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Parthenolide induces apoptosis in colitis-associated colon cancer, inhibiting NF-κB signaling

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Abstract. Recently, the nuclear factor (NF)-κB inhibitor parthenolide (PT) was identified as a promising anticancer agent for the promotion of cancer cell apoptosis. Additionally, our previous study demonstrated that PT administration suppresses tumor growth in a xenograft model of colorectal cancer cells via regulation of the B-cell lymphoma-2 (Bcl-2) family. However, the role of PT in the development of colitis-associated colon cancer (CAC) is poorly understood. Therefore, the aim of the present study was to investigate the effects of PT administration on CAC using a murine model. Azoxymethane (AOM) and dextran sulfate sodium (DSS) were administered to induce experimental CAC in the following three groups of treated mice: i) AOM and DSS plus vehicle; ii) AOM, DSS and 2 mg/kg PT; and iii) AOM, DSS and 4 mg/kg PT. It was demonstrated that the histological acuteness of AOM/DSS-induced CAC was significantly reduced following the administration of PT, resulting in decreased NF-κB p65 expression levels via a blockade of phosphorylation and subsequent degradation of inhibitor of κB-α (IκBα). Furthermore, PT administration appeared to enhance the process of carcinogenesis via the downregulation of the antiapoptotic proteins Bcl-2 and Bcl-extra large, mediated by inhibition of NF-κB activation. Apoptosis and caspase-3 expression were markedly increased in the PT-treated group. These findings indicate that PT inhibits IκBα phosphorylation and NF-κB activation, resulting in the initiation of apoptosis and the eventual suppression of CAC development. The beneficial effects of PT treatment observed in the experimental CAC model indicate the potential chemopreventive and therapeutic role of PT in CAC.

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Introduction

The worldwide incidence of inflammatory bowel disease (IBD), namely ulcerative colitis (UC) and Crohn's disease, has been increasing for a number of decades. One possible explanation for this increase is that advances in treatment strategies have resulted in the prolonged survival of affected patients, however, the number of IBD patients diagnosed with colorectal cancer [CRC; colitis-associated cancer (CAC)] has also increased, particularly in those suffering from UC (1). CAC is considered to develop as a result of chronic inflammation, with a recently conducted meta-analysis estimating the incidence rate of CAC at 7 and 12 per 1,000 individuals per year in UC patients at 20 and 30 years post-diagnosis, respectively. This high incidence rate highlights the importance of preventing the development of CAC in high-risk UC patients (2). Furthermore, the expression of numerous inflammatory cytokines is associated with the development of acute or chronic intestinal inflammation (3-5); for example, the upregulation of the tumor necrosis factor-α (TNF-α) and interleukin (IL)-1β pro-inflammatory cytokines requires the activation of transcription factor nuclear factor κB (NF-κB) (6-8).

Feverfew (*Tanacetum parthenium*), a traditional herb that has been applied medicinally in Europe for the treatment of migraine, fever and arthritis, contains a number of sesquiterpene lactones, including parthenolide (PT) (9). Previously conducted studies have established that the anti-inflammatory mechanisms of feverfew involve the inhibition of IL-1- and TNF-α-mediated NF-κB activation (10-12). PT was recently demonstrated to be the specific agent in feverfew that was responsible for this action, inhibiting NF-κB activation and thus inducing apoptotic cell death in various types of human cancer cells (13,14). Additionally, previous studies have also demonstrated that PT is a potent inhibitor of NF-κB activation and can inhibit the expression of pro-inflammatory cytokines in experimental murine models (11,15-19). Our recent study used xenograft models to reveal that PT is a potential chemopreventive and therapeutic agent for CRC (20); however, to date, no evidence

of the therapeutic effect of PT on CAC exists. Therefore, we hypothesized that PT exerts its anticarcinogenic effect on CAC by inhibiting the activation of the NF-κB signaling pathway. The present study aimed to provide experimental evidence of this hypothesis by evaluating the effect of PT administration on a murine model of azoxymethane (AOM)/dextran sulfate sodium (DSS)-induced CAC.

