

# Apigenin Inhibits Expression of Vascular Endothelial Growth Factor and Angiogenesis in Human Lung Cancer Cells: Implication of Chemoprevention of Lung Cancer

Ling-Zhi Liu, Jing Fang, Qiong Zhou, Xiaowen Hu, Xianglin Shi, and Bing-Hua Jiang

*Institute for Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China (L.-Z.L., J.F., Q.Z., X.H., X.S., B.-H.J.); and Mary Babb Randolph Cancer Center, Department of Microbiology, Immunology and Cell Biology, West Virginia University, Morgantown, West Virginia (B.-H.J.)*

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## ABSTRACT

Apigenin is a natural dietary flavonoid. It has recently been shown to have anticancer effects on prostate and ovarian cancer cells. However, the molecular basis of the effect of apigenin on cancer cells remains to be elucidated. In this study, we found that apigenin inhibited A549 lung cancer cell proliferation and vascular endothelial growth factor (VEGF) transcriptional activation in a dose-dependent manner. In an attempt to understand the mechanism of apigenin-inhibited VEGF expression, we found that apigenin inhibited VEGF transcriptional activation through the hypoxia-inducible factor 1 (HIF-1) binding site and specifically decreased HIF-1 $\alpha$  but not HIF-1 $\beta$  subunit expression in the cells. In our efforts to understand the

signaling pathway that mediates VEGF transcriptional activation, we found that apigenin inhibited AKT and p70S6K1 activation. When testing the effect of apigenin *in vivo*, we found that apigenin significantly inhibited tumor growth in nude mice. Apigenin inhibited HIF-1 $\alpha$  and VEGF expression in the tumor tissues, suggesting an inhibitory effect of apigenin on angiogenesis. To confirm this, we showed that apigenin inhibited angiogenesis in nude mice using the Matrigel assay. HIF-1 $\alpha$  and VEGF are well known inducers of angiogenesis. Our data suggested that apigenin may inhibit human lung cancer angiogenesis by inhibiting HIF-1 $\alpha$  and VEGF expression, thus providing a novel explanation for the anticancer action of apigenin.

Lung cancer is one of the most prevalent forms of cancer worldwide and is a leading cause of death for adults in the United States (Brognard et al., 2001; Khuri and Cohen, 2004). Non-small-cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for 75 to 80% of occurrences (Brognard et al., 2001). Lung cancer mortality rates are high because of ineffective detection methods and the resistance of the disease to some therapeutic treatments (Brognard et al., 2001; Hirsch et al., 2003; Balsara et al., 2004). The seminal studies in cancer chemoprevention were performed by Hong (1999a,b) using retinoids. One innovative approach suggests that the use of synthetic retinoids can reverse premalignancy in the upper aerodigestive tract and prevent secondary tumor growth in head and neck cancers (Hong et al., 1990, 1993; Khuri and Cohen, 2004). However, further research has demonstrated that the preventive effects decline with lower doses, making this treatment ineffective at advanced stages (Karp, 1997;

Khuri and Cohen, 2004). No effective chemotherapeutic agents for treating lung cancer currently exist (Hecht, 1997; Omenn, 2000; Lippman and Spitz, 2001). Recent studies focus on the molecular events leading to lung cancer and the development of molecularly targeted therapies (Chun et al., 2003; Balsara et al., 2004). Studies have demonstrated that AKT is a potential target of lung cancer treatment (Brognard et al., 2001; Balsara et al., 2004). The discovery of a new agent that targets tumorigenic mechanisms such as angiogenesis and vascular endothelial growth factor (VEGF) is a potentially effective chemotherapeutic treatment for human lung adenocarcinoma.

Apigenin (4',5,7-trihydroxyflavone) is a common dietary flavonoid found in fruits and vegetables (Dunnick and Hailey, 1992). It has been used as a dietary supplement and has become an attractive compound in the cancer research community because of its antitumor properties (Fotsis et al., 1997). Apigenin has been shown to possess growth inhibitory abilities against many human cancer cell lines, including breast (Yin et al., 2001), colon (Wang et al., 2004), skin (Li and Birt, 1996), thyroid (Yin et al., 1999a), leukemia (Wang

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**ABBREVIATIONS:** NSCLC, non-small-cell lung cancer; VEGF, vascular endothelial growth factor; HIF-1, hypoxia inducible factor 1; DMSO, dimethyl sulfoxide; bp, base pair(s); PBS, phosphate-buffered saline;  $\beta$ -gal,  $\beta$ -galactosidase; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling; PCNA, proliferating cell nuclear antigen; SABC, streptavidin-biotin-horseradish peroxidase complex.

et al., 1999), and prostate cancer (Shukla and Gupta, 2004). In particular, apigenin has been shown to dramatically decrease CA-HPV-10 prostate cancer cell viability and to inhibit PC-3 prostate cancer cell proliferation with few inhibitory effects on normal prostate epithelial cells (Knowles et al., 2000; Gupta et al., 2001). Although apigenin has been shown to have antitumorigenic effects, its effect on lung cancer cells is not known.

Angiogenesis plays an important role in many physiological and pathological processes, including tumor growth and proliferation (Tanaka et al., 2003; Blagosklonny, 2004). It initiates tumor development, allowing solid tumors to expand beyond 1 to 2 mm in diameter (Folkman, 1995), and induces metastasis (Li et al., 2000; Sandler et al., 2004). VEGF has been recognized as the most important growth factor involved in angiogenesis (Yancopoulos et al., 1998; Ferrara, 1999; Gale and Yancopoulos, 1999). VEGF provides the tissue surrounding the tumor with nutrients for vascular permeability. It has been suggested that VEGF induces tumor metastasis (Bergsland, 2004; Kim et al., 2004). Research has shown a connection in NSCLC between VEGF and tumor vasculature growth, resistance to therapies, and negative prognosis (Kishiro et al., 2002; Sandler et al., 2004; Talbot et al., 2004).

VEGF is regulated by hypoxia inducible factor 1 (HIF-1) (Forsythe et al., 1996). HIF-1 is a heterodimeric transcription factor composed of HIF-1 $\alpha$  and HIF-1 $\beta$  subunits (Wang et al., 1995; Jiang et al., 1997b). HIF-1 $\alpha$  is induced by hypoxia, growth factors, and oncogenes (Fukuda et al., 2002; Amano et al., 2004; Slomiany and Rosenzweig, 2004; Traxler et al., 2004). HIF-1 $\alpha$  is often up-regulated in human cancers and activates the expression of VEGF at the transcriptional level. We recently identified the phosphatidylinositol 3-kinase/AKT signaling pathway as a mediator of VEGF through the induction of HIF-1 $\alpha$  expression in ovarian cancer (Fang et al., 2004; Skinner et al., 2004). The level of HIF-1 $\alpha$  expression has been shown to correspond with tumorigenesis and angiogenesis in animal models (Jiang et al., 1997a; Maxwell et al., 1997; Ryan et al., 1998).

