

Combined Inhibitory Effects of Curcumin and Phenethyl Isothiocyanate on the Growth of Human PC-3 Prostate Xenografts in Immunodeficient Mice

Tin Oo Khor,^{1,2} Young-Sam Keum,^{1,2} Wen Lin,^{1,2} Jung-Hwan Kim,^{1,2} Rong Hu,^{1,2} Guoxiang Shen,^{1,2} Changjiang Xu,^{1,2} Avanthika Gopalakrishnan,^{1,2} Bandaru Reddy,^{1,3} Xi Zheng,^{1,3} Allan H. Conney,^{1,3} and Ah-Ng Tony Kong^{1,2}

¹Center for Cancer Prevention Research, ²Department of Pharmaceutics, and ³Susan Lehman Cullman Laboratory of Cancer Research, Department of Chemical Biology, Ernest Mario School of Pharmacy, Rutgers, The State University of New Jersey, Piscataway, New Jersey

Abstract

Earlier studies using prostate cancer cells in culture showed that phenethyl isothiocyanate (PEITC) and curcumin have significant chemopreventive and possibly chemotherapeutic effects. However, their *in vivo* effects are still lacking. Hence, this study was undertaken to determine the possible *in vivo* efficacy of prostate cancer-prevention as well as cancer-therapeutic treatment by PEITC and curcumin alone or in combination. We evaluated the effects on tumor growth *in vivo*, using NCr immunodeficient (*nu/nu*) mice bearing s.c. xenografts of PC-3 human prostate cancer cells. Molecular biomarkers representing proliferation and apoptosis were determined. Continued i.p. injection of curcumin or PEITC (6 and 5 μmol ; thrice a week for 28 days), beginning a day before tumor implantation significantly retarded the growth of PC-3 xenografts. Combination of i.p. administration of PEITC (2.5 μmol) and curcumin (3 μmol) showed stronger growth-inhibitory effects. Next, we evaluated the cancer-therapeutic potential of curcumin and PEITC in mice with well-established tumors, and the results showed that PEITC or curcumin alone had little effect, whereas combination of curcumin and PEITC significantly reduced the growth of PC-3 xenografts. Immunohistochemistry staining and Western blot analysis revealed that the inhibition of Akt and nuclear factor- κB signaling pathways could contribute to the inhibition of cell proliferation and induction of apoptosis. Taken together, our results show that PEITC and curcumin alone or in combination possess significant cancer-preventive activities in the PC-3 prostate tumor xenografts. Furthermore, we found that combination of PEITC and curcumin could be effective in the cancer-therapeutic treatment of prostate cancers. (Cancer Res 2006; 66(2): 613-21)

Introduction

Prostate cancer is one of the most prevalent types of malignancy in the United States. Prostate carcinogenesis has been viewed as a multistage and complex process consisting of initiation, promotion, and progression. Despite tremendous efforts and resources devoted to treatment, the incidence and mortality of prostate cancer have

not decreased in past decades, because prostate cancer cells are barely responsive to the cytotoxic effects of high concentrations of chemotherapeutic agents or radiotherapy. In addition, although patients with metastatic prostate cancer can benefit from androgen ablation, most of them will die of prostate cancer progression to an androgen-refractory state. Therefore, an effective prevention strategy against prostate cancer is needed to spare the burden of the patients (1). Hence, much attention has been paid to the discovery of chemopreventive substances from various edible and medicinal plants, and a large number of naturally occurring chemopreventive compounds have been identified (2). Actually, a majority of patients with prostate cancer are now combining the conventional therapies with these compounds as alternative, adjuvant, and/or complementary medication. Numerous epidemiologic studies have shown that consumption of cruciferous vegetables is protective against various types of human cancers (3). Cruciferous vegetables are a group of vegetables named by the cross-shaped flowers, which include broccoli, Brussel sprouts, watercress, cabbage, kale, cauliflower, kohlrabi, and turnip. Compared with other families of vegetables, they contain a significant amount of isothiocyanates, and strong anticarcinogenic activities of cruciferous vegetables have thus been attributed to the high abundance of isothiocyanates (4). Phenethyl isothiocyanate (PEITC), a naturally occurring isothiocyanate particularly abundant in watercress, has received much attention as a possible chemopreventive agent. The mechanisms by which PEITC protects against cancer have been shown to involve the deletion of preneoplastic cells through induction of cell cycle arrest and apoptosis (5, 6) and inhibition of carcinogen activation via modulation of cytochrome P450-dependent monooxygenases and enhancement of the antioxidant response element-dependent carcinogen detoxification enzymes (7). In contrast to the high incidence of prostate cancer in the United States, the incidence of this disease is very low in India, which has been attributed to the dietary consumption of large amounts of plant-based foods rich in phytochemicals (8). The powdered root of turmeric (*Curcuma longa* L., Zingiberaceae) has been widely used as a spice and a source of yellow coloring agent (9). Curcumin (diferuloylmethane), a major constituent of turmeric, has been shown to be highly effective in affording protection against cancer in experimental animals induced by a variety of chemical carcinogens (10–12). Treatment of curcumin caused apoptosis and cell cycle arrest but inhibited cell growth, activation of signal transduction, and transforming activities in both androgen-dependent and androgen-independent prostate cancer cells in culture (13). Curcumin also exerted strong antioxidant and anti-inflammatory activities by suppressing both constitutive and inducible nuclear factor- κB (NF- κB) and activator protein-1 activation (14). Moreover, combined treatment of

Note: T.O. Khor and Y-S. Keum contributed equally to this work.

Requests for reprints: Ah-Ng Tony Kong, Department of Pharmaceutics, Rutgers, The State University of New Jersey, 160 Freilinghuysen Road, Piscataway, NJ 08854-8020. Phone: 732-445-3831; Fax: 732-445-3134; E-mail: kongt@rci.rutgers.edu.