Materials and methods

Chemicals and reagents. Parthenolide and z-VAD-fluoromethylketone were obtained from Calbiochem (San Diego, CA, USA), AOM and DSS were purchased from Sigma-Aldrich (St. Louis, MO, USA) and a terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assay kit was obtained from Promega Corporation (Madison, WI, USA). Anti-inhibitor of κBα (IκBα, mouse monoclonal; cat. no. sc-8404), anti-p65 (mouse monoclonal; cat. no. sc-8008), anti-B-cell lymphoma (Bcl)-2 (rabbit polyclonal; cat. no. sc-783), anti-Bcl-extra large (xL, mouse monoclonal; cat. no. sc-8392) and anti-caspase 3 (rabbit polyclonal; cat. no. sc-7148) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Beverly, MA, USA), while anti-actin (rabbit polyclonal; cat. no. A2066) antibodies were purchased from Sigma-Aldrich.

Animal models. A total of 15 six-week-old pathogen-free female Balb/C mice were purchased from Orient Bio Inc., (Seongnam, Korea). Mice were given *ad libitum* access to water and standard rodent food until they reached the desired weight (18–20 g). Mice were maintained on a 12-h:12-h light/dark cycle under specific pathogen free conditions. All procedures using the mice were reviewed and approved by Chonbuk National University Animal Care and Use Committee (Approval no: CBNU 2015-0013). In each group, five mice were randomly assigned after they were weighed. The mice were injected intraperitoneally with 7.4 mg/kg body weight of AOM dissolved in physiological saline. After five days, 3% DSS was administered in the drinking water for five days, followed by 16 days of regular water. This cycle was repeated three times. Following sacrifice by cervical dislocation, the entire colon was removed from the cecum to the anus, and the colon was then opened longitudinally, and the number of macroscopic tumors were counted and measured using calipers. Subsequently, the distal colons were fixed in 10% neutral-buffered formalin for 24 h, and transferred to 70% ethanol for subsequent paraffin embedding and histological analysis.

Histological analysis. The sections (5 μm) were stained with hematoxylin and eosin, and histological analysis was performed by a pathologist in a double-blind manner. The inflammation scores of mucosal inflammation were determined as follows (21): 0, normal morphology; 1, focal inflammatory cell infiltrate around the crypt base; 2, diffuse infiltration of inflammatory cells around the crypts or erosion/destruction of the lower one-third of the glands; and 3, erosion/destruction of the lower two-thirds of the glands or loss of all the glands. Furthermore, invasion depth was scored as follows (22): 0, no invasion; 1, invasion through the mucosa; 2, invasion through the submucosa; and 3, full invasion through the muscularis and into the serosa.

Immunohistochemistry (IHC). IHC was performed in paraffin-embedded, 5-μm tissue sections. For the analysis of NF-κB p65, phospho-IκBα, Bcl-2, Bcl-xL and caspase 3 protein expression levels, the slides were hydrated and endogenous peroxidase activity quenched with 0.03% hydrogen peroxide in MeOH. Antigen retrieval was performed using boiling sodium citrate in a microwave (20 mM sodium citrate pH 6.5) for 16 min at 30% power. Subsequent to blocking, primary antibody was added (dilution ratio, 1:500) overnight at 4˚C. The slides were washed and incubated with secondary antibody for 30 min. After this, the slides were reacted with streptavidin for 20 min, the reaction was visualized with 3,3-diaminobenzidine tetrahydrochloride for 5 min, and the slides were counterstained with Meyer's hematoxylin. Sections were examined under a Olympus BX53 microscope (Olympus America, Hauppauge, NY, USA); images of the representative areas were captured at a magnification of x20 and the number of positively stained cells were counted.

Apoptosis was quantitatively determined by performing a TUNEL assay using an ApopTag® *in situ* Apoptosis Detection kit (EMD Millipore, Temecula, CA, USA), according to the manufacturer's instructions. Four fields at x20 magnification were selected at the proliferation front of each tumor and the number of TUNEL-positive cells were counted.

Western blotting. Colon tissue samples were homogenized in lysis buffer [20 mM Tris HCl (pH 7.5), 1% Triton X100, 0.2 M NaCl, 2 mM EDTA, 2 mM ethylene glycol tetraacetic acid, 1 M dithiothreitol and 2 M aprotinin). Protein samples (50 μg per lane) were electrophoresed on 10% SDS PAGE gels and the separated proteins were electrophoretically transferred onto polyvinylidene difluoride membranes. Subsequently, the membranes were incubated with anti-phospho IκB (1:1,000), anti-p65 (1:1,000), anti-Bcl2 (1:1,000), anti-Bcl-xL (1:1,000) and anti-caspase3 (1:1,000) and anti-β-actin (1:1,000) antibodies. The signal was detected using enhanced WEST-one (iNtRON Biotechnology, Daejeon, Korea) and analyzed with a luminescent image analyzer (LAS-3000; Fuji Film Corporation, Tokyo, Japan).

