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Caudatin Inhibits Carcinomic Human Alveolar Basal Epithelial Cell Growth and Angiogenesis Through Modulating GSK3β/β-Catenin Pathway

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ABSTRACT

In this study, we investigate the anti-cancer activity of caudatin in carcinomic human alveolar basal epithelial cell line A549 and anti-angiogenic activity in human umbilical vein endothelial cells (HUVECs). We show that caudatin impairs the cell viability and induces G_0/G_1 phase arrest in A549 cells with a dose dependent manner. A549 cells, not HUVECs, dealing with caudatin exhibited typical characteristics of apoptosis, which were accompanied by activation of caspase-3, caspase-9 and Poly(ADP–Ribose) Polymerase (PARP). In addition, caudatin treatment resulted in a decrease of β-catenin and increase of phosphorylation of β-catenin, and inhibited phosphorylation levels of GSK3β (Ser 9) in A549 cells. Conditional medium of A549 cells-induced or growth factors-induced tube formation of HUVECs was markedly inhibited by caudatin treatment, which was associated with the inhibiting VEGF secretion from A549 cells by caudatin. Our findings suggest that caudatin inhibits carcinomic human alveolar basal epithelial cell growth and angiogenesis by targeting GSK3β/β-catenin pathway and suppressing VEGF production. J. Cell. Biochem. 113: 3403–3410, 2012.

KEY WORDS: CAUDATIN; CELL GROWTH; ANGIOGENESIS; β-CATENIN; GSK3β; VEGF

Wnt signaling is an evolutionarily conserved signaling cascade with imperative roles in regulating developmental decisions as well as adult tissue homeostasis. The protein β-catenin is the central player in one major arm of the Wnt pathway called the canonical Wnt pathway [MacDonald et al., 2009; Behari, 2010]. Characterization of this pathway has shown that Wnt/β-catenin signaling pathway is indispensible in processes as diverse as cell fate, proliferation, differentiation, growth, and cell survival [El Wakil and Lalli, 2011; Monga, 2011; Xiong et al., 2011]. Hyperactivation of β-catenin signaling has been implicated as a driver of various cancers, including lung cancer. Therefore, there is a great interest in identifying the inhibitors of Wnt/β-catenin signaling pathway.

Natural products represent a rich source of compounds that have been found many applications in various fields of medicines and therapy, which occupy a very important position in the area of cancer chemotherapy due to their excellent pharmacological activities and low toxicity. C-21 steroidal glycosides is one species of important biological active compounds, which are widely found in the plants of asclepiadaceae family and have extensive pharmacological effects such as inhibiting proliferation and invasion of tumor cells [Zhang et al., 2007; Peng et al., 2008].

Our previous studies have shown that caudatin, one species of C-21 steroidal glycosides, induces apoptosis in HepG2 cells [Fei et al., 2012], but the underlying mechanisms are not well documented. Thus, we perform this experiment to elucidate the mechanism by which caudatin might inhibit the growth of human lung cancer cells. Our results show that caudatin modulating GSK3β/β-catenin signaling possesses the activity to inhibit cell proliferation and induce cell apoptosis in A549 cells. In addition, nontoxic dose of

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caudatin suppresses the tube formation of endothelial cells through the inhibition of VEGF secretion in A549 cells.

MATERIALS AND METHODS

MATERIALS

Caudatin was purchased from the Shenzhen Medherb Biotechnology Co., Ltd. (Shenzhen, P.R. China), and dissolved with DMSO.

CELLS AND CELL CULTURE

A549 cells and HepG2 cells were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (Gibco BRL, MD), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in humidified 5% CO_2. HUVECs were purchased from the KeyGen Biotech (Nanjing China) and cultured in M199 containing 20% fetal bovine serum (Biowest, Rue de la Caille, France), 20 ng/ml fibroblast growth factor (Biowest).

MTT ASSAY

Cell viability was measured using the MTT assay. In brief, cells were cultured in 96-well plates (5×10^3 cells) for 24 h. The medium was removed, and cells were treated with either DMSO as a control or various concentrations of caudatin. The final concentration of DMSO in the medium was <0.1% (v/v). After 24 h, a volume of 20 μl of MTT (5 mg/ml, Genview, Houston, TX) was added and incubated at 37°C for 4 h. After removing the supernatant, 150 μl of DMSO was added to resolve formazan crystals, and the value of the optical density was detected at 490 nm. The median inhibitory concentration (IC_{50}) was assessed from the dose–response curves.

FLOW CYTOMETRY ANALYSIS

The DNA contents of cell were measured by the PI (Sigma, USA) staining method. Cells (1×10^6) were harvested by trypsinization and washed twice with PBS, then fixed with 75% ethanol for 24 h at 4°C. Fixed cells were re-suspended in PBS without calcium and magnesium containing 50 μg/ml RNase A (Sigma), and incubated in 37°C for 30 min. PI was added to the cells suspension (50 μg/ml) and incubated in the dark for 20 min. Stained cells were analyzed by a FACScan flow cytometry and CellQuest analysis software (Becton Dickinson, CA). Annexin V-FITC apoptosis detection kit (KeyGEN Biotech, Nan Jing, China) was used to detect cell apoptosis. Annexin V staining was performed according to the manufacturer's protocols, and cells were analyzed by flow cytometry.

