Curcumin Inhibits STAT3 Signaling in the Colon of Dextran Sulfate Sodium–treated Mice

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Turmeric (Curcuma longa L., Zingiberaceae) has a long history of use in medicine for the treatment of inflammatory conditions. One of the major constituents of turmeric is curcumin (diferuloylmethane), which is responsible for its characteristic yellow color. In the present study, we have examined the chemoprotective effects of curcumin on dextran sulfate sodium (DSS)-induced mouse colitis. For this purpose, we pre-treated male ICR mice with curcumin (0.1 or 0.25 mmol/kg in 0.05% carboxymethyl cellulose) by gavage for a week and then co-treated the animals with curcumin by gavage and 3% DSS in drinking water for another 7 days. Our study revealed that administration of curcumin significantly attenuated the severity of DSS-induced colitis and STAT3 signaling in mouse colon. The levels of the cell cycle regulators CDK4 and cylinD1 were significantly reduced by curcumin administration. Moreover, the expression of p53, which is an upstream regulator of the CDK4-cylinD1 complex, was inhibited by curcumin treatment. (J Cancer Prev 2013;18:186–191)

Key Words: Colitis, Curcumin, Dextran sulfate sodium, STAT3, Mouse colon

INTRODUCTION

Colorectal cancer (CRC), the third most common malignant neoplasm with the second leading cause of death worldwide, is related to inflammatory bowel disease (IBD) including ulcerative colitis and Crohn's disease.[1] As chronic inflammation has been recognized as an essential risk factor for a variety of human malignancies,[2] alleviating inflammation is an important strategy for chemoprevention. Chemoprevention is a strategy of use of nontoxic chemicals such as phytochemicals to inhibit, retard, or reverse the process of multistage carcinogenesis.[3]

Curcumin (Fig. 1A), the major yellow colouring pigment found in the household spice turmeric (Curcuma longa Linn, Zingiberaceae), has been used for centuries in food preparation as well as in Ayurvedic traditional medicine to treat inflammatory disorders.[4] Curcumin has low toxicity and has been shown to be effective for the treatment of chronic gut inflammation in an animal model, and also beneficial in a randomised cross-over trial in the treatment of ulcerative colitis.[5]

The transcription factor STAT3 is has been reported to play a role in colonic inflammation.[6] STAT3 is a transcription factor activated by a variety of cytokines and growth factors.[7,8] Upon activation, STAT3 translocates to the nucleus, where it regulates genes involved in apoptosis (e.g., Bcl-X1), cell cycle regulator (e.g., cyclin D1), migration, and survival depending on the cell types.[9,10]

Although the inhibitory effect of curcumin on DSS-induced mouse colitis has been reported,[11] the detailed molecular mechanism is still unclear. This prompted us to investigate the effects of curcumin on activation of STAT3,
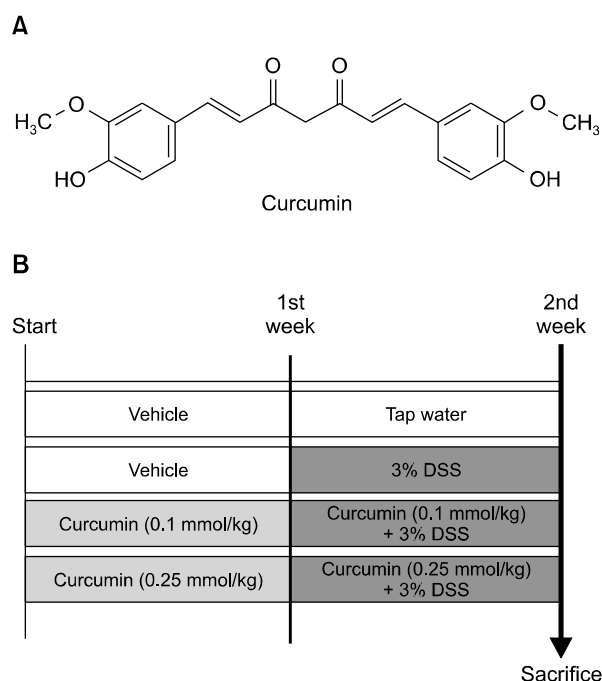
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A

Curcumin

B

Fig. 1. (A) The chemical structure of curcumin, a yellow coloring ingredient of turmeric (*Curcuma longa* L., Zingiberaceae). (B) The experimental protocol for the treatment of mice.

a key transcription factor involved in inflammatory signaling, in the colon of mice challenged with DSS.

