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염증성 장질환 모델에서 Astragalin의 Nuclear Factor-κB 신호전달 차단을 통한 장염 억제효과

Astragalin inhibits Nuclear Factor-κB signaling in human colonic epithelial cells and attenuates experimental colitis in mice

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서울대학교 대학원
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Astragalin inhibits Nuclear Factor-κB signaling in human colonic epithelial cells and attenuates experimental colitis in mice

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ABSTRACT

Background/Aims: Astragalin (kaempferol-3-O-β-D-glucoside) is a flavonoid isolated from the leaves of persimmon or Rosa agrestis. Astragalin exhibits various anti-inflammatory properties; however, little is known about its therapeutic potential for inflammatory bowel disease (IBD). This study aims to investigate the anti-inflammatory effect of astragalin via blockade of the nuclear factor-κB (NF-κB) signaling pathway in human colonic epithelial cells and a murine colitis model.

Methods: HCT-116 and HT-29 human colonic epithelial cells were pretreated with astragalin and stimulated with tumor necrosis factor-α (TNF-α). Cell viability was assessed by the MTS assay. Real-time reverse transcription polymerase chain reaction was used to analyze the messenger RNA expression of the inflammatory cytokines interleukin (IL)-6 and IL-8. The effect of astragalin on the NF-κB pathway was evaluated by Western blot analysis of inhibitor of NF-κB alpha (IκBα) phosphorylation/degradation and by electrophoretic mobility shift assay. Dextran sulfate sodium (DSS)-induced acute murine colitis model was used for in vivo experiments.

Results: Astragalin strongly suppressed the expression of pro-inflammatory cytokines in human colonic epithelial cells in a dose-dependent manner. Western blot analysis showed that astragalin inhibited IκBα phosphorylation/

degradation. Additionally, astragalin reduced the DNA binding activity of NF-κB. Astragalin alleviated colon shortening and improved the pathologic scores in DSS-induced acute murine colitis model. Furthermore, astragalin reduced the level of phosphorylated IκBα and decreased the production of the inflammatory cytokines IL-6, IL-8, and TNF-α in the DSS-treated colon mucosa.

Conclusions: Astragalin exerted an anti-inflammatory effect through NF-κB pathway inhibition and attenuated murine colitis. Astragalin is thus a potential therapeutic agent for IBD.

Keywords: Inflammatory bowel disease; Astragalin; NF kappa B; Human colonic epithelial cells; Colitis

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LIST OF ABBREVIATIONS

IBD: inflammatory bowel disease

NF-κB: Nuclear factor-kappaB

IL: interleukin

LPS: lipopolysaccharide

TNF-α: tumor necrosis factor-alpha

IκBα: inhibitor of Nuclear factor-kappaB alpha

DMSO: dimethyl sulfoxide

mRNA: messenger RNA

RT-PCR: reverse transcription-polymerase chain reaction

EMSA: electrophoretic mobility shift assay

DSS: dextran sulfate sodium

INTRODUCTION

Inflammatory bowel disease (IBD), which is represented by ulcerative colitis and Crohn's disease, is a chronic inflammatory disorder involving gastrointestinal tract. Although the incidences of ulcerative colitis and Crohn's disease have increased significantly in Asia in the past two decades, they are still the highest in the West.[1] Although the pathogenesis of IBD is not clear, multiple factors such as genetic predisposition, host-microbial interaction, and immunologic imbalance are thought to contribute.[2,3] The colonic microenvironment plays an important role, which includes inflammatory cells like neutrophils, monocytes, and T cells as well as cytokines and chemokines secreted by these cells.[4]

Nuclear factor-κB (NF-κB) signaling pathway is one of the dominant signaling pathways involved in pathogenesis of IBD.[5,6] There has been a report that NF-κB overexpression and activation were observed in macrophages and intestinal epithelial cells obtained from inflamed intestinal tissues of IBD patients.[7] Since NF-κB pathway brought about a pro-inflammatory cascade and promoted the production of various pro-inflammatory cytokines, modulation of this inflammatory pathway is important in these patients.

Astragalin (kaempferol-3-O-β-D-glucoside), which is a natural flavonoid extracted from the leaves of Rosa agrestis or persimmon, has been used as a

traditional Chinese prescription as it has shown anti-inflammatory and antioxidant effect. There were several evidences that astragalin has an anti-inflammatory effect by blockage of NF-κB.[8] Previous study reported that astragalin suppressed the production of nitric oxide, prostaglandin E2, and interleukin (IL)-6 in lipopolysaccharide (LPS)-activated RAW 264.7 cells.[9] It also has been reported that astragalin had inhibitory effect of tumor necrosis factor-α (TNF-α) production in RAW 264.7 cells.[10] By inactivation of NF-κB pathway, astragalin showed decreased production of TNF-α, IL-6, IL-1β in murine model of LPS-induced acute lung injury.[11] However, little is known on therapeutic potential of astragalin for IBD. This study aimed to investigate the anti-inflammatory effect of astragalin by blockade of NF-κB signaling pathway in human colonic epithelial cells and murine colitis model.