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doi:10.1158/0008-5472.CAN-05-2708

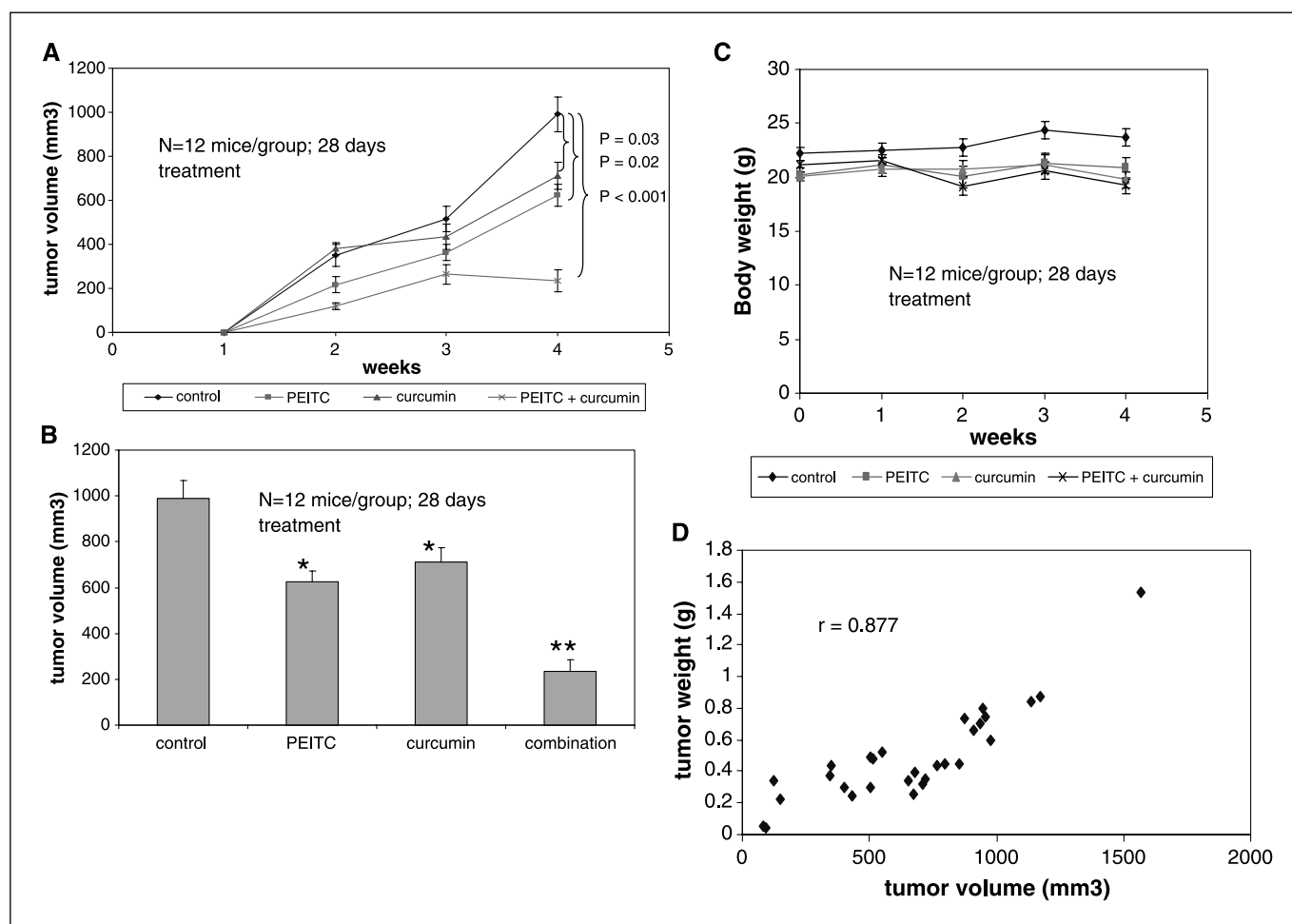


Figure 1. Effects of i.p. injections of curcumin or PEITC alone or in combination on the growth of PC-3 prostate tumors in immunodeficient mice (experiment 1). Male NCr mice received injections with vehicle (100 μ L), curcumin (6 μ mol; 100 μ L vehicle), PEITC (5 μ mol; 100 μ L vehicle), or curcumin (3 μ mol) + PEITC (2.5 μ mol) in 100 μ L vehicle. **A**, growth curve of PC-3 tumors in each group. **B**, tumor volume for each group after 28 days of treatment. **C**, body weight changes of mice during the 28 days of study. Statistical analysis of the percentage of initial body weight changes showed no significant differences between the treatment and control groups. **D**, relationship between tumor volume and tumor weight. Points and columns, mean; bars, SE. *, $P < 0.05$, statistically significant. **, $P < 0.01$, statistically significant.

curcumin sensitized and thus enhanced the cytotoxicity of prostate cancer cells by various chemotherapeutic agents (15). Despite plenty of convincing data from cell culture-based experiments, little is known about the roles of PEITC and curcumin *in vivo*, especially on prostate cancer. In line with this notion, the present study was undertaken to evaluate the potential efficacy of PEITC and curcumin and their combination as prostate cancer-preventive and possibly prostate cancer-therapeutic agents, using NCr immunodeficient mice (*nu/nu*) bearing s.c. xenografts of PC-3 androgen-independent human prostate cancers.

Materials and Methods

Cell culture and reagents. PC-3 cells were purchased from the American Type Culture collection (Manassas, VA). Curcumin and PEITC were obtained from Sigma (St. Louis, MO). Matrigel was obtained from BD Biosciences (Bedford, MA). RPMI 1640 tissue culture medium, penicillin-streptomycin, and fetal bovine serum were from Life Technologies (Grand Island, NY). Rabbit polyclonal antibodies against cleaved poly(ADP-ribose) polymerase (PARP), cleaved caspase-3, phosphorylated Akt (p-Akt; Ser⁴⁷³), p-BAD (Ser136), p-GSK3 β , p-IKK β , and p-I κ B α were purchased from Cell Signaling (Beverly, MA). Anti-actin was a product of Santa Cruz Biotechnology (Santa Cruz, CA). PC-3 cells were maintained in RPMI 1640 containing

10% fetal bovine serum that was supplemented with penicillin (100 units/mL) and streptomycin (100 μ g/mL). Cells were cultured at 37°C in a humidified atmosphere of 5% CO₂ and passaged twice a week.

Growth of PC-3 tumors in immunodeficient mice. Six-week-old male NCr immunodeficient mice were obtained from Taconic Farms, Inc. (Germantown, NY). The animals were housed in sterile filter-capped microisolator cages and provided with sterilized AIN76A diet (Research Diet, New Brunswick, NJ) and water. Prostate cancer PC-3 cells (2×10^6 /0.2 mL/mouse) were suspended in 50% Matrigel in RPMI 1640 and injected s.c. into the right flank of the mice. Curcumin and PEITC were dissolved in vehicle containing polyethylene glycol, benzyl alcohol, ethanol, and water (40:0.1:10:49.1). For experiment 1, i.p. injections of the compounds were given a day before the injection of tumor cells and continued for another 28 days. For experiment 2, i.p. injections of the compounds were given thrice a week for 28 days starting after the injected tumor cells grew to a size of 6.5 to 10 mm (~3 weeks). Tumor size and body weight were measured before i.p. injections of curcumin, PEITC, and the combination at various time intervals throughout the study. At the end of study, mice were sacrificed at 24 to 48 hours after the last administration of compounds. The tumors were excised and weighed. Part of the tumor was snap-frozen for protein assay, whereas the remaining portion was placed in phosphate-buffered formalin and then ethanol before preparing paraffin sections for immunohistochemistry. All animal experiments were carried out under an Institutional Animal Care and Use Committee-approved protocol.

