Chlorogenic acid induces apoptosis to inhibit inflammatory proliferation of IL-6-induced fibroblast-like synoviocytes through modulating the activation of JAK/STAT and NF-κB signaling pathways

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Abstract. Chlorogenic acid (CGA) is the primary constituent of Caulis *Lonicerae*, a Chinese herb used for the treatment of rheumatoid arthritis (RA). The present study aimed to investigate whether CGA was able to inhibit the proliferation of the fibroblast-like synoviocyte cell line (RSC-364), stimulated by interleukin (IL)-6, through inducing apoptosis. Following incubation with IL-6 or IL-6 and CGA, the cellular proliferation of RSC-364 cells was detected by MTT assay. The ratio of apoptosed cells were detected by flow cytometry. Western blot analysis was performed to observe protein expression levels of key molecules involved in the Janus-activated kinase/signal transducer and activator of transcription 3 (JAK/STAT) signaling pathway [phosphorylated (p)-STAT3, JAK1 and gp130] and the nuclear factor κB (NF-κB) signaling pathway [phosphorylated (p)-inhibitor of κB kinase subunit α/β and NF-κB p50). It was revealed that CGA was able to inhibit the inflammatory proliferation of RSC-364 cells mediated by IL-6 through inducing apoptosis. CGA was also able to suppress the expression levels of key molecules in the JAK/STAT and NF-κB signaling pathways, and inhibit the activation of these signaling pathways in the inflammatory response through IL-6-mediated signaling, thereby resulting in the inhibition of the inflammatory proliferation of synoviocytes. The present results indicated that CGA may have potential as a novel therapeutic agent for inhibiting inflammatory hyperplasia of the synovium through inducing synoviocyte apoptosis in patients with RA.

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Introduction

Rheumatoid arthritis (RA) is an inflammatory disease characterized by chronic inflammation of the synovial joints and destruction of cartilage and bone that affects ~1% of the population worldwide (1). Despite the advent of anticytokine therapies that ameliorate the inflammatory manifestations of RA, RA remains incurable (2). Proliferation of synovial cells and infiltration of activated immunoinflammatory cells result in the progressive destruction of cartilage and bone (3). Fibroblast-like synoviocytes (FLSs) have a key role in this process through producing cytokines that perpetuate autoimmune inflammation and proteases that contribute to cartilage destruction (4). Synovial hyperplasia in RA is also considered to impair apoptosis of FLSs (5,6). Proliferation of FLSs in RA occurs as a result of the imbalance between cell proliferation, survival and death. The synovial environment in RA is beneficial to FLS survival and inhibits FLS apoptosis, and is, thus, involved in preventing their elimination. Novel therapeutic strategies targeting FLSs should aim to be effective in inflammatory arthritis without suppressing systemic immunity.

Interleukin-6 (IL-6) is a pleiotropic cytokine that has multiple biological functions, including involvement in the development of the nervous and hematopoietic systems, and acute-phase, inflammatory and immune responses (7). As a proinflammatory cytokine, IL-6 is produced by a variety of cell types in the inflamed RA synovial microenvironment, including macrophages, FLSs and chondrocytes (8). Previous reports have indicated that synovial fluid levels of IL-6 are increased in patients with RA and may be associated with progressive joint damage (9,10). Anti-IL-6 receptor antibody

treatment of patients with RA also revealed a clinically significant therapeutic effect (11,12). In addition, IL-6 was observed to contribute to various models of antigen-induced RA, including collagen-induced and adjuvant-induced arthritis (13,14). gp130 is a receptor subunit and signal transducer for the cytokines of the IL-6 family, and is involved in the Janus-activated kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) signaling pathway. STAT3 has an important role in cell survival, growth and differentiation, and is also associated with osteoclastogenesis (15). Hence, modulation of the activation of the IL-6/gp130/STAT3 signaling pathway is likely to be a potent therapeutic strategy in the treatment of RA. Nuclear factor-κB (NF-κB) is also involved in inflammation, cell survival, proliferation and differentiation. NF-κB regulates the expression levels of several proinflammatory gene clusters, including cytokines, chemokines, adhesion molecules and nitric oxide synthases. In addition, activation of NF-κB is associated with chronic inflammatory disorders in RA. Furthermore, animal models of RA, including collagen- and adjuvant-induced arthritis rat models, indicated its role in synovial inflammation (16,17). Thus, the NF-κB and JAK/STAT signaling pathways appear to be involved in the inflammatory proliferation of FLSs induced by inflammatory cytokines.

