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Ethyl acetate fraction from Cudrania tricuspidata inhibts IL-1β-induced rheumatoid synovial fibroblast proliferation and MMPs, COX-2 and PGE2 production

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ABSTRACT

Objectives: The objective of this study is to determine the effects of Ethyl acetate fraction from Cudrania tricuspidata (EACT) on the interleukin-1β (IL-1β)-induced proliferation of rheumatoid synovial fibroblasts (RASFs) and production of matrix metalloproteinases (MMPs), cyclooxygenase (COX) and prostaglandin E2 (PGE2) by RASFs.
Materials and Methods: The proliferation of RASFs was evaluated with CCK-8 reagent in the presence of IL-1β with/without EACT. The expression of MMPs, TIMP-1, COXs, PGE2 and intracellular MAPK signalings, including p-ERK, p-p38, p-JNK and NF-kB were examined by immunoblotting or semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) and ELISA in conditions as described above.
Results: EACT inhibits IL-1β-induced proliferation of RASFs and MMP-1, 3, COX-2 mRNA and protein expression, PGE2 production induced with IL-1β. EACT also inhibits the phosphorylation of ERK-1/2, p38, JNK and activation of NF-kB by IL-1β.
Conclusions: These results suggest that EACT might be involved in synovial fibroblast proliferation and MMPs, COX-2, and PGE2 production, which are involved in joint destruction in rheumatoid arthritis (RA), indicating that this might be a new therapeutic modality for management of rheumatoid arthritis.

Key terms: Cudrania tricuspidata, COX, IL-1b, MMPs, PGE2, Rheumatoid Arthritis (RA)

INTRODUCTION

Rheumatoid arthritis (RA) is a chronic inflammatory disease of unknown etiology characterized by chronic synovitis with subsequent articular bone and cartilage destruction [Cawston TE., 1995, Han MK et al., 2003]. Histologically, RA joints are characterized by chronic inflammation, with hyperplasia in the synovial lining cells. Growing evidence suggests that activated rheumatoid synovial fibroblasts (RASFs) actively participate in RA synovitis. RASFs in RA joints aggressively proliferate to form a pannus, which produces inflammatory mediators such as cytokines, matrix-metalloproteinases (MMP) and cyclooxygenase-2(COX-2) and eventually destroys articular bone and cartilage [Cawston TE., 1995, Han MK et al., 2003]. IL-1β is considered the most important cytokine in the pathogenic process of inflammation in rheumatoid arthritis. IL-1β induces proliferation of RASFs, production of high levels of matrix metalloproteinase and prostaglandin E2 (PGE2) via COX expression by RASFs [Choy EH et al., 2001, Firestein GS., 1997].

Cudrania tricuspidata (CT) is a deciduous tree distributed over South Korea, China and Japan and cortex and root bark of CT have been frequently used as a traditional medicine for curing inflammation and tumors [Lee IK et al., 1996]. Several previous reports have suggested the roles of CT extract, including antioxidant activity [Cho EJ et al., 2003], inhibitory effects on nitric oxide synthase (NOS) [Kang DG et al., 2002] and on proliferation of inflammatory immune cells and tumor cells [Chang SH et al., 2008, Seo WG et al., 2001]. However, no studies have investigated the effects of ethyl acetate extract of CT (EACT) on the inflammatory reactions including proliferation of RASFs and production of PGE2 by RASFs, which play a crucial role in the pathogenesis of synovitis in RA.

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In the present study we investigated the effects of EACT on the production of proinflammatory mediators produced by RASFs and proliferation of these cells, after stimulation with IL-1β. PGE2 and COX-2 were studied and intracellular signaling factors were evaluated to define the mechanisms of the effects of EACT. Here we show that EACT can inhibit IL-1β-induced proliferation and inflammatory reactions via MAPK, NF-kB pathways in RASFs.

MATERIALS AND METHODS

Reagents and antibodies

Recombinant human IL-1β was purchased from R&D System (Minneapolis, Minnesota, USA). Monoclonal antibody (mAb) against COX-2 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). mAb against ERK, p-ERK, JNK, p-JNK, p38, p-p38, NF-kB (p65), IkBα and β-actin were purchased from Cell Signaling (Beverly, MD, USA). Fetal bovine serum (FBS) was obtained from Gibco BRL (Life Technologies, Grand Island, NY, USA).