Apoptosis and proliferation. The apoptotic cells were detected using an ApopTag *In Situ* Apoptosis Detection kit (Chemicon, Temecula, CA). The assay was done according to the manufacturer's manual. After deparaffinization, the tissue sections were incubated in proteinase K for 15 minutes at room temperature. The sections were then incubated with terminal deoxynucleotidyl transferase enzyme at 37°C for 1 hour, washed in three changes of PBS, and incubated with anti-digoxigenin conjugate in a humidified chamber at room temperature for 30 minutes. The color was developed by incubating the sections with peroxidase substrate and then counterstained with hematoxylin for 30 seconds. For detection of proliferative cells, proliferating cell nuclear antigen (PCNA) antibody (1:50; DAKO, Carpinteria, CA) was used. The assay was done following the manufacturer's protocols. The scoring of apoptotic and proliferative cells was done at $\times 400$. A positive control slide of rat mammary glands provided by the manufacturer was used as positive control for the *in situ* apoptosis detection assay. For the PCNA staining, mouse intestinal crypt cells were used as a positive control.

Western blotting. Tumors dissected from mice of each treatment group were weighed, pooled, and treated with radioimmunoprecipitation buffer [50 mmol/L NaCl, 0.5% Triton X-100, 50 mmol/L Tris-HCl (pH 7.4), 25 mmol/L NaF, 20 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L Na_3VO_4 , protease inhibitor cocktail tablet (Roche, Mannheim, Germany)] at a concentration of 10 $\mu\text{g}/\text{mL}$ for 40 minutes on ice followed by centrifugation at $14,800 \times g$ for 15 minutes. The protein concentrations of the supernatant were measured by using the bicinchoninic acid solution (Pierce, Rockford, IL). Protein (20 μg) was loaded onto NuPAGE 4% to 12% electrophoresis gel (Invitrogen, Carlsbad, CA) and, after electrophoresis, transferred onto polyvinylidene difluoride membrane. The membrane was blocked with 5% nonfat dry milk in 0.1% Tween 20 in PBS (PBST) for 1 hour and incubated with primary antibody in 5% bovine serum albumin/0.1% PBST overnight at

4°C. After three 5-minute washes with PBST, the membrane was then incubated with horseradish peroxidase-conjugated secondary antibody in 5% nonfat dry milk/PBST for 1 hour at room temperature and then washed with PBST thrice. The transferred proteins were visualized with the Super Signal chemiluminescent substrate (Pierce). The intensity of visualized bands was measured with Quantity One software (ver 4.4.0, Bio-Rad, Richmond, CA).

Statistical analysis. Tumor volumes, body weights, and percentage of apoptotic and proliferative cells were represented as mean \pm SE. Relative fold activation/suppression was represented as mean \pm SD. Student's *t* test was used for the statistical analysis. *P*s < 0.05 were considered significant.

Results

Effects of i.p. Injections of Curcumin or PEITC Alone or in Combination on the Growth of PC-3 Prostate Tumors in Immunodeficient Mice

Experiment 1. Male NCr mice received injections with vehicle (100 μL), curcumin (6 μmol ; 100 μL vehicle), PEITC (5 μmol ; 100 μL vehicle), or curcumin (3 μmol) + PEITC (2.5 μmol) in 100 μL vehicle thrice a week for 28 days starting a day before the injection of PC-3 prostate cancer cells. Tumor growth was measured once a week, and tumor volume (*V*) was calculated as $V = (L \times W^2) \times 0.52$, where *L* is the length and *W* is the width of a xenograft (Fig. 1A). The tumor volumes were significantly different between the treatment groups and the control (*P* = 0.02 for test between PEITC and control; *P* = 0.03 for test between curcumin and control and *P* < 0.001 for PEITC + curcumin versus control group).