Statistical analysis. The data are presented as the mean ± standard error of the mean of a minimum of three independent experiments performed in duplicate. Imaging analysis for IHC were performed by cellsense standard software (standard version, Olympus America). Representative blots are included. All data were entered into a Microsoft Excel version 5.0 (Microsoft Corporation, Redmond, WA, USA) spreadsheet, and SPSS software (SPSS, Inc., Chicago, IL, USA) was used to perform the two-tailed t tests or the analysis of variance, where appropriate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

PT inhibits colon carcinogenesis induced by AOM/DSS administration in a murine model. AOM is a procarcinogen that upon metabolic activation, causes the formation of O^6-methylguanine (23). AOM induces the development of tumors in the distal colon of rodents and is commonly used to elicit CRC in experimental animals (24-26). Thus, the present study used a CAC model in which six-week-old mice were injected with

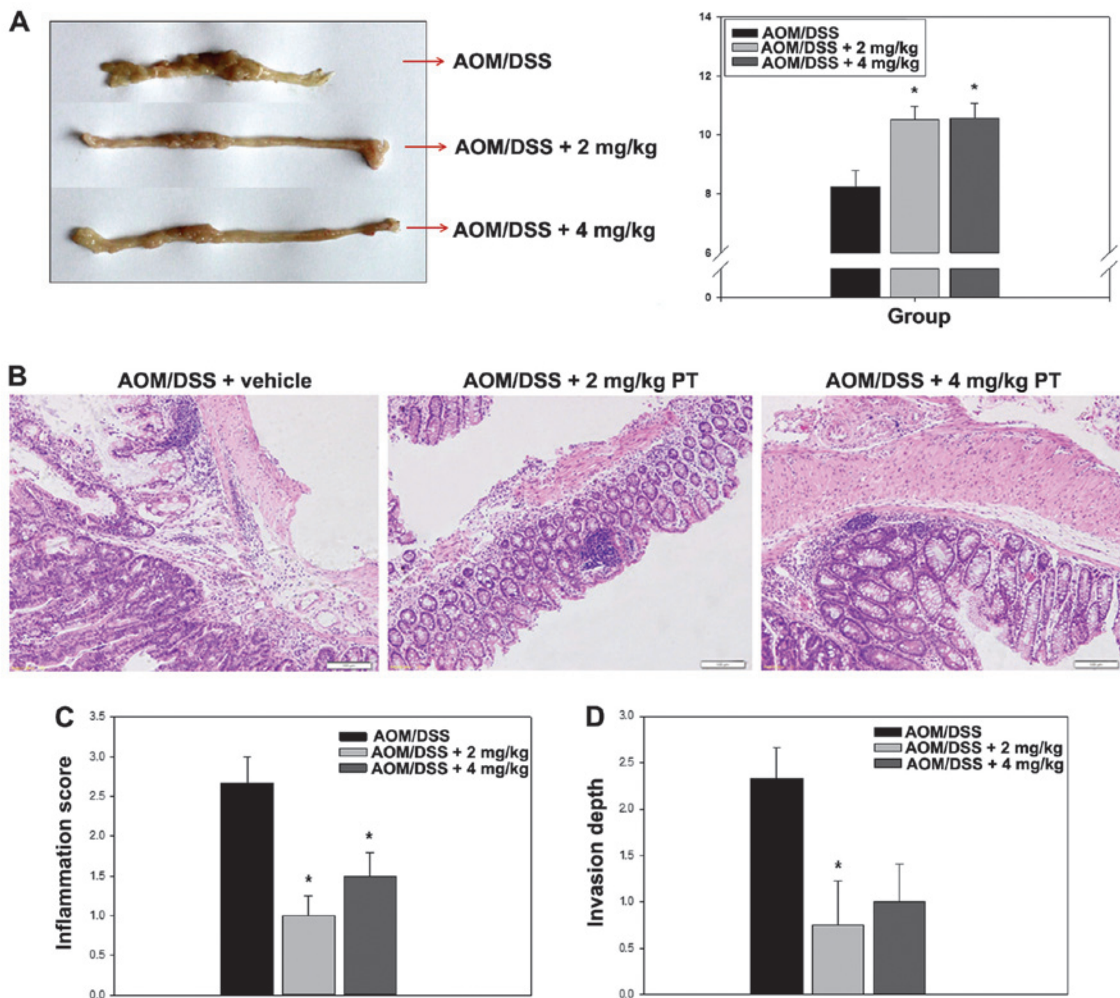

Figure 1. PT reduces colon carcinogenesis in a mouse model of colitis-associated cancer (CAC). (A) Representative images of the gross anatomy of the colon, indicating colon length upon termination of the CAC protocol. (B) Hematoxylin and eosin (H&E) staining of colonic mucosal tissue section from CAC mice (magnification, x10). (C-D) Inflammation scores and invasion depth of H&E-stained tissue specimens obtained from mice at the end of CAC induction, as determined by two pathologists in a blinded manner. *P<0.05 vs. vehicle-treated group. AOM, azoxymethane; DSS, dextran sulfate sodium; PT, parthenolide.