Preparation of crude extract of Cudrania tricuspidata

Cudrania tricuspidata was collected from Jiri Mountain in South Korea and plant material was identified by an authority at the Rheumatology Laboratory and Research Center for Pulmonary Diseases, Chonbuk National University Medical School, Jeonju, Jeonbuk, South Korea. Air-dried stem bark (1 kg) was cut into pieces and extracted with 50% methanol at room temperature for several days. The methanol extract of CT was evaporated and partitioned with n-hexane, benzene, trichloromethane, ethyl acetate and n-butanol, consecutively as described previously [Seo WG et al., 2001]. The ethyl acetate fraction was used in this study after well drying the fraction.

Isolation and culture of RASFs

Synovial tissues obtained at the time of total knee arthroplasty in patients who fulfilled the American College of Rheumatology Criteria for RA [Arnett FC et al., 1988], as previously described [Lee HY et al., 2006]. Synovial fibroblasts from passages 3-7 were used for each experiment and were morphologically homogeneous and had the appearance of RASFs with typical fibroblastoid configuration under inverse microscopy. The purity of the cells was tested by flow cytometry using phycoerythrin (PE)-conjugated anti-Thy-1 (CD90) or anti-CD14 and fluorescein isothiocyanate (FITC)-conjugated anti-CD3 mAb (BD Pharmingen, San Diego, CA). Informed consent was obtained from all patients, and the study protocol was approved by the Chonbuk National University Hospital Ethical Committee.

Cell viability analysis

Cell viability was determined by a CCK-8 kit (Dojindo Laboratories, Japan) according to the manufacturer's instructions. Briefly, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitropenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium (CCK-8) was reduced by dehydrogenases in cells to yield an orange-colored product (formazan) [Hodgkin PD et al., 1990]. The amount of the formazan dye generated by dehydrogenases in cells was directly proportional to the number of living cells. RASFs (1×10^5 cells per well in complete RPMI-1640 media in a 96-well plate) were cultured in 200 mL medium per well without antigen stimulation in the presence or absence of 100 mg/mL EACT for 2 days. CCK-8 (20 mL) was added to each well of the plate and the cells were incubated for 2-3 h. The absorbance was measured at 450 nm using a microplate reader.

RNA Isolation and semiquantitative RT-PCR of COX, MMPs and TIMP

Total RNA was extracted from cultured cells using the TRIsol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's instructions. One microgram of RNA was reverse-transcribed using Maxime RT Premix Kit (iNtRON Biotechnology, Korea). cDNA was amplified using the following primer sets: COX-1 (sense) 5'-GCT ATT CCG GCC CCA ACT-3' (antisense) 5'-GAT GAA GGT GGC ATT GAC AAA CT-3', COX-2 (sense) 5'-TCC TTG CTG TTC CCA CCC ATG-3' (antisense) 5'-CAT CAT CAG ACC AGG CAC CAG-3', MMP-1 (sense) 5'-GAA GGA GAT GAA GCA GCC CAG ATG T-3' (antisense) 5'-CAG TTG TGG CCA GAA AAC AGA AGT GAA A-3', MMP-3 (sense) 5'GAC ACC AGC ATG AAC CTT GTT-3' (antisense) 5'-GGA ACC GAG TCA GGA CTA TG-3', TIMP-1 (sense) 5'-CCT TCT GCA ATT CCG ACC TCG TC-3' (antisense) 5'-CGG GCA GGA TTC AGG CTA TCT GG-3'. PCR products were electrophoresed by using 1% agarose gels and visualized by staining with ethidium bromide. Densitometric analysis was performed on the relative intensity of each band using the Multi Gauge program, version 3.0 (Fuji film, Tokyo, Japan).