In this study, we hypothesized that apigenin inhibited VEGF expression, tumor growth, and angiogenesis. To test this hypothesis, we wanted to determine whether apigenin inhibited A549 cell proliferation, inhibited VEGF expression, inhibited VEGF expression at the transcriptional level through HIF-1 expression, or affected tumor growth and angiogenesis. We also sought to determine the signaling pathways involved in apigenin-inhibited VEGF expression.

## Materials and Methods

**Cell Culture and Reagent.** A549 human NSCLC cells (American Type Culture Collection, Manassas, VA) were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum. Apigenin was purchased from Sigma (St. Louis, MO), dissolved in dimethyl sulfoxide (DMSO), and stored at  $-20^{\circ}\text{C}$ . Matrigel and antibodies against HIF-1 $\alpha$  and HIF-1 $\beta$  were from BD Biosciences (Franklin Lakes, NJ). Anti-proliferating cell nuclear antigen (PCNA) antibody and the In Situ cell apoptosis detection kit were purchased from Boster Inc. (Wuhan, China). Drabkin's reagent kit and anti- $\beta$ -actin antibodies were from Sigma. Antibodies against phosphorylated AKT (Ser-473), total AKT, and phosphorylated p70S6K1 (Thr-421/Ser-424) were obtained from Cell Signaling Technology (Beverly, MA). Antibodies against VEGF and total p70S6K1 were from Santa Cruz Biotechnology (Santa Cruz, CA).

**Construction of Plasmids.** VEGF reporter pGL-StuI containing a 2.65-kilobase pair fragment of human VEGF gene promoter, and

VEGF reporter pMAP11wt, which contains 47 bp of functional VEGF 5'-flanking sequence (from  $-985$  to  $-939$ ), were cloned into the pGL2 basic luciferase vector (Forsythe et al., 1996). The VEGF reporter pMAP11mut was constructed by introducing a 3-bp substitution into pMAP11wt, which abolishes the HIF-1 binding site. Plasmid encoding human HIF-1 $\alpha$  was inserted into pCEP4 vector (Forsythe et al., 1996; Jiang et al., 1996).

**Cell Proliferation Assay.** To determine the effects of apigenin on A549 cells,  $5 \times 10^5$  cells were seeded in a 24-well plate and cultured overnight. The cells were treated with apigenin at 10 and 20  $\mu\text{M}$ , respectively. The cells were treated with DMSO as the solvent control. The cells were then trypsinized, and aliquots of cells were counted using a hemocytometer after 24 and 48 h. Data were from three separate experiments with three replications per experiment.

**DNA Fragmentation Analysis.** For DNA ladder experiments, cells were cultured to 80 to 90% confluence and exposed to apigenin for 48 h. The adherent and floating cells were collected and washed with  $1 \times$  phosphate-buffered saline (PBS). Total DNAs were isolated using the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Isolated DNA was air-dried and dissolved in Tris-EDTA buffer (10 mM Tris/HCl and 1 mM EDTA, pH 8.0). Aliquots of total DNAs were separated on a 1.8% agarose gel and visualized by ethidium bromide staining.

**Flow Cytometry.** A549 cells were treated with 0, 5, 10, and 20  $\mu\text{M}$  apigenin for 24 h. The floating and adherent cells were harvested by trypsinization, washed with PBS, resuspended in 500  $\mu\text{l}$  of PBS, and fixed by the addition of 500  $\mu\text{l}$  of ice-cold ethanol at  $-20^{\circ}\text{C}$ . After incubation for 30 min, cell pellets were collected by centrifugation, resuspended in 0.5 ml of PBS containing 100  $\mu\text{g}/\text{ml}$  RNase, and incubated at  $37^{\circ}\text{C}$  for 30 min. Propidium iodide solution (0.5 ml) (100  $\mu\text{g}/\text{ml}$  in PBS) was added and incubated on ice for 30 min. The cells were analyzed with a FACScalibur flow cytometer (BD Biosciences). Distribution of cell cycles was analyzed using the ModFit LT for Mac software, version 1.01.

**Transient Transfection and Luciferase Assay.** A549 cells were seeded in six-well plates and cultured to 70% confluence. To determine the effects of apigenin on VEGF transcriptional activation, the cells were transiently transfected with VEGF reporter plasmid using Lipofectamine (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The pCMV- $\beta$ -galactosidase ( $\beta$ -gal) plasmid was cotransfected into the cells as an internal control for transfection efficiency. After transfection, the cells were cultured for 20 h, followed by incubation with apigenin for 24 h. Cells were then washed once with PBS and lysed with reporter lysis buffer (Promega, Madison, WI). The luciferase (Luc) activities of the cell extracts were determined using the luciferase assay system (Promega, Madison, WI). The  $\beta$ -gal activity was measured in assay buffer (200 mM phosphate, 2 mM  $\text{MgCl}_2$ , 100 mM  $\beta$ -mercaptoethanol, and 1.33 mg/ml *o*-nitrophenyl  $\beta$ -D-galactopyranoside) as we described previously (Jiang et al., 2001). The relative Luc activity (defined as VEGF reporter activity) was calculated as the ratio of Luc/ $\beta$ -gal activity and normalized to the control.

**Immunoblotting.** A549 cells were cultured in RPMI 1640 media supplemented with 10% fetal bovine serum for 24 h, followed by treatment with apigenin. Cells were then washed with ice-cold  $1 \times$  PBS (140 mM NaCl, 3 mM KCl, 6 mM  $\text{Na}_2\text{HPO}_4$ , and 1 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4), scraped from the dish, and centrifuged at 4000 rpm for 5 min. The cell pellet was incubated for 30 min on ice in radioimmune precipitation buffer (150 mM NaCl, 100 mM Tris, pH 8.0, 1% Triton X-100, 1% deoxycholic acid, 0.1% SDS, 5 mM EDTA, and 10 mM NaF) supplemented with 1 mM sodium vanadate, 2 mM leupeptin, 2 mM aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 2 mM pepstatin A. After centrifugation at 14,000 rpm for 15 min, the supernatant was collected as the total cellular protein extracts and stored at  $-70^{\circ}\text{C}$ . Tumor tissues from nude mice were ground in liquid nitrogen in radioimmunoprecipitation assay buffer, and the total tissue proteins were extracted as described above. The protein concentration was determined using Bio-Rad protein assay reagent (Richmond, CA). The cellular protein extracts were sepa-

rated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane in 20 mM Tris-HCl, pH 8.0, containing 150 mM glycine and 20% (v/v) methanol. Membranes were blocked with 5% nonfat dry milk in TBS buffer containing 0.05% Tween 20 and incubated with antibodies against HIF-1 $\alpha$ , HIF-1 $\beta$ , and  $\beta$ -actin. Protein bands were detected by incubation with horseradish peroxidase-conjugated antibodies (Cell Signaling Technology, Beverly, MA), and visualized through enhanced chemiluminescence reagent (PerkinElmer Life and Analytical Sciences, Boston, MA).