a single dose of AOM followed by DSS administered in the drinking water to analyze the antitumor activities of PT. First, colon length was compared between the control (AOM/DSS plus DMSO as vehicle) and PT-treated (AOM/DSS plus 2 mg/kg PT and AOM/DSS plus 4 mg/kg PT) groups. Numerous nodular, polypoid and caterpillar-like tumors were observed in the middle and distal colon of mice in the control group. By contrast, shortening of the colon, which is a characteristic of colon carcinogenesis, was significantly improved in the PT-treated group (Fig. 1A). Histological analysis revealed that the severity of inflammation and the invasion depth of the ulcerated areas in the colons of the PT-treated mice were significantly lower compared with that in the non-PT-treated mice (P<0.05; Fig. 1B-D).

Administration of PT suppresses NF-κB signaling by blocking IκBα phosphorylation. Degradation of IκB proteins via a phosphorylation and ubiquitination-dependent pathway is an essential step for NF-κB activation (27). To evaluate the molecular basis of NF-κB inactivation by PT, the present study examined the effects of PT on the phosphorylation and degradation of IκBα protein. As indicated in Fig. 2A, AOM/DSS mice treated with PT displayed significantly reduced phospho-IκBα positively-stained cells compared with the AOM/DSS control mice treated with vehicle. Furthermore, the protein expression level of phospho-IκBα was increased in the AOM/DSS-treated mice, but was markedly inhibited in the PT-treated mice (Fig. 2B).

Anti-NF-κB p65 (RelA) is one of the subunits of NF-κB (27). Numerous anti-NF-κB p65 positively-stained cells were detected in the mice with AOM/DSS-induced CAC, whereas treatment with PT resulted in a significant reduction in the number of NF-κB p65 positively-stained cells (Fig. 2A; P<0.05). Furthermore, the protein expression level of NF-κB p65 was markedly inhibited in the PT-treated mice and was highly correlated with the IHC results (Fig. 2B).

Administration of PT downregulates anti-apoptotic proteins Bcl-2 and Bcl-xL. The regulation of Bcl-2 and Bcl-xL expression by PT administration was examined in the CAC mice. As indicated in Fig. 3A, cells positively immunostained for Bcl-2 were detected in the AOM/DSS-induced CAC group; however, the number of Bcl-2 positively-immunostained cells was significantly reduced by PT treatment (Fig. 3A; P<0.05). Furthermore, the data correlated well with the western