Immunoblotting

RASFs (1×10^6 cells) were seeded on 100-mm culture dishes and harvested in phosphate buffered

saline (PBS) after stimulation as described above. Cells were lysed in lysis buffer containing 50 mM Tris-CL, 150 mM NaCl, 5 mM EDTA 1% Triton X-100, 1 mM sodium fluoride, 1 mM sodium vanadate, 1% deoxycholate, and protease inhibitors. To determine the membrane COX-2 expression on RASFs, cell membranes were prepared from isolated RASFs, as described previously [Nakagawa T et al., 2002]. To analyze NF-kB (p65), nuclear extract was prepared using a previously described method [Lee HY et al., 2006]. To determine the cytoplasmic IkBα, cytoplasmic extracts were prepared prepared from isolated RASFs as described previously [Lee HY et al., 2006]. The protein concentration was determined by the Bio-Rad protein assay regent (Bio-Rad Laboratories, USA). Samples (50 mg) were prepared with the four volume of 0.5 M Tris buffer (pH 6.8) containing 4% SDS, 20% glycerol and 0.05% bromophenol blue at 95°C for 5 min. SDS-PAGE was performed in 10% slab gel. Proteins were transferred to nitrocellulose paper. The membrane was washed in blocking buffer (10 mM Tris-HCl pH 8.0, 150 mM NaCl, 5% fat-free milk) for 60 min at room temperature with shaking and then washed with TBST (TBS, 0.01% Tween 20). Primary antibodies (10 mg/ml) against MMP-1, -3, TIMP-1, COX-1, -2, ERK, p-ERK-1/2, p-38, p-p38 MAPK, JNK, p-JNK, NF-kB (p65), IkBα and β-actin was incubated at 4°C for 4 hr. The secondary HRP-conjugated antibody was goat anti-mouse IgG (Stressgen Bioreagents, Ann Arbor, MI, USA). Reactive proteins were detected using enhanced chemiluminescence (ECL, Amersham Life Sciences, Arlington, IL, USA) using Fuji film LAS-3000 (Tokyo, Japan).

Assay of PGE2 production

RASFs were grown in 25 cm^2 tissue-culture flasks for 48 hours before treatment. After washing with PBS (pH 7.4), cells were pretreated with IL-1β (1.0 ng/ml) or EACT (100 mg/ml) at 37°C for 48 hours in DMEM containing 10% (v/v) FCS in an atmosphere of 5% CO_2. The culture supernatant described above was collected at 2 days. The level of PGE2 in the medium was determined by ELISA kit (R&D Systems) in accordance with the instructions of the manufacturer.

Statistical analysis

All data were expressed as the mean ± SD of triplicates and all data were analyzed by the SPSS 12.0 program. Group mean values were compared by Student's t test or ANOVA as appropriate. The significance of difference was defined as p values < 0.05.

RESULTS

EACT inhibits IL-1β-induced proliferation of RASFs

To evaluate the effect of EACT on the growth properties of RASFs, we initially measured the cell proliferation with IL-1β for 3 days. IL-1β is well known as potent growth-promoting factors for RASFs. Cell proliferation was assayed as described in Materials and Methods. As shown in Figure 1A, IL-1β accelerated the proliferation of RASFs dose-dependently (from 0.1 to 10 ng/ml) over time. To know the effect of EACT on IL-1β-induced proliferation of RASFs, EACT (100 μg/ml) was added to the RASFs cultures with/without IL-1β (1.0 ng/ml) for 2 days and CCK-8 assay was performed. As shown in Figure 1B, IL-1β significantly increased the proliferation of RASFs compared to the results of control without IL-1β and EACT ($p < 0.05$). EACT also significantly inhibits the proliferation of RASFs compared

