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Consumption of Lycopene Inhibits the Growth and Progression of Colon Cancer in a Mouse Xenograft Model

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ABSTRACT: A previous study indicated that lycopene could significantly inhibit the proliferation of human colon cancer cells in vitro. However, the in vivo anticancer effects of lycopene against colon cancer have not been demonstrated yet. Therefore, this study investigated whether consumption of lycopene could prevent the growth and progression of colorectal tumor in a mouse xenograft model. Bioluminescence imaging, histopathological, immunofluorescence (IFC), and immunohistochemical (IHC) staining results indicated that lycopene could effectively suppress the growth and progression of colon cancer in tumor-bearing mice. The results demonstrated that lycopene significantly suppressed the nuclear expression of PCNA and β-catenin proteins in tumor tissues. Consumption of lycopene could also augment the E-cadherin adherent molecule and nuclear levels of cell cycle inhibitor $p21^{CIP1/WAF1}$ protein. The chemopreventive effects of lycopene were associated with suppression of COX-2, PGE_2, and phosphorylated ERK1/2 proteins. Furthermore, the inhibitory effects of lycopene were inversely correlated with the plasma levels of matrix metalloproteinase 9 (MMP-9) in tumor-bearing mice. These results suggested that lycopene could act as a chemopreventive agent against the growth and progression of colorectal cancer in a mouse xenograft model.

KEYWORDS: lycopene, β-catenin, proliferating cell nuclear antigen, matrix metalloproteinase 9, tumor growth, human colon cancer cells

INTRODUCTION

Colorectal cancer is one of the leading causes of cancer death in many countries. In the United States alone, thousands of deaths are attributed to this cancer every year.[1] Several oncoproteins such as KRAS/RAF/mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3 kinase (PI-3K)/Akt are frequently mutated in cancer.[2−4] RAS mutations augment the GTPase activity and transmit signals to RAF. RAF mutations could also drive the MAPK signaling pathway and induce tumor growth and progression. Meanwhile, PI3K mutations could activate Akt and mTOR cascades to enhance cell survival and escape from cell apoptosis. Suppression of apoptotic pathways including the downstream caspase-3 molecule could avoid cell death.[5] RAS, RAF, and PI3K mutations occur in 37, 13, and 33% of colon cancer patients, respectively.[6] The incidence of these cancer-specific point mutations is particularly high in colorectal tumors and has been linked to poor outcomes.[2,3,6] The aberrant activation of RAS/RAF/MEK/MAPK signaling pathways stimulates key processes involved in tumor growth and progression, including proliferation, angiogenesis, invasion, and metastasis.[7] The activation of MAPK/ERK signaling pathway could induce the expression of cyclooxygenase-2 (COX-2) protein, its principal metabolite prostaglandin E_2 (PGE_2), and inflammatory response.[8,9] More than 50% of colorectal carcinomas have elevated levels of COX-2 protein.[9] The aberrant activation of the MAPK/ERK signaling pathway also plays an important role in the disassembly of E-cadherin adherent complex and augments nuclear accumulation of β-catenin transcription factors in several types of cancer.[10−12] Abnormal accumulation of β-catenin is correlated with tumor growth and progression.[13] Moreover, suppression of β-catenin could block the formation of colorectal carcinoma.[14] Therefore, a recent study implicated that β-catenin could be an important biomarker of human cancer.[15] During the activation of these signaling pathways, cell cycle related proteins such as proliferating cell nuclear antigen (PCNA) are major regulators for DNA replication. Up-regulation of PCNA is strongly correlated with tumor growth. Recent studies indicated that $p21^{CIP1/WAF1}$ protein may serve as an inhibitor protein and suppress the function of PCNA protein.[16,17]

During the progression of colorectal tumor, overexpression of matrix metalloproteinases (MMPs) is highly correlated with inflammatory response, tumor growth, angiogenesis, and metastasis.[18,19] MMPs could degrade the extracellular matrix and create a microenvironment supporting maintenance of tumor development. Invasion of colon cancer cells into surrounding stroma occurs through the augmented expression of MMPs.[20,21] Previous studies suggested that elevated expression of MMP-9 was strongly correlated with poor prognosis and low survival rate

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in colon cancer patients.[22] However, suppression of MMPs could prevent the development of colorectal tumors.[23]

Epidemiological studies have shown that people consuming traditional Mediterranean diets with good amounts of vegetables, especially tomato, fruits, olive oil, grains, beans, and fish, have lower rates of chronic diseases such as heart disease and cancer. Lycopene, a major component in tomato, exhibited potential anticarcinogenesis activity against several types of cancer cell lines.[24−26] Our previous study demonstrated that lycopene could have anticancer effects to inhibit the proliferation of human colon cancer HT-29 cells (RAF mutation and PI3K mutation) in vitro.[27,28] Epidemiologic studies reported statistically significant inverse associations between tomato consumption and risk of several types of cancer such as lung and prostate cancer.[25,29] However, the in vivo anticancer effects of lycopene on the growth of colon cancer have not been demonstrated yet. The lack of results across preclinical studies may not be able to demonstrate the chemopreventive effects of lycopene against human colon cancer. Therefore, the current study would investigate whether consumption of lycopene could inhibit the tumor growth and progression of colorectal cancer in nude mice inoculated with human colon cancer HT-29 cells (RAF mutation and PI3K mutation).

MATERIALS AND METHODS

Reagents and Antibodies. Lycopene dry powder (Lycovit 10%) was kindly provided by Dr. Xiang-Dong Wang (Tufts University). Anti-$p21^{CIP1/WAF1}$, anti-β-catenin, anti-E-cadherin, anticleaved caspase-3, and anti-PCNA antibodies were purchased from Cell Signaling Technology, Inc. (Danvers, MA). Antiphosphorylation ERK1/2, antitotal ERK1/2 (t-ERK1/2), anticleaved caspase-3, and anti-COX-2 antibodies were purchased form Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin antibody, avidin−biotin complex (ABC), diaminobenzidine (DAB), 4,6-diamidino-2-phenyindole (DAPI), L-glutamine, sodium bicarbonate, and RPMI-1640 medium were purchased from Sigma (St. Louis, MO). Fetal bovine serum (FBS) was purchased from Thermo Fisher Scientific Inc. (Pittsburgh, PA). Luciferin and human colon cancer cells HT-29 cell line with luciferase reporter gene were purchased from Caliper Life Sciences Inc. (Hopkinton, MA). Phosphate-buffered saline (PBS), fluorescein isothiocyanate (FITC), and rhodamine were purchased from Invitrogen Inc. (Carlsbad, CA). MMP-9 and PGE_2 ELISA kits were purchased from R&D Systems, Inc. (Minneapolis, MN).

Cell Culture. Briefly, HT-29 colon cancer cells were cultured in a 37 °C humidified incubator with 5% CO_2 and grown to confluency using RPMI-1640 media. RPMI-1640 medium was supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and 2 g/L sodium bicarbonate.

