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ORIGINAL PAPER

Homeopathic *Rhus toxicodendron* treatment increased the expression of cyclooxygenase-2 in primary cultured mouse chondrocytes

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Background: **Rhus toxicodendron (*Rhus tox*) is a homeopathic remedy with anti-inflammatory activities used for arthritis pain.**
Methods: **We studied the effects of 4×, 30×, 30c and 200c homeopathic dilutions of *Rhus tox* in primary cultured mouse chondrocytes. We examined the expression of collagen type II, a marker protein of chondrocytes, and cyclooxygenase-2 (COX-2), which is responsible for the biosynthesis of prostaglandin E2 (PGE2) and the regulation of the inflammatory response. We assessed the expression of collagen type II and COX-2 using biochemical and immunological methods, such as reverse transcription polymerase chain reaction (RT-PCR), quantitative (or real-time) RT-PCR (qRT-PCR) and immunoblot assays.**
Results: **Stimulation with different concentrations of *Rhus tox* increased the mRNA expression of COX-2, and stimulation with 30× *Rhus tox* showed the most prominent mRNA expression in both RT-PCR and qRT-PCR analyses. We also observed that homeopathic dilutions of 4×, 30× and 30c *Rhus tox* inhibited collagen type II expression, suggesting that *Rhus tox* induced the dedifferentiation of chondrocytes. In addition, treatment with 30× *Rhus tox* significantly increased PGE2 release compared with other homeopathic dilutions of *Rhus tox*.**
Conclusions: **Taken together, these results suggest that homeopathic treatment with *Rhus tox* induced chondrocyte dedifferentiation and inflammatory responses, such as COX-2 expression and PGE2 production, in primary cultured chondrocytes.** *Homeopathy* (2013) **102**, 248–253.

Keywords: *Rhus toxicodendron* (*Rhus tox*); Cyclooxygenase-2 (COX-2); Type II collagenase (col-II); Prostaglandin E2 (PGE2); MTT assay; RT-PCR; Immunoblot analysis

Introduction

Rhus toxicodendron (*Rhus tox*) is another name for the plant *Toxicodendron pubescens*.[1] *Rhus tox* has been extensively used as a traditional homeopathic remedy for inflammatory conditions, including skin eruption, back pain and stiffness, irritability and restlessness, and rheumatoid arthritis.[2–4] *Rhus tox* contains a potent allergen called Urushiol, which is present in the resinous sap of this plant and causes contact dermatitis.[5,6] Recent studies have demonstrated the anti-inflammatory and immunomodulatory effects of *Rhus tox* in experimental animal models.[1,4,6] These studies reported that homeopathic dilutions of 6c, 30c and 200c *Rhus tox* show anti-inflammatory activity, involving histamine and prostaglandin, thus modulating anti-arthritis.[1,4] The previously reported effects of homeopathic dilutions of *Rhus tox*

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were primarily demonstrated through clinical trials using animal models.

Here, we investigated the gene expression of primary cultured mouse chondrocytes stimulated with *Rhus tox* using reverse transcription polymerase chain reaction (RT-PCR) and quantitative (or real-time) RT-PCR (qRT-PCR). Nucleic amplification methods are important in basic research, pharmacogenomics and molecular diagnostics.[7] Reverse transcription (RT), followed by PCR, is a technique used for the analysis of mRNA expression.[8] This method uses gene-specific primers and internal competitor template sets to amplify the target gene and generate conventional RT-PCR products. The quantification of mRNA levels through RT-PCR is an exquisitely sensitive technique, facilitating the analysis of gene expression from small amounts of RNA.[9] The target gene mRNA levels are measured relative to their respective competitor templates, and the measurement of the expression of each gene expression mediates the direct comparison between experiments.[7] Therefore, we applied RT-PCR and qRT-PCR to examine the mRNA expression of collagen type II, which is a unique marker protein for differentiated chondrocytes, and the expression and activity of cyclooxygenases-2 (COX-2), a major inflammatory mediator in arthritis, in primary cultured mouse chondrocytes. COX exists as two isoforms, COX-1 and COX-2.[10] COX-1 is constitutively expressed in most tissues,[10] and COX-2 is stimulated through inflammatory signals; thus, COX-2 expression is primarily involved in inflammation. COX-2 is responsible for the synthesis of the prostanoids (prostaglandins and thromboxanes) involved in pathological processes.[10–12] COX-2 promotes the release of the pro-inflammatory mediator prostaglandin E2 (PGE2), and COX-2 inhibitors suppress PGE2 production.[13] The results of the present study showed that the stimulation of *Rhus tox* in mouse chondrocytes increased COX-2 mRNA and protein expression and enhanced PGE2 production.