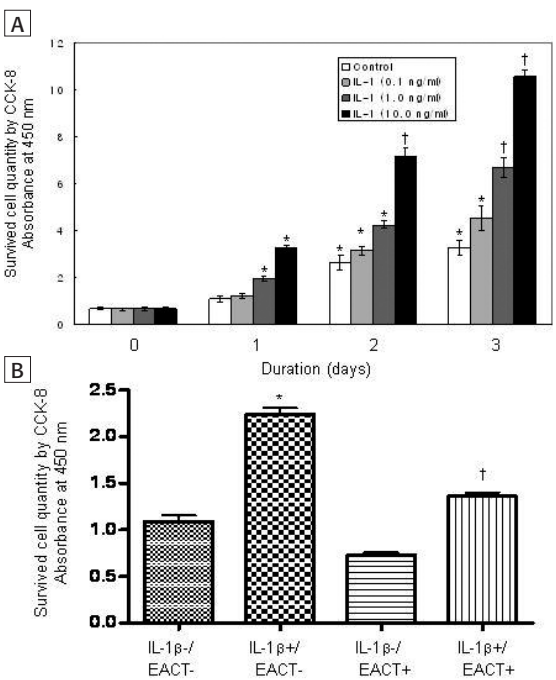

Figure 1: IL-1β increased the poliferation of RASFs dose-dependently and EACT inhibits IL-1β-induced proliferation of RASFs. The proliferation of RASFs was evaluated with CCK-8 kit after culture for 3 days with different concentration of IL-1β (0.1 - 10 ng/mL) (A). The proliferation of RASFs was also evaluated after culture for 2 days with CCK-8 kit with/without IL-1β (1.0 ng/mL) and EACT (100 ug/mL) (B). Results are Presented as mean ± SD, n = 3, *p<0.01 versus no IL-1β and EACT, †p<0.05 versus IL-1β without EACT.

to the results with IL-1β ($p < 0.05$). To ascertain dose-dependent effect of EACT on IL-1β-induced proliferation of RASFs, different dose of EACT (50, 100, 200 μg/ml) was added to the RASFs cultures with IL-1β (1.0 ng/ml) for 2 days and CCK-8 assay was performed. The inhibitory effects of EACT were significantly enhanced by increasing concentration of EACT (data not shown).

Effects of EACT on IL-1β-induced MMPs, TIMP-1 and COX mRNA expression in RASFs

Real-time PCR was performed to evaluate the expression of MMP-1, 3 and TIMP-1 mRNA in the monocultured RASFs. RASFs were stimulated with IL-1β (1.0 ng/ml) for 12 h in the presence or absence of EACT (100 μg/ml). IL-1β enhanced the expression of MMP-1, 3 mRNA in RASFs ($p < 0.05$), but not TIMP-1. EACT inhibited the effects of IL-1β on the expression of MMP-1, 3 mRNA ($p < 0.05$ and $p < 0.01$, Fig. 2A). IL-1β also enhanced the expression of COX-2 mRNA in RASFs ($p < 0.01$), but not COX-1 (data not shown). EACT inhibited IL-1β-induced expression of COX-2 mRNA ($p < 0.05$, Fig. 2A).

Effects of EACT on IL-1β-induced MMPs, TIMP-1 and COX protein expression in RASFs

To further evaluate the expression of MMPs, TIMP-1 and COX proteins in mono-cultured RASFs, we performed immunoblotting. RASFs were stimulated with IL-1β (1.0 ng/ml) for 48 h in the presence or absence of EACT (100 μg/ml). IL-1β enhanced the expression of MMP-1, 3 proteins in RASFs ($p < 0.05$), but not TIMP-1 protein, as with the results with mRNA expression. EACT inhibited IL-1β-induced expression of MMP-1, 3 proteins ($p < 0.05$ and $p < 0.01$, Fig. 2B). IL-1β also enhanced the expression of COX-2 protein in RASFs ($p < 0.05$), but not COX-1 (data not shown). EACT inhibited IL-1β-induced expression of COX-2 protein ($p < 0.05$, Fig. 2B).

EACT inhibits IL-1β-induced PGE2 production in RASFs

To confirm the effect of EACT on the role of IL-1β in PGE2 production by RASFs, we examined the concentration of PGE2 in culture supernatant. RASFs (1×10^4 cells) were grown in 25 cm^2 tissue-culture flasks for 48 hours before and after treatment with IL-1β (1.0 ng/ml) or/and EACT (100 μg/ml). PGE2 production was increased after IL-1β treatment ($p < 0.05$) in comparison to controls, and it was significantly inhibited by treatment with EACT at 48 hours, as was expected with the results of COX-2 expression (Fig. 3).

Effects of EACT on IL-1β-induced signal pathways in RASFs

To demonstrate the involvement of the signal transduction and mechanisms of the effects of EACT on IL-1β-induced RASFs proliferation, MMPs, COX-2 expression and PGE2 production, activation of MAPKs and NF-kB were evaluated in RASFs.

