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Nobiletin, a citrus polymethoxy flavonoid, suppresses gene expression and production of aggrecanases-1 and -2 in collagen-induced arthritic mice

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ABSTRACT

Aggrecanase-1/a disintegrin and metalloproteinase with thrombospondin-like motifs (ADAMTS)-4 and aggrecanase-2/ADAMTS-5 have been shown to play crucial roles in cartilage destruction in arthritic diseases, including rheumatoid arthritis and osteoarthritis. In this study, we examined the effects of nobiletin, a citrus polymethoxy flavone, on the expression and production of ADAMTS-4 and -5 *in vitro* and *in vivo*. Nobiletin (16–64 μM) interfered with the interleukin (IL)-1β-mediated ADAMTS-4 and -5 mRNA expression in cultured human synovial fibroblasts. Furthermore, intraperitoneal administration of nobiletin (15, 30, and 60 mg/kg) also suppressed ADAMTS-4 and -5 mRNA expression in the joint tissues of collagen-induced arthritic (CIA) mice. Immunohistochemical analysis using an antibody against aggrecan neoepitope ($NVTEGE^{373}$) revealed that aggrecanase-mediated degradation of aggrecan in cartilage was effectively inhibited by nobiletin. These results provide novel evidence that nobiletin effectively interferes with gene expression of ADAMTS-4 and -5, and thereby prevents cartilage destruction in CIA mice.

Articular cartilage destruction is a common feature in arthritic diseases including rheumatoid arthritis (RA) and osteoarthritis (OA) [1]. The extracellular matrix in cartilage is mainly composed of type II collagen, hyaluronan, and a large aggregating proteoglycan, aggrecan [1,2]. Among these extracellular matrix components, the loss of aggrecan is an early event in the destruction of articular cartilage [3]. Normal cartilage function depends on high aggrecan content, high glycosaminoglycan substitution, and large aggregate size, resulting in compressive and tensile strength properties [1,2]. Therefore, the loss of aggrecan results in the disruption of the structural and functional integrity of cartilage.

The aggrecan core protein is subdivided into the N-terminal globular G1 and G2 domains, the keratan sulfate and chondroitin sulfate attachment regions, and the C-terminal globular G3 domain [1,2]. Aggrecan is mainly destroyed by proteolysis at a site in the interglobular domain (IGD) between the G1 and G2 domains [4]. Two kinds of aggrecanases, aggecanase-1/a disintegrin and metalloproteinase with thrombospondin-like motifs (ADAMTS)-4 and aggrecanase-2/ADAMTS-5 have been identified as the enzymes that cleave the aggrecan core protein at the Glu^{373}-Ala^{374} bond in IGD [4]. The products of this cleavage have been found in synovial fluid of patients with RA and OA [5], indicating that aggrecanase activity is predominantly responsible for the cartilage matrix catabolism in arthritic diseases. In particular, ADAMTS-5 has been shown to be the most important aggrecanase responsible for pathological cartilage destruction by experiments using ADAMTS-5 knock-out mice [6].

Nobiletin (5,6,7,8,3′,4′-hexamethoxyflavone), a citrus polymethoxylated flavonoid, has been reported to exhibit pharmacological actions including anti-inflammation, antitumor

Abbreviations: ADAMTS, a disintegrin and metalloproteinase with thrombospondin-like motifs; CIA, collagen-induced arthritis; COX, cyclooxygenase; FBS, fetal bovine serum; GAPDH, glyceraldehydes-3-phosphate dehydrogenase; IGD, interglobular domain; IL, interleukin; MEK, mitogen-activated protein/extracellular signal-related kinase; MMP, matrix metalloproteinase; OA, osteoarthritis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PG, prostaglandin; RT, reverse transcription; RA, rheumatoid arthritis; TIMP, tissue inhibitor of metalloproteinases; TNF, tumor necrosis factor.