**Mouse Tumorigenesis Experiments.** Male nude mice (6 weeks old; BALB/cA-nu (nu/nu)) were purchased from Shanghai Experimental Animal Center (Chinese Academy of Sciences, China) and maintained in pathogen-free conditions. Twenty mice were randomly divided into two groups. A549 cells were injected subcutaneously into both flanks of nude mice ( $1.25 \times 10^6$  cells in 100  $\mu$ l). For the apigenin group, the cells were injected into the mice with 15  $\mu$ M apigenin. Three days after the injection, the mice in the apigenin-treated group were i.p. injected with apigenin in PBS (3 mg/kg of body weight) in a 0.2-ml volume. The control group was treated with PBS at an equal volume. Bidimensional tumor measurements were measured with calipers three times weekly, and the mice were euthanized after 40 days. Tumor volumes were calculated according to the formula ( $\text{width}^2 \times \text{length}/2$ ).

**Terminal Deoxynucleotidyl Transferase-Mediated dUTP Nick End-Labeling.** Tumor tissues were employed to assess apoptosis using the terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method using the In Situ cell death detection kit (Boster) according to the manufacturer's instructions. In brief, parts of the tumor tissues were fixed in 4% paraformaldehyde and 3% saturated picric acid and stored at 4°C. Dehydrating cells were treated in 30% sucrose for 12 h, and serial 5- $\mu$ m frozen sections were prepared and mounted on slides coated with 3-amino propyltriethoxy silane. The sections were washed three times in 1 $\times$  PBS and water, respectively, and then permeabilized in 3% acetic acid, pH 2.5, at room temperature for 10 min. The ends of the DNA fragments were labeled by incubation with digoxin-labeled deoxyuridine triphosphate and terminal deoxynucleotidyl transferase, and exposed to streptavidin-conjugated alkaline phosphatase through the development with nitro blue tetrazolium and bromochloroindolyl phosphate. Sections were counterstained with nucleic red and examined under light microscopy (Olympus, Tokyo, Japan). The section incubated without terminal deoxynucleotidyl transferase alone was used as a negative control.

**Immunohistochemistry.** Parts of the tumor tissues were fixed in 4% paraformaldehyde and 3% saturated picric acid, then stored at 4°C. Dehydrating cells were treated in 30% sucrose for 12 h, and serial 5- $\mu$ m frozen sections were prepared and mounted on slides coated with 3-amino propyltriethoxy silane, and one was stained with hematoxylin and eosin. Adjacent sections were stained for the expression of VEGF, PCNA, and HIF-1 $\alpha$  and detected through streptavidin-biotin-horseradish peroxidase complex (SABC) formation. In brief, the sections were washed three times in 1 $\times$  PBS and incubated for 20 min in 0.3% hydrogen peroxide/methanol. The slides were washed three times with 1 $\times$  PBS, then incubated with 10% normal goat serum/0.3% Triton X-100 at 37°C for 20 min. The sections were incubated with the following primary antibodies: mouse anti-human VEGF (1:200), PCNA (1:200), and HIF-1 $\alpha$  (1:100). The samples were incubated in a humid chamber at 4°C for 16 h. After three washes in 1 $\times$  PBS, the slides were incubated for 2 h with goat anti-mouse secondary IgG, and detected by incubation with SABC compound for 1 h at 37°C. Sections were rinsed several times in 1 $\times$  PBS. Sections incubated with preimmune IgG instead of the primary antibodies were used as a negative control.

**Matrigel Plug Angiogenesis Assay.** Male BALB/cA-nu nude mice (6 weeks old) were purchased from Shanghai Experimental Animal Center (Chinese Academy of Sciences, China) and maintained in pathogen-free conditions. Eight mice were randomly divided into two groups. A549 cells were trypsinized and resuspended at  $3 \times 10^7$  cells/ml in serum-free medium. Aliquots of cells (0.1 ml,

$3 \times 10^6$  cells) were mixed with 0.2 ml of phenol red-free Matrigel (BD Biosciences) and injected into both flanks of each nude mouse. For the apigenin-treated group, the cells were injected with 15  $\mu$ M apigenin. Cells in equal volumes of solvent were used as the control. The Matrigel mixed with the medium alone was used as a negative control. The Matrigel plugs were removed 15 days after the implantation, weighed, and used for the measurement of the hemoglobin content. Hemoglobin was measured using a Drabkin's reagent kit according to the manufacturer's instructions. The concentration of hemoglobin was calculated based on a set of hemoglobin standards. The data represented the mean  $\pm$  S.E. from duplicate experiments.

**Statistical Analysis.** All values in the present study were reported as mean  $\pm$  S.E. Student's unpaired *t* test was used for statistical analyses. Differences between values were considered significant at  $p < 0.05$ .

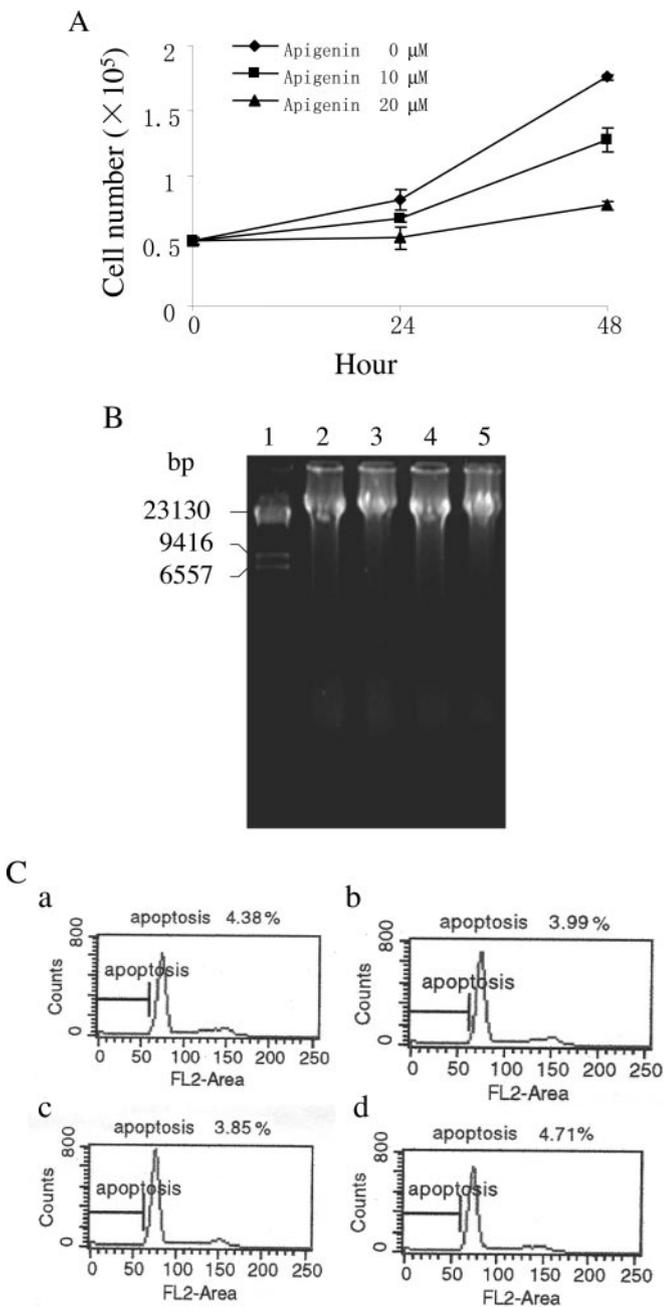
## Results

**Apigenin Inhibited A549 Lung Cancer Cell Proliferation.** To determine the effect of apigenin on A549 cell proliferation, the cells were treated with fresh medium with or without various doses of apigenin. Cell numbers were counted using a hemocytometer. The apigenin treatment inhibited cell proliferation in a dose-dependent manner (Fig. 1A). To study whether the decrease of cell proliferation was due to apoptosis, we examined the effect of apigenin on A549 cell apoptosis. A549 cells were treated with various doses of apigenin for 48 h, and DNA fragmentation was not observed in the cells treated with apigenin up to 20  $\mu$ M (Fig. 1B). A similar result was observed when we assayed the cellular apoptosis by flow cytometry (Fig. 1C). These data indicate that the inhibition of proliferation was not caused by apoptosis induced by apigenin.