MATERIALS AND METHODS

Cells and materials

The HCT-116 and HT-29 cells were purchased by the Korean Cell Line Bank (Seoul, Korea). Astragalin and LPS (Escherichia coli 0127:B8) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). MTS kit was supplied by Promega (CellTiter 96® Aqueous, Madison, WI, USA). Anti-inhibitor of NF-κB alpha (IκBα), anti-phosphorylated IκBα antibodies were purchased from Cell Signaling (Danvers, MA, USA) and anti-β-actin and anti-NF-κB p50 antibodies were supplied by Santa Cruz Biotechnology (Santa Cruz, CA, USA). LightShift Chemiluminescent EMSA Kit from Thermo Scientific Inc. (Rockford, IL, USA) was used for electrophoretic mobility shift assays (EMSAs).

Mice

The ethical approval of this study was granted by the Institutional Animal Care and Use Committee of Seoul National University, Seoul, Korea (IACUC number: SNU-170012). All procedures involving animals were in accordance with the Guide for the Care and Use of Laboratory Animals (NIH publication no. 80-23, revised in 1978). Six-week-old C57BL/6 wild-type mice were purchased from Koatech (Pyeongtaek, Korea) and breed in specific pathogen free conditions. A standard liberal diet was provided and mice were grown

under 12/12-hour day/night cycle till they became desired age (7 to 9 weeks) and body weight (19 to 22 g). Ventilated cages with 50% ± 5% relative humidity and 24°C ± 2°C temperature under specific pathogen free conditions were maintained for mice.

Cell viability

The cell viability was assessed by MTS assay. The HCT-116 and HT-29 cells were planted in 96-well plates and manipulated with various concentrations of astragalin for 24 hours. Cells were incubated with MTS for 4 hours and then 150 μL of dimethyl sulfoxide (DMSO) was applied to solubilize formazan. Cell viability was measured as relative absorbance at 570 nm compared to control.

Real-time reverse transcription polymerase chain reaction

The expression of messenger RNA (mRNA) for IL-6, IL-8 and TNF-α were analyzed by using real-time reverse transcription polymerase chain reaction (RT-PCR). The HCT-116 and HT-29 cells were preconditioned with and without astragalin for 24 hours and stimulated with 10 ng/mL of TNF-α for 30 minutes. Intracellular RNAs were extracted from HCT-116 and HT-29 cells using TRIzol (Gibco/BRL, Gaithersburg, MD, USA). The mRNAs of IL-6, IL-8, TNF-α and β-actin were amplified by RT-PCR. Primers were constructed using Primer Express version 2.0 (Applied Biosystems, Foster City, CA, USA).

The fold changes of IL-6, IL-8, and TNF-α mRNA expression were compared to that of β-actin.

Western blot

Cells were pretreated with and without astragalin, stimulated by 10 ng/mL of TNF-α for 30 minutes. Change of phosphorylated IκBα and IκBα after treatment by TNF-α were evaluated by using anti-IκBα, anti-phosphorylated IκBα and anti-β-actin antibodies.[12] Image Gauge version 3.12 (Fuji Photo Film, Tokyo, Japan) and Luminescent Image analyzer LAS 1000-plus (Fuji Photo Film) were used to analyze the density of protein bands.[13] The phosphorylation and degradation of IκBα were measured by comparing density of the phosphorylated IκBα band to that of the IκBα band.

Electrophoretic mobility shift assay

Changes in the DNA binding activity of NF-κB were detected by using EMSA analysis.[14] Pretreated cells with and without astragalin were stimulated with TNF-α (10 ng/mL) for 1 hour. A biotin labeled DNA oligonucleotide probe for NF-κB consensus site was added to nuclear extracts to measure DNA binding activity of NF-κB. Anti-NF-κB p50 antibodies were used for a supershift assay. Bounded and unbounded DNA samples were loaded on to a 5% polyacrylamide gel and electrophoresis was done. We transferred separated DNAs to a nylon

membrane, and detected target DNA labeled with biotin using chemiluminescence.

