ingredient(s) is critical to understand precise molecular mechanism.

Conclusion

GMHGST has been used in the oriental medicine for the cure of diverse inflammatory disease including RA. To examine whether GMHGST is effective for controlling chemical mediators in RA, FLS isolated from RA patients were used to determine possible regulatory effects of GMHGST on levels of inflammatory cytokines, NO production, and cell proliferation. Major findings are summarized as follows.

GMHGST was not lethal to cultured human fibroblast cells at less than 125 ㎍/㎖. IL-1β, IL-6 and IL-8 mRNA expression levels in FLS activated by TNF-α and IL-1β co-treatment were largely inhibited by 100 ㎍/㎖ of GMHGST treatment. MMP-3 and TIMP mRNA expression levels in activated FLS were strongly inhibited by 100 ㎍/㎖ of GMHGST treatment, showing the similar levels of mRNA in MTX-treated positive control cells. At 10 ㎍/㎖ of GMHGST, only TIMP mRNA expression was partially inhibited.

Levels of both NOS-II mRNA expression and NO production in activated FLS were decreased by increasing GMHGST concentration to 100 ㎍/㎖. Similarly, ICAM-1 mRNA synthesis in FLS were strongly blocked by 100 ㎍/㎖ of GMHGST treatment, which was lower than that in

MTX-treated cells. FLS proliferation was increased by IL-1β and TNF-α, and then strongly inhibited by GMHGST treatment in a dose dependent manner.

These results suggest that GMHGST is effective for inhibiting inflammatory responses induced in activated FLS. Further in vivo studies using an experimental animal should be helpful for characterizing active herbal components among GMHGST, which could provide an insight into therapeutic development for clinical application.