Chlorogenic acid (CGA) is one of the most abundant polyphenols and exists widely in medicinal herbs. Several studies have shown that CGA exhibits a wide range of biological activities, including potent immunoprotective, anti-inflammatory, anti-bacterial and anti-oxidant activities (18-20). However, there are no reports regarding the inhibitory effects of CGA on the proliferation of FLSs through inducing apoptosis in RA, or the associated molecular mechanism.

In the present study, a rat FLS cell line (RSC-364) stimulated by IL-6 was used to observe the ability of CGA to suppress the proliferation of FLSs induced by proinflammatory cytokines by triggering apoptosis. The modulated function of the aforementioned complex on the activation of JAK/STAT and NF-κB signaling pathways were also detected. Therefore, the present study aimed to investigate the effect and mechanism of CGA on inhibiting IL-6-mediated proliferation of FLSs through inducing apoptosis, and investigate the potent inhibitory therapeutic function of CGA on FLS proliferation in inflammatory hyperplasia of the synovium in RA.

Materials and methods

Preparation of IL-6 and CGA. The purities of CGA (National Institutes for Food and Drug Control, Beijing, China) were detected using an API 3200 LC/MS/MS System (AB SCIEX, Ontario, Canada). A total of 1 mol/l CGA was dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA) and 100 mg/l IL-6 was dissolved in Dulbecco's modified Eagle's medium (DMEM; both Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The solutions were diluted to final concentrations of 0, 5, 10, 25, 50 and 100 μmol/l CGA, and 0, 0.1, 1, 2.5, 5 and 10 μg/l IL-6.

Cell culture. The rat FLS cell line, RSC-364 (21,22), used in the present study was a generous gift from Dr Jingxiang Huang (Chinese People's Liberation Army General Hospital, Bejing, China). Cells were cultured in DMEM containing 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich) at 37˚C in a humidified atmosphere of 5% CO_2 in air. When cells reached 90% confluency, they were harvested with trypsin/EDTA (both Thermo Fisher Scientific, Inc.) and subcultured at a split ratio of 1:3 into new flasks.

Cell viability assay. Cell viability assays were detected using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method. Briefly, RSC-364 cells were seeded into a 96-well plate at a density of 4,000 cells/well. Following treatment with various concentrations of CGA (5, 10, 25, 50 and 100 μmol/l) and IL-6 (0.1, 1, 2.5, 5 and 10 μg/l) for 24 h at 37˚C, cells were added to wells with 20 μl MTT (5 mg/ml) per well and incubated for an additional 4 h. Subsequently, after discarding the supernatant, cells were lysed in 100 μl DMSO and absorbance was detected at 492 nm using a Varioskan Flash microplate reader (Thermo Fisher Scientific, Inc.).

Flow cytometric analysis. RSC-364 cells were plated at a density of $1x10^5$ cells in 25 cm^2 flasks and incubated for 15-17 h in a tissue culture incubator at 37˚C with an atmosphere of 5% CO_2. The media was discarded and cells were washed twice with phosphate-buffered saline. Cells were incubated for 24 h in DMEM containing 3% FBS with 2.5 μg/l IL-6 alone, or 2.5 μg/l IL-6 in combination with 25 μmol/l CGA. The final concentrations of IL-6 and CGA were determined by the results of the cell viability assay. RSC-364 cells were stained with fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI). Cells were trypsinized and collected by centrifugation for 5 min at 800 x g for the detection of apoptotic cells using an Annexin V-FITC Apoptosis Detection kit (eBioscience, Inc., San Diego, CA, USA). Briefly, RSC-364 cells were washed twice with cold PBS at 4˚C and resuspended in 500 μl binding buffer [10 mmol/l HEPES-NaOH (pH 7.4), 140 mmol/l NaCl, 2.5 mmol/l $CaCl_2$] at a concentration of $1x10^6$ cells/ml. After the addition of 5 μl Annexin V-FITC solution and PI (1 μg/ml), cells were incubated for 15 min at room temperature and then analyzed by flow cytometry (FC 500; Beckman Coulter, Inc., Fullerton, CA, USA). All experiments were performed in triplicate with three replicates each.