DSS-induced acute murine colitis model

Seven-week-old wild-type C57BL/6 mice, approximately 20 g in weight, were used for the acute murine colitis model. Dextran sulfate sodium (DSS) of 4% was used to induce colitis. After body weight check, 24 mice were randomly allocated into four groups (control, vehicle, astragalin 2 mg/kg, and astragalin 5 mg/kg). Filtered water was supplied for 7 days for the control group. The vehicle group administered DMSO for 7 days. Astragalin was dissolved in the same volume of DMSO and received by oral gavage once daily over 7 days in astragalin group. The vehicle group and astragalin group received drinking water mixed with 4% DSS during 5 days after prior administration of DMSO or astragalin for 2 days.[12,14,15] We checked the body weight daily and sacrificed all mice on day 8. After sacrifice, entire colon was extracted and colon length was measured. The colon was dissected into two pieces representative for proximal and distal colon and incised longitudinally. Hematoxylin and eosin staining was done for formalin-fixed paraffin-embedded slides. The severity of inflammation was scored by a pathologist who was blinded to the details of study. We measured the extent of crypt damage and inflammation by a score (score from 1 to 4) for the involved area and calculated the sum as a histologic

score.[16] IκBα phosphorylation was evaluated using immunohistochemical staining to figure out the protective effect of astragalin in the colon. Intensity of immunoreactivity for phospho-IKBα immunohistochemistry was evaluated according to a 0 to 4+ scale for each slide. The scoring system for overall intensity of the staining reaction was measured accordingly: 0 revealed no immunoreactivity, no positive cells; 1+ revealed weak immunoreactivity, less than 10% cells with positivity; 2+ revealed mild immunoreactivity,10%–30% cells with positivity; 3+ revealed moderate immunoreactivity, 31%–60% cells with positivity; and 4+ revealed strong immunoreactivity, 61%–100% cells with positivity. The total percentage of cells with positivity (0 through 4+) was documented for each case.[15] The expression of mRNA for IL-6, IL-8 and TNF-α in mice colon tissue were analyzed using real-time RT-PCR. Total RNAs were extracted from tissue samples. The mRNA of IL-6, IL-8, TNF-α and β-actin were amplified by real-time RT-PCR. Primers were constructed by Primer Express version 2.0 (Applied Biosystems). The fold changes of IL-6, IL-8, and TNF-α mRNA expression were compared to that of β-actin.

Statistical analysis

Data are presented as mean ± standard deviation. GraphPad Prism software version 5.0 (GraphPad, La Jolla, CA, USA) was used for statistical analysis. For the analysis of continuous variables, an independent t-test or one-way

analysis of variance was used. Repeated measured analysis of variance was performed to compare body weight changes among the groups. Pairwise comparison was conducted using Tukey's post hoc analysis. p-values< 0.05 were considered statistically significant.

RESULT

Astragalin inhibits colon cell proliferation

MTS assay was performed to evaluate the effects of astragalin on colonic epithelial cell proliferation. The growth of HCT-116 and HT-29 cells was significantly prohibited dose-dependently.

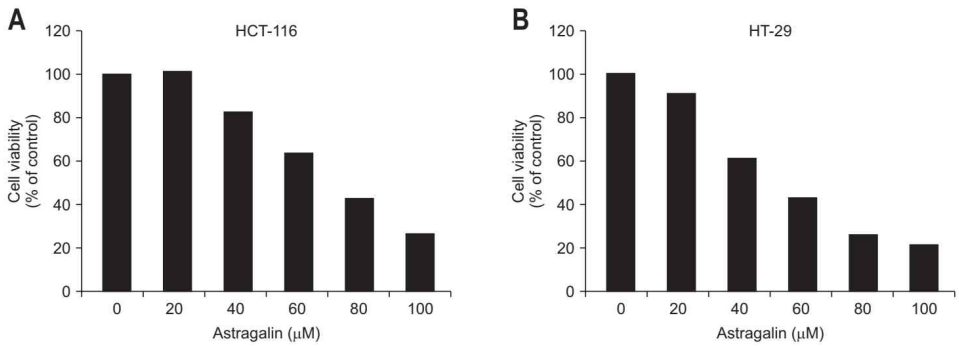

Figure 1 Effect of astragalin on the growth of colonic epithelial cells. (A) HCT-116 and (B) HT-29 cells were treated with astragalin at different concentrations (0, 20, 40, 60, 80, and 100 μM) for 24 hours, and cell viability was evaluated by an MTS assay

Astragalin inhibits the production of inflammatory cytokines in TNF-α-stimulated HCT-116 and HT-29 cells

To investigate anti-inflammatory effect of astragalin, mRNA expressions for IL-6, IL-8, and TNF-α were analyzed using RT-PCR. Preconditioning with astragalin markedly reduced TNF-α induced IL-6 mRNA expression in HCT-116 and HT-29 cells. IL-8 mRNA levels were significantly down-regulated by astragalin pretreatment in both HCT-116 and HT-29 cells. The mRNA expression of TNF-α is also reduced by the treatment of astragalin.