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References

1. Aho, K., Koskenvuo, M., Tuominen, J., Kaprio, J. Occurrence of rheumatoid arthritis in a nationwide series of twins. J Rheumatol. 13(5):899-902, 1986.
2. Ahrens, D., Koch, A.E., Pope, R.M., Stein-Picarella, M., Niedbala, M.J. Expression of matrix metalloproteinase 9 (96-kd gelatinase B) in human rheumatoid arthritis. Arthritis Rheum. 39, pp 1576-1587, 1996.
3. Allen, J.B., Manthey, C.L., Hand, A.R. Rapid onset of synovial inflammation and hyperplasia induced by transforming growth factor beta. J. Exp. 171, pp 231-247, 1990.
4. American College of Rheumatology Ad Hoc Committee on Clinical Guidelines, 1Guidelines for the management of rheumatoid arthritis. Arthritis Rheum. 38, pp 713-722, 1996.
5. Aruoma, O.I. Use of DNA damage as a measure of pro-oxidant actions of antioxidant food additives and nutrient components. In: B. Halliwell and O.I. Aruoma, Editors, DNA and Free Radicals, Ellis Horwood, London, pp 315-327, 1993.
6. Dasgupta, B., Corkill, M., Kirkham, B., Gibson, T., Panayi, G. Serial estimation of interleukin 6 as a measure of systemic disease in rheumatoid arthritis. J. Rheumatol. 19, pp 22-25, 1992.
7. Genestier, L., Paillot, R., Fournel, S., Ferraro, C., Miossec, P., Revillard, J.P. Immunosuppressive properties of methotrexate: Apoptosis and clonal deletion of activated peripheral T cells. J Clin Invest. 102, pp 322-328, 1998.
8. Gribi, R., Tanaka, T., Harper-Summers, R., Yu, J. Expression of activin A in inflammatory arthropathies. Mol. Cell. Endocrinol. 180, pp 163-167, 2001.
9. Hwang, S.Y., Kim, J.Y., Kim, K.W., Park, M.K., Moon, Y., Kim, W.U., Kim, H.Y. IL-17 induces production of IL-6 and IL-8 in rheumatoid arthritis synovial fibroblasts via NF-kappaB- and PI3-kinase/Akt-dependent pathways. Arthritis Res. 6, pp 120-128, 2004.
10. Huang, W., Li, W.Q., Dehnade, F., Zafarullah, M. Tissue inhibitor of metalloproteinases-4 (TIMP-4) gene expression is increased in human osteoarthritic femoral head cartilage. J. Cell. Biochem. 85, pp 295-303, 2002.
11. Jungel, A., Distler, J.H., Kurowska-Stolarska, M., Seemayer, C.A., Seibl, R., Forster, A., Michel, B.A., Gay, R.E., Emmrich, F., Gay, S., Distler, O. Expression of interleukin-21 receptor, but not interleukin-21, in synovial fibroblasts and synovial macrophages of patients with rheumatoid arthritis. Arthritis Rheum. 50, pp 1468-1476, 2004.
12. 김호철, 이상인 안덕균, Human monocyte의 IL-8생산억제에 미치는 류마토이드관절염 치료제로서의 오가피의 효과 . 대한본초학회지. 10(1):49-58, 1996.
13. Kirwan, J.R. The relationship between synovitis and erosions in rheumatoid arthritis. Br. J. Rheumatol. 36, pp 225-228, 1997.
14. Kojima, F., Naraba, H., Sasaki, Y., Beppu, M., Aoki. H., Kawai, S. Prostaglandin E2 is an enhancer of interleukin-1beta-induce expression of membrane-associated prostaglandin E syntase in rheumatoid synovial fibroblasts. Arthritis Rheum. 48, pp 2819-2828, 2003.
15. Kontoyiannis, D., Kollias, G. Fibroblast biology. Synovial fibroblasts in rheumatoid arthritis: leading role or chorus line?. Arthritis Res. 2, pp 342-343, 2000.
16. Konttinen, Y.T., Ainola, M., Valleala, H., Ma, J., Ida, H., Mandelin, J., Kinne, R.W., Santavirta, S., Sorsa, T., Lopez-Otin, C., Takagi, M. Analysis of 16 different matrix metalloproteinases (MMP-1 to MMP-20) in the synovial membrane: different profiles in trauma and rheumatoid arthritis. Ann Rheum Dis. 58, pp 691-697, 1999.
17. Kramer, I., Wilbulswas, A., Croft, D., Genot, E. Rheumatoid arthritis: targeting the proliferative fibroblast. Prog. Cell Cycle Res. 5, pp 59-70, 2003.
18. Krause, D., Schleusser, B., Herborn, G., Rau, R. Response to methotrexate treatment is associated with reduced mortality in patients with severe rheumatoid arthritis. Arthritis and Rheumatism. 43, pp 14-21, 2000.
19. Lee, D.M., Weinblatt, M.E. Rheumatoid arthritis. Lancet. 15, pp 903-911, 2001.
20. Manabe, N., Oda, H., Nakamura, K. Involvement of fibroblast growth factor-2 in joint destruction of rheumatoid arthritis patients. Rheumatology. 38, pp 714-720, 1999.
21. Martel-Pelletier, J., Welsch, D.J., Pelletier, J.P. Metalloproteases and inhibitors in arthritic diseases. Best Pract. Res., Clini.

Rheumatol. 15, pp 805-829, 2001.