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proliferation, and antitumor invasion and metastasis *in vitro* and *in vivo* [7–9]. In addition, we have previously reported that nobiletin suppresses the production of interstitial procollagenase/promatrix metalloproteinase (proMMP)-1, prostromelysin-1/proMMP-3, and progelatinase B/proMMP-9, and augments that of tissue inhibitor of metalloproteinases (TIMP)-1 in synovial fibroblasts and chondrocytes from humans and rabbits [10,11]. Therefore, nobiletin is likely to prevent cartilage destruction in arthritic diseases. However, the effects of nobiletin on the expression of aggrecanases have yet to be elucidated. Moreover, the *in vivo* therapeutic action of nobiletin against arthritic diseases remains unclear. In this study, we provide novel evidence that nobiletin suppresses the expression of ADAMTS-4 and -5 and the subsequent degradation of cartilage aggrecan in collagen-induced arthritic (CIA) mice.

Materials and methods

Cell culture. Normal human synovial fibroblasts (Cell Systems Co., Kirkland, WA) were cultured in Dulbecco's modified Eagle's medium (DMEM)/10% fetal bovine serum (Thermo ELECTRON Co., Melbourne, Australia) until confluence. Then, cells were treated with interleukin (IL)-1β (10 ng/ml) and/or nobiletin (16, 32, and 64 μM) in DMEM/0.2% lactalbumin hydrolysate for 24 h, and total RNA was extracted using Isogen (Nippon Gene Co., Toyama, Japan). Nobiletin isolated from the juice of *Citrus depressa* Hayata (Rutaceae) as described previously [11] was dissolved in dimethylsulfoxide (DMSO), and the final concentration of DMSO was 0.1% in all cultures.

Collagen-induced arthritic (CIA) mice. Male DBA/1J mice (8–10 weeks old, Charles River Laboratory Japan, Kanagawa, Japan) were intradermally immunized at the base of the tail with 100 μl of the emulsion containing 100 μg of bovine type II collagen (Sigma, St. Louis, MO) and Freund's complete adjuvant (Sigma) [12]. On day 21 after the initial collagen immunization, the mice were intraperitoneally boosted with 100 μg of bovine type II collagen dissolved in phosphate buffered saline (PBS). Nobiletin (15, 30, and 60 mg/kg) was dissolved in 20% DMSO/80% polyethyleneglycol 300, and was intraperitoneally administered daily from day 21 to 41 after initial collagen immunization. The same volume (2.34 ml/kg) of vehicle was administered as a control. Age-matched and non-immunized male mice were used as a normal control. Mice were euthanized by cervical dislocation on day 42 after initial collagen immunization. The hind limb including the paw, ankle, and knee was dissected, fixed immediately in 4% paraformaldehyde, decalcified in 10% EDTA, and then embedded in paraffin. Another hind limb was homogenized in TRIzol (Invitrogen, Carlsbad, CA) for the extraction of total RNA. The procedures used in this study were approved by the Committee on the Care and Use of Experimental Animals in China Academy of Chinese Medical Sciences.

Reverse transcription (RT) and real-time polymerase chain reaction (PCR). Total RNA (1 μg) was subjected to RT reaction using QuantiTect Reverse Transcription Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instruction. Aliquots (an equivalent of 25 ng of total RNA) of the transcript were subjected to real-time PCR using QuantiTect SYBR Green PCR Kit (Qiagen) and QuantiTect Primer Assays [Cat No. QT00032949 for human ADAMTS-4, Cat No. QT00011088 for human ADAMTS-5, QT00079247 for human glyceraldehydes-3-phosphate dehydrogenase (GAPDH), Cat No. QT00118818 for mouse ADAMTS-4, Cat No. QT00131376 for mouse ADAMTS-5, and Cat No. QT01036875 for mouse 18S rRNA, Qiagen]. The amplification cycle was performed at 94 °C for 15 s, 55 °C for 30 s, and 72 °C for 30 s using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems Japan Ltd., Tokyo, Japan). Obtained threshold cycle (C_T) value of each sample was normalized by that of GAPDH or 18S rRNA, and relative expression level is expressed as the mean value of vehicle group as 1.