MATERIALS AND METHODS

1. Materials

Curcumin was obtained from LKT laboratories (Minneapolis, MN, USA). DSS with a molecular weight of 36,000-50,000 was obtained from MP Biomedical Inc. (Solon, OH, USA). Rabbit polyclonal COX-2 antibody was the product of Cayman Chemical Co. (Ann Arbor, MI, USA). Primary antibodies for pSTAT3, STAT3 and cyclinD1 were procured from Cell Signaling Technology (Danvers, MA, USA). p53 and CDK4 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-actin was purchased from Sigma Aldrich (St. Louis, MO, USA). Anti-rabbit and anti-mouse horseradish peroxidase-conjugated secondary antibodies were products of Zymed Laboratories (San Francisco, CA, USA). Oligonucleotide probe containing the STAT3 consensus sequence (5'-GAT CCT TCT GGG AAT TCC TAG ATC-3') was purchased from Santa Cruz Biotechnology.

Polyvinylidine difluoride (PVDF) membrane was supplied by Gelman Laboratory (Ann Arbor, MI, USA). BCA reagent is a product of Pierce (Rockford, USA). The enhanced chemiluminescence (ECL) detection kit and [γ -^{32}P] ATP were purchased from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other chemicals used during the experiment were obtained in the purest from available at the commercial grades.

2. Animal treatment

Male Institute of Cancer Research (ICR) mice (5 weeks of age) were purchased from Central Lab. Animal Inc. (Seoul, Korea). The animals were housed in climate-controlled quarters (24°C at 50% humidity) with a 12-h light/12-h dark cycle. Male ICR mice were divided into 4 groups (Fig. 1B). Curcumin suspended in 0.05% carboxymethyl cellulose was given orally (0.1 or 0.25 mmol/kg body weight daily) for 7 days. After one week of treatment with either vehicle or curcumin, animals were given 3% DSS in drinking water, except those of the control group, for 7 days with or without curcumin treatment. Control mice were treated with vehicle (0.05% CMC) only.

3. Western blot analysis

Collected colon tissue was homogenized in ice-cold lysis buffer [150 mM NaCl, 0.5% Triton-X 100, 50 mM, Tris-HCl (pH 7.4), 20 mM ethylene glycol tetraacetic acid (EGTA), 1 mM dithiothreitol (DTT), 1 mM Na$_3$VO$_4$ and protease inhibitor cocktail tablet] and lysed for 30 min at 0°C followed by centrifugation at 14,000 rpm for 15 min. Supernatant was collected and total protein concentration was quantified by using the BCA protein assay kit. Cell lysates (30-50 μg protein) were boiled in sodium dodecyl sulfate (SDS) sample buffer for 5 min before electrophoresis on 8-12% SDS-polyacrylamide gel. After transfer to PVDF membrane, the blots were blocked with 5% fat-free dry milk phosphate-buffered saline containing 0.1% Tween-20 (PBST) or 1 % BSA in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 1 h at room temperature and then washed with PBST or TBST buffer. The membranes were incubated for 2 h at room temperature with 1 : 4,000 dilutions of primary antibody for actin, for 12 h at 4°C with 1 : 1,000 dilutions of primary antibodies for p53, cyclinD1, CDK4, pSTAT3, STAT3, pAkt. Blots were