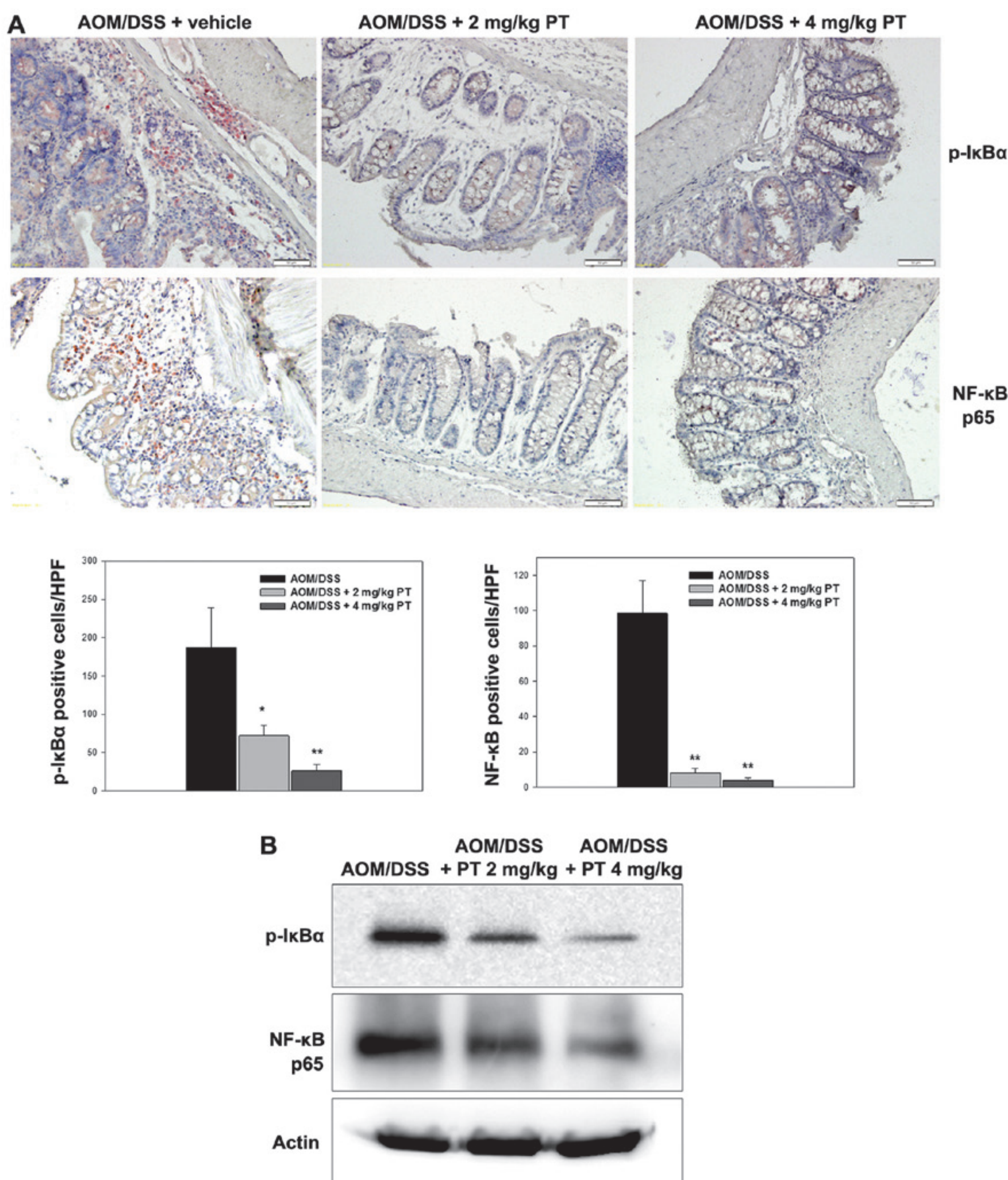

Figure 2. Administration of PT suppresses NF-κB activation by blocking IκBα phosphorylation. (A) Sections of colon from vehicle- and PT-treated colitis-associated cancer mice are stained with a p-IκBα or NF-κB p65 antibodies (magnification, x20). The number of positively-stained cells in the colon tissues was counted and represented as a graph. Error bars indicate the standard error of the mean. **P<0.01 vs. vehicle-treated group. (B) Western blot results demonstrate the protein expression levels of p-IκBα and NF-κB p65 in the colon tissues. Actin was used as the loading control. AOM, azoxymethane; DSS, dextran sulfate sodium; PT, parthenolide; p-IκBα, phosphorylated inhibitor of κBα; NF-κB, nuclear factor-κB; HPF, high-power field.

blotting results (Fig. 3B). Similarly, IHC with the Bcl-xL antibody resulted in markedly fewer positively-stained cells in the PT-treated group compared with the vehicle-treated group (Fig. 3A), and Bcl-xL protein expression levels were significantly downregulated following PT treatment (Fig. 3A; P<0.05).

Administration of PT increases apoptosis in dysplasia lesions in a murine CAC model. To perform additional evaluation of the effects of PT on apoptosis, caspase 3 expression was examined by IHC, and apoptotic cells were detected in the colonic epithelium using a TUNEL assay. The results revealed that the number of cells positively stained with caspase 3 and TUNEL (Fig. 4) was significantly increased in the two PT-treated groups compared with the vehicle-treated group, indicating that PT promotes apoptosis in the colons of CAC mice.