Animals, Xenograft Implantation of Tumor Cells, Diet, and Lycopene Supplementation. Female adult (3−4 weeks old) BALB/cAnN-Foxn1 nude mice (22−25 g) were obtained from the National Laboratory Animal Center (Taipei, Taiwan). Mice were maintained under specific pathogen-free conditions in facilities approved by the National Laboratory Animal Center in accordance with current regulations and standards. During the entire experimental period, mice were fed a standard Lab 5010 diet purchased from LabDiet Inc. (St. Louis, MO). The standard diet contains 4.5% crude fat and had no detectable amounts of lycopene as indicated by the supplier.

To produce colon tumors, subconfluent cultures of colon cancer HT-29 cells were given fresh medium 24 h before being harvested by a brief treatment with 0.25% trypsin and 0.02% EDTA. Trypsinization was stopped with medium containing 10% FBS, and the cells were washed twice with PBS and resuspended in serum-free RPMI-1640 medium. Only single-cell suspensions with a viability of >90% were used for injections.

Mice anesthetized with an inhalation of isofluorane were placed in a supine position. Human colon cancer HT-29 cells (1×10^6/0.1 mL medium) transfected with luciferase reporter gene were injected subcutaneously into the right flank of thigh tissues. A well-localized bleb was a sign of a technically satisfactory injection.

After the inoculation, mice were divided into three subgroups ($n = 9$ for each subgroup). Lycopene was dissolved in corn oil at dosages of 3 and 6 mg/kg of body weight (BW). Mice received lycopene by gavage at a dose of 0, 3, or 6 mg/kg of BW per day. Body weights were also measured weekly. Following 5 weeks of supplementation, mice were sacrificed and different tissues including muscle, lung, liver, intestine, kidney, spleen, pancreas, and tumors were frozen immediately.

Bioluminescence Imaging of Colon Cancer. Documentation of bioluminescence imaging was also performed weekly during the 5 week experimental period by using a noninvasive in vivo imaging system 200 (IVIS 200) (Caliper Life Sciences). Briefly, luciferin (15 mg/mL), the substrate of luciferase, was injected into the tumor-bearing nude mice peritoneally. Results of bioluminescence imaging were collected and analyzed by accessory software. The intensity of bioluminescence was expressed as $p/s/cm^2$. Bioluminescence intensities compared to the control group (tumor-bearing mice without lycopene supplementation at week 2) represented proliferation indices.

Histopathological, Immunohistochemical, and Immunofluorescent Staining of Tumor Tissues. Frozen tumor tissues were cut in 5 μm sections and fixed with 4% paraformaldehyde immediately. Sections were stained with Mayer's hematoxilin−eosine for light microscopic analysis. Negative controls did not show staining. In a blinded manner, three hot spots were examined per tumor section (high power fields × 200) of nine different tumors in each group. Images of tumor sections were acquired on an Olympus BX-51 microscope using an Olympus DP-71 digital camera and imaging system.

For immunohistochemical staining, frozen tissue sections were treated with 0.3% hydrogen peroxide to block the endogenous peroxide activity. Nonspecific protein bindings were blocked with 10% normal goat serum (NGS) for 1 h followed by the incubation of anti-COX-2 primary antibody (1:300). Tissue sections were washed with 0.1 M PBS and incubated with biotinylated immunoglobin G (1:300 secondary antibody) at room temperature for 1 h. Tissue sections were stained with ABC, DAB, and hydrogen peroxide. Images of tumor sections were acquired on an Olympus BX-51 microscope using an Olympus DP-71 digital camera and imaging system.

For immunofluorescence staining, primary colon cancer tissue were frozen, sectioned, and subjected to anti-E-cadherin and anti-β-catenin antibodies. The sectioned tissues probed with anti-E-cadherin antibody were further subjected to secondary antibody with anti-IgG conjugated FITC label. The sectioned tissues probed with anti-β-catenin antibody were further subjected to secondary antibody with rhodamine label. Cell nuclei were stained with DAPI. Images of tumor sections were acquired on a Leica SP2 confocal spectral microscope using an accessory digital camera and imaging system. Imaging was documented at 600× magnification. To investigate the apoptotic levels, sectioned tissues were probed with anticleaved caspcase-3 antibody and further subjected to secondary antibody with anti-IgG conjugated FITC label. Cell nuclei were stained with DAPI. Imaging was documented at 200× magnification.

Quantitative Real-Time PCR (qPCR). Tissue RNA samples were prepared, extracted from each group, and reversely converted into cDNA for further analysis. Briefly, cDNA samples were used in PCR reaction mix containing gene-specific primers: COX-2 forward, 5′-CGCTCAGCCATACAGCAA-3′, and reverse, 5′-GAATCCTGTCCGGGTACAATC-3′; and GAPDH (used as an internal control gene) forward, 5′-ACGGATTTGGTCGTATTGGG-3′, and reverse, 5′-TGATTTTGGAGGGATCTCGC-3′. The cDNA was amplified in the following conditions: denaturation, 95 °C for 2 min; amplification, 40 cycles (94 °C for 30 s); annealing, 1 min for 30 s; and extension, 1 min

for 30 s. Quantitative PCR experiments were performed using the real-time PCR detection system (ABI 7900). The relative COX-2 mRNA expression levels in lycopene treatment groups were presented as percentage of the corresponding tumor control group.

Western Blotting Analysis. Nuclear and cytoplasma fractions from animal tissues were prepared by using a Nuclear and Cytoplasmic Extract Reagent Kit (NE-PER) with protease inhibitor and phosphatase inhibitors (Pierce Biotechnology, Rockford, IL).

Briefly, tissues were extracted according to the manufacturer's instruction. Tissue lysates were cleared by centrifugation. Cross-contamination between nuclear and cytoplasma fractions was not found (data not shown).

Nuclear proteins (100 μg) were fractioned on 10% SDS-PAGE, transferred to nitrocellulose membrane, and blotted with anti-p21$^{CIP1/WAF1}$ monoclonal antibody, according to the manufacturer's instructions. The blots were stripped and reprobed with lamin A/C antibody as loading control. Levels of PCNA and β-catenin were measured by using the same procedure described above.

Cytoplasma proteins (100 μg) were fractioned on 10% SDS-PAGE, transferred to nitrocellulose membrane, and blotted with anti-phosphorylation ERK1/2 monoclonal antibody, according to the manufacturer's instructions. The blots were stripped and reprobed with total-ERK1/2 antibody as loading control.

Detection of MMP-9 and PGE2 by Enzyme-Linked Immunosorbent Assay (ELISA). The plasma levels of MMP-9 protein were measured by ELISA kit according to the manufacturer's instruction (R&D Systems). Briefly, 100 μL of diluted plasma sample (1:100 dilution) was added to each well and analyzed according to the instructions. Upon completion of the ELISA process, the plate was read at 450/570 nm wavelength using a microplate reader.

Serum levels of PGE_2 were measured by ELISA kit according to the manufacturer's instruction (R&D Systems). Briefly, 150 μL of diluted serum sample was added to each well and analyzed according to the instructions. Upon completion of the ELISA process, the plate was read at 450/570 nm wavelength using a microplate reader.