Western blot analysis. Cells were harvested and stimulated, as described in the previous paragraph. Following treatment, whole cell lysates from $1x10^6$ RSC-364 cells were generated using a Total Protein Extraction kit (Thermo Fisher Scientific, Inc.) supplemented with 1X complete protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The protein concentration was determined using a Pierce BCA Protein Assay kit (Thermo Fisher Scientific, Inc.). Equal quantities of protein samples (40 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Sigma-Aldrich). Electrophoresis was initially performed at 40 V constant voltage. Once the dye had passed through the stacking gel, the voltage was increased to 80 V until the dye ran off the bottom of the gel (<2 h). The protein samples were subsequently transferred to nitrocellulose membranes (EMD Millipore, Billerica, MA, USA) and the membranes were blocked with 5% skimmed

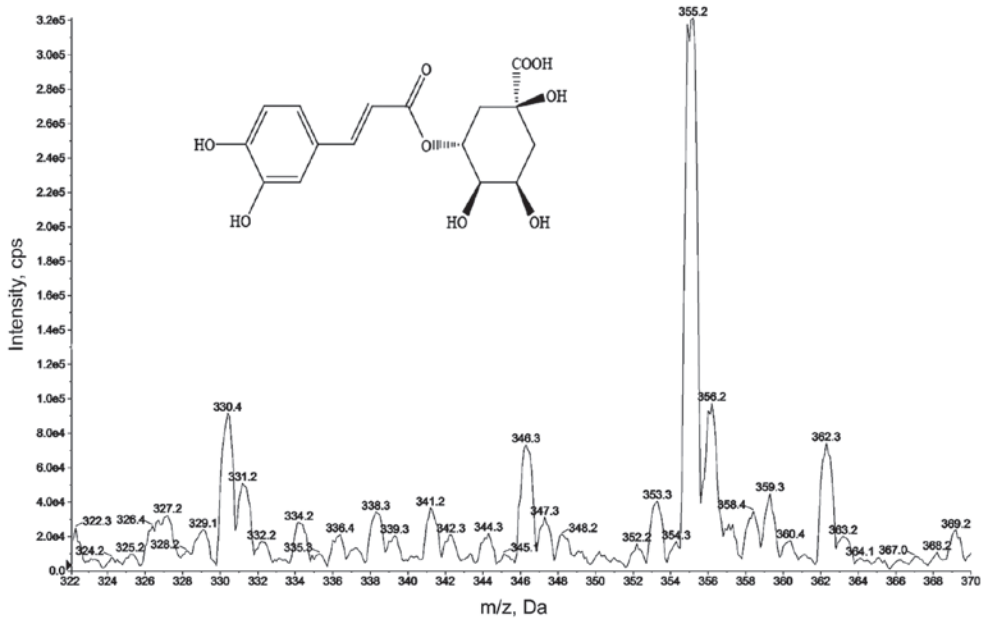

Figure 1. Structural formula and purity of chlorogenic acid (CGA). CGA is a natural phenolic acid with chemical formula $C_{16}H_{18}O_9$. The purity of CGA was determined by mass spectrometric analysis. cps, counts per second; m/z, mass-to-charge ratio.

milk powder (Applygen Technologies Inc., Beijing, China) and incubated with primary antibodies at 4˚C for 12 h. Antibodies involved in i) JAK/STAT signaling, including gp130 (1:1,000; 3732), JAK1 (1:1,000; 3344), phosphorylated (p)-STAT3 (1:1,000; 4093) and p-inhibitor of κB kinase subunit α/β (IKKα/β; 1:1,000; 2697) antibodies; and ii) NF-κB signaling, including NF-κB p50 antibody (1:1,000; 4717), were used. Membranes were incubated with glyceraldehyde 3-phosphate dehydrogenase (GAPDH; 1:1,000; 2118) antibody as the loading control. All primary rabbit monoclonal antibodies were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA). Then, the membranes were incubated with the horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:5,000; BA1055; Boster Biological Technology, Inc., Wuhan, China) for 1 h at room temperature. The immunoreactive proteins were enhanced by SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, Inc.). Each experiment was performed in triplicate with three replicates each. Densitometry values were quantified for each band using Image-Pro Plus version 4.0 (Media Cybernetics, Rockville, MD, USA).