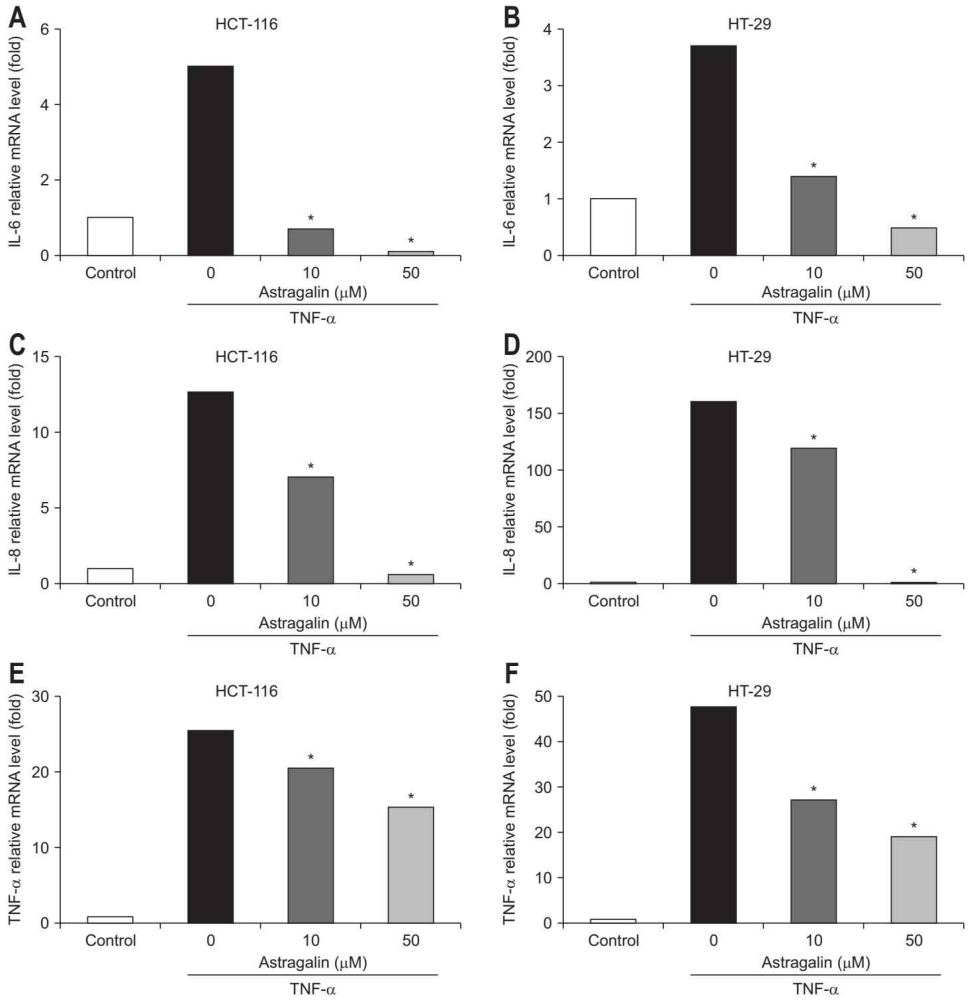

Figure 2 Effect of astragalin on the messenger RNA (mRNA) expression of inflammatory cytokines in colonic epithelial cells. HCT-116 and HT-29 cells were pretreated with astragalin at different concentrations (0, 10, and 50 μM) and stimulated with 10 ng/mL tumor necrosis factor α (TNF-α) for 30 minutes. Reverse transcription polymerase chain reaction was performed to detect the mRNA expression levels of interleukin-6 (IL-6) and IL-8. (A) IL-6 in HCT-116

and (B) HT-29 cells. (C) IL-8 in HCT-116 and (D) HT-29 cells. (E) TNF-α in HCT-116 and (F) HT-29 cells. *$p < 0.05$ compared with TNF-α alone

Astragalin suppressed IκBα phosphorylation in TNF-α stimulated HCT-116 and HT-29 cells

The levels of phosphorylated and non-phosphorylated forms of IκBα were estimated by Western blot analysis. When stimulated by TNF-α, the phosphorylated IκBα markedly increased and IκBα decreased in HCT-116 and HT-29 cells. However, the pretreatment with astragalin suppressed the phosphorylation and degradation of IκBα in dose-dependent manner.