Discussion

Our previous study demonstrated that PT induces apoptosis in a number of human CRC cell lines, including the HT-29, LS174T and SW480 cell lines, and that it reduces tumor growth and

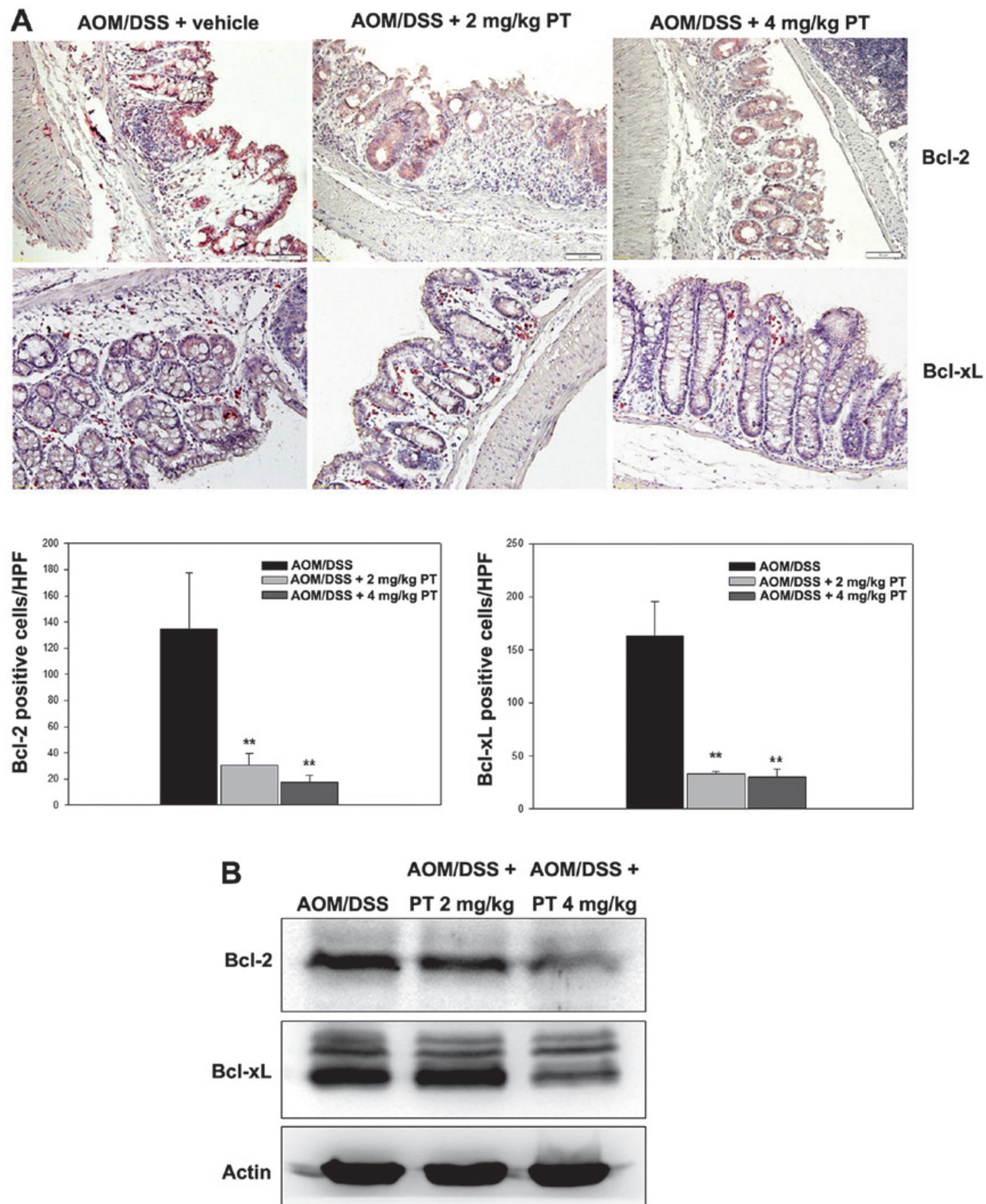

Figure 3. Administration of PT downregulates anti-apoptotic proteins. (A) Sections of colon from vehicle- and PT-treated colitis-associated cancer mice are stained with a Bcl-2 or Bcl-xL antibodies (magnification, x20). The number of positively-stained cells in the colon tissue was counted and represented as a graph. Error bars indicate the standard error of the mean. **$P<0.01$ vs. vehicle-treated group. (B) Western blotting results demonstrate the protein expression levels of p-IκBα and NF-κB p65 in the colon tissues. Actin was used as the loading control. AOM, azoxymethane; DSS, dextran sulfate sodium; PT, parthenolide; p-IκBα, phosphorylated inhibitor of κBα; NF-κB, nuclear factor-κB; HPF, high-power field.