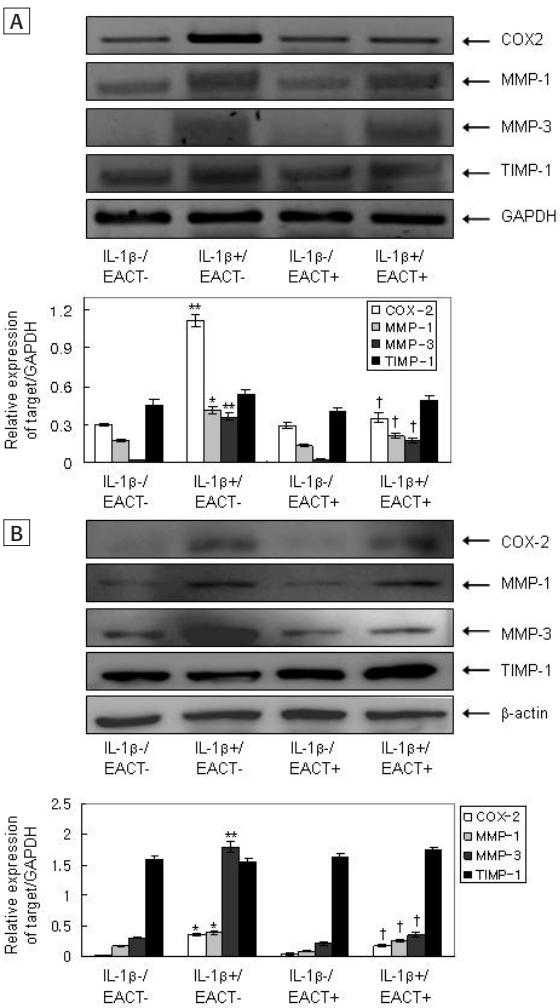

Figure 2: EACT inhibited IL-1β-induced production of MMPs and COX-2 in RASFs. To evaluate the expression of MMP-1, 3, TIMP-1 and COX-1, 2 protein, immunoblotting was performed. RASFs were cultured for 2 days with/without IL-1β (1.0 ng/mL) and EACT (100 ug/mL). The RNA and protein were extracted from RASFs and analysed by RT-PCR (A) and Western blotting (B). IL-1β enhanced the expression of MMP-1, 3, TIMP-1 and COX-2 protein compared with the results without IL-1 β. EACT inhibited the IL-1β-induced expression of MMP-1. 3 and COX-2 protein. Results are presented as mean ± SD. n = 3. *p < 0.05 or

IL-1β activated the intracellular MAPKs including ERK, p-38 and JNK and EACT significantly inhibits the IL-1β-induced intracellular MAPKs activation (Fig. 4A). Activation of NF-kB p65 and degradation of cytoplasmic IkBα was observed in RASFs treated with IL-1β. These effects of IL-1β on NF-kB activation were abrogated by EACT (Fig. 4B). These results indicate that EACT might inhibit IL-1β-induced proliferation of RASFs, expression of COX-2 and production of PGE2 via intracellular MAPKs and NF-kB pathways.

DISCUSSION

We show here that ethyl acetate extract of Cudrania tricuspidata (EACT) inhibits IL-1β-induced cell proliferation, COX-2 expression and PGE2 production in RASFs by inhibition of activation of the MAP kinases ERK1/2, p-38 and JNK and NF-kB signaling pathways. These findings suggest that EACT can be used as a new therapeutic agent for management of RA by decreasing inflammation.

Cudrania tricuspidata (CT) is a deciduous tree distributed over South Korea, China and Japan and cortex and root bark extract of CT have been frequently used as a traditional medicine for curing inflammation, gastritis, and tumors in East Asian countries [Lee IK et al., 1996]. The pharmacological action of CT extract has seldom been examined. Several previous reports suggested the roles of CT extract, including antioxidant activity [Cho EJ et al., 2003], inhibitory effects on nitric oxide synthase (NOS) [Kang DG et al., 2002] and on proliferation of tumor cells [Seo WG et al., 2001]. There have been limited reports on the function and action of CT extract in immune mechanisms. Chang et al. [Chang SH et al., 2008] reported that CT extract could inhibit the proliferation of antigen-mediated spleen and T cells, which can accelerate inflammation and also decrease the production of the pro-inflammatory cytokines IL-2 and IFN-γ in antigen-mediated T cells. Although these results suggest that CT extract has effects on immune-mediated inflammatory joint