Statistical analysis. All data are presented as mean ± standard deviation. The statistical analyses were performed using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance followed by the Tukey-Kramer test for multiple comparisons were used to compare the treatment groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Chemical structures of CGA. CGA is the major component of several medicinal herbs, such as Caulis *Lonicera*, which is commonly used in treatment of RA by traditional Chinese medical practitioners. CGA ($C_{16}H_{18}O_9$) is one of the most abundant phenolic acids in nature (23) and its molecular weight is 354.31 Da. The purity of CGA was detected using an API 3200 LC/MS/MS System. As revealed in Fig. 1, the greatest peak is evident at 355.2 Da, providing confirmation that the purity of CGA used in the present study was suitable for use in experimental applications.

Selection of the appropriate stimulating concentrations of IL-6 and CGA. To investigate the effect of IL-6 on the proliferation of RSC-364 cells and to determine the appropriate concentration of CGA, an MTT assay was performed to detect the degree of proliferation of RSC-364 cells stimulated by IL-6 at different concentrations, in addition to the cytotoxicity experienced by cells treated with CGA. As displayed in Fig. 2A, RSC-364 cells proliferated significantly after 24 h of incubation with varying concentrations of IL-6 compared with those cultured in normal medium. The degree of proliferation of RSC-364 cells treated with IL-6 (2.5 μg/l) increased most significantly, as compared with untreated cells ($P=0.003$). The toxicity to cells was evident after 24 h of treatment with CGA at concentrations of 50 and 100 μmol/l (Fig. 2B). The results indicated that concentrations of 2.5 μg/l IL-6 and 25 μmol/l CGA were most suitable for stimulating RSC-364 cells in the present study.

Ability of CGA to induce apoptosis of RSC-364 cells stimulated by IL-6. RSC-364 cells were treated with IL-6 alone or in combination with CGA, and apoptosis was detected in the cells by Annexin V/PI staining. The ratios of apoptosis were observed by flow cytometry. As displayed in Fig. 3, CGA appeared to significantly induce apoptosis in RSC-364 cells stimulated by IL-6 compared with IL-6 stimulation alone (P-0.011). By contrast, the ratio of apoptosis in RSC-364 cells was significantly reduced after 24 h of IL-6 treatment compared with untreated cells ($P=0.037$). The results revealed that CGA was able to increase the ratio of apoptosis significantly in RSC-364 cells stimulated by IL-6 compared with those incubated with IL-6 alone.

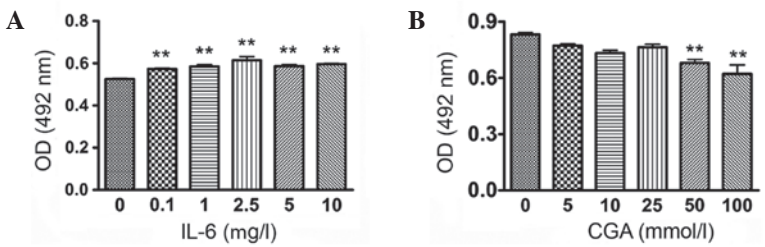

Figure 2 Determination of appropriate treatment concentrations of IL-6 and CGA. Viability of RSC-364 cells was determined following stimulation with (A) IL-6 (0, 0.1, 1, 2.5, 5, 10 μg/l) and (B) CGA (0, 5, 10, 25, 50 and 100 μmol/l) at different concentrations. Data are expressed as the mean ± standard deviation of individual groups (n=6 per group). **P<0.01 vs. cells cultured in normal medium. OD, optical density; IL-6, interleukin-6; CGA, chlorogenic acid.

