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Methanolic Extract of Adlay Seed Suppresses COX-2 Expression of Human Lung Cancer Cells via Inhibition of Gene Transcription

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Previous results demonstrated that the methanolic extract of adlay seed exerted an antiproliferative effect on human lung cancer cells in vitro and in vivo and might prevent tobacco carcinogen-induced lung tumorigenesis. In this study, the methanolic extract of adlay seed was tested for its regulation of COX-2 expression of human lung cancer cells. Western blot analysis showed that the methanolic extract of adlay seed inhibited basal and TPA-induced COX-2 expression in a dose-dependent fashion, whereas COX-1 expression was not affected. By using a promoter activity assay, it was found that the methanolic extract inhibited basal and TPA-stimulated COX-2 expression at the transcription level. The effect of the methanolic extract on COX-2 expression in vivo was then investigated. The data demonstrated that treatment of the methanolic extract reduced the PGE_2 level in serum and inhibited COX-2 expression of tumor tissues in nude mice. Taken together, these results suggest that inhibition of COX-2 is one of the mechanisms by which the methanolic extract of adlay seed inhibits cancer growth and prevents lung tumorigenesis.

KEYWORDS: Adlay seed; COX-2; tumorigenesis

INTRODUCTION

Adlay (*Coxi lachryma-jobi* L. var. *ma-yuen* Stapf) is a grass crop that has long been used in traditional Chinese medicine and as a nourishing food. Adlay seed has been reported to exhibit anti-inflammatory, stomachic, diuretic, and antispastic effects in vivo and has been used in China for the treatment of warts, rheumatism, and neuralgia (*1*, *2*). Previous studies have demonstrated that a number of components isolated from adlay exhibited an anticancer effect in vitro (*3*−*7*). Additionally, adlay seed is the major effective component of a traditional drug named Chen-Chie-Wan, which was commonly used for the treatment of lung cancer in ancient China. Our and others results demonstrated that adlay seed could inhibit the growth of human non-small cell lung cancer cells and Ehrlich ascites sarcoma cells and exert anticancer effects in experimental animals (*8*, *9*).

Recent studies indicate that the development of tumors is frequently associated with deregulation of cell cycle control (*10*, *11*), and alteration of expression of cell cycle regulatory genes is frequently found in human lung tumor tissues or cancer cell lines (*12*−*14*). Our previous data have shown that the methanolic extract of adlay seed may inhibit proliferation of A549 lung cancer cells by inhibiting cyclin A expression and by inducing apoptotic cell death (*8*).

Emerging evidence suggests that prostaglandins (PGs) play a critical role in various physiological and pathophysiological processes, including blood clotting, kidney function, wound healing, cardiovascular disease, and inflammation (*15*, *16*). Cyclooxygenases (COXs) are the rate-limiting enzymes involved in the conversion of arachidonic acid to PGs. Two isoenzymes of COXs have been identified (*17*): a constitutive isoform COX-1 and an inducible isoform COX-2. COX-2 expression is undetectable in most tissues and is induced by mitogens, cytokines, or tumor promoters (*18*). Recent works suggest that COX-2 is involved in the steps of carcinogenesis. First, elevated expression of COX-2 is frequently found in human cancers including gastric cancer, esophageal cancer, lung cancer, colon cancer, hypopharynx squamous cell carcinoma, and head and neck cancers (*19*−*24*). Second, enforced expression of COX-2 is sufficient to induce tumorigenesis in transgenic mice (*25*). Third, nonsteroidal anti-inflammatory drugs (NSAIDs), potent inhibitors for COXs, exert anticancer and chemopreventive effects on tumor development (*26*−*28*). Fourth, COX-2 expression may induce tumor metastasis and angiogenesis (*29*, *30*).

In this study, we investigate the effect of the methanolic extract of adlay seed on the expression of COX-2 in human non-small cell lung cancer cells and elucidate the molecular mechanism of this action.

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MATERIALS AND METHODS

Cell Culture and Reagents. A549 human lung cancer cells were cultured in DMEM/F12 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37 °C. Antibodies against COX-1 and COX-2 were obtained from Santa Cruz (Santa Cruz, CA). TPA was purchased from Sigma Chemical Co. Methanolic extract of whole adlay seed was prepared as described previously (*31*).