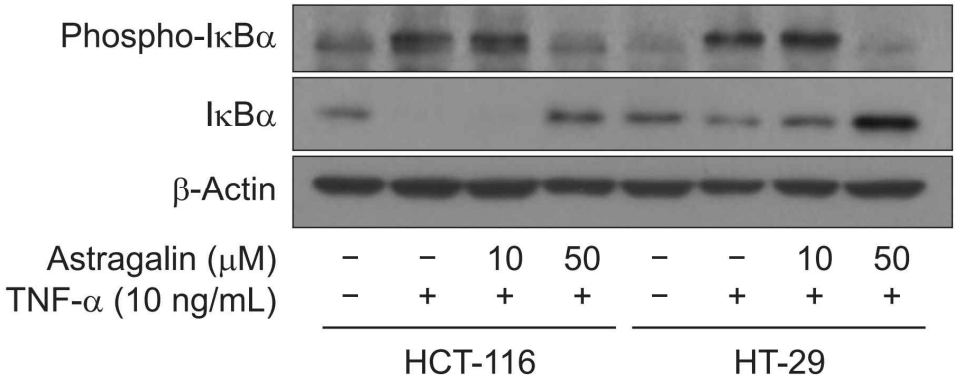

Figure 3 Effect of astragalin on inhibitor of nuclear factor κB-alpha (IκBα) phosphorylation/degradation in colonic epithelial cells. HCT-116 and HT-29 cells were treated with astragalin at different concentrations (0, 10, and 50 μM) and stimulated with 10 ng/mL tumor necrosis factor-α (TNF-α) for 30 minutes. Whole-cell extracts were prepared and analyzed for IκBα and phospho-IκBα expression.

Astragalin reduces DNA binding activity of NF-κB in HCT116 cells

EMSA was performed to detect the changes in the DNA binding activity of NF-κB. Strong DNA binding activity was observed in the nuclear extract of HCT-116 cells after TNF-α stimulation, however, this activity was markedly prohibited after astragalin pretreatment.

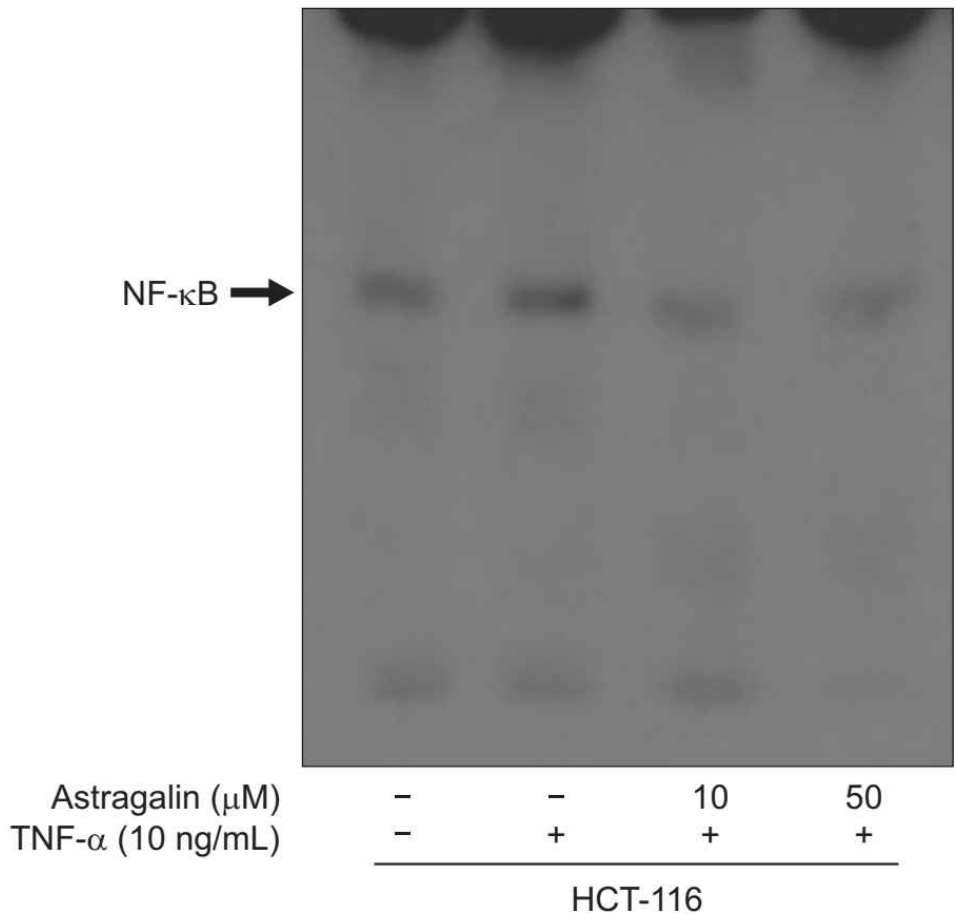

Figure 4 Effect of astragalin on the DNA binding activity of nuclear factor κB (NF-κB) in colonic epithelial cells. HCT-116 cells were treated with astragalin at different concentrations (0, 10, and 50 μM) and stimulated with 10 ng/mL tumor necrosis factor-α (TNF-α) for 30 minutes. The DNA binding activity was evaluated using electrophoretic mobility shift assay

Astragalin attenuates experimental colitis in DSS-induced acute murine colitis model

The mice in the vehicle group showed the most severe body weight loss, whereas the control group showed higher body weight compared with vehicle group. Astragalin showed tendency of reduced body weight loss, albeit statistically insignificant. Mice in the vehicle group showed shortened colon length, whereas the oral treatment with astragalin improved colon shortening. On histologic exam, oral administration of astragalin induced a significant improvement of colonic inflammation compared with vehicle groups.