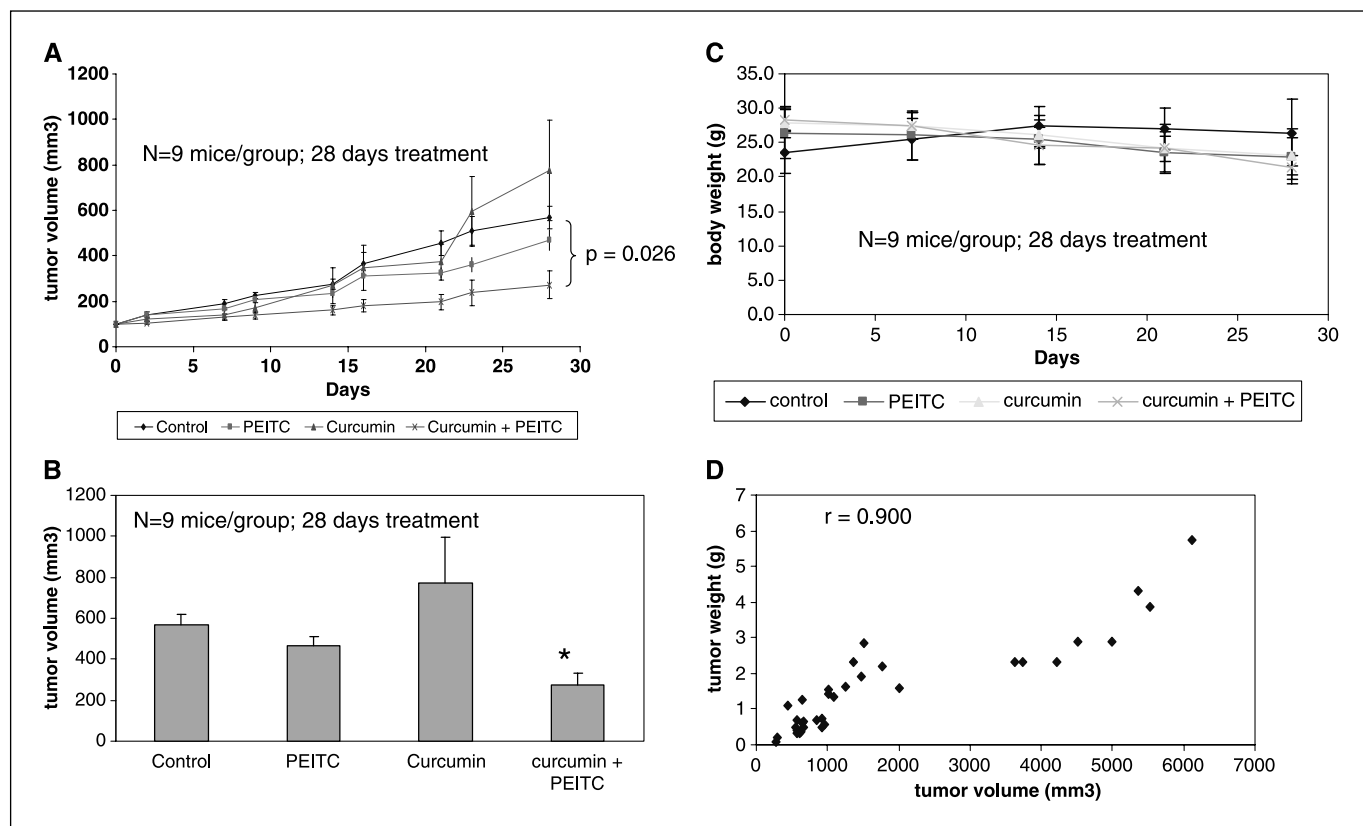


Figure 2. Effects of i.p. injections of curcumin or PEITC alone or in combination on the growth of PC-3 prostate tumors in immunodeficient mice (experiment 2). Male NCr mice received injection with vehicle (100 μL), curcumin (6 μmol ; 100 μL vehicle), PEITC (5 μmol ; 100 μL vehicle), or curcumin (3 μmol) + PEITC (2.5 μmol) in 100 μL vehicle. A, growth curve of PC-3 tumors in each group. B, tumor volume for each group after 28 days of treatment. C, body weight changes of mice during the 28 days of study. D, relationship between tumor volume and tumor weight. Points and columns, mean; bars, SE. *, *P* < 0.05, statistically significant.

The mean \pm SE for tumor volumes was $990 \pm 78 \text{ mm}^3$ for the control group, 624 ± 49 , 711 ± 61 , and $234 \pm 49 \text{ mm}^3$ for PEITC, curcumin, and PEITC + curcumin group, respectively (Fig. 1B).

The effect of various treatments on body weight is shown in Fig. 1C. Statistical analysis of the percentage of initial body weight changes showed no significant differences between the treatment and control group. A good relationship between tumor volumes (as calculated using measurement from live animals) and tumor weights obtained in individual mice at the time of sacrifice was observed ($r = 0.877$; Fig. 1D).

Experiment 2. Male NCr mice received injections with vehicle (100 μL), curcumin (6 μmol ; 100 μL vehicle), PEITC (5 μmol ; 100 μL vehicle), or curcumin (3 μmol) + PEITC (2.5 μmol) in 100 μL vehicle thrice a week for 28 days starting after the xenografts were well established (6.5-10 mm). Tumor growth was measured twice a week, and the tumor volume was calculated as $V = (L \times W^2) \times 0.52$, where L is the length and W is the width with shorter diameter of a xenograft (Fig. 2A). The tumor volumes were only significantly different between the PEITC + curcumin and the control group ($P = 0.026$). The mean \pm SE for tumor volumes was $568 \pm 50 \text{ mm}^3$ for the control group, 469 ± 43 , 775 ± 220 , and $271 \pm 60 \text{ mm}^3$ for PEITC, curcumin, and PEITC in combination with curcumin group, respectively (Fig. 2B).

The effect of various treatments on body weight is shown in Fig. 2C. Statistical analysis of the percentage of initial body weight changes showed statistically significant differences between the treatment and control group ($P < 0.05$ for control versus curcumin, PEITC alone, and combination). An excellent relationship between tumor volumes (as calculated using the measurement from live animals) and tumor weight in individual mice obtained at the time of sacrifice was observed ($r = 0.900$; Fig. 2D).

Effect of i.p. Injections of Curcumin or PEITC Alone or in Combination on Apoptosis and Proliferation in PC-3 Prostate Tumors in Immunodeficient Mice

Experiment 1. The apoptotic and proliferative cells in tumors were evaluated as described in Material and Methods. The percentage of apoptotic cells in tumors of control mice was significantly lower compared with tumors from the treatment groups ($P = 0.029$, 0.048, and 0.005 for PEITC versus control, curcumin versus control, and PEITC + curcumin versus control, respectively). The mean \pm SE for percentage of apoptotic cells in control tumors was $1.5 \pm 0.2\%$, whereas $3.1 \pm 0.3\%$, $3.0 \pm 0.4\%$, and $5.3 \pm 0.6\%$ of apoptotic cells were detected in tumors from the PEITC, curcumin, and PEITC + curcumin groups, respectively (Fig. 3A). In contrast, the percentage of proliferative cells was