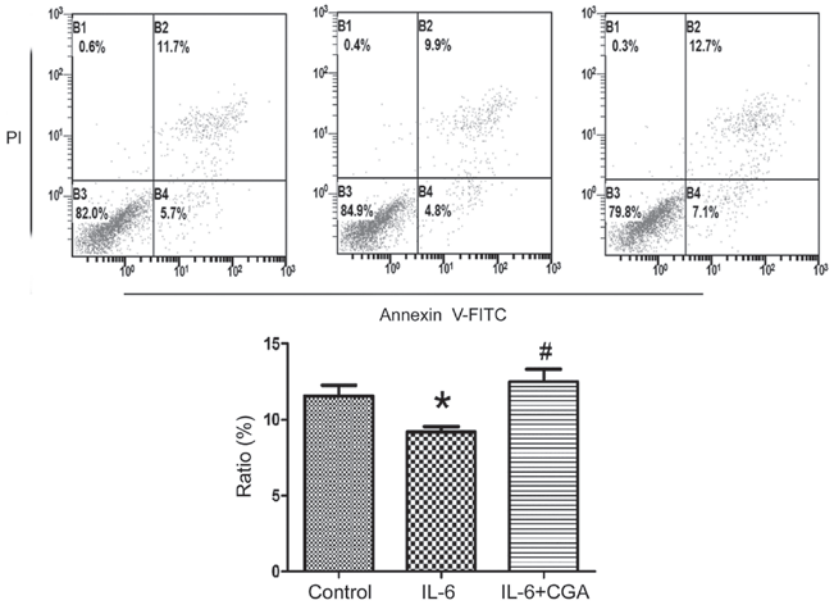

Figure 3. CGA induces apoptosis in RSC-364 cells stimulated by IL-6. RSC-364 cells were stained with FITC-conjugated Annexin V and PI after 24 h of treatment. The Annexin V$^+$PI$^+$ cells indicated apoptotic cells. The apoptosis rates of RSC-364 cells were detected by flow cytometry. Data are expressed as the mean ± standard deviation of individual groups (n=3 per group). *P<0.05 vs. cells cultured in normal medium (control); $^\#$P<0.05 vs. cells stimulated by IL-6 alone. PI, propidium iodide; FITC, fluorescein isothiocyanate IL-6, interleukin-6; CGA, chlorogenic acid.

CGA modulates the activation of the JAK/STAT signaling pathway. Following the observation of the effects of CGA on the apoptosis of RSC-364 cells, protein expression levels of key molecules of JAK/STAT signaling were detected in RSC-364 cells after 24 h of treatment. As is evident in Fig. 4, significantly increased expressions levels of p-STAT3, JAK1 and gp130 were detected in RSC-364 cells after 24 h of treatment with IL-6, compared with those cultured in normal medium (P=0.013, P=0.002 and P=0.001, respectively). However, the high expression levels of the p-STAT3, JAK1 and gp130 were inhibited significantly by CGA, compared with IL-6 treatment alone (P=0.002, P=0.048 and P=0.002, respectively).

Effect of CGA on suppressing the activation of the NF-κB signaling pathway. The ability of CGA to modulate the expression levels of key molecules of the NF-κB signaling pathway in RSC-364 cells stimulated by IL-6 was also investigated. The expression levels of NF-κB p50 and p-IKKα/β were increased significantly after 24 h of IL-6 stimulation (P=0.001 and P=0.003, respectively; Fig. 5) compared with cells cultured in normal medium. Following incubation with IL-6 and CGA for 24 h, the expression levels of NF-κB p50 and IKKα/β in RSC-364 cells were suppressed significantly compared with those cultured in IL-6 alone (P=0.003 and P=0.026, respectively; Fig. 5).