Astragalin attenuated the amount of phosphorylated IκBα in DSS-induced acute murine colitis model

In vehicle group, cells in destroyed epithelium and lamina propria of colon tissues showed strong staining of phosphorylated IκBα. Oral administration of astragalin ameliorated IκBα phosphorylation in colon tissue.

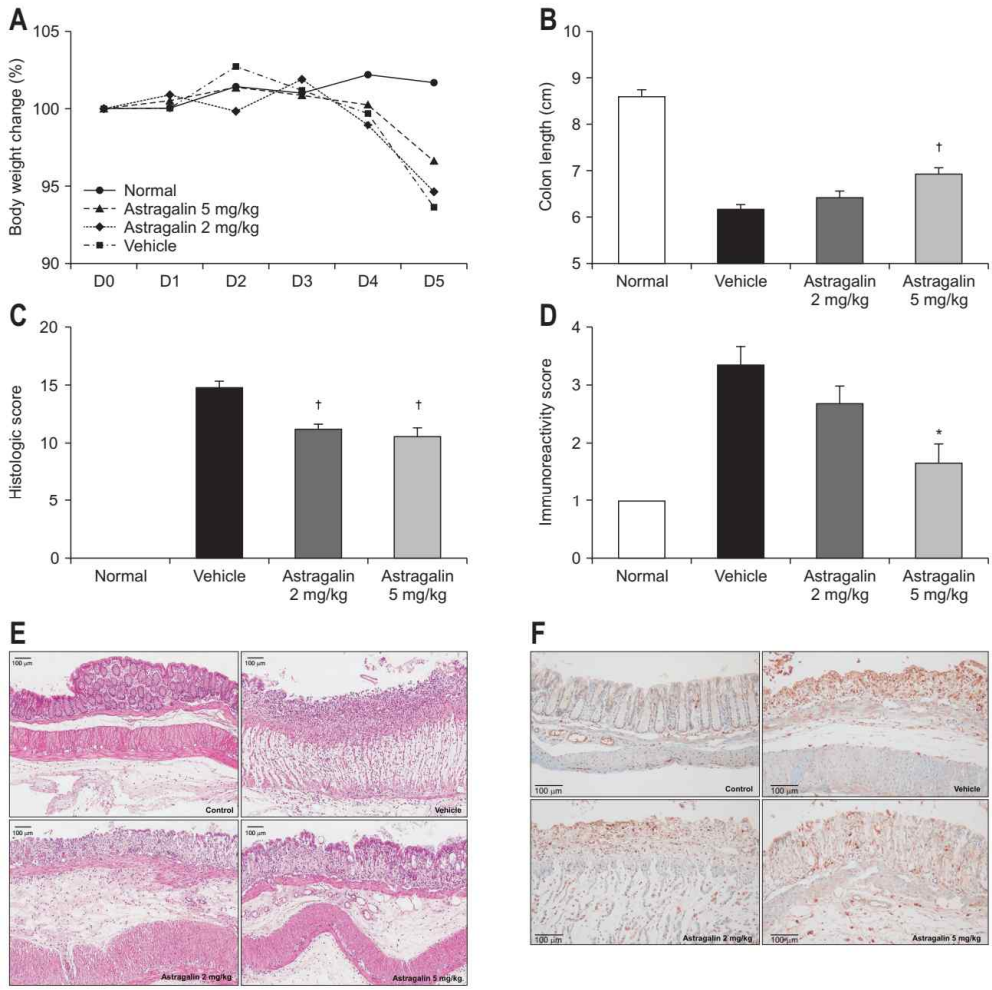

Figure 5 Effect of astragalin on dextran sulfate sodium (DSS)-induced acute murine colitis. (A) Oral administration of astragalin significantly reduced the degree of body weight loss compared with that of vehicle-treated mice. (B) Colon shortening improved with oral administration of astragalin. (C) Treatment with astragalin significantly improved the histologic scores of mice with colitis compared with those of vehicle-treated mice. (D) Astragalin

reduced colitis-induced IκBα phosphorylation in the colonic mucosa. (E) Oral administration of astragalin attenuated the destruction of crypts, damage to the epithelium and infiltration of inflammatory cells (H&E). (F) Oral administration of astragalin reduced the level of phosphorylated IκBα in both destroyed epithelial cells and inflammatory cells (immunohistochemical staining). *$p < 0.05$ and † $p < 0.01$ compared with vehicle

Astragalin inhibits the production of inflammatory cytokines in DSS-induced acute murine colitis model

To investigate anti-inflammatory effect of astragalin in DSS-induced acute murine colitis model, mRNA expressions for IL-6, IL-8 and TNF-α were analyzed using RT-PCR. After treatment with astragalin, mRNA expression of IL-6, IL-8 and TNF-α in mice colonic extracts were markedly reduced dose-dependently.