WESTERN BLOTTING ASSAY

Western blotting assay was performed after treatment with caudatin for 24 h. Growing cells were washed twice with PBS and lysed in ice-cold lysis buffer (150 mM NaCl, 20 mM Tris–HCl, (pH 7.4), 0.1% SDS, 1% NP-40, 0.5% Na-DOC, 0.2 mM PMSF, and protease inhibitor and phosphates inhibitor cocktails) for 20 min on ice. Lysates were centrifuged at 13,000*g* for 20 min and the supernatants were used as total cell lysates. A quantity of 30 μg total proteins per lane was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Bedford). Membranes were incubated with the specific primary antibodies such as mouse anti-β-actin (Sigma,1:20,000 dilution), rabbit anti-pro-caspase-9 (Cell Signaling, MA, 1:600 dilution), rabbit anti-caspase-9 (Cell Signaling, 1:400 dilution), rabbit anti-pro-caspase-3 (Cell Signaling, 1:400 dilution), rabbit anti-PARP (Cell Signaling, 1:600 dilution), rabbit anti-β-catenin (Cell Signaling, 1:1,000 dilution); rabbit anti-p-β-catenin(Thr41/Ser45) (Cell Signaling, 1:600 dilution); rabbit anti-p-GSK3β-Ser^9 (Cell Signaling, 1:800 dilution); mouse anti-p-GSK3β-Tyr^{216} (BD Biosciences, 1:400 dilution), mouse anti-cyclinD1 (Santa Cruz, 1:500 dilution), rabbit anti-VEGF (Santa Cruz, 1:600 dilution) and the secondary antibody was diluted in 3% BSA/Tween-TBS. Detection of the target proteins on the membranes was performed using the ECL Detection Reagents (Thermo Scientific Pierce, USA).

IMMUNOFLOURESCENCE ASSAY

Cultured cells were fixed with cold 100% methanol at −20°C for 10 min and permeabilized with PBS-0.1%Triton X-100 for 10 min. After blocked with 3% BSA, the cells were incubated with anti-β-catenin antibody (Bioworld, 1:100 dilution). After washing, cells were incubated with FITC-conjugated secondary antibody (Bioworld, 1:200 dilution), and DAPI was used to stain the nuclei. The signals were detected by fluorescence confocal microscope with appropriate filter sets.

ENDOTHELIAL TUBE FORMATION ASSAY

In vitro angiogenesis was assayed using MatrigelTM matrix (BD-354234 and BD-354230, Two Oak Park, Bedford, MA). BD-354234 is a solubilized basement membrane preparation including various growth factors (such as TGF-beta, epidermal growth factor, insulin-like growth factor, fibroblast growth factor, tissue plasminogen activator and other growth factors which occur naturally in the EHS tumor), and growth factor reduced BD Matrigel (BD-354230) was used to detect the inhibitory effect of caudatin on the tumor induced angiogenesis. Formation of capillary like structures in a three-dimensional setting was used to assess the tube-forming activity of endothelial cells. In brief, 60 μl of cold Matrigel per well was transferred with a cold tip using a 96-well plate. HUVEC was seeded onto the Matrigel-precoated well present with or without caudatin. Tube-forming activity was estimated by counting the number of complete capillaries connecting individual points of the polygonal structures in a light microscope 4–10 h after transferring the cells to Matrigel. Three fields in the central area were chosen randomly in every well.

VEGF ASSAY

Secreted VEGF levels were determined by enzyme-linked immunosorbent assay (ELISA). In brief, A549 cells were seeded in 12-well plates and grown to 75–80% confluence. The cells were switched to fresh serum-free medium in the presence or absence of caudatin and incubated for another 12 h. After the treatment, the cell culture supernatant was harvested and spun at 4°C to remove cell debris. The supernatant culture medium was quantitated and assayed for

secreted VEGF using commercially available ELISA kits (R&D Systems, Minneapolis, MN).

STATISTICAL ANALYSIS

All the data were expressed as mean ± standard deviation (SD). Statistical analysis was performed using the Student's *t*-test and $P < 0.05$ was indicated to be statistical significance.

RESULTS

CAUDATIN INHIBITS A549 CELL AND HUVEC GROWTH

To determine whether caudatin inhibits cell survival and growth in human lung cancer cell and endothelial cell, we first examined cell viability to increasing concentrations of caudatin by the MTT assay. As shown in Figure 1A, caudatin inhibited the viability of A549 cells ($IC_{50} = 91.2$ μg/ml) in a dose dependent manner, and inhibited HUVEC growth with an IC_{50} of 121.1 μg/ml.