Gelatin Zymography. Plasma samples from tumor and lycopene treatment groups were performed as follows. Gels were copolymerized with 0.1% gelatin. For each group, equal amounts of plasma sample were loaded. Electrophoresis was carried out using the minigel slab apparatus Mini Protean 2 (Bio-Rad) at a constant voltage of 150 V, until the dye reached the bottom of the gel. Following electrophoresis, gels were washed in buffer solution (2.5% Triton X-100 in 50 mM Tris-HCl (pH 7.5)) for 1 h in an orbital shaker. Then the zymograms were incubated for 24 h at 37 °C in incubation buffer (0.15 M NaCl, 10 mM $CaCl_2$, 0.02% NaN_3 in 50 mM Tris-HCl (pH 7.5)). Gels were then stained with Coomassie blue and destained with 7% methanol and 5% acetic acid. Areas of enzymatic activity appeared as clear bands over the dark background.

Statistical Analysis. Quantitative methodology was used to determine the difference in tumor growth among experimental sets and tumor control sets (n = 9) of colon cancer HT-29 cell xenograft tumor tissue. All data were expressed as the mean ± SEM. In brief, statistical analyses of the differences in tumor growth among experimental and control conditions were performed using SYSTAT. Confirmation of difference in proliferation indices as being statistically significant requires rejection of the null hypothesis of no difference between mean weight obtained from sets of experimental and control groups at the p = 0.05 level with the one-way analysis of variance (ANOVA). The Bonferroni post hoc test was used to determine differences among different groups.

RESULTS

Consumption of Lycopene Suppressed the Growth and Progression of Colorectal Tumor in a Mouse Xenograft Model. Our previous studies already showed that lycopene could act as an anticancer compound. However, no studies have investigated whether consumption of lycopene could inhibit the tumor growth and progression of colorectal tumors in vivo yet. Therefore, we examined the effects of lycopene on the growth of human colon cancer HT-29 cells in a mouse xenograft model. As shown in Figure.1A, supplementation of lycopene at low (3 mg/kg of BW per day; Lyc3) or high (6 mg/kg of BW per day; Lyc6) dosage could effectively inhibit the growth of colorectal tumors in a mouse xenograft model. Bioluminescent imaging results also suggested that intake of lycopene could effectively suppress the growth of colon cancer by using a noninvasive in vivo imaging system (IVIS) during the 5 week experimental period (Figure1B,C). Moreover, histopathological staining results indicated that consumption of lycopene at low (Lyc3) or high (Lyc6) dosage could reduce proliferative levels of colon cancer cells in tumor-bearing mice (Figure.1D). No hepatoxicity of lycopene at doses of 3 or 6 mg/kg of BW was observed in this study (data not shown). These results suggested that consumption of lycopene could effectively prevent the growth of colorectal cancer in a mouse xenograft model.

Inhibitory Effects of Lycopene on the Growth of Colon Cancer Were Associated with Augmented Apoptosis, Up-regulation of p21$^{CIP1/WAF1}$ Inhibitory Proteins, and Suppression of PCNA Expression in Tumor-Bearing Mice. The results described above already demonstrated the inhibitory effects of lycopene on the growth of colon cancer cells in a mouse xenograft model. We further examined the molecular actions of lycopene in tumor-bearing mice. It is well-known that PCNA protein plays an important role during the tumor growth and development. Up-regulation of p21$^{CIP1/WAF1}$ protein could interrupt cell cycle progression and cell proliferation. Therefore, we investigated whether consumption of lycopene could modulate the expression of PCNA and p21$^{CIP1/WAF1}$ proteins. As shown in the Figure 2A, consumption of lycopene could effectively suppress the nuclear accumulation of PCNA protein in tumor-bearing mice. The results further demonstrated that lycopene could induce the expression of p21$^{CIP1/WAF1}$ protein. Taken together, the results indicated that lycopene could effectively suppress cell proliferation in part through the modulation of PCNA and p21$^{CIP1/WAF1}$ proteins. Thus, one plausible mechanism by which lycopene effectively inhibits the proliferation of colon cancer cells is associated with the induction of cell cycle inhibitor p21$^{CIP1/WAF1}$ protein and the suppression of PCNA protein in tumor-bearing mice. To further validate the importance of lycopene, we investigated its chemopreventive effects on apoptotic pathways. As shown in Figure 2B, consumption of lycopene significantly augmented the levels of cleaved caspase-3 proteins in tumor-bearing mice. It is plausible that lycopene consumption could inhibit cell proliferation accompanied with cell apoptosis in tumor tissues.

Consumption of Lycopene Could Modulate the Expression of β-Catenin and E-Cadherin Proteins in Tumor Tissues. Several studies demonstrated the important role of E-cadherin/β-catenin adherent complex in the maintenance of cell integrity and the suppression of cell invasion.[30] Our previous study also indicated that lycopene could effectively inhibit cell proliferation in part through the suppression of nuclear β-catenin protein in vitro.[27] To explore the possible mechanism of actions, we further investigated the effects of lycopene on the expression of β-catenin and E-cadherin proteins. As shown in Figure 3A, consumption of lycopene could moderately induce the expression of E-cadherin in a mouse xenograft model. Lycopene could