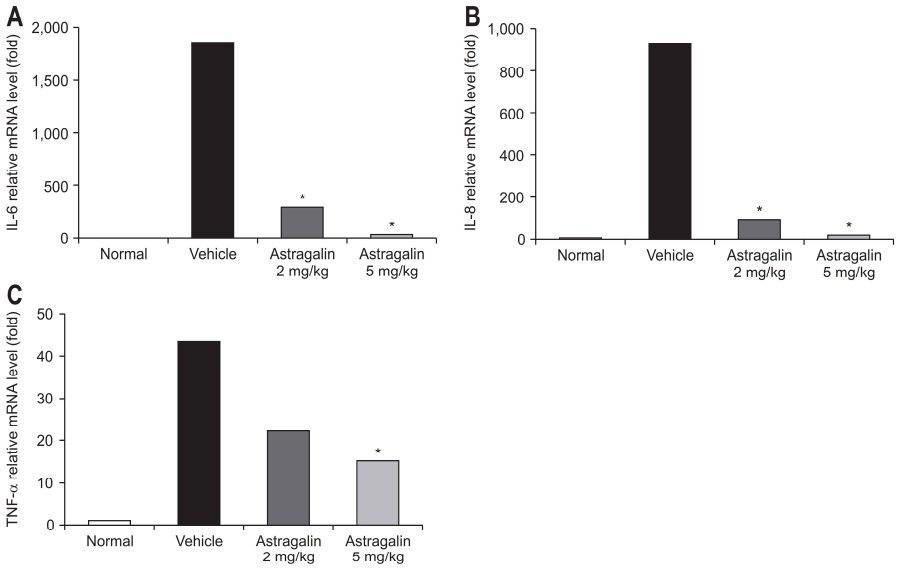

Figure 6 Effect of astragalin on the messenger RNA (mRNA) expression of inflammatory cytokines in dextran sulfate sodium (DSS)-induced acute murine colitis. Oral administration of astragalin significantly and dose-dependently reduced mRNA expression of (A) IL-6, (B) IL-8, and (C) TNF-α in murine colonic extracts. IL, interleukin; TNF, tumor necrosis factor. *p< 0.05 compared with vehicle

DISCUSSION

In the present study, we proved that astragalin shows anti-inflammatory effect through the NF-κB pathway inhibition and attenuated murine colitis. When treated with astragalin, the phosphorylation of IκBα decreased and degradation of IκBα also lowered. Astragalin reduced DNA binding activity of NF-κB, and as a result, the expression of inflammatory cytokines decreased. We aimed to demonstrate anti-inflammatory effect of astragalin in colonic epithelial cell, because anti-inflammatory effect induced by NF-κB down regulation could be different depending on the cell types. The novel finding is that our study demonstrated the anti-inflammatory effects of astragalin on human colonic epithelial cell for the first time. Furthermore, we showed that astragalin ameliorates experimental colitis by down regulation NF-κB pathway.

NF-κB pathway activation promoted the production of many pro-inflammatory cytokines and triggered a pro-inflammatory cascade. NF-κB pathway is a key pathway of IBD. NF-κB is bound by IκBα, which is an inhibitory molecule. When inflammatory cascade was triggered, phosphorylation of IκBα increased, resulting in the increased degradation of IκBα. This promotes NF-κB being free to bind DNA, thus, DNA binding activity of NF-κB is enhanced in both intestinal epithelial cells and macrophages. Finally, the NF-κB-induced inflammatory cytokine production promoted. Many established therapeutic agents, such as corticosteroids, 5-

aminosalycilic acid, methotrexate, and anti-TNF-α agents, exerted its effect through the inhibition of NF-κB pathway.[17-20]