washed three times with PBST or TBST at 10 min intervals followed by incubation with 1 : 5,000 dilution of respective horseradish peroxidase conjugated secondary antibodies (rabbit, goat or mouse) for 1 h and again washed in PBST or TBST for three times. The transferred proteins were visualized with an ECL detection kit according to the manufacturer's instructions.
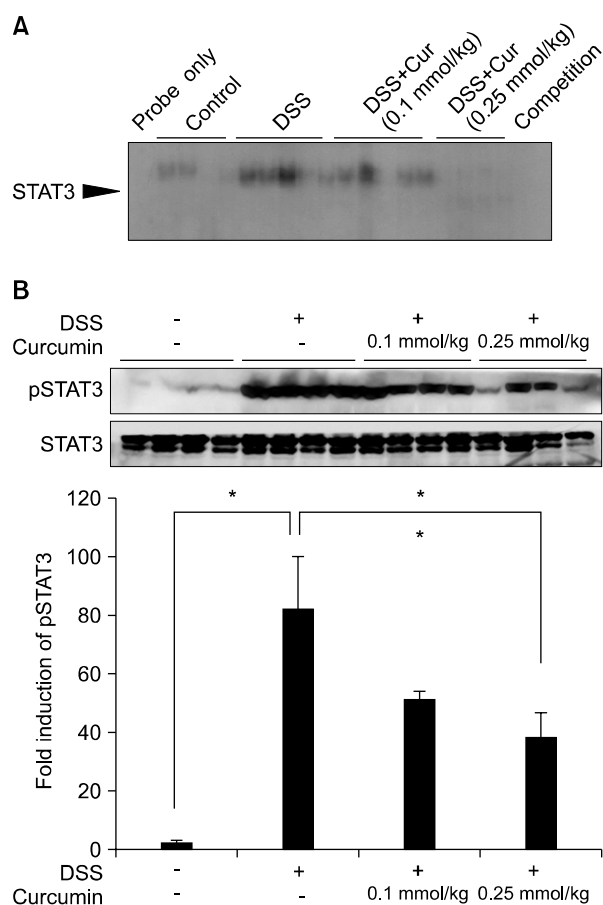
4. Preparation of cytosolic and nuclear extracts from mouse colon

The nuclear extract from mouse colon was prepared by following procedure. In brief, collected colon of mice was
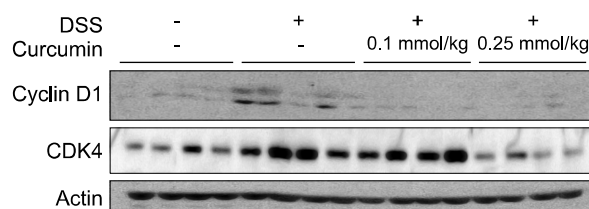
A

B

Fig. 2. Inhibitory effects of curcumin on DSS-induced DNA binding and phosphorylation of STAT3 in nuclear extracts of mouse colon. (A) Nuclear extracts were prepared from colon of each treatment group. STAT3 DNA binding was measured by EMSA as described in Materials and Methods. To ensure the specific STAT3 DNA binding, a competition assay was performed with excess unlabeled oligonucleotides. (B) Colon tissue lysates from different treatment groups were separated by10% SDS-polyacrylamide gel and immunoblotted by using pSTAT3 (Tyr705) antibody.

homogenized in hypotonic buffer A [10 mM HEPES (pH 7.8), 10 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA and 0.1 mM phenylmethylsulfonylfluoride (PMSF)]. 10% Nonidet P-40 (NP-40) solution was added into homogenate and the mixture was then centrifuged for 2 min at 14,000 rpm. The supernatant was collected as a cytosolic fraction. The precipitated nuclei were washed twice with buffer A plus 40 μl of 10% NP-40, centrifuged, resuspended in 200 μl of buffer C [50 mM HEPES (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 20% glycerol] and centrifuged for 5 min at 14,000 rpm. The supernatant containing nuclear proteins was collected and stored at $-70^{\circ}C$ after determination of protein concentration.