angiogenesis in a CRC xenograft model (28). PT is considered to be a promising candidate as a novel type of chemotherapeutic agent for cancer treatment; however, to the best of our knowledge, no study has yet explained the mechanism of the anticancer effect of PT on CAC. Therefore, the present study selected an AOM/DSS mouse model to assess the role of PT in CAC and in NF-κB inactivation. In agreement with our hypothesis, the results indicated that PT administration clinically and histologically improves AOM/DSS-induced CAC in mice, as assessed by histological injury scores. Furthermore, the beneficial effect of PT treatment appears to inhibit NF-κB activation via blocking IκBα phosphorylation and is associated with the downregulation of the apoptosis-associated molecules, Bcl-2, Bcl-xL and caspase 3. These findings indicate that PT may be a useful therapeutic approach to treat CAC and that PT may block IκBα phosphorylation, thus silencing NF-κB activation.

The oncogenic potential of AOM is markedly augmented in the setting of chronic inflammation, such as that induced by repeated cycles of DSS treatment (29). A recent study

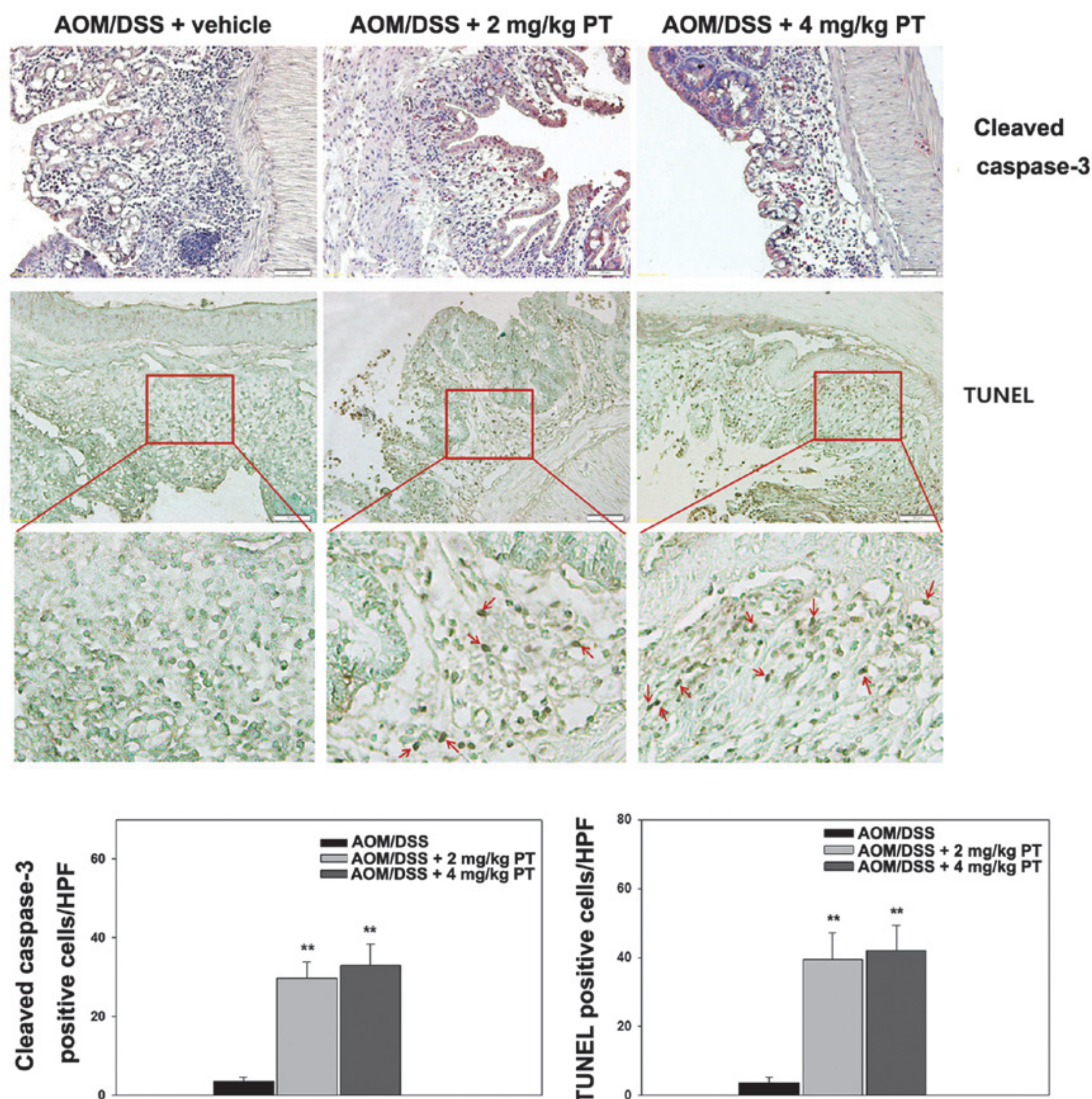

Figure 4. Administration of PT increases apoptosis in dysplasia lesions in colitis-associated cancer mice. Sections of colon from vehicle- and PT-treated mice are stained with caspase 3 antibody and analyzed using TUNEL (magnification, x20). Red arrows indicate TUNEL positively-stained cells. The number of positively-stained cells in the colon tissue was counted and represented as a graph. Error bars indicate the standard error of the mean. $^{**}P<0.01$ vs. vehicle-treated group. AOM, azoxymethane; DSS, dextran sulfate sodium; PT, parthenolide; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling assay; HPF, high-power field.

demonstrated the power of this model by deciphering the epithelial versus myeloid cell contribution of IκB kinase β to polyp formation in the setting of inflammation (30), while a different study investigated the contributions of IL-6 and its downstream mediator, signal transducer and activator of transcription-3 (STAT-3) (31). PT is an effective inhibitor of IL-6-type cytokines, which activate the phosphorylation of STAT3 on Tyr 705. PT has been demonstrated to prevent the activity of IL-6-type cytokines by inhibiting the phosphorylation of STAT3 on Tyr 705 (32,33), indicating that PT, as an inhibitor of IL-6, is able to provide protection from carcinogenesis in experimental CAC models. Although the present study did not demonstrate the role of PT in regulating and suppressing carcinogenesis via the IL-6/STAT3 pathway, additional important insights into the mechanism were obtained.