**Apigenin Inhibited VEGF Expression at the Transcriptional Level through the HIF-1 DNA Binding Site.** To determine whether apigenin inhibits VEGF expression at the transcriptional level, we analyzed the effects of apigenin on a VEGF luciferase reporter with 2.6 kilobase pairs of human VEGF gene promoter. The A549 cells were cotransfected with the VEGF reporter and  $\beta$ -gal plasmids and cultured in the absence or presence of apigenin, which did not induce apoptosis. Apigenin treatment inhibited VEGF transcriptional activation in a dose-dependent manner (Fig. 2A). To determine whether apigenin inhibits VEGF transcriptional activation through the HIF-1 binding site, the VEGF reporter plasmid pMAP11wt encoding 47 bp of VEGF 5'-flanking sequence and pMAP11mut containing three base pair substitutions at the HIF-1 binding site were used in the study. The cells were treated with apigenin as described above. Treatment with apigenin inhibited the activity of the pMAP11wt VEGF reporter in a dose-dependent manner (Fig. 2B), which is similar to the result obtained with the full-length VEGF reporter. However, apigenin treatment did not inhibit activity of the mutant VEGF reporter pMAP11mut (Fig. 2C). These results suggest that apigenin inhibits VEGF transcriptional activation through the HIF-1 DNA binding site in the VEGF promoter region, and the mutation of the HIF-1 binding site abolished the inhibitory effect of apigenin.

**Apigenin Specifically Inhibited Expression of HIF-1 $\alpha$  but Not HIF-1 $\beta$ .** To determine whether apigenin inhibited VEGF transcriptional activation through the decrease of HIF-1 $\alpha$  and HIF-1 $\beta$  expression, A549 cells were treated with apigenin to analyze HIF-1 protein levels. The cells were treated with different doses of apigenin for 6 h, and HIF-1 $\alpha$  and HIF-1 $\beta$

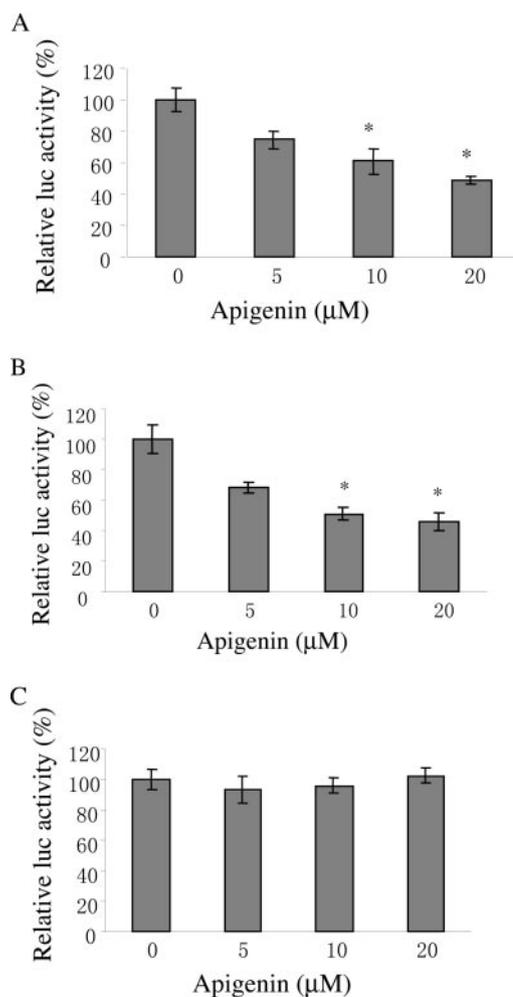
expression was detected by immunoblotting. HIF-1 $\alpha$  expression in A549 cells was inhibited by apigenin in a dose-dependent manner, whereas HIF-1 $\beta$  and  $\beta$ -actin levels were not affected



**Fig. 1.** Apigenin inhibited A549 cell proliferation. A, A549 cells were seeded at  $5 \times 10^5$  cells/well on a six-well plate and cultured overnight the day before the treatment. The old medium was discarded and 500  $\mu$ l of fresh medium was added with various doses of apigenin (0, 10, and 20  $\mu$ M). After 24 and 48 h, aliquots of cells were counted in triplicate using a hemocytometer. B, A549 cells were incubated with various doses of apigenin (5, 10, and 20  $\mu$ M) for 48 h. The adherent and floating cells were collected, total DNA was isolated, and 8  $\mu$ g of DNA was resolved on 1.8% agarose gel. Lane 1,  $\lambda$ DNA/HindIII marker; lane 2, A549 cells treated without apigenin; lane 3, with apigenin at 5  $\mu$ M, lane 4, with apigenin at 10  $\mu$ M, and lane 5, with apigenin at 20  $\mu$ M. C, A549 cells were cultured to 80% confluence and then exposed to solvent (a), 5 (b), 10 (c), and 20 (d)  $\mu$ M apigenin for 24 h. Cells were trypsinized, harvested, washed once with PBS buffer, and stained using propidium iodide. Stained cells were then subjected to flow cytometry assay by a FACSCalibur system (BD Biosciences) to analyze the apoptotic cells.

by apigenin (Fig. 3). Apigenin at 20  $\mu$ M significantly inhibited HIF-1 $\alpha$  protein levels, although it did not induce apoptosis. There was no detectible HIF-1 $\alpha$  protein in the cells when the cells were treated with 40  $\mu$ M apigenin (Fig. 3). These results suggest that apigenin may inhibit VEGF transcriptional activation specifically through the inhibition of expression of HIF-1 $\alpha$  but not HIF-1 $\beta$ .