5. Electrophoresis mobility shift assay (EMSA)

The EMSA for STAT3 DNA binding was performed using a DNA-protein binding assay kit, according to the manufacturer's protocol (Gibco BRL, Grand Island, NY, USA). Briefly, STAT3 oligonucleotide probe 5'-GAT CCT TCT GGG AAT TCC TAG ATC- 3' was labeled with [γ-^{32}P] ATP by T4 polynucleotide kinase and purified on a Nick column (Amersham Pharmacia Biotech). The binding reaction was carried out in 25 μl of the mixture containing 5 μl of incubation buffer [10 mm Tris-HCl (pH 7.5), 100 mm NaCl, 1 mm DTT, 1 mm EDTA, 4% glycerol, and 0.1 mg/ml sonicated salmon sperm DNA], 10 μg of nuclear extracts, and 100,000 c.p.m. of [γ-^{32}P] ATP-end labeled oligonucleotide. After 50 min incubation at room temperature, 2 μl of 0.1% bromophenol blue was added, and samples were electrophoresised through 6% non-denaturing polyacrylamide gel at 150 V in a cold room for 2 h. Finally,

Fig. 3. Effects of curcumin on DSS-induced expression of cell cycle regulator cyclin D1 and CDK4. Whole tissue lysates from different treatment groups were separated by 10% SDS-polyacrylamide gel and immunoblotted by using antibody specific for detecting cyclin D1 or CDK4.

the gel was dried and exposed to X-ray film.

6. Statistics

Values were expressed as the mean ± SEM of at least three independent experiments. Statistical significance was determined by Student's t-test and $P < 0.05$ was considered to be statistically significant.

RESULTS

1. Curcumin inhibits DSS−induced DNA binding of STAT3 in mouse colon
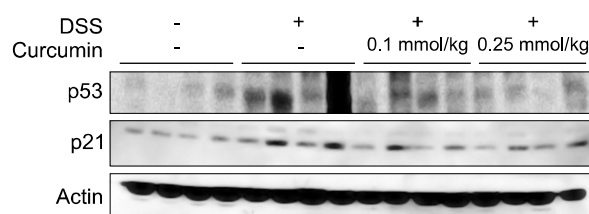
STAT3 contributes to colitis-associated colorectal cancer through transcriptional activation of diverse pro-inflammatory and proliferative genes.[12] Our study revealed that the DNA binding of STAT3 was markedly increased in colon tissue of mice challenged with DSS alone, while administration of curcumin by gavage significantly negated DSS-induced STAT3 DNA binding (Fig. 2A). Mechanistically, STAT3 is activated through phosphorylation of tyrosine 705 residue by a variety of protein tyrosine kinases.[13] Western blot analyses revealed that curcumin suppressed DSS-induced phosphorylation of STAT3 in mouse colon (Fig. 2B).

2. Curcumin attenuates DSS−induced expression of cyclin D1 and CDK4 in mouse colon

Colitis, which often turns into colorectal cancer, is associated with increased cell proliferation. One of the cell cycle regulatory proteins is cyclin D1, which by forming a complex with Cdk4, promotes cell cycle progression. The expression of both Cyclin D1 and Cdk4 is transcriptionally regulated by STAT3. DSS treatment elevated the expression of cyclin D1 in mouse colon, which was attenuated by administration of curcumin (Fig. 3). Likewise, curcumin inhibited DSS-induced expression of CDK4 in mouse colon (Fig. 3).

3. Curcumin inhibits DDS−induced expression of p53

p53 is a key biosensor of inflammatory stress. Since p53 is activated by phosphorylation during inflammation stress,[14] we investigated the level of p53 to measure the degree of acute inflammation. Expression levels of p53 and its target

Fig. 4. Effects of curcumin on DSS−induced expression of p53 and p21. Whole tissue lysates from different treatment groups were separated by 10% SDS−polyacrylamide gel and immunoblotted by using antibody specific for detecting p53 or p21.

protein p21 were elevated by DSS treatment and it was inhibited by curcumin (Fig. 4).