Immunoblotting. Investigation of alteration of the protein level was performed as described previously (*32*). In brief, cells were treated with different doses of methanolic extract for various times. After treatment, cells were rinsed with ice-cold phosphate-buffered saline (PBS) and harvested in a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethanesulfonyl fluoride, 1 mg/mL aprotinin, 2 μg/mL pepstatin A, and 2 μg/mL leupeptin) for 30 min on ice. Cellular lysates were centrifuged at 12000*g* for 10 min, and protein concentrations of the lysates were determined by using a BCA protein assay kit (Pierce, Rockford, IL). Equal amount of proteins were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The blots were blocked in 5% nonfat milk in TBST (20 mM Tris-HCl, pH 7.4, 137 mM NaCl, and 0.05% Tween-20) overnight at 4 °C. After blocking, the blots were washed in TBST and incubated with various primary antibodies for 1 h at room temperature, followed by incubation of peroxidase-conjugated secondary antibody for another 1 h. The blots were developed by using the ECL chemiluminescence system (Amersham Pharmacia Biotech) and were reprobed with actin antibody to confirm equal loading of proteins in each lane.

Promoter Activity Assays. The effect of the methanolic extract on COX-2 promoter activity was analyzed as described previously (*33*). For analysis of basal promoter activity, cells were plated onto six-well plates at a density of 100000 cells/well and grown overnight. Cells were then transfected with 2 μg of COX-2 promoter−luciferase plasmid for 5 h by the LipofectAMINE method. After transfection, cells were incubated in 10% FCS medium containing different doses of methanolic extract for 24 h, and luciferase activity was determined by using an assay system according to the procedures of the manufacturer (Promega). For analysis of TPA-stimulated promoter activity, cells were treated as described above and incubated with vehicle or various doses of methanolic extract for 2 h and stimulated with or without 10 nM TPA treatment for another 6 h. Luciferase activity was determined, normalized for protein concentration in cell lysate and expressed as an average of three independent experiments.

Nude Mouse Experiments. All experiments on mice were performed according to the guidelines for our institute (*Guide for Care and Use of Laboratory Animals*, Kaohsiung Medical University). Male BALB/c-nu nude mice (8 weeks old) were housed in barrier facilities on a 12-h light/dark cycle and received food and water ad libitum. Tumors were induced by subcutaneous (sc) injection of A549 cells (2×10^6 cells in 0.1 mL of phosphate-buffered saline) at one site of the right flank. Tumors (visualized as small nodules at the sites of injection) appeared ~20 days after injection, and the animals were randomly distributed into a control group, which received vehicle, or a treatment group, which received 3 mg/kg of methanolic extract. Administration of vehicle or methanolic extract via ip injection was initiated from day 21 after cell inoculation. Animals were injected every day, and treatment was continued for 45 days. At the end, mice were sacrificed for analysis of COX-2 expression in tumor tissues and serum prostaglandin E_2 (PGE_2) concentration.

Determination of Serum PGE_2. After sacrifice, blood was collected from the mice by cardiac puncture, and serum PGE_2 was extracted and measured using an ELISA kit according to the procedures of the manufacturer (Neogen Co.).

COX-2 Protein Level in Tumor Tissues. After sacrifice, tumor tissues were removed and stored at −70 °C until use. Tissues were homogenized in a tissue homogenization buffer (50 mmol/L Tris-HCl, pH 7.4, 250 mmol/L NaCl, 1 mmol/L EDTA, 0.1% sodium dodecyl sulfate, 2 mmol/L sodium orhovanadate, 50 mmol/L NaF, 1 mmol/L phenylmethanesulfonyl fluoride, 1 mg/mL aprotinin, 2 μg/mL pepstatin A, and 2 μg/mL leupeptin). An equal amount of tissue proteins was separated by SDS-PAGE, and immunoblotting was performed as described previously.

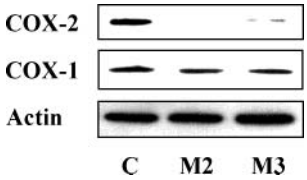

Figure 1. Effect of methanolic extract of adlay seed on basal levels of COX-2 protein in A549 lung cancer cells. Cells were cultured in various doses of methanolic extract (M2, 33 μg/mL; M3, 11 μg/mL) for 24 h. COX-2 protein level was investigated by immunoblotting.

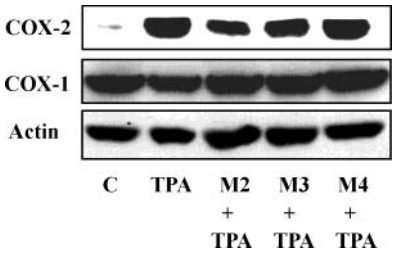

Figure 2. Effect of methanolic extract of adlay seed on TPA-stimulated COX-2 expression in A549 lung cancer cells. Cells were cultured in various doses of methanolic extract (M2, 33 μg/mL; M3, 11 μg/mL; M4, 3.7 μg/mL) for 12 h followed by treatment of 10 nM TPA for another 2 h. COX-2 expression was investigated by immunoblotting.