**Apigenin Inhibited AKT and p70S6K1 Activation.** To determine the signaling pathway regulating VEGF transcriptional activation, we found that apigenin inhibited AKT activation (Fig. 4) and p70S6K1 expression and activation in the cells (Fig. 5). AKT and p70S6K1 were recently shown to regulate HIF-1 and VEGF transcriptional activation. This result indicates that inhibition of AKT and p70S6K1 activa-

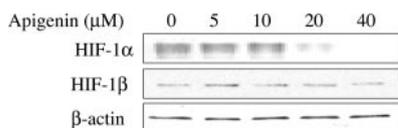


**Fig. 2.** Apigenin inhibited VEGF transcriptional activity through the HIF-1 DNA binding site. A549 cells were seeded on a six-well plate. When they reached 60 to 70% confluence, the cells were transfected using Lipofectamine reagent. A, A549 cells were cotransfected with 1  $\mu$ g of pGL3-StuI VEGF reporter and 0.3  $\mu$ g of  $\beta$ -gal plasmid as described. The cells were cultured for 20 h, followed by treatment with apigenin at various doses (5, 10, and 20  $\mu$ M) for 24 h. Cells were then lysed and the supernatants were used to assay Luc and  $\beta$ -gal activities. The relative luciferase activities in the cell extracts were assayed by the ratio of Luc/ $\beta$ -gal activity and normalized to the value in the solvent DMSO control. B, cells were cotransfected with 1  $\mu$ g of pMAP11wt VEGF reporter and 0.3  $\mu$ g of  $\beta$ -gal plasmid as described. Cells were treated with different doses of apigenin as described above. C, cells were transfected with 1  $\mu$ g of pMAP11mut VEGF reporter and 0.3  $\mu$ g of  $\beta$ -gal plasmid. The cells were cultured and treated with apigenin as described above. Luc and  $\beta$ -gal activities were assayed. \*, significant difference compared with the solvent control ( $p < 0.05$ ).

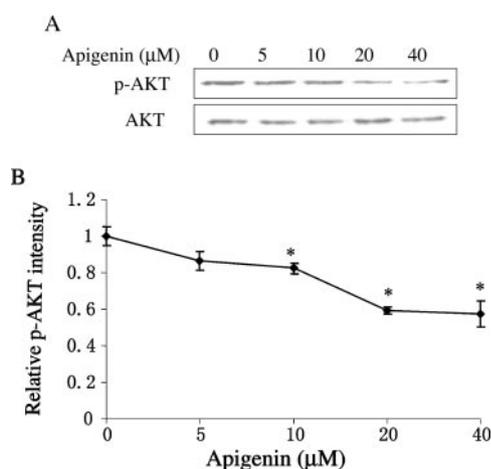
tion may be responsible for decreasing VEGF transcriptional activation in the cells.

**Apigenin Inhibited Tumor Growth in Nude Mice.** To determine whether apigenin inhibits tumor growth, equal numbers of A549 cells were injected subcutaneously into both flanks of the nude mice. The tumor growth was measured 14 days after the injection. The tumors from the mice treated by apigenin were smaller than those tumors treated by the solvent control (Fig. 6A). The mean volumes of the tumors from the mice treated by apigenin were significantly smaller than those treated by the solvent control (Fig. 6B). The mice were euthanized at the end of the experiment. Tumor volume and weight were measured. Similar results were obtained from duplicate experiments based on the volume and tumor weight (Fig. 6, C and D). These results suggest that apigenin significantly inhibits the tumor growth in nude mice xenografts.

**Apigenin Inhibited Expression of HIF-1 $\alpha$  but Not HIF-1 $\beta$  in Tumors.** We also determined the effects of apigenin on HIF-1 $\alpha$  and HIF-1 $\beta$  protein levels in the tumors. Total proteins were extracted from the tumors, and HIF-1 $\alpha$  and HIF-1 $\beta$  proteins were detected by immunoblotting. HIF-1 $\alpha$  expression in the tumors was inhibited by apigenin (Fig. 7A). The relative HIF-1 $\alpha$  protein levels were analyzed by densitometry from five tumors per treatment. The ratio of HIF-1 $\alpha$  to HIF-1 $\beta$  protein was calculated and normalized to the solvent control. Apigenin treatment decreased HIF-1 $\alpha$  expression three times less than that of the solvent control (Fig. 7B). To determine



**Fig. 3.** Apigenin inhibited HIF-1 $\alpha$  protein expression. A549 cells were seeded in 60-mm plates and cultured to 80 to 90% confluence. The cells were then treated with various doses of apigenin (5, 10, 20, and 40  $\mu$ M) for 6 h. The cells treated with solvent alone were used as the control. Whole-cell extracts were subjected to immunoblotting analysis using antibodies specific for HIF-1 $\alpha$ , HIF-1 $\beta$ , or  $\beta$ -actin.



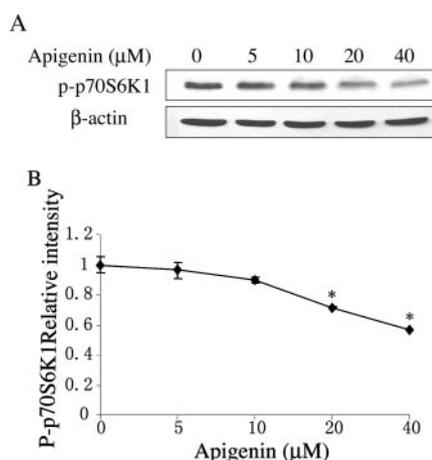
**Fig. 4.** Apigenin inhibited AKT activation. A, cells were treated as described above and the extracts were subjected to immunoblotting analysis using phospho-AKT (p-AKT) or total AKT antibodies. B, the intensity of p-AKT protein signals obtained in A was quantified using Chem Doc densitometry software (Quantity One, ver. 4.5.0; Bio-Rad, Hercules, CA). The p-AKT densitometry data were normalized to that of the control (lane 1) and total AKT levels. The plots represented mean  $\pm$  S.D. from three independent experiments. \*, significant difference compared with the untreated cells ( $p < 0.05$ ).

whether apigenin treatment affects the location of HIF-1 $\alpha$  protein in the tissue, we performed tissue staining with HIF-1 $\alpha$  antibodies. HIF-1 $\alpha$  staining exhibited a granular cytoplasmic pattern in the tissue sections (Fig. 8A). The number of positive cells expressing HIF-1 $\alpha$  in tumor sections treated by apigenin was significantly less than those treated by the solvent control (Fig. 8A). This result showed that apigenin specifically inhibited HIF-1 $\alpha$  expression in tumors in vivo.

**Apigenin Inhibited VEGF and PCNA Expression in the Tumors.** To determine whether apigenin inhibits VEGF and PCNA expression, tumor sections were stained using antibodies against VEGF or PCNA. VEGF was expressed mainly in the cytoplasm, whereas proliferation marker PCNA was expressed in the nuclei (Fig. 8B). The sections stained with pre-immune serum were used as a negative control. The number of cells expressing VEGF and PCNA in the tumors treated by apigenin was significantly less than those treated by the solvent control. Because PCNA is a proliferation marker, this result indicates that apigenin treatment also inhibits cell proliferation in the tumors (Fig. 8B).

**Apigenin Induced Apoptosis in the Tumors.** To determine whether apigenin induces cellular apoptosis in the tumors, tumor sections were assayed for apoptosis using the TUNEL assay. The number of apoptosis-positive cells in the tissues treated with apigenin was significantly greater than in those treated with the PBS solvent (Fig. 9A). These data indicate that apigenin induces cellular apoptosis in the tumors. Because apigenin did not induce apoptosis in cultured cells, it may induce apoptosis in tumor tissues through the inhibition of angiogenesis in vivo.