Discussion

RA is a chronic autoimmune joint disease affecting ~1% of the global population, and involves the small joints of the hands and feet. The etiology of RA has yet to be elucidated, however, genetic and environmental influences have been demonstrated to participate. RA is characterized by persistent inflammation of the synovial tissues of joints, resulting in loss of joint function (24). FLSs have an important role in the initiation and perpetuation of RA, and are characterized by resistance to apoptosis, consequential overexpansion and the destruction of articular cartilage. Synovial tissue is comprised of two layers; the intimal lining and the sublining. The intimal lining of the synovium displays marked changes during RA, characterized by an increase in cellularity. There are two cell types present in the aforementioned structure, termed type A macrophage-like cells and type B fibroblast-like cells. Numerous reports indicate that type A cells predominate in RA by assisting the migration

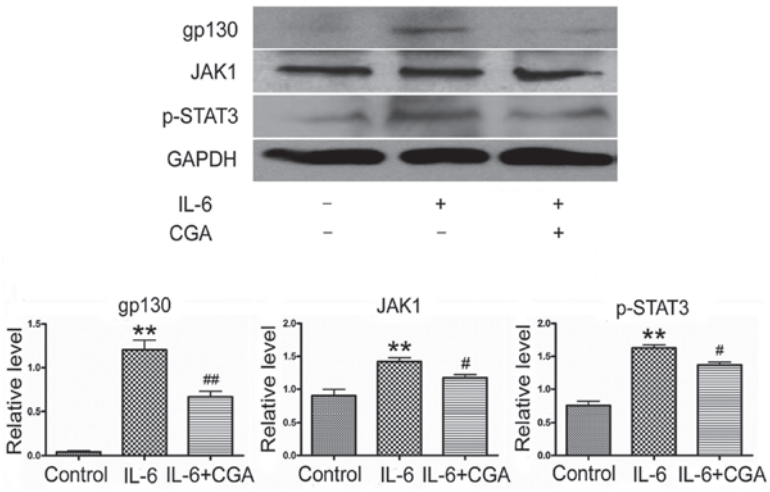

Figure 4. Effect of CGA on blocking the JAK/STAT-mediated proinflammatory responses through IL-6-mediated signaling. Western blotting was used to detect the protein expression levels of p-STAT3, JAK1 and gp130 in RSC-364 cells after 24 h of treatment. Data are expressed as the mean ± standard deviation from three separate experiments. **P<0.01 vs. cells cultured in normal medium (control); $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. cells stimulated with IL-6 alone. JAK1, Janus-activated kinase 1; p-STAT3, phosphorylated-signal transducer and activator of transcription 3; IL-6, interleukin-6; CGA, chlorogenic acid.

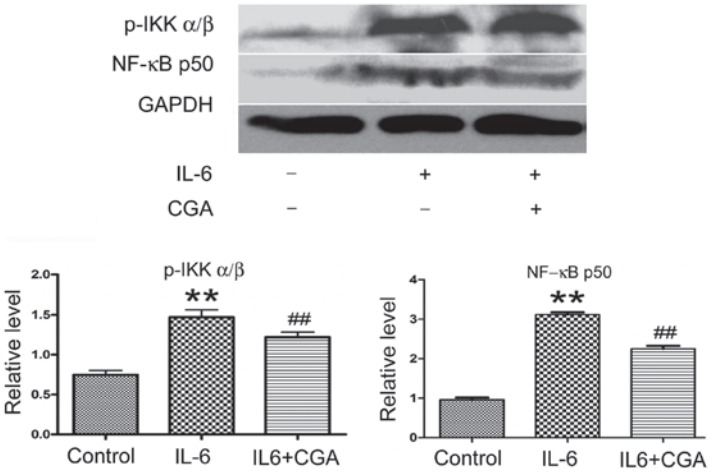

Figure 5. Effect of CGA on suppressing the activation of the NF-κB signaling pathway in RSC-364 cells through IL-6 stimulation. Western blotting was used to detect the protein expression levels of p-IKKα/β, NF-κB p50 in RSC-364 cells after 24 h. Data represent means ± standard deviation from three separate experiments. **P<0.01 vs. cells activated in normal medium (control); $^{\#\#}$P<0.01 vs. cells stimulated with IL-6 alone. NF-κB, nuclear factor κB; p-IKKα/β, phosphorylated-inhibitor of κB kinase subunit α/β; IL-6, interleukin-6; CGA, chlorogenic acid.

of new cells from the bone marrow through the circulation, and producing proinflammatory cytokines, chemokines and growth factors (5,6). The aforementioned factors activate FLSs in the lining and subsequently produce array of mediators, including IL-6, prostanoids and matrix metalloproteinases (25). Therefore, the pathological reaction is likely to perpetuate synovitis, recruit new cells to the joint and contribute to the destruction of the extracellular matrix (26). The synovial microenvironment in RA supports FLS survival and inhibits their elimination through apoptosis (27). As a result, proliferation of FLS is difficult to control, leading to progressive aggravating inflammation in the lining of synovium, and the destruction of cartilage and bone.