DISCUSSION

Despite many anticancer treatments have been developed, cancer is still a global health problem with severe morbidity and mortality. As cancer is a multi-step process typically occurring over an extended period beginning with initiation followed by promotion and progression, the preventive strategy to reduce the risk of cancer development is important.[15] One such example is chemoprevention. The goals of chemoprevention are to block, retard or reverse the process of carcinogenesis, using non-toxic substances of natural or synthetic origin.[15,16] Dietary phytochemicals such as capsicin, resveratrol, curcumin have been known to prevent the initiation and promotion of multistep carcinogenesis.[15,16]

Curcumin is a polyphenol and pharmacologically active compound of the perennial herb *Curcuma longa* (commonly known as turmeric). Traditionally, turmeric has been used for a foodstuff, cosmetic, and medicine. As a spice, turmeric has been used to provide curry with its distinctive yellow color and flavor.[17] In folk medicine, turmeric and natural curcuminoids have been used as therapeutic treatment over the centuries in different parts of the world. In traditional Chinese herbal medicine, it is used to treat diseases associated with abdominal pain.[18] In ancient Hindu medicine, it was used to treat sprains and swelling.[19] Throughout the Orient history, it has traditionally been used as a good therapeutic medication, particularly as an anti-inflammatory,[20] and many of its therapeutic effects have been confirmed by modern scien-

tific research. The most remarkable and key rationale for the continuing traditional therapeutic use of curcumin is its extremely good safety profile that was reported in several studies.[21,22] Anti-carcinogenic and anti-inflammatory effects of curcumin have been observed in various experimental models. Oral administration of curcumin suppressed adenocarcinoma formation in dimethylhydradine-initiated and DSS-promoted mouse colon carcinogenesis.[23] Dietary curcumin also suppressed the production of cytokines such as tumor necrosis factor-α and IFN-γ and expression of cyclooxygenase-2 and inducible nitric oxide synthase induced by DSS in moue colon.[24]

Recently, STAT3 has been reported to be strongly phosphorylated in patients with IBD such as ulcerative colitis and Crohn's disease[25] as well as in DSS-induced colitis in mice.[26] STAT3 is reported to be involved in colonic inflammation and play an important role in colitis-associated cancer.[6,12] During the early stage of colitis-associated tumorigenesis, the activation of STAT3 as well as NF-κB promotes cell proliferation and survival through the upregulation of their target genes including cyclin D1 and Bcl-xL.[27] In this study, we found that curcumin suppressed DNA binding of STAT3 in DSS-stimulated mouse colon. In addition, we also found that curcumin inhibited DSS-induced phosphorylation of STAT3 in mouse colon. Cyclin D1 has been reported to be associated with cell proliferation and regulated by NF-κB and STAT3.[13,28] Moreover, CDK4 is a protein that forms a complex with cyclin D1 and thereby regulates G1 to S cell cycle progression. So, we determined the levels of cyclin D1 and CDK4 in each group and found that curcumin inhibited DSS-induced expression of cyclin D1 and CDK4.

p53 is a key biosensor of inflammatory stress and activated by phosphorylation during inflammation.[14] Therefore, we investigated the level of p53 to measure the degree of acute inflammation. p53 was induced by DSS treatment and it was inhibited by curcumin in a dose-dependent manner.

Taken together, the above findings suggest that oral administration of curcumin provokes anti-inflammatory effects by inhibiting the STAT3. In addition, curcumin also elicits anti-proliferative effects through suppression cyclin D1, CDK4 expression, which is likely to be mediated by

blocking STAT3 signaling. Considering STAT3 as a potential molecular link between inflammation and cancer and the role of aberrantly overexpressed cyclin D1 in tumor promotion, the present study provides the molecular basis for the previously reported chemopreventive effects of curcumin.

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