Next, we assessed effect of caudatin on the A549 cell cycle distribution using FACS analysis. As shown in Figure 1B, similar to the function in the HepG2 cells, caudatin caused an accumulation of A549 cells in the G_0/G_1 phase, and cell numbers in S-phase were decreased significantly, suggesting that caudatin blocks G_0/G_1 to S phase transition in A549 cells.

CAUDATIN INDUCES CELL APOPTOSIS IN A549 CELL BUT NOT IN HUVEC

To verify the apoptotic effect of caudatin on the lung cancer cells and endothelial cells, permeability of the cell membrane was analyzed by Annexin V-FITC and PI staining. Cells were incubated with increasing concentrations of caudatin for 24 h and harvested for further procession according to the manufacturer's instructions. Flow cytometry analysis results showed that the induction of apoptosis by caudatin occurred in A549 cells (Fig. 2A). In contrast, at the concentration of 100 μg/ml, caudatin exerted no significant effect on cell apoptosis in HUVECs (Fig. S1).

Caspase activation is a common feature of cells undergoing apoptosis. Therefore, we evaluated the effect of caudatin on the activation of caspases. Western blotting assay revealed that cleavage of apical pro-caspase-3, pro-caspase-9 and PARP into the characteristic activate fragments in A549 cells was evident when treatment with 50 μg/ml caudatin (Fig. 2B). However, higher dosage of caudatin (100 μg/ml) did not induce the activation of caspase-3 and caspase-9 in HUVECs (data not shown).