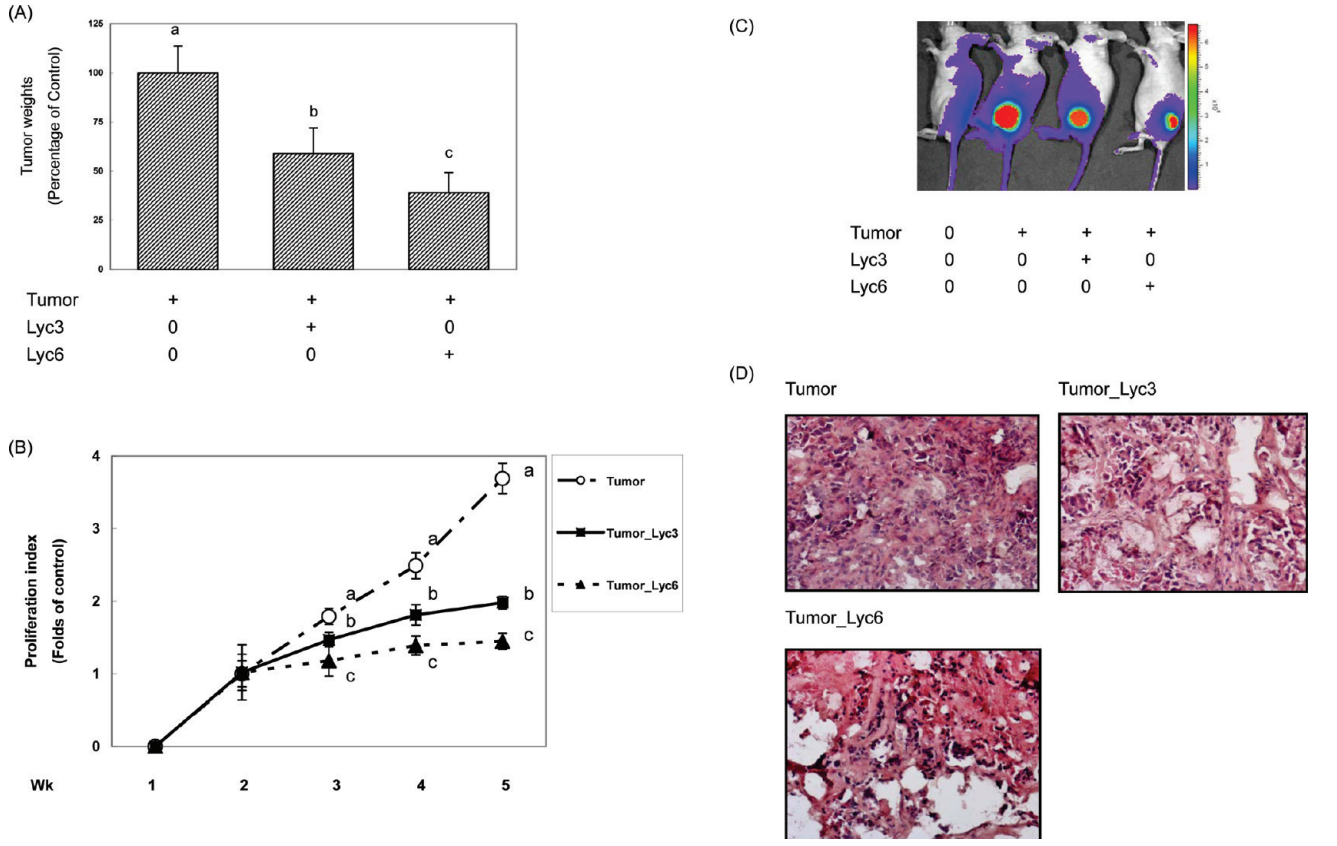

Figure 1. Consumption of lycopene suppressed the growth and progression of colorectal tumor in a mouse xenograft model. (A) Xenograft nude mice ($n = 9$ for each group) were divided into three groups (tumor, tumor with Lyc3, tumor with Lyc6) and given lycopene (0, 3, and 6 mg/kg of body weight (BW)/day) for 5 weeks. Data represent the change of tumor weight among tumor group (control group), tumor with 3 mg/kg of BW/day lycopene supplementation (Lyc3), and tumor with 6 mg/kg of BW/day lycopene supplementation (Lyc6). Different letters represent statistically significant difference, $p < 0.05$. (B) The extent of tumor growth was evaluated by using a bioluminescent imaging system (IVIS) model 200 (Caliper Life Science, Hopkinton, MA). Data of bioluminescent intensity represented the proliferation index of primary tumor tissues. Different letters at the same time point represent statistically significant difference, $p < 0.05$. (C) Xenograft nude mice were given lycopene (at dosages of 3 and 6 mg/kg of BW per day) for 5 weeks. The extent of tumor growth was evaluated by using a bioluminescent imaging system. Data represent the bioluminescent imaging of primary tumor tissues at week 5. (D) Tumor tissues were formalin-fixed, embedded in paraffin, sectioned, and subjected to hematoxylin and eosin (H&E) staining as described under Materials and Methods. Imaging was documented at 200× magnification. Blue spots represent the nuclei stained with hematoxylin. Red spots represent cytoplasma stained with eosin.

also significantly inhibit the nuclear levels of β-catenin protein in tumor tissues (Figure 3B). Immunofluorescence staining results demonstrated that consumption of lycopene could induce expression of E-cadherin (indicated with green fluorescence) and prevent the nuclear translocalization of β-catenin (indicated with red fluorescence) (Figure 3C). These results suggested that lycopene could effectively prevent the disassembly of E-cadherin/β-catenin adherent complex in tumor-bearing mice. They also provided novel evidence of lycopene on the prevention of growth and progression of colon cancer through the stabilization of E-cadherin/β-catenin adherent complexes.

Inhibitory Effects of Lycopene on Tumor Progression Were Associated with the Suppression of COX-2, PGE_2, and Phosphorylation ERK1/2 Proteins in Tumor-Bearing Mice. The activation of MAPK/ERK cascades is frequently observed in cancer and involves the down-regulation of the E-cadherin molecule. A previous study also reported that suppression of MAPK/ERK cascade could prevent the loss of the E-cadherin molecule in several types of cancer cell lines.[11] Thus, we further examined the effects of lycopene on the MAPK/ERK cascade in tumor-bearing mice. As shown in the Figure 4A, mice inoculated with colon cancer HT-29 cells had augmented levels of phosphorylation ERK1/2 protein. However, consumption of lycopene could effectively suppress the activation of ERK1/2 cascade in tumor-bearing mice. To prove the chemopreventive effects of lycopene on tumor progression, we further examined the effects of lycopene on COX-2 expression. As shown in Figure 4B, lycopene could effectively inhibit the expression of COX-2 (brown area) in the cytoplasma of human colon cancer HT-29 cells in a mouse xenograft model. The results of real time-PCR demonstrated that lycopene consumption effectively suppresses COX-2 mRNA levels (Figure 4C). To further examine the inhibitory effects of lycopene consumption on the major metabolites (PGE_2) of COX-2, we analyzed the serum levels of PGE_2 in tumor-bearing mice. As shown in Figure 4D, lycopene consumption could effectively inhibit serum PGE_2 levels in a xenograft mouse model.

Chemopreventive Effect of Lycopene Was Inversely Correlated with Plasma Levels of MMP-9. Previous studies have already shown that overexpression of MMP-9 protein was correlated with tumor angiogenesis and invasion in colorectal carcinomas. In the current studies, we would investigate whether