**Apigenin Inhibited A549-Induced Angiogenesis in Nude Mice.** The above studies suggest that apigenin may inhibit angiogenesis in vivo. To test whether apigenin inhibits angiogenesis, A549 cells were mixed with Matrigel and injected into both flanks of the nude mice. The mice were sacrificed 15 days after implantation. The cancer cells greatly increased tumor growth compared with the volume of the cancer cells with Matrigel alone (Fig. 9B). The tumors from



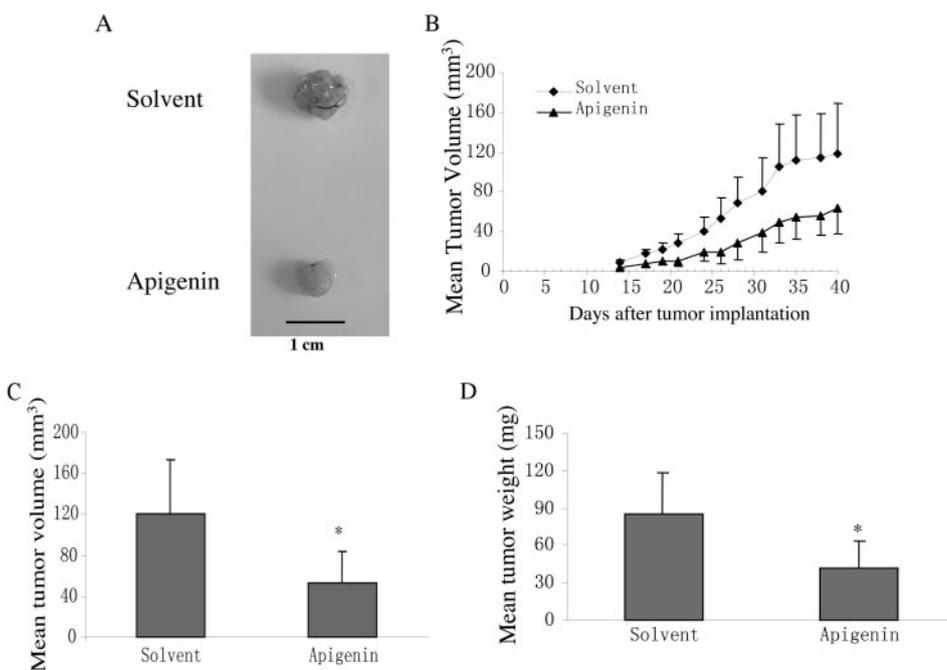
**Fig. 5.** Apigenin inhibited p-p70S6K1 protein expression. A, A549 cells were treated as above and the extracts were subjected to immunoblotting analysis using phospho-p70S6K1 (p-p70S6K1) or  $\beta$ -actin antibodies. B, intensity of p-p70S6K1 and  $\beta$ -actin protein signals obtained in A was quantified using Chem Doc densitometry software (Quantity One, ver. 4.5.0; Bio-Rad). The densitometry data were normalized to  $\beta$ -actin levels and those of the control (lane 1). The plots represented mean  $\pm$  S.D. from three independent experiments. \*, significant difference compared with the untreated cells ( $p < 0.05$ ).

the mice treated by apigenin were significantly smaller than the tumors treated by the solvent control (Fig. 9C). The relative angiogenesis was assayed by the hemoglobin content of the Matrigel plug. Compared with the Matrigel mixed with the medium alone, A549 cells greatly induced angiogenesis, and the hemoglobin levels in the A549 cell-treated plugs were 6-fold higher than those in the Matrigel alone because of the increased amounts of mouse hemoglobin. Apigenin treatment inhibited A549 cell-induced angiogenesis, and the hemoglobin levels in the apigenin-treated plug were significantly lower than those in the solvent-treated plugs (Fig. 9, B and D). These results suggest that A549 cells indeed induced tumor angiogenesis in nude mice, and that apigenin treatment greatly decreased angiogenesis, which may be responsible for apigenin-inhibited tumor growth in nude mice.

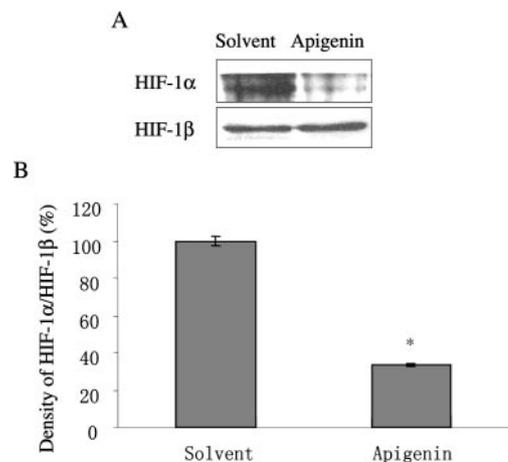
## Discussion

Non-small-cell lung cancer is the most prevalent type of lung cancer. Despite treatment advances in surgery, chemotherapy, and radiotherapy, the overall survival rate is poor (Chun et al., 2003; Soria et al., 2003). Thus, the search for an effective chemotherapeutic approach is important to lung cancer treatment. Apigenin, a low toxicity and nonmutagenic flavonoid, has garnered attention as an inhibitor of certain signal transduction pathways (Kuo et al., 1992; Chaumontet et al., 1994). Apigenin is a chemopreventive compound that inhibits protein kinase by competing with ATP (Geahlen et al., 1989; Yin et al., 1999b, 2001). However, the effects of apigenin on lung cancer cells remain to be elucidated. We found in this study that apigenin inhibited lung cancer cell proliferation and VEGF expression. Overexpression of VEGF is associated with tumor growth and angiogenesis, which is inversely correlated to the resistance of non-small-cell lung cancer (Volm and Rittgen, 2000). Recent studies showed that VEGF levels correspond with advanced lung cancer (Kishiro et al., 2002). VEGF is known to be specific for vascular endothelial cells. Our study showed that apigenin inhibited

VEGF expression at the transcriptional level through HIF-1 $\alpha$  expression, which suggests that apigenin may inhibit lung cancer angiogenesis. To understand the mechanism of apigenin-inhibited VEGF expression, we found that apigenin treatment inhibited the VEGF transcriptional activation through the HIF-1 DNA binding site in the VEGF promoter region. Apigenin inhibited the VEGF reporter with the normal HIF-1 binding site but not with the mutation of the HIF-1 binding site, suggesting that apigenin may inhibit HIF-1 expression. To test whether apigenin affects HIF-1 levels, we analyzed HIF-1 $\alpha$  and HIF-1 $\beta$  expression by immunoblotting and showed that apigenin specifically inhibited HIF-1 $\alpha$  but not HIF-1 $\beta$  expression in the cancer cells. HIF-1 expression is known to play an important role in VEGF



**Fig. 6.** Apigenin inhibited tumor growth in vivo. Nude mice were injected subcutaneously with  $1 \times 10^6$  A549 cells in PBS buffer or with  $15 \mu\text{M}$  apigenin. Each treatment group contained 10 mice. The mice were treated by intraperitoneal injection of PBS, or 3 mg/kg apigenin three times a week starting on day 4 to 40. The mice were euthanized on day 40. A, representative tumors from the control and apigenin-treated groups. Scale bar, 1 cm. B, tumor volumes were measured by the width and length of tumors from 10 mice in each treatment when tumors were visible. The mean  $\pm$  S.D. were obtained from 10 mice. C, the tumors were removed from mice, measured by the width and length, and analyzed as above. D, the mean  $\pm$  S.D. of the tumor weight. \*, significant difference when compared with the solvent control ( $p < 0.05$ ).