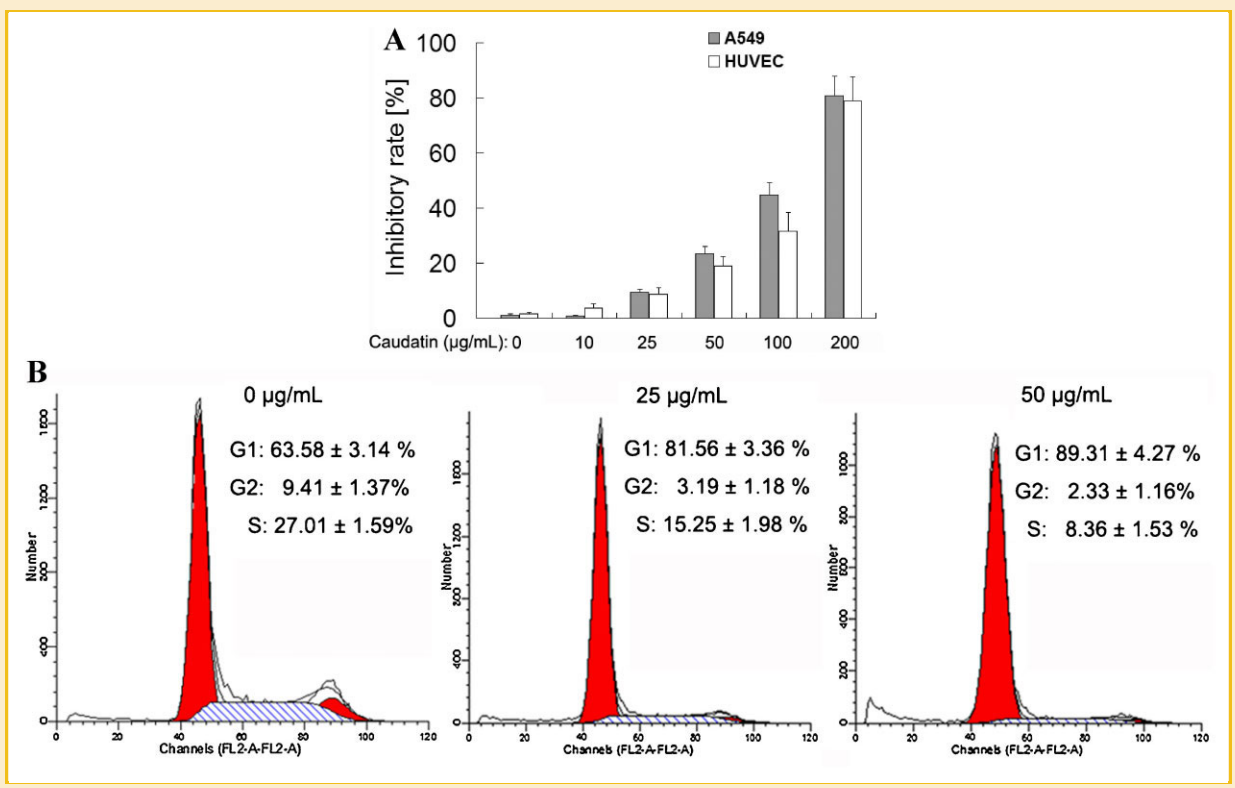

Fig. 1. Caudatin inhibits cell growth of A549 and HUVECs. A: MTT assay to detect the viability of A549 cells and HUVECs. Cells were incubated with 0, 10, 25, 50, 100, or 200 μg/ml of caudatin for 24 h, and cell growth was determined by the MTT assay. The graph displays the mean ± SD of the three independent experiments. B: Caudatin induces cell cycle arrest in A549 cell. Cells were treated with different content of caudatin for 24 h and stained with PI. The DNA content was analyzed by means of flow cytometry. Control cells yielded 63.58 ± 3.14% cells in the G1 phase, 27.01 ± 1.59% cells in the S phase, and 9.41 ± 1.37% cells in G2/M phase of cell cycle 24 h after plating. Treatment of A549 cells with 25 and 50 μg/ml caudatin increased the percentage of cells in the G1 phase to 81.56 ± 3.36% and 89.31 ± 4.27%, respectively. In contrast, the percentage of cells in the S phase and G2/M phase was decreased significantly. Data shown are from representative experiments repeated three times with similar results.