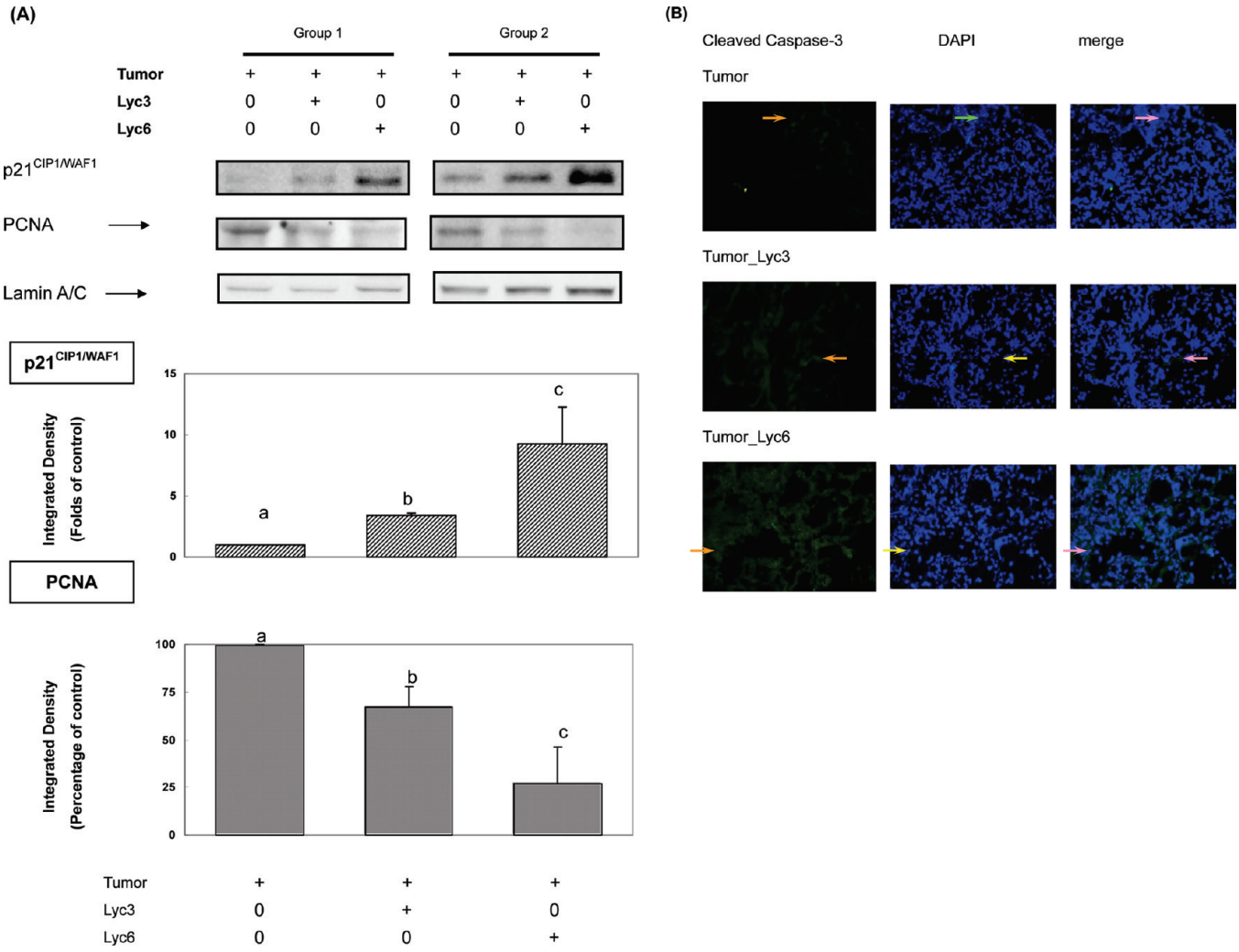

Figure 2. Inhibitory effects of lycopene on the growth of colon cancer were associated with augmented apoptosis, up-regulation of $p21^{CIP1/WAF1}$ inhibitory proteins, and suppression of PCNA expression in tumor-bearing mice. (A) Cell lysates from animal tissues (groups 1 and 2) were prepared by using a Tissue Extract Reagent Kit with protease inhibitor and phosphatase inhibitors according to the manufacturer's instruction. Cross-contamination between nuclear and cytoplasma fractions was not found (data not shown). Cell lysates were blotted with anti-$p21^{CIP1/WAF1}$ and anti-PCNA monoclonal antibodies as described under Materials and Methods. The levels of detection in cell lysate represent the amount of $p21^{CIP1/WAF1}$ and PCNA in human colon cancer cells. The blots were stripped and reprobed with antilamin A/C antibody as loading control. The immunoreactive bands are noted with arrows. The integrated density of $p21^{CIP1/WAF1}$ and PCNA proteins adjusted with internal control protein (lamin A/C) is shown in the bottom panel. Different letters represent statistically significant difference, $p < 0.05$. (B) Tumor tissues were frozen, sectioned, and subjected to anticleaved caspase-3 antibody by immunofluorescence staining described under Materials and Methods. Imaging was documented at 200× magnification. The green fluorescence area represented distribution of cleaved caspcase-3 protein in HT-29 cells stained with monoclonal antibody. The blue fluorescence area represents the location of cell nuclei stained with 4,6-diamidino-2-phenylindole (DAPI). Merged imaging represents the colocalization of cleaved caspase-3 and nuclei in tumor tissues. These results are representative of nine different experiments.

consumption of lycopene could modulate the expression of MMP-9 protein in HT-29 cancer cell inoculated nude mice. As shown in Figure 5A, consumption of lycopene could significantly inhibit the plasma levels of MMP-9 protein in a dose-dependent manner. The results demonstrated that lycopene could also suppress the activity of MMP-9 by using zymogram analysis (Figure 5B).

These results suggested that the inhibitory effects of lycopene on tumor progression of colon cancer were correlated with the diminished plasma levels and activities of MMP-9 proteins in tumor-bearing mice. Taken together, it is probable that lycopene consumption could inhibit the growth and progression of colon cancer in part through suppression of MMP-9 proteins.

DISCUSSION

Most of the papers concerning the anticancer activities of phytochemicals are based on their ability to inhibit the expression of oncoproteins, which has been associated with cancer prognosis. Among them, overexpressions of biomarkers such as β-catenin, COX-2, and MMP-9 proteins may play crucial roles in tumor growth and progression of colon cancer. Lycopene and its metabolites have been demonstrated as chemopreventive agents against many types of cancer.[24,25,31,32] Our previous in vitro studies demonstrated that lycopene could inhibit the proliferation of human colon cancer HT-29 cells in a dose-dependent manner (0, 2, 5, and 10 μM).[27] Lycopene could inhibit the Akt/β-catenin signaling cascades in human colon cancer HT-29 cells.

The current study demonstrated that consumption of lycopene could significantly inhibit the growth of human colon cancer in a mouse xenograft model. At low dosage (3 mg/kg of BW per day), lycopene could effectively suppress the growth of colon cancer up to 40%. At a high dosage (6 mg/kg of BW per day), lycopene could effectively suppress the proliferation of colon cancer up to 60% in tumor-bearing mice. No hepatoxicity of lycopene at doses of 3 or 6 mg/kg of BW was observed in this study (data not shown). In this study, lycopene (Lycovit 10%) consumption at a dose of 3 mg/kg of BW per day in mice is equivalent to consumption of lycopene at dose of 0.0243 mg/kg of BW per day for humans (or 1.7 mg lycopene per day for humans with 70 kg BW).[33] Lycopene (Lycovit 10%) consumption at a dose of 6 mg/kg of BW per day in mice is equivalent to consumption of lycopene at dose of 0.0486 mg/kg of BW per day for humans (or 3.4 mg lycopene per day for humans with 70 kg BW).[33] The average daily intake of lycopene for humans is highly variable from 0.6 to 6.2 mg in many countries, including North America.[24,34] Results from the current study were consistent with our previous findings and suggested chemopreventive roles of lycopene against human colon cancer.

We further demonstrated that lycopene could significantly suppress the expression of PCNA proteins. The anticancer effects of lycopene were associated with the up-regulation of $p21^{CIP1/WAF1}$ cell cycle inhibitor protein. The results suggested that lycopene could effectively inhibit cell progression and the proliferation of colon cancer cells in tumor-bearing mice. Our results further demonstrated that consumption of lycopene could increase cleaved caspase-3 levels and augment apoptotic cascades in tumor tissues.

The results not only demonstrated the chemopreventive effects of lycopene in preclinical studies but also suggested a potential application in cancer prevention. Therefore, supplementation of lycopene could probably inhibit tumor growth and progression in tumor-bearing mice. To identify the additional roles of lycopene in cancer prevention, we further examined their inhibitory effects on the progression of colorectal tumor. The results demonstrated that lycopene significantly inhibit the expression of β-catenin, E-cadherin, COX-2, and phosphorylation ERK1/2 proteins in tumor tissues. Recent studies also reported that increased phosphorylation of ERK1/2 proteins was strongly correlated with the induction of COX-2 and suppression of E-cadherin adherent complex during tumor progression in several types of cancer.[8,10] β-Catenin has come onto the scene and reached central status as an important regulator of several important oncogenes including *cyclin D1* and *c-Myc* during tumor development. Increasing evidence implicates that β-catenin is an important biomarker of malignant colon cancer. Thus, suppression of β-catenin proteins would hinder the progression of colorectal tumor.