Materials and methods

Preparation of reagents

The liquid dilutions of *Rhus tox* at 4×, 30×, 30c, and 200c were purchased from Boiron (Newtown Square, PA, USA) and used for 1:10 dilutions in cell culture media according to the manufacturer's instructions. In addition, the liquid form of *Rhus tox* was supplied in 20% ethanol (EtOH), and we prepared a 20% (v/v) EtOH solution for use in the 1:10 dilution. A final concentration of 2% (v/v) EtOH solution in the cell culture media was used in the control group. Unless otherwise mentioned, all chemicals were purchased from Sigma–Aldrich.

Primary cultures of mouse chondrocytes

The mice were maintained under specific pathogen-free conditions, and all animal experiments were approved through the Gwangju Institute of Science and Technology (Gwangju, Korea, Animal Care and Use Committee). Mouse articular chondrocytes were obtained from 8-day-old mice,[14] and cartilage was isolated from the femoral heads, femoral condyles, and tibial plateau. The cartilage was digested using 0.2% (w/v) collagenase type II, and individual cells were grown in Dulbecco's modified Eagle's media (DMEM; Invitrogen, Carlsbad, CA, USA) containing 10% (v/v) fetal bovine serum and 100 units/100 μg/ml penicillin–streptomycin.

Cell proliferation assay

Cell proliferation was measured using a CellTiter 96 Non-radioactive cell proliferation assay kit to measure changes in absorbance at a specific wavelength using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) according to the manufacturer's instructions (Promega, Madison, WI, USA). Briefly, the cells were plated onto 96-well plates at a density of 1.0×10^4 cells/wells and were cultured for up to 24 h. For the homeopathy remedy, 4×, 30×, 30c, or 200c *Rhus tox* or 2% EtOH, as a control, was added to the cells and co-cultured for 48 h at 37°C in a humidified 5% CO_2 atmosphere. MTT-phenozine methosulfate solution (15 μl/well) was added, and the cells were incubated for 4 h at 37°C in a humidified 5% CO_2 atmosphere. Subsequently, the reaction was terminated upon the addition of 100 μl of Solubilization Solution/Stop Mix. The absorbance was measured at 570 nm using an ELISA reader (BioTek Instrument, Winooski, VT, USA). The data represent the average of three wells in one independent experiment repeated four times.

RT-PCR and qRT-PCR

To examine the COX-2 expression under treatment with various concentrations of *Rhus tox*, we analyzed COX-2 gene expression using RT-PCR and qRT-PCR. Chondrocytes were grown in media containing 4×, 30×, 30c, or 200c homeopathic dilutions of *Rhus tox* or 2% EtOH for 48 h, and total RNA was extracted using an RNeasy kit (Qiagen, Austin, TX, USA) according to the manufacturer's instructions. After extraction, the RNA was converted to cDNA through RT. RT was performed using 1 μg of total RNA with TOPscript RT DryMIX (Enzynomics, Seoul, Korea). The PCR reactions were performed using a GeneAmp PCR System 9700 (Applied Biosystems, Carlsbad, CA, USA) with Ampli*Taq* DNA polymerase (PE Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The following primers (Macrogen, Seoul, Korea) were used for the RT-PCR and qRT-PCR reactions: Glyceraldehyde phosphate dehydrogenase (GAPDH, 587 bp): Sense 5′-TCACGCCACCCAGAAGAC-3′, Antisense 5′-TCAC TGCCACCCAGAAGAC-3′; COX-2: Sense 5′-GGTCTG GTGCCTGGTCTGATGAT-3′, Antisense 5′-GTCCTTTCA AGGAGAATGGTGC-3′. The PCR conditions included denaturation (95°C, 3 min), then amplification and quantification (95°C, 20 s; 62°C, 10 s; 72°C 30 s; 22 cycles for GAPDH and 95°C, 20 s; 63°C, 10 s; 72°C 30 s; 28 cycles for COX-2), followed by a final elongation (72°C for 5 min). The amplified PCR products were visualized on 1.5% agarose gels. The qRT-PCR was performed using an iCycler™ thermal cycler (Bio-Rad Laboratories, Berkeley, CA, USA) and SYBR Premix Ex Taq™ (TaKaRa Bio Inc.,