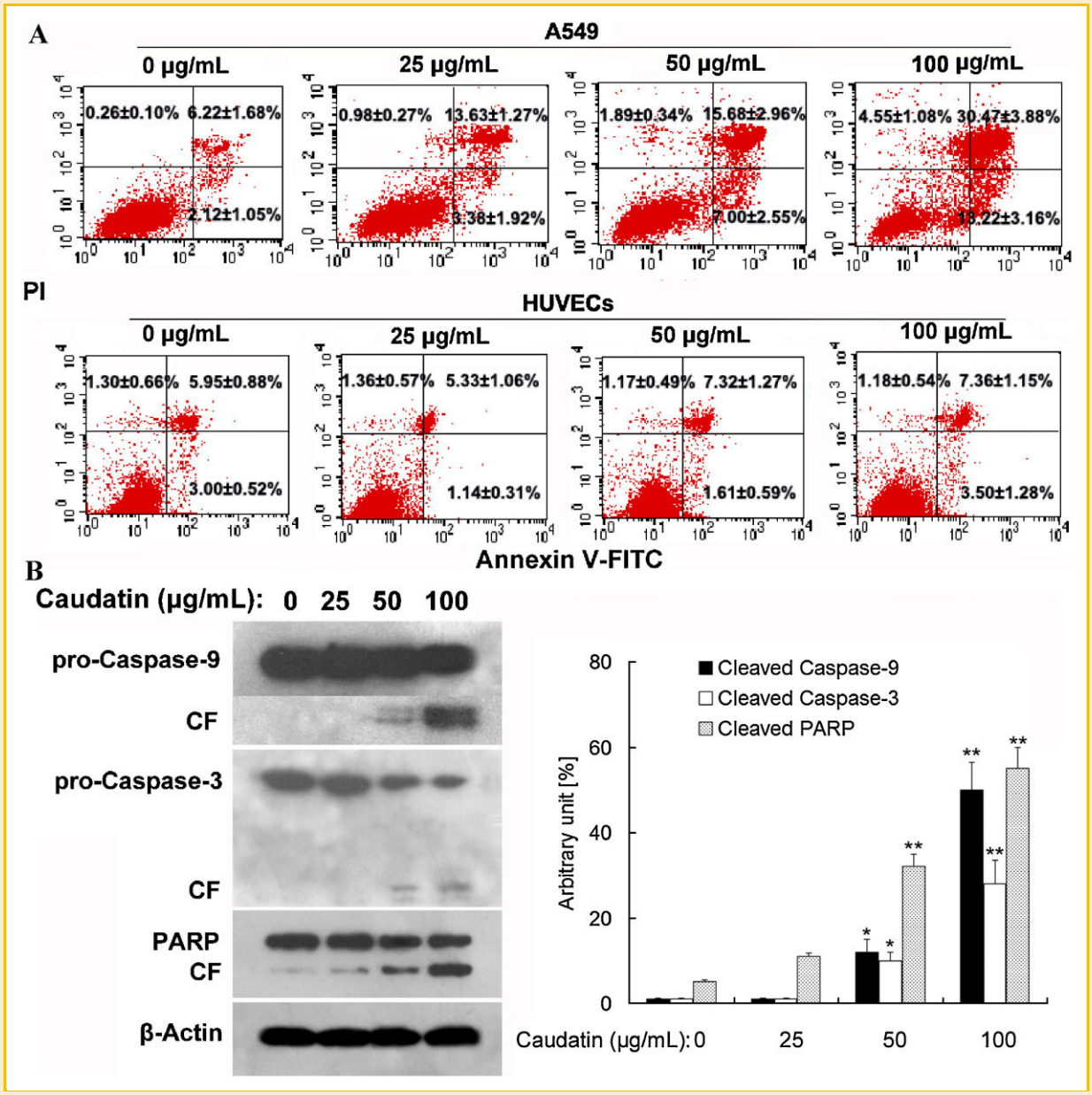

Fig. 2. Caudatin induces cell apoptosis in A549 cells but not HUVECs. A: Cells were treated with different content of caudatin for 24 h, and then analyzed for apoptosis by flow cytometry for Annexin V and PI staining. After treatment with 100 μg/ml caudatin, 13.22 ± 3.16% of the A549 cells were in early apoptotic stage (low-right phase), 30.47 ± 3.88% in late apoptotic/necrotic stage (up-right phase), and 4.55 ± 1.08% in necrotic stage (up-left phase). There was no difference in apoptosis between the HUVECs and caudatin treatment. B: Western blotting analysis showing cleavage of caspase-9, caspase-3 and PARP in response to different content caudatin in A549 cells. Each bar corresponds to the mean ± SD for three independent experiments. *indicates that $P < 0.05$ and **indicates that $P < 0.01$ versus control cells using the Student's *t*-test.