Previous studies have demonstrated that NF-κB, a central molecule involved in inflammation, regulates the expression of a diverse array of target genes that are involved in promoting cell proliferation, regulating immune and inflammatory responses and contributing towards the pathogenesis of various diseases, including cancer (34-36). In chronic inflammation, NF-κB has a specific role in coupling inflammation to cancer. In total, >15% of all malignancies are initiated by chronic inflammatory disease; for example, skin inflammation initiates squamous cell carcinoma, viral hepatitis initiates liver cancer and inflammatory bowel disease (IBD) initiates CRC (31,37-41). Previous studies have indicated that constitutive NF-κB activation in IBDs may be the cause of the increased risk of developing CRC (42-44). It has been shown that PT is a potent inhibitor of NF-κB activation, which is able to effectively inhibit pro-inflammatory cytokine expression in cultured cells and experimental models (11,15-19,45). Furthermore, a previous study using experimental murine colitis demonstrated that the administration of PT significantly reduces the severity of DSS-induced colitis, as assessed by NF-κB p65 and the blockage of IκBα protein

phosphorylation, and resulting in a reduction in the expression of inflammatory mediators, including myeloperoxidase activity, TNF-α and IL-1β (46). The present study has demonstrated that PT suppresses IκBα phosphorylation and NF-κB p65 in a CAC mouse model, providing experimental evidence of the potential application of PT in CAC patients.

The association between NF-κB and apoptosis was recently elucidated in studies demonstrating that the inhibition of NF-κB activation, by the IκB super repressor (47,48) or in Rel A (p65) knock-out cells (49), results in increased apoptosis. In particular, NF-κB appears to be responsible for the activation of genes involved in proliferation and tumor survival, such as those of the apoptotic proteins Bcl-2 and Bcl-xL (50,51). The results of the present study indicated that PT is essential for AOM/DSS-induced colon cancer carcinogenesis via the apoptotic route-associated Bcl-2 family members, which cause inhibition of NF-κB activation. Additionally, the PT-treated cells appeared to undergo greater levels of apoptosis compared with the vehicle-treated group, as evaluated by caspase 3 antibody staining and TUNEL assay. Thus, the present study indicates that NF-κB is the key regulator of PT-mediated apoptosis via the apoptosis signaling cascade.

In conclusion, the present study demonstrated that the administration of PT appears to significantly inhibit the inflammation-carcinoma sequence and may be a crucial regulator of experimental CAC. Possibly via the negative regulation of NF-κB, PT reduces IκBα phosphorylation, and Bcl-2 and Bcl-xL expression, increases the expression of caspase 3, and promotes cell apoptosis, resulting in the suppression of tumorigenesis. Therefore, PT may be a novel chemopreventive agent for the treatment of CAC.

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