Astragalin has been investigated as an anti-inflammatory drug due to suppression of inflammatory cascade in various cell lines, although the exact mechanisms of astragalin are lacking. In recent, potential relation between astragalin and NF-κB pathway has been revealed. Astragalin efficiently inhibited inflammatory mediator such as TNF-α, IL-1β, and IL-6 and macrophage derived chemokine such as macrophage inflammatory protein-1α, monocyte chemotactic protein-1 in macrophages.[8] Astragalin showed its anti-inflammatory activity by protecting mice from lethal sepsis and defending mice against acute lung injury induced by LPS.[11] In vivo studies using LPS-induced mastitis murine model also reported that astragalin attenuated inflammatory cell infiltration and expression of inflammatory mediators.[21,22] Another study proved that astragalin suppressed NF-κB on IL-1β-induced inflammation in chondrocytes.[23] Several previous studies which showed anti-inflammatory effect of astragalin raised expectation for the possibility of anti-colitis effect of astragalin. Herein, we aimed to prove the anti-inflammatory effect of astragalin in colonic epithelial cell and experimental colitis model, thus, to see the potential therapeutic effect for IBD through the modulation of inflammatory process. We proved that astragalin act as a potent inhibitor of NF-κB pathway in colonic epithelial cell. We also proved that astragalin showed anti-colitic effect in murine colitis model. Considering these findings, astragalin showed a

possibility of a therapeutic option for IBD.

To apply these results from bench to clinics, further supporting studies are warranted. First, anti-inflammatory effect of astragalin could be further validated in vivo therapeutic models. We used DSS-induced acute murine colitis model[7,16] and proved that astragalin was effective in preventing acute colitis. Additional experiment using chronic colitis model of IL-10 -/- mice would be appropriate to prove therapeutic effect of astragalin in vivo. IL-10 -/- mice revealed marked chronic colonic inflammation by the administration of nonsteroidal anti-inflammatory drug and widely used to discover the potential treatment effects of new drugs on chronic colitis.[24] Second issue is a safety problem. Astragalin is a natural flavonoid that widely found in fruit and vegetables, and until now, no specific adverse effect or toxicity was reported. However, further experiments are mandatory to evaluate the safety according to the dose and duration, because there are remaining concern about potential harmful effect. Although multiple therapeutic administrations of astragalin in variable diseased status have been reported, further investigations are still needed to ultimately lead towards potent drug candidates, for example, structural optimization to upgrade its absorption profiles, improve its chemical accessibility, and to synthesize more effective analogues.[25]

In conclusion, astragalin showed anti-inflammatory effect through the inhibition of NF-κB pathway and attenuated murine colitis. Astragalin might be potential therapeutic agent for IBD.

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국문 초록

서론: Astragalin (kaempferol-3-O-β-D-glucoside) 은 감잎에서 추출한 flavonoid로 항염증 효과를 가지고 있다. 본 연구에서는 장상피 세포에서 Astragalin의 nuclear factor-κB (NF-κB) 신호 전달에 미치는 영향과 대장염 마우스 모델에서의 항염증 효과에 대해서 알아보고자 하였다.

방법: 장상피 세포주인 HCT-116 와 HT-29를 astragalin 용매로 전처치한 후에 tumor necrosis factor-α (TNF-α)를 이용하여 염증반응을 유발하였다. MTS assay를 시행하여 세포 생존력을 확인하였다. 이후 염증 반응의 변화를 보기 위해 염증성 사이토카인인 인터루킨 6,8 messenger RNA(mRNA)의 변화를 Real-time reverse transcription polymerase chain reaction을 통해 측정하였다. NF-κB 신호전달에 미치는 영향을 확 인하기 위해 IκBα 인산화의 정도를 western blot으로, NF-κB의 DNA 결합 정도를 electrophoretic mobility shift assay로 확인하였다. In vivo 실험에서는 dextran sodium sulfate (DSS)로 대장염을 유발시킨 급성 예방 모델을 이용하였다. 대장염의 정도는 적출한 대장의 병리학적 소견으로 평가하였다.

결과: Astragalin은 장상피세포에서 염증성 사이토카인의 mRNA 발현을 유의하게 억제하였으며 IκBα의 인산화와 NF-κB의 DNA 결합을 억제하였다. 또한 astragalin은 급성 예방 모델에서 대장염의 완화 효과를 보여주었다. DSS를 이용한 급성 예방모델에서 대장 단축 및 병리 소견의 호전을 보여주었다. 적출한 대장 조직에서 시행한 인산화된 IκBα의 면역화학염색에서, astragalin을 투약했을 때 인산화된 IκBα의 염색이 유의하게 감소하였고 염증성 사이토카인의 mRNA 발현 억제를 확인할 수 있었다.

결론: Astragalin은 장상피세포에서 NF-κB의 신호전달을 억제하며 대장염 마우스 모델을 완화시키는 효과를 보였다. 향후 astragalin이 염증성 장질환의 새로운 잠재적인 치료제로 이용될 수 있을 것으로 기대된다.

주요어: 염증성 장질환, 아스트라갈린 (astragalin), 대장 상피 세포, NF kappa B, 대장염 마우스 모델, 대식세포, N F-κB

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