Assessment of arthritis. The extent of arthritis was assessed by two independent blinded observers. Clinical scores expressed arthritis severity on a scale of 0–4 for each paw according to changes in erythema and edema as previously described [12]. Briefly, score 0, normal; score 1, detectable arthritis with erythema at least in some digits; score 2, significant swelling and redness; score 3, severe swelling and redness from joint to digit; and score 4, maximal swelling with ankylosis. The total score was the cumulative value for the four paws of each mouse with a maximum of 16. Arthritis was considered to be present if the score for a paw was >2. The incidence of arthritis was defined as the percentage of arthritic mice in each group.

Histological analysis. Paraffin sections (6 μm) of the joint tissues were stained with hematoxylin and eosin. All sections were randomized and evaluated by a trained blinded observer. The severities of inflammation, pannus, cartilage damage, and bone damage were scored on a scale from 0 to 3 as follows: score 0, absent; score 1, weak; score 2, moderate; and score 3, severe [12].

Immunohistochemical staining. Paraffin sections (6 μm) of the joint tissues were reacted with Aggrecan Neo (Affinity Bioreagents, Golden, CO), a rabbit polyclonal antibody against C-terminal peptide (CGGNITEGE^{373}) of aggrecanase-mediated aggrecan fragment, at a dilution of 1:200 in PBS overnight at 4 °C. This antibody also recognizes mouse aggrecan neoepitope (NVTEGE^{373}). After incuba-

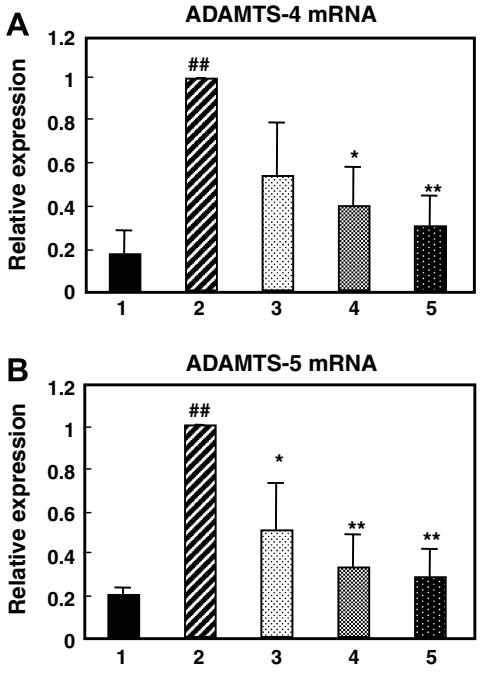

Fig. 1. Nobiletin interfered with IL-1β-mediated ADAMTS-4 and -5 mRNA expression in human synovial fibroblasts. Confluent human synovial fibroblasts were treated with IL-1β (10 ng/ml) and/or nobiletin (16, 32, and 64 μM) for 24 h. Total RNA was subjected to quantitative real-time RT-PCR for ADAMTS-4 and -5, and GAPDH mRNA as described in the text. Lane 1, untreated control cells; lane 2, cells treated with IL-1β (10 ng/ml); and lanes 3–5, cells treated with IL-1β (10 ng/ml) plus nobiletin (16, 32, and 64 μM, respectively). Relative expression is expressed in IL-1β-treated cells (lane 2) as 1 after normalized by GAPDH mRNA. (A) ADAMTS-4 mRNA, and (B) ADAMTS-5 mRNA. Data are represented as means ± SEM for four independent experiments. Statistical significance was examined by one-way ANOVA followed by the LSD test. $^{\#\#}P < 0.01$ compared with untreated control cells, and $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with cells treated with IL-1β.