CHAPTER II

MATERIALS AND METHODS

2.1 Materials

2.1.1 Reagents used in this study and their sources

All chemicals used were analytical grade or equivalent. The chemicals show below are listed in groups according to supplier.

BDH AnalaR® (Pooled, England)

Dimethyl sulfoxide, HEPES, Sodium chloride, Sodium hydrogen carbonate

Fermentas (EU)

DreamTaq® Green, Master Mix RevertAid ™First Strand cDNA synthesis kit

Gibco (New York, USA)

Dulbecco's Modified Eagle Medium (DMEM), Leibovitz's L-15 Media (L-15), 0.5% Trypsin-EDTA

Hyclone® (Waltham, MA, USA)

Hank's balanced salt solution (HBSS) without sodium bicarbonate, Penicillin/Streptomycin (10,000 U/10,000 µg/ml)

Merck (Darmstadt, F.R. Germany)

Citric acid monohydrate, Di-sodium hydrogen phosphate anhydrous, Ethanol (absolute), Hydrogen peroxide, Potassium chloride, Sodium carbonate anhydrous, Sodium dihydrogen phosphate monohydrate

Pacific Science

Nucleospin ® RNAII kit

Sigma-Aldrich (USA)

Bovine serum albumin, Hyaluronic acid (from human umbilical cord), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Lipopolysaccharide form *Escherichia Coli* O26:B6, *o*-phenylene-diamine, polyoxyethylene sorbitan monolaurate (Tween-20)

Vivantis (Malaysia)

Agarose, DNA ladders and markers (VC 100bp plus DNA ladder)

Zymed Laboratory, Inc. Ca, U.S.A.

Peroxidase conjugated anti-biotin monoclonal antibody

2.1.2 List of the cis-3-(2',4',5'-trimethoxyphenyl)-4-{(E)-2''',4''',5''' trimethoxylstyryl}cyclohex-1-ene (compound C) used in this study

Compound C isolated from *Zingiber cassumunar* Roxb. were provided from Prof. Vichai Reutrakul Department of Chemistry, Faculty of Science, Mahidol University, Thailand.

2.1.3 List of the cells used in this study

Human synovial fibroblast was prepared from human synovium tissue that was provided from Maharaj Nakorn Chiang Mai Hospital, (Department of Orthopidic, Faculty of Medicine, Chiang Mai University, Thailand).

Human synovial fibroblast SW982 cell line (SW982) were obtained from ATCC ® number HTB-93

2.2 Method

2.2.1 Human synovial fibroblast SW982 cell line (SW982) culture

Human synovial fibroblast SW982 cell line (SW982) was obtained from ATCC ® number HTB-93. SW982 cell line was cultured in L-15 containing 10% fetal bovine serum and 1% penicillin/streptomycin as high density primary monolayer cultures until 80% confluence (Initiation of number cell is 2 x 10^5 cells / 25 cm^2 flask). The cells were maintained in culture in humidified incubator without 5% CO_2 at 37 ^oC.

To examine the effect of LPS on HASs gene expression and HA synthesis in human synovial fibroblast SW982 cell line (SW982). LPS with vary concentration 0.03-0.3 μg/ml were added to the culture or left untreated as a control for 0-24 hours. Treatments were performed in triplicate with cell line.

To evaluate the effect of compound C on LPS-induced HASs gene expression and HA synthesis in human synovial fibroblast SW982 cell line (SW982). Compound C with various concentrations (1-100 μM) and 0.1 μg/ml LPS were added to culture or left untreated as a control. Treatments were performed in triplicate with cell line.

2.2.2 Human synovial fibroblast culture

Non-inflammatory human synovial tissue was provided with fully informed patient consent from the arthroscopic diagnosis of a flat pad syndrome patient at Maharaj Nakorn Chiang Mai Hospital, (Department of Orthopidic, Faculty of Medicine, Chiang Mai University, Thailand). Primary human synovial fibroblasts were isolated by overnight cultures in 0.25% trypsin at 4 ^oC for 1 hour at 37 ^oC. After that tissue was digested from 2.0 μg/ml collagenase type IA for 3 hours at 37 ^oC.