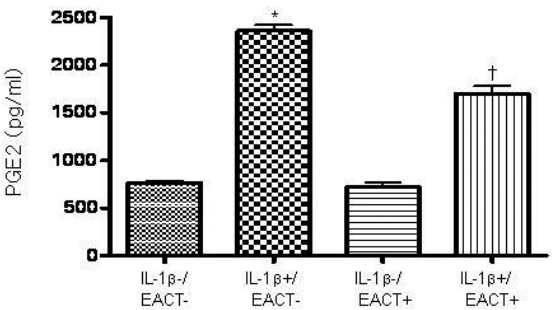

Figure 3: PGE2 production was inhibited by treatment with EACT. The production of PGE2 by RASFs was evaluated with ELISA kit. RASFs were cultured for 2 days with/without IL-1β (1.0 ng/mL) and EACT (100 mg/mL). Results are presented as mean ± SD, n = 3, *p < 0.05 versus no IL-1β and EACT, †p < 0.05 versus IL-1β without EACT.

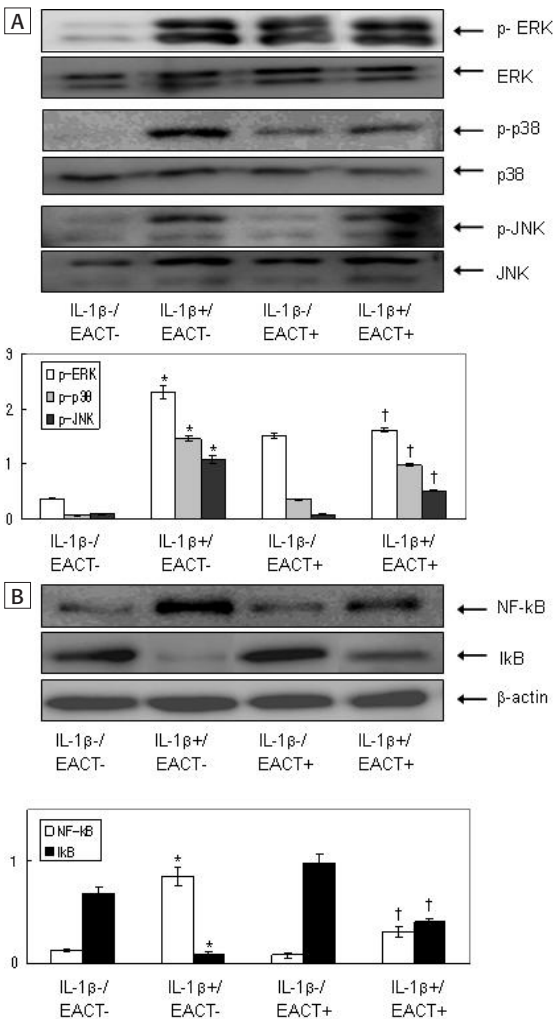

Figure 4: Effects of EACT on IL-1β-induced MAPK signal pathways and NF-kB activation in RASFs. To evaluate the mechanism of EACT on IL-1β in RASFs, MAPKs signaling pathways (ERK, p38 and JNK) and NF-kB and IkBα were evaluated by immunoblotting. RASFs were cultured for 10 min with/without IL-1β (1.0 ng/mL) and EACT (100 ug/mL). IL-1β enhanced the phosphorylation of ERK, p38 and JNK and EACT inhibited the IL-1β-induced activation of p38 and JNK (A). IL-1β activated nuclear NF-kB and decreased cytoplasmic IkBα and EACT inhibited the IL-1β-induced activation of NF-kB (B). Results are presented as mean ...

diseases, including RA, a possible pharmacological mechanism by which CT can cure inflammation is still unknown and remains to be established. No studies have investigated the effects of CT on inflammatory reactions, including proliferation of RASFs and production of PGE2 by RASFs, which play a crucial role in the pathogenesis of synovitis in RA.