DECREASES EXPRESSION OF β-CATENIN BY CAUDATIN TREATMENT

The prominent role of Wnt/β-catenin signaling in tumorigenesis has attracted considerable interest in the drug discovery research community, and identification of inhibitors for this signaling pathway has been performed by researchers. To assess whether caudatin affects the expression of β-catenin, A549 cells were exposed to various concentrations of caudatin for 24 h. Western blotting results indicated that caudatin significantly downregulated the expression of β-catenin and increased the phosphorylation of β-catenin in A549 cells. Inhibition the level of cyclin D1, a downstream protein of β-catenin, was also observed in A549 cells following treatment with caudatin. We then examined level of phospho-GSK3β in the A549 cells. As shown in Figure 3A, caudatin downregulated phosphorylation of GSK3β at Ser 9, but did not influence the tyrosine phosphorylation of GSK3β (Tyr 216). In addition, incubation with 80 μg/ml of caudatin was also associated with a significant decrease in β-catenin levels in HepG2 cells (Fig. 3B). Immunofluorescence assays in the A549 cells treated with DMSO revealed nuclear location of β-catenin. After the 50 μg/ml of

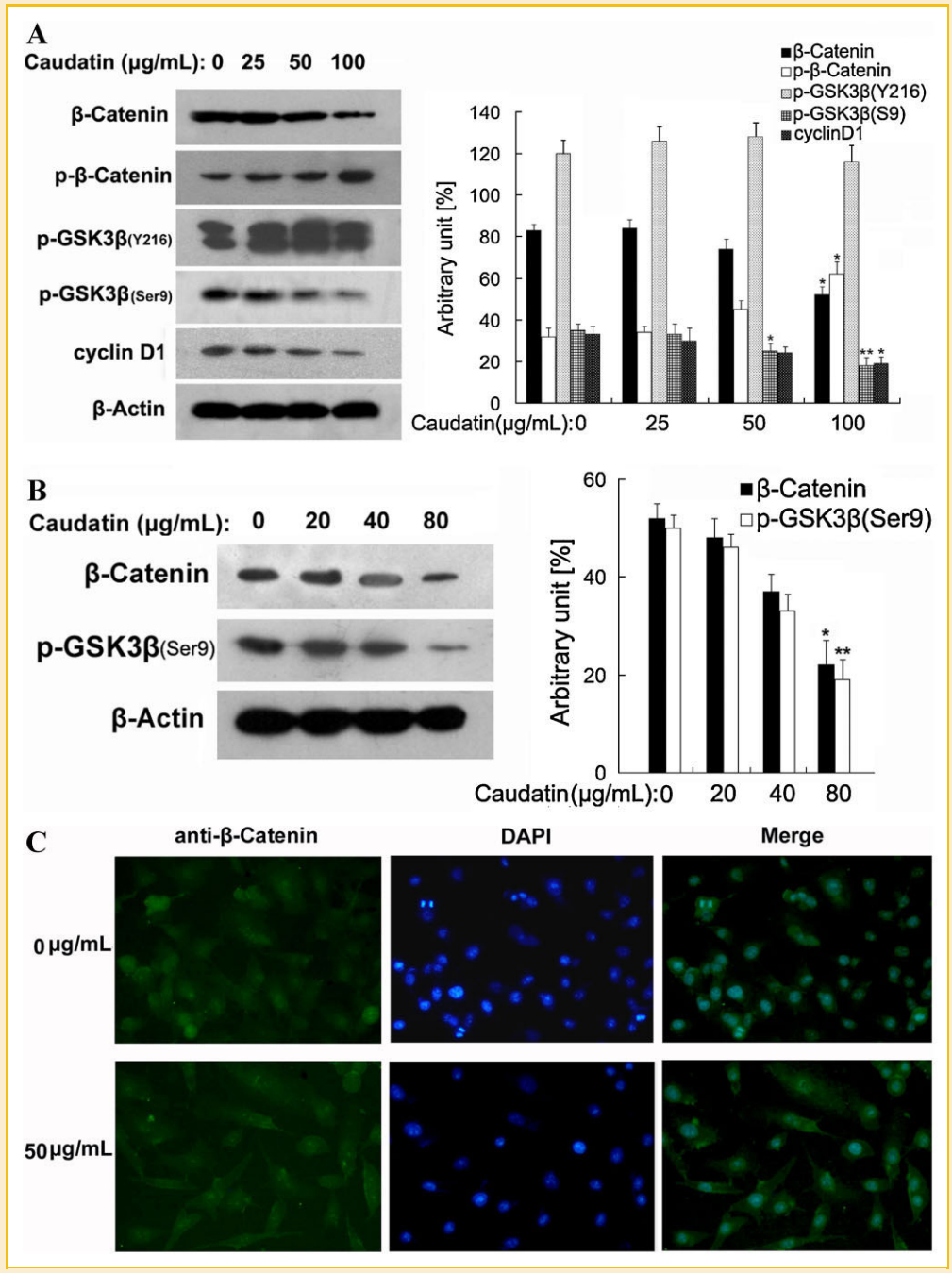

Fig. 3. Caudatin treatment decreases the expression of β-catenin. Examination of phosphorylation and total level of β-catenin, phosphorylation of GSK3β and cyclinD1 by Western blotting assay in A549 cell (A) and HepG2 cell (B). Each bar corresponds to the mean ± SD for three independent experiments.*indicates that $P < 0.05$ and **indicates that $P < 0.01$ versus control cells using the Student's *t*-test. C: β-catenin location in A549 cells was assessed by immunofluorescence assays. Cultured A549 cells were subjected to immunofluorescence staining with anti-β-catenin antibody. The green indicated the expression of β-catenin, and the blue denoted nuclei stained DAPI.

caudatin treatment, β-catenin was mainly located under the plasma membrane (Fig. 3C), suggesting that caudatin regulated the β-catenin pathway by inducing β-catenin degradation.

CAUDATIN INHIBITS TUBE FORMATION OF HUVEC

Tumor growth is closely dependent on the formation of new blood vessels, so the tube formation assay of endothelial cells was used to determine the anti-angiogenic action of caudatin. HUVEC incubated on Matrigel substratum for 10 h resulted in the formation of tube-like structure in the control group, whereas HUVEC exposed to caudatin formed incomplete tube networks (Fig. 4A). We also assessed the effect of caudatin on cancer angiogenesis induced by condition medium of A549 cells. As shown in Figure 4B, the condition medium of A549 cells caused the formation of the capillary-like HUVEC structures, but this phenomenon was blocked by 100 μg/ml caudatin treatment.

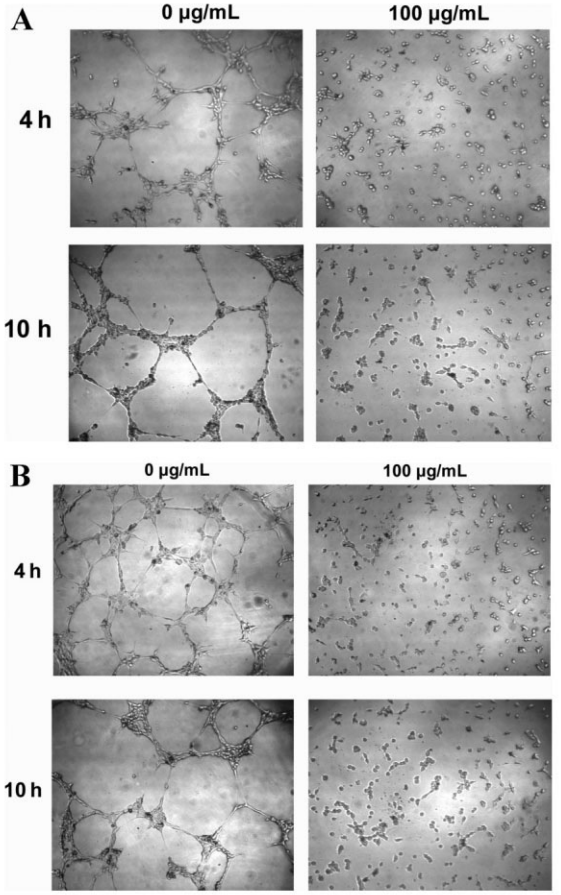

Fig. 4. Caudatin inhibits the tube formation of HUVECs. Cells (1×10^5 cells/well) were treated with or without the indicated concentration of caudatin in the presence of growth factors (A) or conditional medium of A549 cells (B), then plated on Matrigel for 4 or 10 h. Tube formation was observed under the microscope at 40× magnification and photographed.