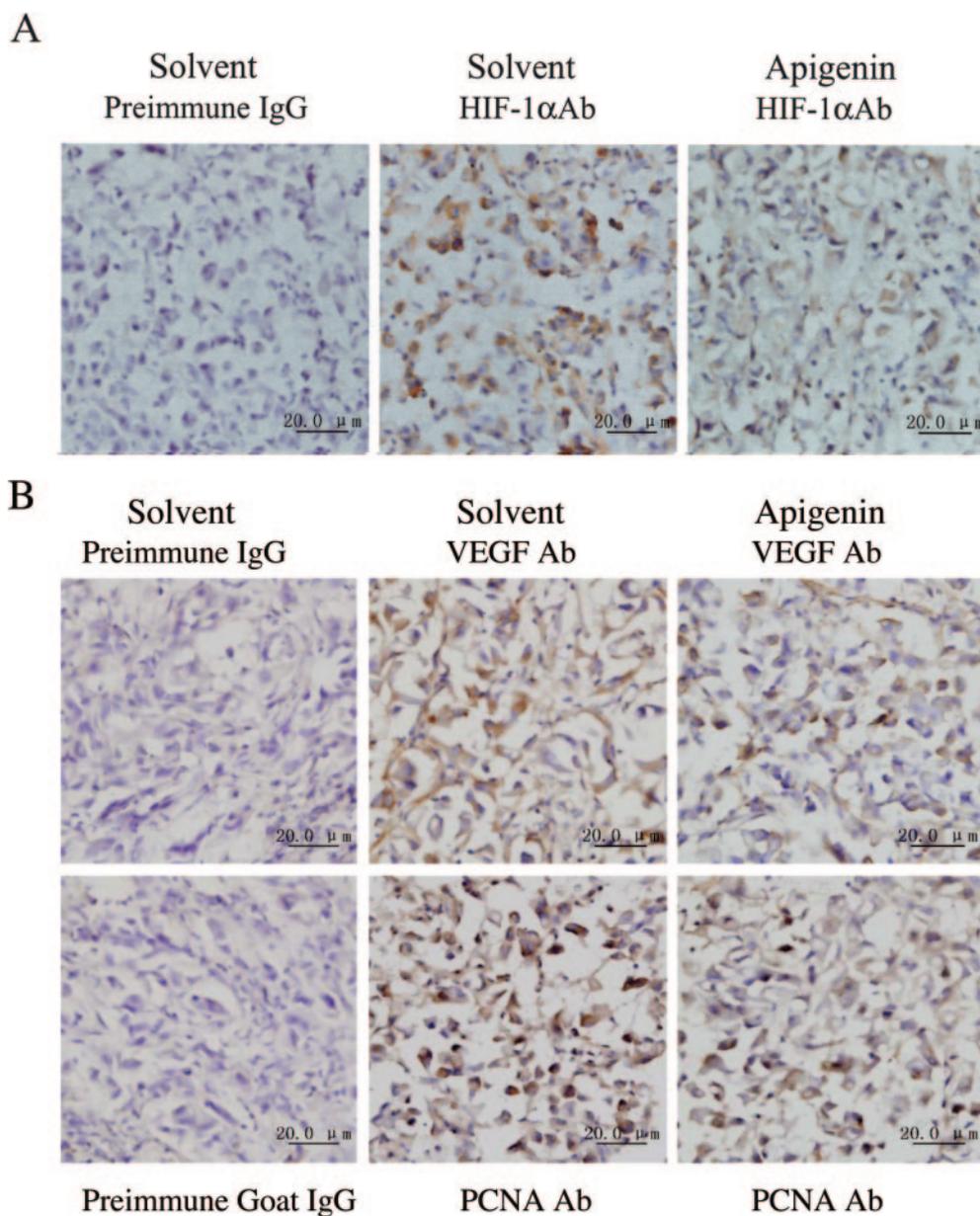


**Fig. 7.** Apigenin inhibited HIF-1 $\alpha$  expression in tumor tissues. Tumor tissues from nude mice were ground in liquid nitrogen and the total proteins were extracted. A, immunoblotting analysis was performed as described above. Lane 1 and 2 are tumor tissues from the control and apigenin-treated groups, respectively. B, the relative levels of HIF-1 $\alpha$  and HIF-1 $\beta$  were quantified by densitometry, and the ratio of HIF-1 $\alpha$  to HIF-1 $\beta$  signal was calculated and normalized to the control from three replicate experiments. \*, significant difference compared with the solvent control ( $p < 0.05$ ).

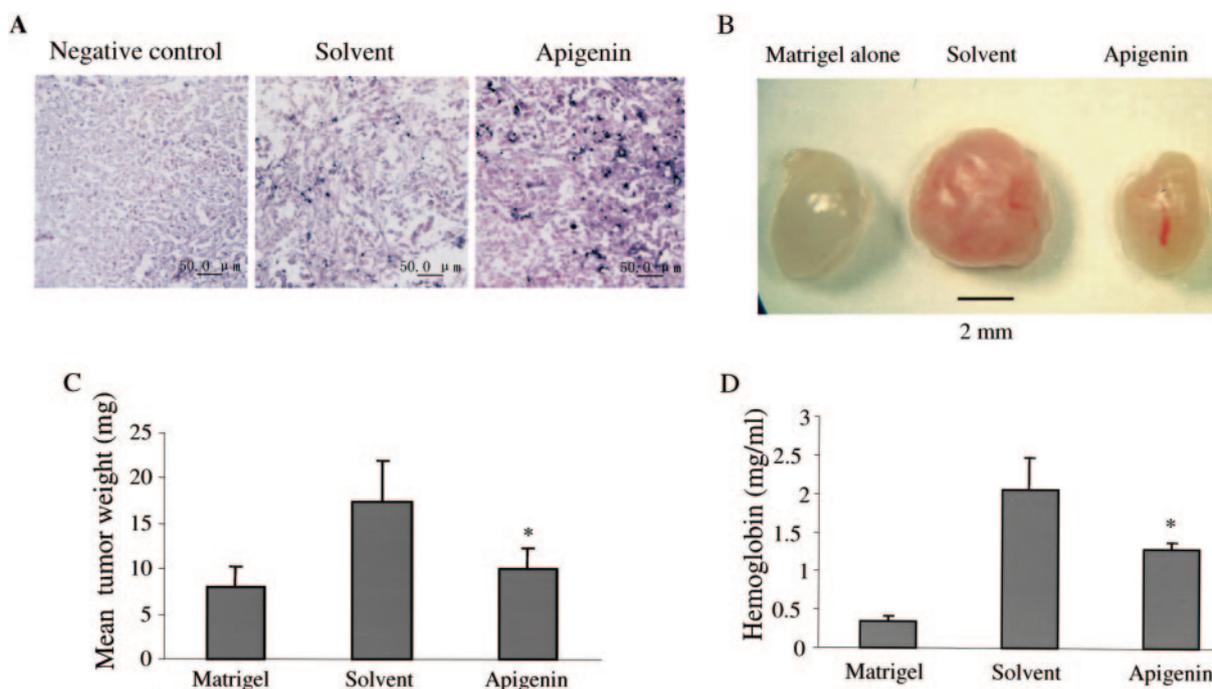
transcriptional activation in response to hypoxia (Forsythe et al., 1996; Jiang et al., 1997a). Thus, apigenin may inhibit VEGF transcriptional activation through the decrease of HIF-1 $\alpha$  expression in cancer cells. The increased VEGF levels were found to be associated with poor prognosis in patients with non-small-cell lung cancer (Iwasaki et al., 2004; Kaya et al., 2004). Apigenin may be used as a chemotherapy agent for lung cancer in the future. HIF-1 is induced by hypoxia, and activates the transcription of many genes including VEGF, endothelin-1, and inducible nitric oxide synthase, which are implicated in vasodilation, neovascularization, and tumor metastasis (Kerbel, 1998; Ryan et al., 1998; Semenza, 1999). The results suggest that apigenin may inhibit lung cancer growth at multiple levels by interfering with HIF-1 $\alpha$  expression (Hasebe et al., 2003; Kim, 2003). To identify signaling molecules that regulate apigenin-inhibited VEGF expression, we found that apigenin inhibited AKT and p70S6K1 activation in the lung cancer cells. Our recent studies demonstrated that phosphatidylinositol 3-kinase and