22. McCarey, D.W., McInnes, I.B., Madhok, R., Hampson, R., Scherbakov, O., Ford, I., Capell, H.A., Sattar, N. Trial of atorvastatin in rheumatoid arthritis (TARA): double-blind, randomised placebo-controlled trial. Lancet. 363, pp 2015-2021, 2004.
23. Melnyk, V.O., Shipley, G.D., Sternfeld, M.D., Sherman, L., Rosenbaum, J.T. Synoviocytes synthesize, bind, and respond to basic fibroblast growth factor. Arthritis Rheum. 33, 493 - 500, 1990.
24. Miranda-Carus, M.E., Balsa, A., Benito-Miguel, M., Perez de Ayala, C., Martin-Mola, E. IL-15 and the initiation of cell contact-dependent synovial fibroblast-T lymphocyte cross-talk in rheumatoid arthritis: effect of methotrexate. J. Immunol. 173, pp 1463-1476, 2004.
25. Morel, J.C., Park, C.C., Zhu, K. Signal transduction pathways involved in rheumatoid synovial fibroblast interleukin-18-induced vascular cell adhesion molecule-1 expression. J. Biol. Chem. 38, pp 34679-34691, 2002.
26. Mottonen, T.T. Prediction of erosiveness and rate of development of new erosions in early rheumatoid arthritis. Annals Rheum Dis. 47, pp 648-665, 1988.
27. Nishimoto, N., Yoshizaki, K., Miyasaka, N., Yamamoto, K., Kawai S., Takeuchi, T., Hashimoto, J., Azuma, J., Kishimoto, T. Treatment of rheumatoid arthritis with humanized anti-interleukin-6 receptor antibody. Arthritis Rheum. 50, pp 1761-1769, 2004.
28. Ota, F., Maeshima, A., Yamashita, S. Activin A induces cell proliferation of fibroblast-like synoviocytes in rheumatoid arthritis. Arthritis Rheum. 48, pp 2442-2449, 2003.
29. Pap, T., Muller-Ladner, U., Gay, R.E. Fibroblast biology. Role of synovial fibroblasts in the pathogenesis of rheumatoid arthritis. Arthritis Res. 2, pp 361-367, 2000.
30. Pincus, T., Callahan, L.F. What is the natural history of rheumatoid arthritis?. Rheumatic Diseases Clinics of North America. 19, pp 123-151, 1993.
31. Plant, M.J., Saklatvala, J., Borg, A.A., Hones, P.W., Dawes, P.T. Measurement and prediction of radiological progression in early rheumatoid arthritis. J Rheumatol. 21, pp 1808-1813, 1994.
32. Ramsey, S.D., Spencer, A.C., Topolski, T.D., Belza, B., Patrick, D.L. Use of alternative therapies by older adults with osteoarthritis. Arthritis Rheum. 45, pp 222-227, 2001.
33. Scartezzini, D., Speroni, E. Review on some plants of Indian traditional medicine with antioxidant activity. Journal of Ethnopharmacology. 71, pp 23-43, 2000.
34. Schulz, J.B., Lindnau, J., Seyfriend, J., Dichgans, J. Glutathione oxidative stress and neuro-degeneration. European Journal of Biochemistry. 267, pp 4904-4911, 2000.
35. Silman, AJ., MacGregor, A.J., Thomson, W., Holligan, S., Carthy, D., Farhan, A., Ollier, W.E. Twin concordance rates for rheumatoid arthritis: results from a nationwidestudy. Br J Rheumatol, 32(10):903-907, 1993.
36. Smeets, T.J., Kraan, M.C., van Loon, M.E. Tumor necrosis factor α blockade reduces the synovial cell infiltrate early after initiation of treatment, but apparently not by induction of apoptosis in synovial cells. Arthritis Rheum. 48, pp 2155-2162, 2003.
37. Stransky, G., Vernon, J., Aicher, W.K. Virus-like particles in synovial fluids from patients with rheumatoid arthritis. Br. J. Rheumatol. 32, pp 1044-1048, 1993.
38. Tak, P.P., Thurkow, E.W., Daha, M.R., Kluin, P.M., Smeets, T.J., Meinders, A.E., Breedveld, F.C. Expression of adhesion molecules in early rheumatoid synovial tissue. Clin Immunol Immunopathol. 77(3):236-242, 1995.
39. Wendling, D., Racadot, E., Wijdenes, J. Treatment of severe rheumatoid arthritis by anti-interleukin 6 monoclonal antibody. J. Rheumatol. 20, pp 259-262, 1993.
40. Wilske, K.R., Healey, L.A. Remodeling the pyramid: a concept whose time has come. J Rheumatol. 16, pp 505-507, 1989.
41. Yelin, E., Callahan, L. The economic cost and social and psychological impact of musculoskeletal conditions. Arthritis Rheum. 38, pp 1351-1362, 1995.