In RA, one of the most striking features is the hyperplasia of synovial fibroblasts in the lining layer [Cawston TE., 1995, Han MK et al., 2003]. RASFs play a crucial role in the physiopathology of rheumatoid arthritis, as these cells are involved in inflammation and joint destruction. The tumor-like proliferation of RASFs is considered to be the major mechanism for the hyperplasic growth of the RA synovium and eventually destroy the articular bones and cartilage [Choy EH et al., 2001, Firestein GS., 1997]. IL-1β played an important role in the pathogenesis of inflammatory synovitis joint destruction in RA by inducing the proliferation of fibroblast cell lines [Gitter BD et al., 1989]. This study shows that EACT significantly inhibits IL-1β-induced proliferation of RASFs in a dose- and time-dependent manner. This probably means that EACT would be an ideal treatment for RA. However, further study is required to know the effects of EACT on RASF proliferation in the absence or presence of stimulating factors and to define exact mechanism.

It has been well known that both COX-1 and COX-2 are expressed by human RASFs and that the expression of COX-2 is enhanced by proinflammatory cytokines, such as IL-1β [Crofford LJ et al., 1994]. COX-2 converts free arachidonic acid into prostaglandins, including a variety of bio-active products (PGI2, thromboxane A2 (TXA2), PGE2 and PGD2). PGE2, a pleiotropic mediator of inflammation, provides certain homeostatic functions, but its excessive production in the joints of RA patients is associated with many pathologic processes and plays a critical role in eliciting the signs and symptoms of inflammation [Martel-Pelletier J et al., 2004]. This study found that EACT inhibits both IL-1β-induced COX-2 protein expression and PGE2 synthesis dose-dependently over time. There were more than 30 volatile components in EACT and 8 compounds were reported to have anti-inflammatory effects [Chang SH et al., 2008].Thus, further studies are also needed to define which components from the EACT are responsible for the our results. It also necessary to demonstrate the above effects on inflammatory joint disease with *in vivo* systems, such as animal model of RA, collagen-induced arthritis.

NF-kB and MAPKs participate in inflammation and destruction of joints in RA. It is known that the inactive NF-kB normally binds to IkB in the cytosol, and NF-kB can be activated by proinflammatory cytokines, IL-1β and TNF-α [Verma IM et al., 1995]. JNK, p38 and ERK are expressed in cultured RASFs and are readily activated by IL-1β [Han Z et al., 2001]. Prostaglandins have also been described as being under the influence of p38 MAPK [Guan Z et al., 1997]. This has been confirmed in a study in which it was reported that glucocorticoids destabilize cyclo-oxygenase-2 (COX-2) mRNA by inhibiting the p38 MAPK route [Lasa M et al., 2001]. Numerous studies have demonstrated that inhibitors of MAPKs or NF-kB decrease synovial inflammation, bone destruction, and cartilage damage in animal models of arthritis, including adjuvant arthritis in rats and CIA in mice [McIntyre KW et al., 2003, Nishikawa M et al., 2003]. To define the mechanisms of the effects of EACT on IL-1β-induced RASFs proliferation, expression of COX-2 and production of PGE2, the activation of the MAPKs and NF-kB was examined. This study shows that EACT inhibits the IL-1β-induced activation of NF-kB and phosphorylation of ERK, p38 and JNK. However, further studies are needed to define what components of EACT are more important and how EACT can inhibit the effects of IL-1β on RASFs. Further investigation is also needed to discern how EACT suppresses NF-kB activation, which components of NF-kB are suppressed, and what kind of intracellular signaling factors is specifically or directly involved in the effect of EACT on the proliferation and PGE2 production in RASFs.

This is the first study to report that CT can inhibit the IL-1β-induced proliferation, expression of COX-2 and production of PGE2 in RASFs. This study also shows that CT also inhibits the activation of NF-kB and phosphorylation of MAPKs pathways. Taken together, these findings suggest that CT may be useful in the treatment of inflammatory diseases, including RA. However, further studies are required to define the exact mechanism underlying the inhibition of synovial cell proliferation and inflammatory reactions, and to find active components in the EACT.

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