RESULTS

Methanolic Extract of Adlay Seed Inhibits Basal and TPA-Stimulated COX-2 Expression. To evaluate the effect of the methanolic extract of adlay seed on COX-2 expression of human lung cancer cells, exponentially growing A549 cancer cells were cultured in 10% FCS medium containing different amounts of methanolic extract of adlay seed for 24 h. After incubation, whole cell lysates were subjected to SDS-PAGE and analyzed by immunoblotting to examine COX-1 and COX-2 protein levels. As demonstrated in **Figure 1**, the methanolic extract inhibited the COX-2 expression of A549 cells in a dose-dependent manner but did not show any inhibitory effect on the COX-1 protein level. Moreover, pretreatment of the methanolic extract suppressed TPA-stimulated COX-2 expression (**Figure 2**). On the contrary, the methanolic extract did not affect COX-1 expression under similar experimental condition.

Methanolic Extract of Adlay Seed Down-Regulates Basal and TPA-Activated COX-2 Promoter Activity. After transfection with full-length COX-2 promoter-luciferase construct, A549 cells were incubated in 10% FCS medium containing different amounts of methanolic extract for 24 h and luciferase activity was assayed. As shown in **Figure 3**, the methanolic extract inhibited basal COX-2 promoter activity in a dose-dependent manner. The methanolic extract at concentrations of 33 and 11 μg/mL could decrease the basal COX-2 promoter activity to 16.7 and 78.4% of the control level. We also tested the effect of methanolic extract on TPA-stimulated COX-2 promoter activity. Cells were transfected with COX-2 promoter−luciferase construct and cultured in 10% FCS medium for 24 h. Cells were then treated with vehicle or different doses of methanolic extract for 2 h followed by stimulation with 10 nM TPA for another 6 h. After treatment, cells were harvested for assaying the luciferase activity. We found that a 2.5-fold increase of the luciferase activity was observed after TPA stimulation. Pretreatment of the methanolic extract could repress TPA-

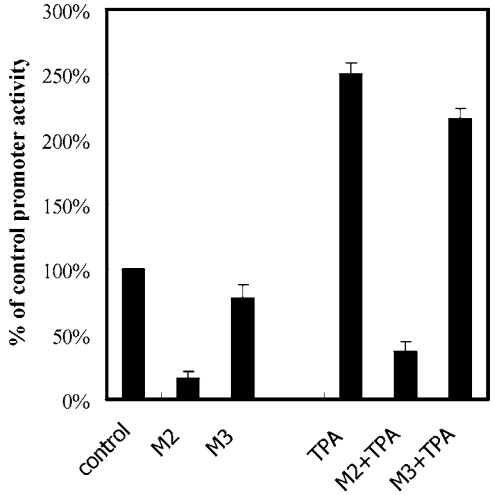

Figure 3. Effect of methanolic extract of adlay seed on basal and TPA-stimulated COX-2 promoter activity. A549 cancer cells were transfected with 2 μg of COX-2 promoter–luciferase plasmid for 6 h. After transfection, cells were cultured in 10% FCS medium for 24 h and then treated with vehicle (MeOH) or various concentrations (M2, 33 μg/mL; M3, 11 μg/mL) of methanolic extract of adlay seed for 2 h followed by treatment with or without 10 nM TPA for another 6 h. Cells were harvested for the determination of luciferase activity. Luciferase activity was normalized for protein concentration in cell lysates, and results from three independent experiments were expressed as mean ± SD.

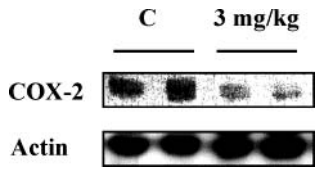

Figure 4. Effect of methanolic extract of adlay seed on COX-2 expression in tumor tissues. Animals were treated as described under Materials and Methods. Tumor tissues were homogenized in a tissue homogenization buffer, and the homogenates were centrifugated at 12000*g* for 15 min at 4 °C. Protein concentrations were determined, and equal amounts of tissue proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. COX-2 protein level was investigated by immunoblotting.

stimulated COX-2 promoter activity in a dose-dependent manner.