AKT signaling mediates angiogenesis and VEGF expression (Jiang et al., 2000; Gao et al., 2002, 2004).

We examined the effects of apigenin on tumor growth induced by lung cancer cells and found that apigenin significantly inhibited tumor growth in nude mice. Immunohistochemistry staining indicated that the expression of PCNA, HIF-1 $\alpha$ , and VEGF was inhibited by apigenin in tumor tissues. The PCNA, which is involved in DNA excision repair, is considered to be a reliable index of the proliferation rate (Sarac et al., 1998; Caputi et al., 1999). The *in vivo* results are consistent with those obtained in the cultured cells. These data suggest that apigenin is a potential chemoprevention agent for lung cancer through inhibiting cell proliferation, VEGF and HIF-1 $\alpha$  expression both *in vitro* and *in vivo*. It was an interesting observation that apigenin did not induce cell apoptosis in cultured cells (Fig. 1B) but induced apoptosis in tumor tissues (Fig. 9A). Our explanation of this is that apigenin induced the apoptosis *in vivo* possibly through the inhibition of angiogenesis *in vivo*, which is re-



**Fig. 8.** Apigenin inhibited HIF-1 $\alpha$ , VEGF, and PCNA expression in tumor sections. A, tumor sections from solvent- and apigenin-treated mice were stained by preimmune serum or HIF-1 $\alpha$  antibodies. The signals were detected using the SABC method. B, tumor sections were processed for immunohistochemical staining with monoclonal antibodies against VEGF, PCNA, or preimmune IgG. All immunostained sections were developed using the SABC method with diaminobenzidine as the chromogen. Negative control sections were incubated with preimmune mouse IgG instead of the primary antibodies.



**Fig. 9.** Apigenin treatment induced cellular apoptosis in tumor tissues and inhibited A549 cell-induced tumor angiogenesis. **A**, tumor tissue sections were cut and stained by TUNEL assay. Sections were lightly counterstained with nuclei red. Representative tumor tissue sections are from the unstained negative control which was incubated without terminal deoxynucleotidyl transferase, the control group, and the apigenin-treated group. Scale bar, 50  $\mu$ m. **B**, nude mice were injected subcutaneously with Matrigel plugs on both sides, and each plug contained  $3 \times 10^6$  A549 cells (0.1 ml) with 0.2 ml of Matrigel in the absence or presence of 15  $\mu$ M apigenin. Each treatment group contained four mice. The mice were euthanized 15 days after implantation. Representative Matrigel plugs from the solvent- and apigenin-treated groups. **C**, the mean tumor weight was obtained from eight Matrigel plugs as described above. **D**, the relative angiogenesis was analyzed by the levels of RBC hemoglobin, a surrogate marker, in the Matrigel plugs. The hemoglobin content was analyzed by adding  $1 \times$  RBC lysis buffer (1 mM EDTA and 5 mM potassium/sodium phosphate buffer, pH 8–8.5) to a plug, followed by incubation at 4°C overnight. Hemoglobin levels were determined by the Drabkin method by a commercial assay kit (Sigma) according to the manufacturer's instruction with some modification. Each treatment had four mice, each of which had an injection on two sides. Relative hemoglobin content is the hemoglobin level (milligrams) divided by the final volume of each plug. The data are the mean  $\pm$  S.E. of eight Matrigel plugs for each experiment. \*, significant difference compared with the solvent control ( $p < 0.05$ ).

quired for the delivery of oxygen and nutrients in the tumor tissues. To test the effect of apigenin on angiogenesis, we assayed angiogenesis using the Matrigel plug in nude mice. Our data showed that apigenin significantly inhibited angiogenesis in the mice (Fig. 9, B and D), indicating that apigenin may indeed inhibit tumor growth through the inhibition of angiogenesis. This result is consistent with the data showing the inhibition of HIF-1 and VEGF expression by apigenin because HIF-1 and VEGF are important for tumor angiogenesis.

According to the daily dietary consumption of flavonoids, the concentration of apigenin used in this work is physiologically relevant in humans and does not cause toxic side effects (Hollman et al., 1999a). Flavonoids are widely distributed in fruits and vegetables such as parsley, onions, oranges, tea, and wheat sprouts (Duthie and Crozier, 2000). Epidemiological surveys indicate that frequent consumption of foods rich in flavonoids is associated with a decrease in cancer incidence (Trock et al., 1990; Hollman et al., 1999b). Thus, apigenin may be a promising chemoprevention agent for lung cancer. Taken together, our results demonstrate that 1) apigenin is a potent agent to inhibit VEGF expression in the cultured cells and tumors, 2) apigenin inhibited VEGF expression at the transcriptional level specifically through the HIF-1 DNA binding site at VEGF promoter region, 3) apigenin specifically inhibited expression of HIF-1 $\alpha$  but not HIF-1 $\beta$  subunit, 4) apigenin inhibited tumor growth and angiogenesis, and 5) apigenin inhibited HIF-1 expression in

vivo, which is important for tumor growth and angiogenesis. These findings suggest that apigenin may be a promising chemoprevention agent against human lung adenocarcinoma.

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**Address correspondence to:** Bing-Hua Jiang, Institute for National Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China. E-mail: bhjiang@sibs.ac.cn