CGA is the major effective component in Caulis *Lonicera*, a Chinese herb commonly used in Chinese medicine for the treatment of RA. According to several reports, CGA has been identified to possess pharmacological activity, including anti-inflammatory, anti-oxidative and anti-carcinogenic effects (28-30). In the present study, the ability of CGA to induce apoptosis for the purpose of inhibiting inflammatory proliferation of IL-6-induced FLS was investigated. The results of the present study revealed that CGA was able to inhibit IL-6-mediated proliferation of RSC-364 cells significantly through promoting cell apoptosis. The aforementioned results indicate that CGA may be a promising agent for the control of inflammatory hyperplasia of the synovium in RA.

IL-6 is a pleiotropic proinflammatory cytokine produced by several cell types in the RA synovial microenvironment, including macrophages, FLSs and chondrocytes (8). IL-6 exerts its function via the signal transducer, gp130, leading to the activation of the JAK/STAT signaling cascade. The complex of IL-6 and its receptor homodimerizes with gp130 at the cell surface, and subsequently transduces the signal for the activation of intracytoplasmic JAK tyrosine kinase. JAK tyrosine kinase is recruited and preferentially induces tyrosine

phosphorylation of STAT3. Subsequently, p-STAT3 is dimerized. The dimers of p-STAT3 translocate to the nucleus where they bind to the promoter regions and initiate the transcription of their target genes. Previous studies have revealed that JAK inhibition has a prominent effect on autoimmune diseases, including RA (31,32) The present results revealed that the expression levels of gp130, JAK1 and p-STAT3 in FLS cells induced by IL-6 were suppressed significantly after 24 h of treatment with CGA. Therefore, the results of the present study indicate that CGA may be useful for blocking JAK/STAT-mediated proinflammatory responses through IL-6-stimulated signaling in RA.

NF-κB is also involved in the pathophysiology of RA and has an important role in the induction of proinflammatory cytokines. NF-κB proteins include NF-κB2 p52/p100, NF-κB1 p50/p105, c-Rel, RelA/p65 and RelB. These proteins function as dimeric transcription factors that control several genes, regulating a broad range of biological processes, including innate and adaptive immunity, in addition to inflammation. NF-κB proteins are bound and inhibited by inhibitor of κB (IκB) proteins. Proinflammatory cytokines activate the IKK complex (IKKα and IKKβ), which can induce the phosphorylation of IκB proteins. The phosphorylation of IκB results in its ubiquitination and proteasomal degradation, releasing the NF-κB dimers. Active NF-κB dimers are further activated by phosphorylation and translocate to the nucleus where they induce target gene expression (33). Therefore, the present study determined whether CGA was able to modulate the activation of the NF-κB signaling pathway in IL-6-stimulated FLS. The results indicated that CGA was able to downregulate expression levels of NF-κB p50 and inhibit the phosphorylation of IKKα/β, compared with those induced by IL-6 alone. Considering the current results, we hypothesize that CGA has a suppressive function on the activation of the NF-κB signaling pathway for transmitting proinflammatory responses in FLSs stimulated by IL-6.

In conclusion, the present study demonstrated that CGA has an inhibitory function on the proliferation of FLSs in the inflammatory microenvironment through inducing apoptosis. Thus, it may be beneficial to inhibit the IL-6-induced proinflammatory responses meditated by the JAK/STAT and NF-κB signaling cascades. Therefore, treatment with CGA may be an efficacious therapy for preventing inflammatory hyperplasia of the synovium in patients with RA.

Acknowledgements

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