To further identify the molecular actions of lycopene, we examined the inhibitory effects on the ERK1/2 signaling cascades and E-cadherin/ β-catenin adherent complex. Our results demonstrated that lycopene significantly inhibits the ERK1/2

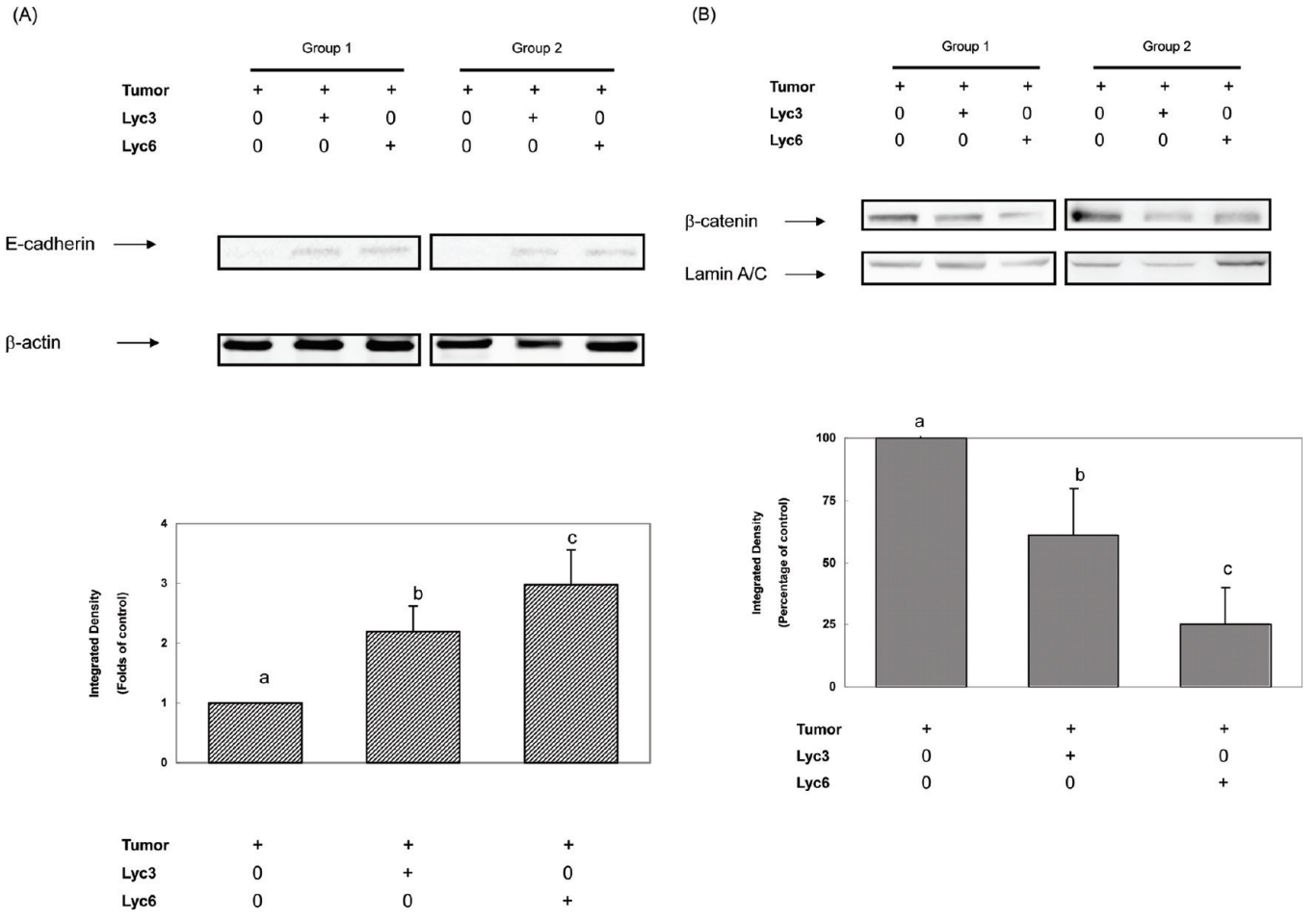

Figure 3. Continued

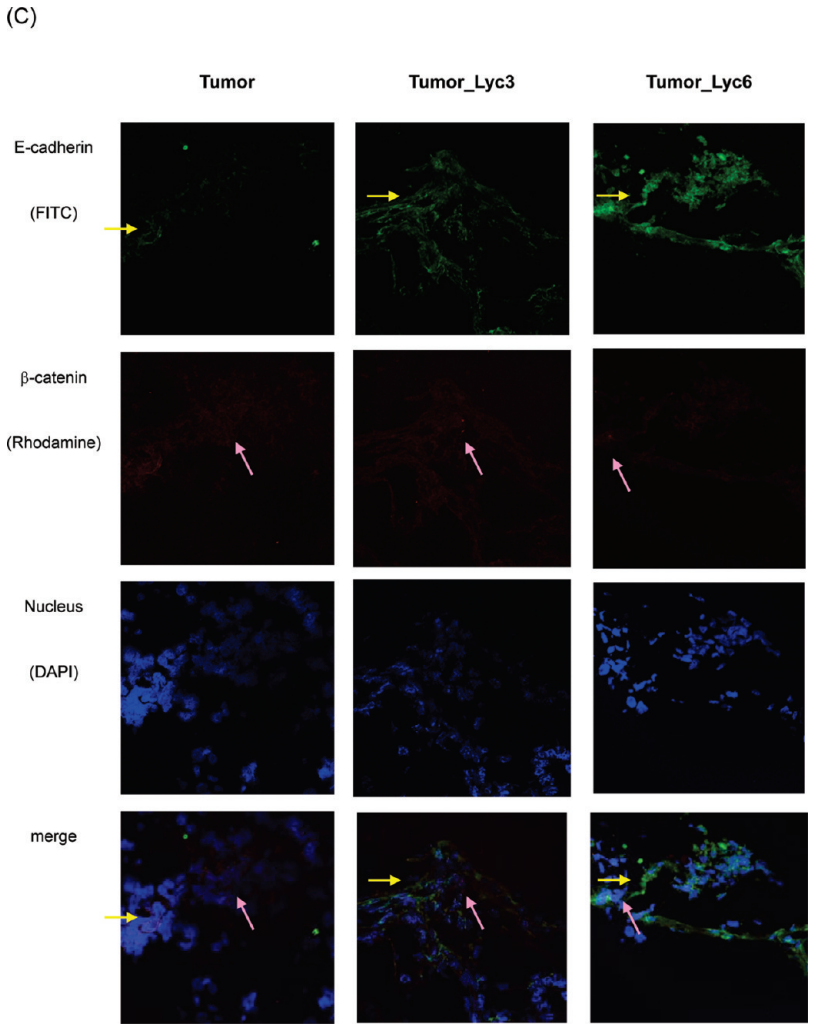

Figure 3. Consumption of lycopene could modulate the expression of β-catenin and E-cadherin proteins in tumor tissues. (A) For the detection of E-cadherin molecule, cell lysates from animal tissues (groups 1 and 2) were blotted with anti-E-cadherin monoclonal antibody as described under Materials and Methods. The levels of detection in cell lysate represent the amount of E-cadherin in human colon cancer cells. The blots were stripped and reprobed with anti-β-actin antibody as loading control. The immunoreactive bands are noted with arrows. The integrated density of E-cadherin protein adjusted with internal control protein (β-actin) is shown in the bottom panel. Different letters represent statistically significant difference, $p < 0.05$. (B) Cell lysates were prepared by using a Tissue Extract Reagent Kit with protease inhibitor and phosphatase inhibitors according to the manufacturer's instruction. Cross-contamination between nuclear and cytoplasma fractions was not found (data not shown). Cell lysates were blotted with anti-β-catenin monoclonal antibody as described under Materials and Methods. The levels of detection in cell lysate represent the amount of β-catenin in human colon cancer cells. The blots were stripped and reprobed with anti-lamin A/C antibody as loading control. The immunoreactive bands are noted with arrows. The integrated density of β-catenin protein adjusted with internal control protein (lamin A/C) is shown in the bottom panel. Different letters represent statistically significant difference, $p < 0.05$. (C) Tumor tissues were frozen, sectioned, and subjected to anti-E-cadherin and anti-β-catenin antibodies by immunofluorescence staining described under Materials and Methods. Cell nuclei were stained with 4,6-diamidino-2-phenyindole (DAPI). Imaging was documented at 600× magnification. The green fluorescence area represents distribution of E-cadherin protein in HT-29 cells stained with monoclonal antibody (indicated with yellow arrows). The red fluorescence area represents distribution of β-catenin protein in HT-29 cells stained with monoclonal antibody (indicated with pink arrows). The blue fluorescence area represents the location of cell nuclei. Merge images indicate the colocalization of nuclei and E-cadherin/ β-catenin proteins in tumor tissues.

signaling cascades and prevents the nuclear translocalization of β-catenin in tumor tissues. A recent study also demonstrated that lycopene could suppress the Ras level and inhibit the activation of ERK1/2 signaling cascades.[35] Results from the current in vivo study were consistent with those previous findings from other studies and ours. Our results suggested chemopreventive roles of lycopene in the modulation of the E-cadherin/β-catenin adherent molecule. The results suggested that lycopene may block tumor progression through the maintenance of cell integrity. Our results further demonstrated that lycopene at either low dosage (Lyc3) or high dosage (Lyc6) could significantly inhibit tumor progression of colon cancer through suppression of COX-2 and PGE_2 production in a mouse xenograft model.

Recent study has indicated that MMP-9 play important roles in the progression of colon cancer.[20] Here, we demonstrated that consumption of lycopene suppressed the plasma levels of MMP-9 and the progression of colon cancer cells in tumor-bearing mice. These lines of evidence suggest that lycopene could modulate cell signaling pathways to suppress the growth and progression of colorectal tumors.