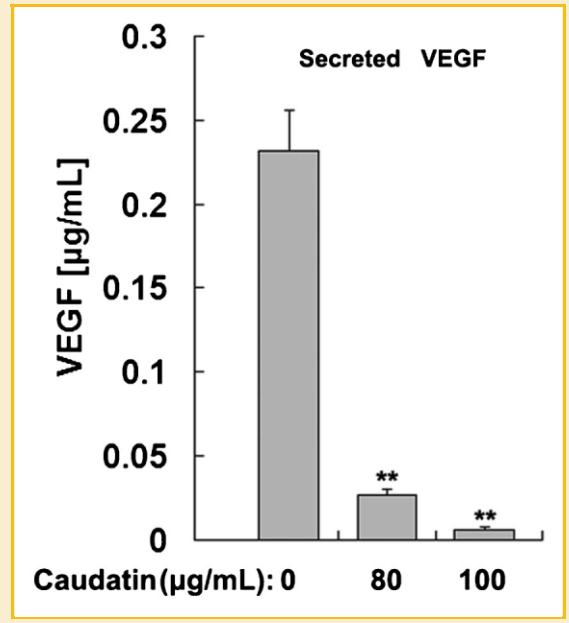

Fig. 5. Caudatin suppresses the secretion of VEGF in human lung cancer cells. A549 cells were treated with the indicated concentrations of caudatin in serum-free medium for 12 h. ELISA assay was used to detect VEGF expression in the culture supernatants ($n = 3$). Each bar corresponds to the mean ± SD for three independent experiments. **indicates that $P < 0.01$ versus control cells using the Student's *t*-test.

VEGF is one of the most important tumor angiogenic factors, and VEGF production in cancer cells is believed to be most specific and critical regulators of angiogenic signaling cascades. Therefore, we intend to elucidate whether the caudatin inhibits the secreted VEGF levels in A549 cells. By using ELISA assay, we found that caudatin significantly suppressed VEGF secretion in human alveolar basal epithelial cells (Fig. 5).

CAUDATIN INHIBITS THE EXPRESSION OF β-CATENIN IN HUVECS

At last, we investigate whether caudatin also inhibits the levels of β-catenin and p-GSK3β in HUVECs. As indicated in Figure 6, caudatin dramatically reduced the expression of β-catenin and p-GSK3β in HUVECs with a concentration dependent manner. In addition, treatment HUVECs with caudatin caused a significant reduction in the level of cyclin D1 and VEGF, two downstream targets of β-catenin.

DISCUSSION

Caudatin is mainly isolated from the root of *Cynanchum bungei Decne*, a traditional Chinese medicine and health food, which has been used to nourish the blood and enhance immunity in China and other Asian countries for a long time [Zhang et al., 2006]. In a previous study, we observed that caudatin induced cell growth arrest and apoptosis in human hepatoma cells. However, the molecular mechanisms are still unclear. In this study, we used carcinomic human alveolar basal epithelial cell as experimental material to confirm the anti-tumor effect of caudatin, and to illustrate the underlying mechanisms of anti-cancer activity. We first examined the cell cycle and cell apoptosis by flow cytometry assay, and found that caudatin induced A549 cell apoptosis and arrested cell cycle in G1 phase. Caspase activation plays a central role in the execution of apoptosis. Caspases are unique proteases that are synthesized as inactive zymogens (or proenzymes) and work in a controlled proteolytic cascade to activate themselves and one another. Once activated, initiator caspases cleave and activate effector caspases, such as caspase-3 and -9, which in turn cleave a variety of cellular protein substrates, ultimately leading to cell apoptosis[Crawford and Wells, 2011; Florentin and Arama, 2012]. Mechanistic studies showed that caudatin-induced A549 cell apoptosis was mediated through activation of caspase-3, caspase-9 and PARP cleavage, same as the activity in HepG2 cell. However, higher dosage of caudatin did not trigger activation of caspase-9 and caspase-3 in HUVECs, which elucidated why the caudatin did not induced the HUVECs apoptosis.

To further investigate the mechanism of caudatin on the regulation of cell proliferation and apoptosis, protein level of β-catenin was determined by Western blotting assay. We found that caudatin treatment inhibited the expression of β-catenin and