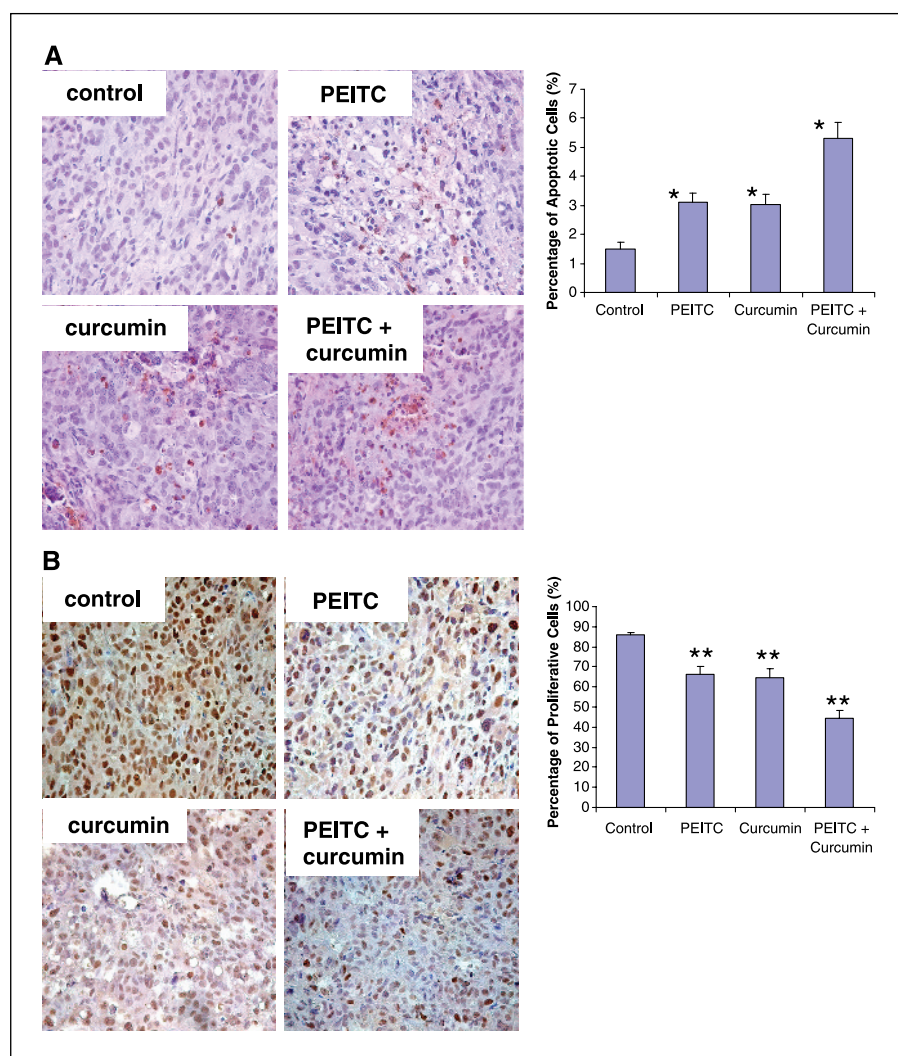


Figure 3. Effect of i.p. injections of curcumin or PEITC alone or in combination on apoptosis and proliferation in PC-3 prostate tumors in immunodeficient mice. The presence of apoptotic (A and C) and proliferative cells (B and D) in tumors from the different groups was evaluated using an ApopTag *In Situ* Apoptosis Detection kit and PCNA labeling, respectively. A and B, experiment 1; C and D, experiment 2. Columns, mean; bars, SE. *, $P < 0.05$, statistically significant. **, $P < 0.01$, statistically significant.

found to be significantly higher in tumors from the control group compared with the treatment groups ($P = 0.001, 0.002, \text{ and } <0.001$ for PEITC versus control, curcumin versus control, and PEITC + curcumin versus control, respectively). The mean \pm SE for the percentage of proliferative cells in tumors from the control, PEITC, curcumin, and the PEITC + curcumin group was $86 \pm 0.8\%$, $66.1 \pm 3.9\%$, $64.8 \pm 4.4\%$, and $44.6 \pm 4.1\%$, respectively (Fig. 3B).

Experiment 2. The percentage of apoptotic cells in tumors of control mice was significantly lower compared with tumors from the PEITC + curcumin-treated mice but not in the PEITC- nor curcumin-treated mice ($P = 0.5, 0.11, \text{ and } <0.001$ for PEITC versus control, curcumin versus control, and PEITC + curcumin versus control, respectively). The mean \pm SE for the percentage of apoptotic cells in control tumors was $1.1 \pm 0.2\%$, whereas $1.4 \pm 0.2\%$, $1.8 \pm 0.3\%$, and $4.9 \pm 0.7\%$ of apoptotic cells were detected in tumors from PEITC, curcumin, and PEITC + curcumin groups, respectively (Fig. 3C). The percentage of proliferative cells was found to be significantly higher in tumors from the controls compared with the PEITC + curcumin-treated mice, but neither the PEITC- nor curcumin-treated mice were found to be statistically different from the controls ($P = 0.7, 0.5, \text{ and } <0.001$

for PEITC versus control, curcumin versus control, and PEITC + curcumin versus control, respectively). The mean \pm SE for percentage of proliferative cells in tumors from the control, PEITC, curcumin, and PEITC + curcumin group was $79.7 \pm 4.4\%$, $76.8 \pm 4.2\%$, $74.9 \pm 2.8\%$, and $46 \pm 4.3\%$, respectively (Fig. 3D).

Effect of i.p. Injections of Curcumin or PEITC Alone or in Combination on the Expression Levels of Cleaved Caspase-3, Cleaved PARP, p-Akt (Ser⁴⁷³), p-GSK3 β , p-BAD (Ser¹³⁶), p-I κ B α , and p-IKK β in PC-3 Prostate Tumors in Immunodeficient Mice

To gain insights into the mechanisms for increased apoptosis in tumors of treated mice, the tumors were analyzed for the expression of cleaved caspase-3 and PARP that is known to be activated during programmed cell death. Biomarkers from the prosurvival/proliferation Akt and NF- κ B signal transduction pathways were also examined.

Experiment 1. Western blots of control and treated mice are shown in Fig. 4A. Densitometric scanning of the immunoreactive bands followed by normalization with actin for differences in protein loading indicated that a 7.3-, 9.4-, and 13.8-fold induction of cleaved caspase-3 was observed in tumors of PEITC-, curcumin-,