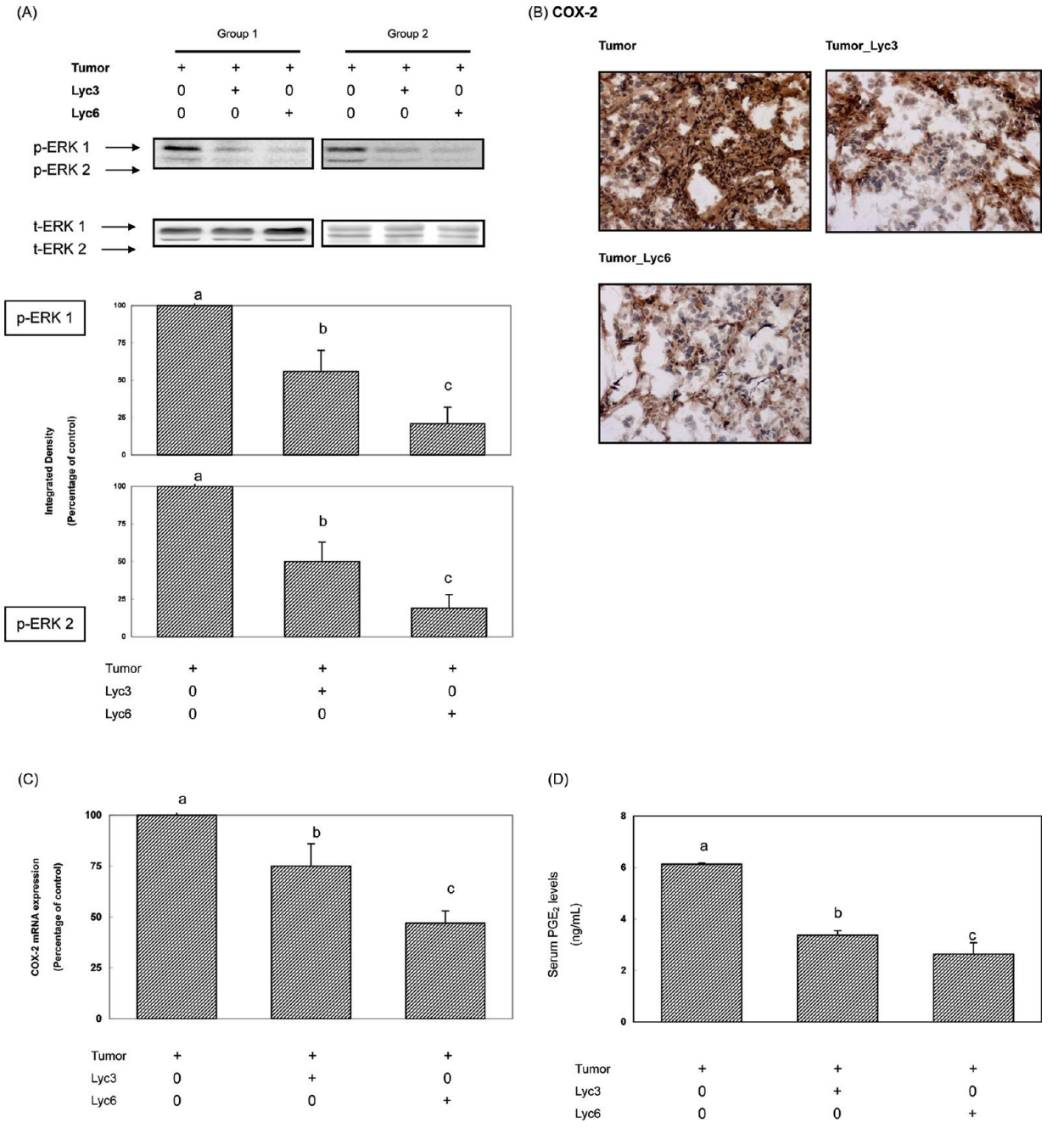

Figure 4. Inhibitory effects of lycopene on tumor progression were associated with the suppression of COX-2, PGE_2, and phosphorylation ERK1/2 proteins in tumor-bearing mice. (A) Cell lysates from animal tissues (groups 1 and 2) were prepared using Tissue Extract Reagent Kit containing protease inhibitor and phosphatase inhibitors according to the manufacturer's instruction. Cell lysates were blotted with anti-phosphorylation ERK1/2 monoclonal antibody as described under Materials and Methods. The levels of detection in cell lysate represent the amount of phosphorylation ERK1/2 in human colon cancer cells. The blots were stripped and reprobed with antitotal ERK1/2 (t-ERK1/2) antibody as loading control. The immunoreactive bands are noted with arrows. The integrated density of phosphorylation ERK1/2 protein adjusted with internal control protein (t-ERK1/2) is shown in the bottom panel. Different letters represent statistically significant difference, $p < 0.05$. (B) Tumor tissues were frozen, sectioned, and subjected to anti-COX-2 antibody by immunohistochemical staining described under Materials and Methods. Imaging was documented at 400× magnification. Dark brown intensity represented distribution and levels of COX-2 protein in cytoplasma of HT-29 cells stained with monoclonal antibody. The blue area represents the location of cell nuclei. (C) The relative COX-2 mRNA expressions in tumor tissues were analyzed by using quantitative RT-PCR. Relative COX-2 mRNA levels in different treatment groups were compared to the corresponding tumor control group (presented as percentage of control). Different letters represent statistically significant difference, $p < 0.05$. (D) The serum levels of PGE_2 were determined by using ELISA Kit (R&D systems). Briefly, equal amounts of diluted serum sample (150 μL) from each group were added to each well and reacted with PGE_2 conjugate molecule according to the manufacturer's instructions. Upon completion of the ELISA process, fluorescence intensities were read using a wavelength of 450/570 nm. The results presented are representative of nine different experiments and presented as serum PGE_2 levels. Different letters represent statistically significant difference, $p < 0.05$.

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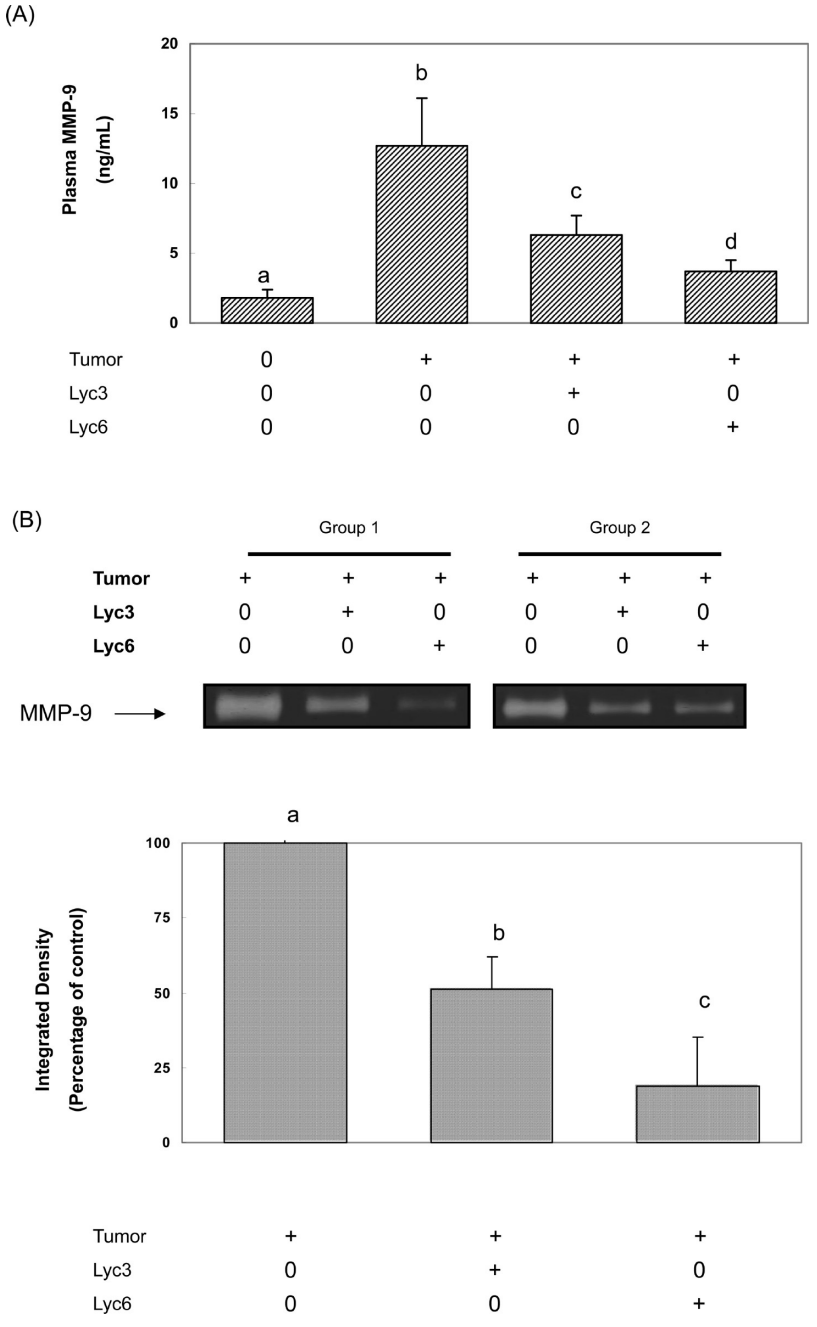