Synovial fibroblasts were washed with PBS and grown in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin as high density primary monolayer cultures until 80% confluence (Initiation of number cell is 2 x 10^5 cells / 25 cm^2 flask). The cells were maintained in culture in humidified incubator with 5% CO_2 at 37 ^oC (86).

To investigate the effect of LPS on HASs gene expression and HA synthesis in human synovial fibroblast cell. LPS with vary concentration 0.03-0.3 µg/ml were added to the culture or left untreated as a control for 0-24 hours. Treatments were performed in triplicate with cell.

2.3 Analytical methods

2.3.1 Cell viability by MTT assay

Cell viability was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay). SW982 cell lines were plated at a density of 1.0 x 10^4 cells per well into 96-well plates. After overnight precultured, cells were treated with various concentration of compound C as described above for 24 hours. At the end of treatment, 10 µl MTT solution (5 mg/ml MTT dissolved in PBS) were added to each well. The plate was incubated in a CO_2 incubator at 37 ^oC for 4 hours. The medium was removed and formazan crystals were dissolved in 100 µl of DMSO. After incubation for 10 minutes, absorbance at wavelength 540 nm was recorded using an ELISA plate reader (87).

2.3.1.1 Calculation and statistical analysis

The cytotoxicity of compound C on SW982 cell line was estimated using the calculation:

$$\% \text{ of control} = \frac{\text{Sample medium}}{\text{Untreated control}} \times 100$$

The significance of the difference between groups of data was tested using Student's t-test. Statistical significance was considered when $p<0.05$.

2.3.2 A competitive inhibition based Enzyme-linked immunosorbent assay (ELISA) for HA

Microtiter plates (Maxisorp®, Nunc) were coated with umbilical cord HA (100 µl/well) in coating buffer at 4 ^{o}C overnight. Uncoated area was then blocked with 150 µl/well of 1% (w/v) BSA in the incubating buffer for 60 min at 25 ^{o}C. After washing, 100 µl of the mixture, sample or standard competitor (HA Healon®: range 39.06-10,000 ng/ml) in B-HABPs (1:100), were added. After incubation for 60 min at 25 ^{o}C, plates then were washed and the mouse monoclonal anti-biotin-peroxidase conjugate (100 µl/well; 1:2,000) was added and incubated for 60 min at 25 ^{o}C. The plates were washed again and then the peroxidase substrate (100 µl/well) was added and incubated at 37 ^{o}C for 20 min to allow the color to develop. The reaction was stopped by addition of 50 µl of 4 M sulfuric acid (H_2SO_4). The absorbance ratio 492/690 nm was measured using the Titertek Multiskan M340 multiplate reader (88).

2.3.2.1 Calculation and statistical analysis

The release of extracellular matrix (ECMs) biomolecules from culture medium of human synovial fibroblasts and SW982 cell line were estimated using the calculation:

$$\% \text{ of control} = \frac{\text{Sample medium}}{\text{Untreated control}} \times 100$$

The significance of the difference between groups of data was tested using Student's t-test. Statistical significance was considered when $p<0.05$.

2.3.3 Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted by use of RNA isolated reagent (illustra RNAspin mini kit). Reverse transcription reaction was performed using 1.0 μg of total RNA and reverse-transcribed into cDNA using RevertAid ™ First Strand cDNA synthesis kit, then amplified 35 cycles using two oligonucleotide primers derived from published HAS2, HAS3, TLR-4, IL-1β, ICE and GAPDH sequence (see appendix; Table 1). PCR is carried out with temperature cycle (see appendix; Table 1). The PCR products were subjected to 1.5% agarose gel electrophoresis. Quantitative data normalized to GAPDH were obtained using Scion Image software (Scion Corporation, Frederick, Maryland, USA), working in the Gel Plot 2 mode.

2.3.3.1 Calculation and statistical analysis

The expression of mRNA from human synovial fibroblasts and SW982 cell line were quantified by densitometry and were estimated by the calculation.

$$\text{Expression coefficient} = \frac{\text{Density of sample}}{\text{Density of GAPDH}}$$

$$\%\ \text{of control} = \frac{\text{Expression coefficient of sample}}{\text{Expression coefficient of untreated control}} \times 100$$

The significance of the difference between groups of data was tested using Student's t-test. Statistical significance was considered when $p<0.05$.