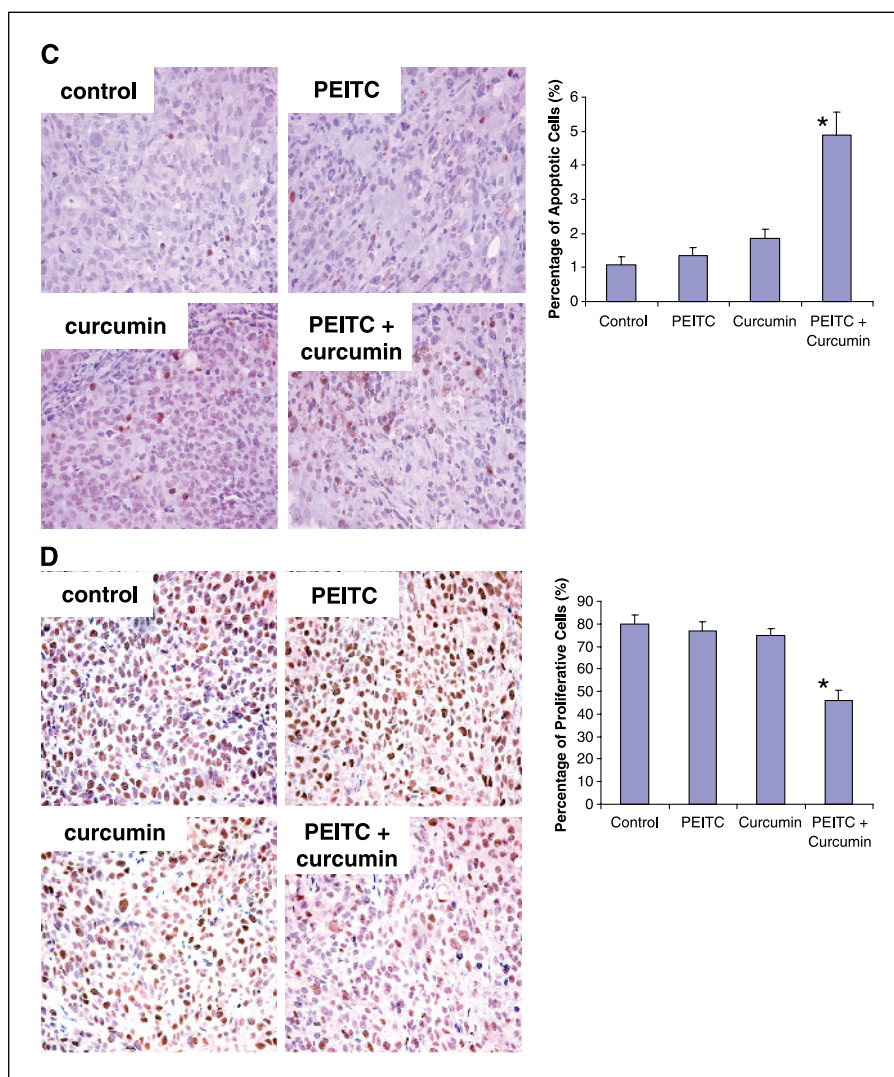


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and PEITC + curcumin-treated mice, respectively, compared with the controls ($P = 0.024$, <0.01 , and 0.047 , respectively). In addition, there was a 2.7-, 4-, and 8.1-fold induction of PARP detected in the tumors of PEITC-, curcumin-, and PEITC + curcumin-treated mice, respectively, compared with the controls ($P = 0.118$, 0.032 , and 0.05 , respectively; Fig. 4A). In addition, we also examined the expression level of p-Akt, p-GSK3 β , p-BAD, p-IKK β , and p-I κ B α , which are known to be important regulator of the Akt and NF- κ B signal transduction pathway. The result shows that expression of p-Akt and its downstream targets p-GSK3 β as well as p-BAD was suppressed in the treatment group (Fig. 4B), except p-BAD for PEITC. Statistical analysis reveals that the expression of p-GSK3 β was significantly suppressed in all the treatment groups compared with the control group ($P = 0.03$ for PEITC- and curcumin-treated

mice and $P = 0.01$ for the combination versus control group). In addition, suppression of p-IKK β and p-I κ B α was also observed in the treatment groups compared with the control counterparts.

Experiment 2. Western blots of cleaved caspase-3 and PARP for control and treated mice are shown in Fig. 4C. A 1.2-, 2.2-, and 9.3-fold induction of cleaved caspase-3 was observed in tumors of PEITC-, curcumin-, and PEITC + curcumin-treated mice, respectively, compared with the controls ($P = 0.08$, 0.07 , and 0.01 , respectively; Fig. 4D). In addition, there was a 1.7-, 2.2-, and 5.8-fold induction of PARP detected in the tumor of PEITC-, curcumin-, and PEITC + curcumin-treated mice, respectively, compared with the controls (Fig. 4D). A marked reduction in the expression of p-Akt, p-GSK3 β , p-BAD, p-IKK β , and p-I κ B α was only observed in the combination group versus the controls

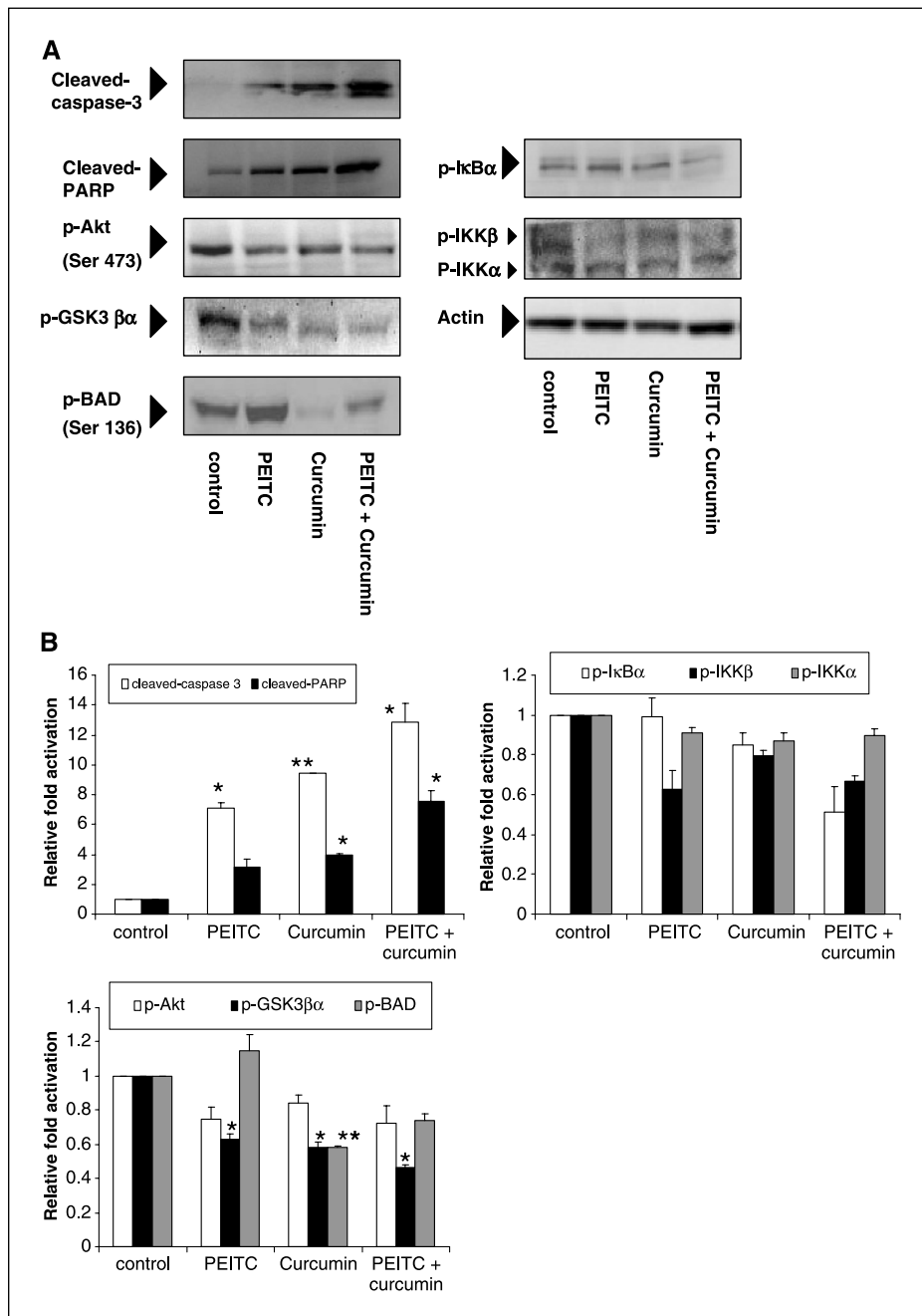


Figure 4. Effect of i.p. injections of curcumin or PEITC alone or in combination on expression level of cleaved caspase-3, cleaved PARP, p-Akt (Ser⁴⁷³), p-GSK3 β , p-BAD (Ser¹³⁶), p-IKK β , and p-I κ B α in PC-3 prostate tumors in immunodeficient mice. A and B, experiment 1; C and D, experiment 2. Columns, mean from two separate experiments; bars, SD. *, $P < 0.05$, statistically significant. **, $P < 0.01$, statistically significant.