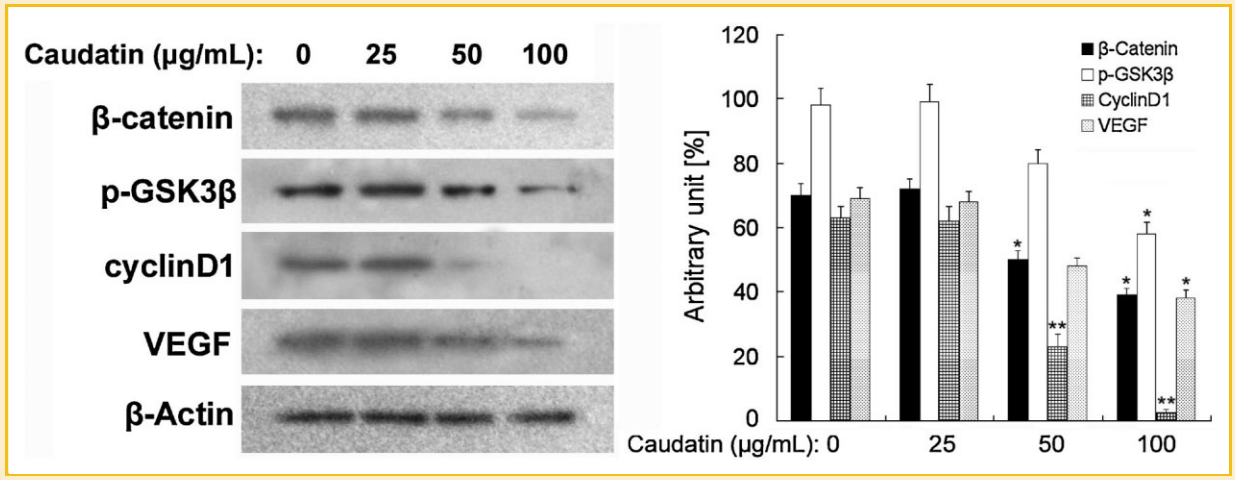

Fig. 6. Caudatin treatment inhibits the expression of β-catenin and p-GSK3β. HUVECs were treated with indicated concentrations of caudatin for 24 h, and the levels of β-catenin, phosphorylation of GSK3β, cyclinD1 and VEGF were examined by Western blotting assay. Each bar corresponds to the mean ± SD for three independent experiments. *indicates that $P < 0.05$ and **indicates that $P < 0.01$ versus control cells using the Student's *t*-test.

increased the level of phospho-β-catenin. Wnt/β-catenin signaling pathway is highly conserved during evolution and regulates cellular processes such as cellular proliferation, survival, and differentiation; dysregulation of this signaling cascade is heavily implicated in the development and progression of cancers [Naito et al., 2010; Prosperi and Goss, 2010; Kwon et al., 2011]. In the absence of Wnt/Wingless ligand activation, β-catenin is sequestered in the cytoplasm by a multiprotein complex and phosphorylated at the NH2-terminal serine and threonine residues by GSK3β, which targets it for ubiquitination and proteolytic degradation [Dodge and Lum, 2011]. Activation of Wnt signaling by binding of Wnt ligands to a Frizzled receptor inhibits GSK3β-mediated phosphorylation of β-catenin, resulting in an accumulation of hypophosphorylated β-catenin in the cytosol [Baryawno et al., 2010]. Stabilized hypophosphorylated or depophosphorylated β-catenin eventually translocates to the nucleus, leading to modulated expression of a broad range of genes, such as cyclinD1 and Myc [Tung et al., 2010; Zhang et al., 2009].

Glycogen synthase kinase 3 (GSK3) is a serine/threonine kinase consisting of two isoforms, GSK3α and GSK3β. The activities of GSK3 are regulated negatively by serine phosphorylation but positively by tyrosine phosphorylation [Liang and Chuang, 2007]. In cancer cells, increased activation of GSK-3β is pro-apoptotic, whereas its inhibition is anti-apoptotic [Li et al., 2008]. Our studies showed that caudatin inhibited GSK3β Ser^9 phosphorylation and increased the phosphorylation of β-catenin in A549 cells, thus implying degradation of β-catenin by antagonizing the inactivation of GSK3β is involved in the caudatin anti-cancer activity. Although caudatin also inhibited the phosphorylation of GSK3β in HUVECs, but no obvious change in apoptosis was observed. This may be due to the fact that caudatin suppressing the GSK3β/β-catenin pathway in HUVECs is not involved in the cell apoptosis, which may play a crucial role in other physiological processes such as regulating the cell proliferation [Liu et al., 2008; Eom and Jope, 2009].

Tumor angiogenesis, the central process of new blood vessels forming a pre-existing blood supply system, promotes tumor progression by supporting cancer cell survival and metastasis. A growing body of evidence indicates blockade of cancer angiogenesis can decrease cancer progression by inhibiting tumor growth and metastasis, and development angiogenesis inhibitors is a desirable anticancer target for which few side effects might be expected [Folkman and Shing, 1992; Tsai et al., 2010]. We therefore detect the anti-angiogentic activity of caudatin in vitro by tube formation assay, and found that caudatin inhibited HUVEC angiogenesis induced by both growth factors and conditional medium of A549 cells. It is notable that the anti-angiogenesis of caudatin is not due to its cytotoxic effect. Until now, VEGF has commonly been accepted as a crucial growth factor to promote the angiogenesis. Results of ELISA assay showed that caudatin inhibited secretion of VEGF from A549 cells. In addition, treatment of the HUVECs with caudatin decreased the level of β-catenin and inhibited GSK3β Ser^9 phosphorylation in a dose dependent manner.

In conclusion, our results suggest that caudatin inhibits carcinomic human alveolar basal epithelial cell growth as well as angiogenesis, and induces of cell G1 phase arrest in a dose dependent manner. A549 cell, not HUVECs, treatment with caudatin shows typical characteristics of apoptosis. Our findings suggest that caudatin-mediated anti-angiogenic effects are not likely due to cell toxicity, but largely due to modulation of VEGF production or angiogenic process. We also demonstrate that caudatin increases GSK3β activity by decreasing its phosphorylation on ser 9 residue, which in turn phosphorylates β-catenin and induces degradation of it. Our results indicate that caudatin may prove to be a valuable tool for inhibition of cancer angiogenesis.

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