Methanolic Extract of Adlay Seed Attenuates Serum PGE_2 Level and COX-2 Expression of Tumor Tissues in Nude Mice. We next investigated the effect of the methanolic extract in vivo. Tumors were induced by sc injection of human lung cancer cells into BALB/c-nu nude mice. Animals were randomly distributed into a control group, which received vehicle, or a treatment group, which received 3 mg/kg of methanolic extract. Administration of vehicle or methanolic extract via ip injection was initiated from day 21 after cell inoculation. Animals were injected every day, and treatment was continued for 45 days. Tumor tissues were collected for assaying COX-2 protein levels by immunoblotting. We found that the methanolic extract suppressed COX-2 expression of tumor tissues (**Figure 4**). Blood collected from the mice by cardiac puncture was subjected to analysis of PGE_2 level. Our data showed that the methanolic extract significantly lowered the PGE_2 level of nude mice when compared with that of the control group (**Figure 5**).

DISCUSSION

Our and others results have demonstrated that COX-2 is involved in the tumorigenesis of many types of cancer (*19*−*24*). Our recent study showed that the methanolic extract of adlay seed might inhibit proliferation of cultured lung cancer cells and prevent tobacco carcinogen-induced lung tumorigenesis (*8*). In this study, we demonstrate for the first time that the methanolic extract of adlay seed may inhibit COX-2 protein expression at the transcription level in human lung cancer cells. These results indicate that inhibition of COX-2 expression is one of the mechanisms by which the methanolic extract of adlay seed inhibits tumor growth. As aforementioned, COX-2 expression may provide a number of growth advantages including stimulation of proliferation, inhibition of apoptosis, and enhancement of angiogenesis for cancer cells. Moreover, a very recent finding indicates that COX-2-derived PGE_2 may increase tumor invasion via activation of CD44 and MMP-2 in NSCLC (*34*). Our results suggest that adlay seed has the potential to be developed as a chemopreventive food.

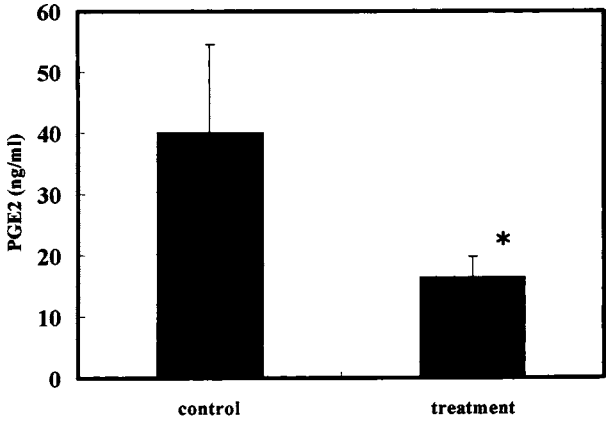

Figure 5. Effect of methanolic extract of adlay seed on serum PGE_2 level in experimental animals. Animals were treated as described under Materials and Methods, and serum samples were obtained by cardiac puncture after treatment. The PGE_2 level in the serum of the control group, which received vehicle, and the treatment group, which received 3 mg/kg methanolic extract of adlay seed, was determined by using an ELISA kit according to the procedures of the manufacturer.

The active components in the methanolic extract of adlay seed that may inhibit COX-2 expression are unclear at present. Several classes of compound including phenolic compounds (such as vanillic acid, syringic acid, and *trans-p*-coumaric acid) and flavonoids (such as naringenin and tricin) were found in adlay seed (*35*, *36*). Previous studies have shown that a number of transcription factor binding sites including AP-1, NF-κB, c-myc, and PEA3 are identified in COX-2 promoter and are known to mediate the effect of extracellular stimuli on COX-2 expression (*37*). Therefore, blocking of upstream signaling pathways that control the activity of these transcription factors may potently inhibit COX-2 expression. Indeed, recent works have demonstrated that curcumin and resveratrol, two natural compounds isolated from food products, might suppress COX-2 expression by inhibiting AP-1 activity (*38*, *39*). Because TPA-induced COX-2 is also strongly suppressed by methanolic extract, it is possible that the active components may affect the AP-1-mediated COX-2 expression. We are now investigating the critical elements in the COX-2 promoter that mediate the effect of the methanolic extract of adlay seed. Our results are of clinical significance because many human lung cancers harbor K-*ras* mutation or neu overexpression, which are known to activate AP-1 transcription activity. Inhibition of AP-1-mediated COX-2 may be helpful for the treatment of lung cancer.

Collectively, these results indicate that the methanolic extract of adlay seed can selectively inhibit COX-2 gene expression

and tumor-induced production of PGE_2. Identification of the active components in the methanolic extract may lead to the development of a novel class of drugs that may inhibit and/or prevent COX-2-overexpressing cancers.

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