($P = 0.16, 0.03, 0.03, 0.06,$ and $0.11,$ respectively; Fig. 4D). Statistical significant suppression of p-BAD was also observed in the curcumin treated group ($P = 0.02$).

Discussion

Our current study presents evidence that continued i.p. injection of naturally occurring PEITC, starting 1 day ahead of implantation of PC-3 prostate xenografts in nude mice, significantly retarded the growth of tumors compared with that of mice treated with vehicle (Fig. 1A-D). In agreement with our results, Chiao et al. have shown

that daily administration of diets supplemented with PEITC-NAC ($8 \mu\text{mol/g}$), a major metabolite of PEITC, inhibited the growth of xenografted tumors of PC-3 prostate cancer cells (16). Xiao et al. have also reported that oral administration of 9 and $12 \mu\text{mol}$ of PEITC inhibited the growth of established TRAMP-C1 xenografts in nude mice (6). Moreover, administration of other naturally occurring isothiocyanates, such as sulforaphane and AITC, have been shown to exhibit significant inhibitory effects on the growth of PC-3 prostate tumor xenografts (5, 17). Together, these results indicate that the growth inhibitory effect of PEITC on PC-3 tumor xenografts may not be a unique property but rather a general event

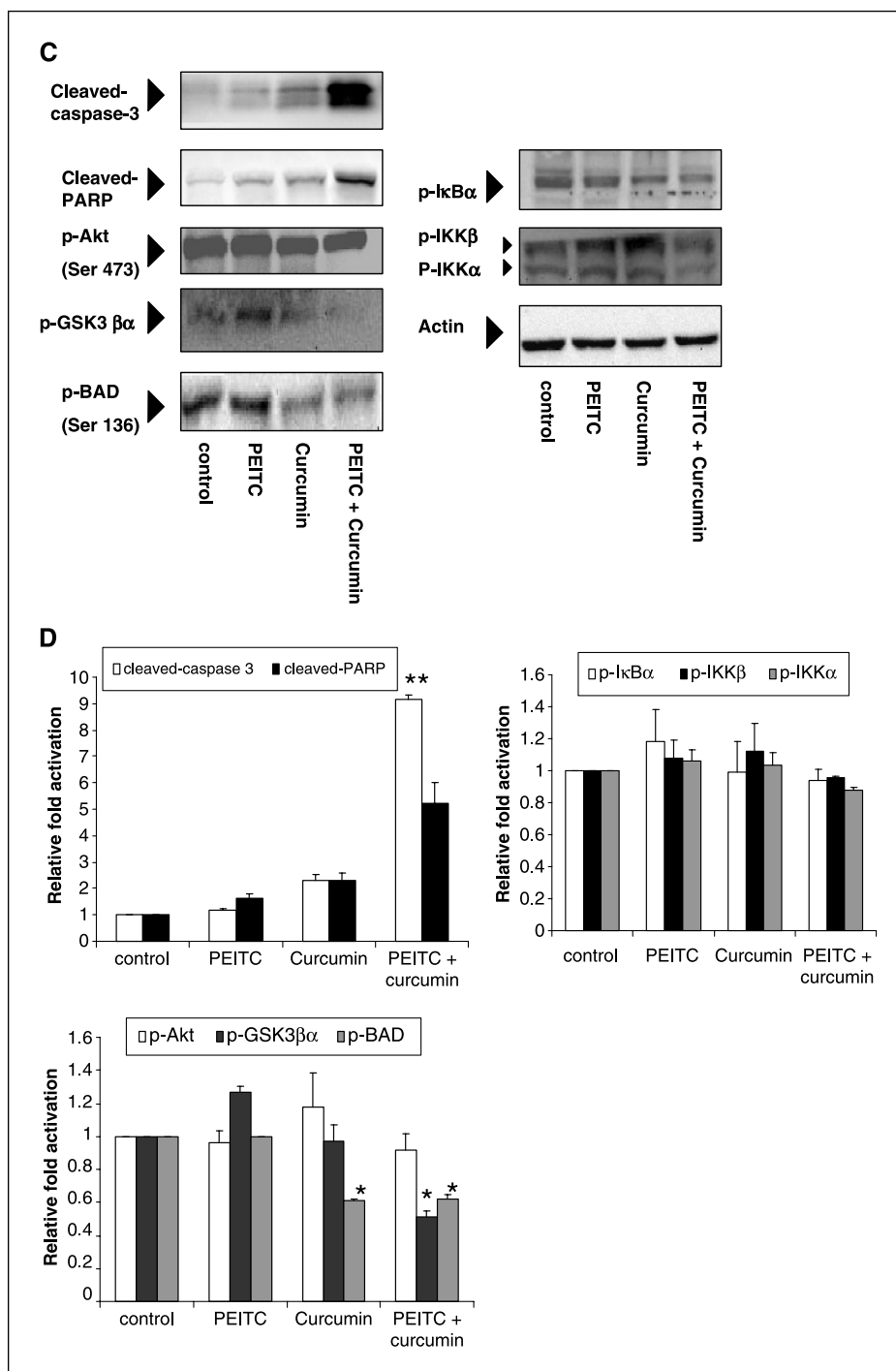


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exerted by the isothiocyanate class of compounds. The inhibitory effect of curcumin on carcinogenesis was first shown in a 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-, BP-, and 7,12-dimethylbenz(a)anthracene-induced mouse skin tumor model (10, 11). Since then, inhibitory effects of dietary curcumin on colon, duodenal, stomach, esophageal, and oral carcinogenesis were also reported (12). However, the effects of curcumin on prostate cancer cells *in vivo* are still lacking. In the present study, we have shown that continued i.p. injection of curcumin before implantation of PC-3 prostate cancer cells significantly retarded the growth of these cells on a xenograft, implying that curcumin possesses inhibitory effects on the growth of prostate cancer *in vivo*. Although the opinion about *in vivo* chemopreventive effects by curcumin is generally held, there exist some reports that contradict this view. For example, Imaida et al. have shown that curcumin failed to prevent the formation of ventral prostate carcinogenesis in rats, induced by 3,2'-dimethyl-4-aminobiphenol and 2-amino-1-methylimidazo[4,5-*b*]pyridine (18). Somasundaram et al. also have shown that administration of dietary curcumin did not augment cyclophosphamide-mediated growth inhibition of BT-474 breast cancer xenografts but rather contributed to an increase tumor size (19). Together, these facts suggest that the growth-inhibitory effect of curcumin might vary, depending on the animal tumor models, *in vivo* oral absorption, and/or target organs and that chemopreventive efficacy of curcumin *in vivo* thus needs to be further evaluated.