Figure 5. Chemopreventive effect of lycopene was inversely correlated with plasma levels of MMP-9. (A) The plasma levels of MMP-9 were quantified by ELISA Kit (R&D systems). Briefly, equal amounts of diluted plasma sample (100 μL) from each group were added to each well and reacted with primary antibody against MMP-9 molecule according to the manufacturer's instructions. Upon completion of the ELISA process, fluorescence intensities were read using a wavelength of 450/570 nm. The results presented are representative of nine different experiments. Different letters represent statistically significant difference, $p < 0.05$. (B) Equal amounts of plasma samples from tumor and lycopene treatment groups (groups 1 and 2) were loaded into gelatin-containing gel. After incubation and staining of gelatin gel, the photographs of zymogram bands are noted with arrows. Areas of enzymatic activity appeared as clear bands over a dark background. The results presented are representative of nine different experiments. Different letters represent statistically significant difference, $p < 0.05$.

We speculated this inhibitory effect to be evolved with lycopene, in a possible model of antisurvival and antimalignant signal transduction pathways. One of the mechanisms by which lycopene may exert its antitumorgenic effect is in part through the inhibition of PCNA protein and through modulation of $p21^{CIP1/WAF1}$ protein. These findings provide a novel mechanistic insight into the inhibitory effects of lycopene on the growth of human colorectal cancer.

Furthermore, lycopene could moderately augment the expression of E-cadherin protein. In this study, we first demonstrated that lycopene may specifically inhibit malignant biomarker β-catenin protein during tumor growth and progression. In the present study, we also demonstrated for the first time that

lycopene effectively inhibited the expression of MMP-9, COX-2 proteins, and PGE_2 in colorectal tumors in a mouse xenograft model. These results suggested chemopreventive roles of lycopene on the blockade of tumor progression in colorectal cancer.

Taken together, consumption of lycopene could effectively inhibit tumor growth and progression of colorectal tumor in a mouse xenograft model. In conclusion, lycopene may effectively inhibit the proliferation and progression of transformed colonocytes and may prove to be a potent new anticancer compound with improved selectivity toward transformed colorectal cells.

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