It is reasonable to expect that appropriate combinations of chemopreventive agents might provide greater efficacy than the administration of individual agents. For example, a combination of piroxicam, a nonsteroidal anti-inflammatory agent, and difluoromethylornithine, a synthetic ornithine decarboxylase inhibitor, produced synergistic inhibitory effects on colon cancer development in male F344 rats (20). Likewise, a combination of clinically achievable concentrations of TPA and all-*trans*-retinoic acid showed synergistic inhibitory effects on the growth of well-established LNCaP cells in athymic nude mice (21). Narayanan et al. have also reported that a combination of docosahexaenoic acid and 1,4-phenylene bis(methylene) selenocyanate suppressed colon cancer cell growth and induced apoptosis more effectively than high doses of individual agents (22). Consistent with these observations, we also found that a combination of PEITC and curcumin at lower doses inhibited the growth of PC-3 prostate tumor xenografts more effectively than administration of PEITC or curcumin alone at a higher dose.

As seen in Fig. 2A-D, i.p. injection of PEITC and curcumin alone failed to inhibit the growth of fully established PC-3 tumor xenografts in nude mice. This fact suggests that the effect of PEITC or curcumin may be effective in the early stages of carcinogenesis (preventive effect) but may not inhibit tumor growth at later stages, when tumors are fully established to certain sizes. However, contrary to our results, Dorai et al. have observed that administration of diet containing 2% curcumin right after the implantation of LNCaP prostate cancer cells significantly inhibited the growth of LNCaP tumor xenografts in nude mice (23). The reason for this discrepancy is unclear at present. One possibility is that because LNCaP cells are androgen-responsive cell lines, reduced androgen level in mice due to aging might contribute to the growth inhibition of implanted LNCaP tumor xenografts, or it could be due to differences in the size of the tumors and/or curcumin concentration levels achieved in the tumors.

Although single administration of PEITC and curcumin failed to affect the growth of PC-3 prostate tumor xenografts, combined i.p. injections of PEITC and curcumin strongly inhibited the growth of well-established PC-3 tumor xenografts. This finding is quite intriguing, and it is tempting to speculate that a combination of PEITC and curcumin might have efficiently influenced the cell death-related signaling pathways, ultimately leading to the suppression of well-established PC-3 tumor xenografts *in vivo*, which might not possibly be achieved by a single administration of PEITC or curcumin alone. In addition, it should be further noted that the combined administration of nontoxic doses of PEITC and curcumin inhibited the growth of this PC-3 tumor xenograft in mice. This finding is significant, because it could provide the basis for the development of combination dose regimens of PEITC and curcumin in preclinical models against prostate carcinogenesis. Continued i.p. injection of curcumin, PEITC (6 and 5 μmol), and the combination (3 and 2.5 μmol of curcumin and PEITC, respectively) thrice a week for 28 days in the present study caused some loss in body weight (Fig. 1C and Fig. 2C). However, gross autopsy of these animals failed to reveal any abnormalities. Therefore, it is very unlikely that the weight loss observed in the present study is due to the nonspecific toxicity.

Recent studies have shown that PEITC and curcumin are strong inducers of apoptosis in PC-3 cells *in vitro*. Xiao et al. have shown that PEITC-induced apoptosis involved extracellular signal-regulated kinase (ERK) activation and the cleavage of procaspase-3, procaspase-8, and procaspase-9 in PC-3 cells (6, 24), whereas apoptotic induction of PC-3 cells by curcumin involved p21^{WAF1/CIP1} and CCAAT/enhancer binding protein β and inhibition of cell survival signal protein kinase B/Akt (13, 15). From earlier studies, it is still unclear whether apoptosis of prostate cancer cells occurs *in vivo* in response to treatment with these compounds and/or whether the mode of actions occurred *in vitro* during apoptosis is identical to those *in vivo*. In the present study, we have shown that suppression of proliferation and induction of apoptosis are indeed occurred *in vivo*, which could be responsible for the inhibitory effects on tumor growth by PEITC and curcumin. In particular, Western blot analysis showed that apoptosis induction of PC-3 tumor xenografts involved caspase-3 activation and PARP cleavage. This fact suggests that apoptotic pathways involving caspase-3 and PARP may likely be the major molecular target(s) in apoptotic responses of PC-3 prostate xenografts to PEITC and curcumin treatment. To dissect the possible mechanisms in which the apoptotic and/or antiproliferation signaling were triggered, we also examined the expression levels of the Akt/NF- κ B signal transduction pathway-related biomarkers. Our result suggests that suppression of the p-Akt, p-GSK3 β , p-BAD, p-IKK β , and p-I κ B α was closely correlated with the reduction of PC-3 tumor xenografts. Because the inhibition of NF- κ B and Akt are both associated with suppression of angiogenesis, invasion, and metastasis in prostate cancer cells (25, 26), it can be surmised that suppression of the activity of these proteins might have also contributed to a delay of the growth of PC-3 xenografts. A puzzling aspect of our study is, however, that some observations previously made *in vitro* did not occur in our current *in vivo* study. For example, although the activation of mitogen-activated protein kinase was responsible for the induction of apoptosis in PC-3 cells *in vitro* (24, 27), changes in the phosphorylation level of ERK and c-Jun NH₂-terminal kinase were not observed in pooled extracts of PC-3 xenografts by PEITC or curcumin (data not shown). In addition, although PEITC- or curcumin-mediated inhibition of epidermal growth factor receptor

(EGFR) phosphorylation was clearly observed in cultured PC-3 cells in our preliminary study, no change in EGFR phosphorylation was observed *in vivo* (data not shown). Hence, it seems that further *in vivo* and *in vitro* correlation studies are needed to better understand the pharmacologic efficacy of PEITC and curcumin.

Acknowledgments

Received 8/1/2005; revised 10/10/2005; accepted 10/25/2005.

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