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Evodiamine inhibits in vitro angiogenesis: Implication for antitumorgenicity

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Abstract

Evodiamine, the major bioactive compound isolated from Chinese herbal drug named Wu-Chu-Yu, has been reported to exhibit anti-tumor growth and metastasis. However, the effect of evodiamine on angiogenesis remains to be investigated. We used the fresh medium containing evodiamine or human lung adenocarcinoma cell (CL1 cells) derived conditioned media free of evodiamine to test their capability to induce in vitro angiogenesis, i.e., human umbilical vein endothelial cells (HUVECs) tube formation and invasion. We demonstrated that evodiamine could directly inhibit in vitro HUVECs tube formation and invasion. Locally administered evodiamine also inhibited the in vivo angiogenesis in the chick embryo chorioallantoic membrane (CAM) assay. The gene expression of vascular endothelial growth factor (VEGF) and the p44/p42 mitogen-activated protein kinase (MAPK, ERK) that correlated with endothelial cells angiogenesis were inhibited by evodiamine. We found that the evodiamine-treated CL1 cells derived conditioned medium showed decreased VEGF release and reduced ability of inducing in vitro tube formation. After the collection of conditioned media, the VEGF expression of remaining CL1 cells were determined by Western analyses and revealed that evodiamine decreased VEGF expression. Moreover, administration of recombinant human $VEGF_{165}$ ($rhVEGF_{165}$) induced tube formation and ERK phosphorylation by HUVECs, and partially attenuated inhibitory effect of evodiamine. From these results, we suggested that evodiamine is a potent inhibitor of angiogenesis. The mechanism might involve at least the inhibition of VEGF expression, probably through repression of ERK phosphorylation.
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Keywords: Evodiamine; VEGF; ERK; HUVECs; Angiogenesis

Introduction

Wu-Chu-Yu is a longstanding Chinese herbal used for a syndrome characterized by cold hand and feet, migraines and vomiting. It has been demonstrated that the effectiveness of Wu-Chu-Yu in maintaining body temperature was due to evodiamine (Kano et al., 1991), which is the major bioactive alkaloid isolated and purified from Wu-Chu-Yu. It has also been shown that evodiamine exerts a positive iontropic effect on the isolated left atrium of the guinea pig (Shoji et al., 1986), and an attenuated contractile response on the rat isolated mesenteric arteries (Chiou et al., 1992). Meanwhile, evodiamine exhibited anti-inflammation on altering nitric oxide production in the murine macrophage (Chiou et al., 1997).

Recently, evodiamine has been shown to alter the balance of Bcl-2 and Bax gene expression and induce apoptosis through the caspase pathway in Hela cells (Fei et al., 2003). Kan et al. (2004) also showed that evodiamine inhibits the growth of prostate cancer cell line, LNCaP, through an accumulation of cell cycle at G2/M phase and an induction of apoptosis. Ogasawara et al. (2001a) demonstrated that evodiamine had a remarkable anti-migratory activity with an IC_{50} value of 1.25 μg/ml (equal to 4.12 μM), suppressed the growth of colon 26-L5 cells and had a marked reduction in tumor metastasis formation in vivo (Ogasawara et al., 2001b). Some agents effective in inhibiting tumor invasion have been revealed to possess anti-angiogenic activity based on inhibition of endothelial cell invasion (Kohn et al., 1995; Belotti et al., 1996; Cai et al., 1999; Singh et al., 2002).

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However, the effects of evodiamine on angiogenesis remain to be investigated.

Angiogenesis is the growth of new vascular capillary from pre-existing vessels, and is important to a number of physiological processes such as embryonic development, placenta formation, and endometrial maturation and cycling. Angiogenesis is also initiated in response to tissue repair or uncontrolled pathological conditions, such as rheumatoid arthritis, diabetic retinopathy, atherosclerosis, and tumor growth. Experimental evidence suggested involvement of angiogenesis in expansion of primary tumors and their metastasis to distant organs (Folkman and Shing, 1992). The factors in controlling tumor angiogenesis include fibroblast growth factor (FGF) (Tanaka et al., 1999), vascular endothelial growth factor (VEGF) (Veikkola and Alitalo, 1999; Eriksson et al., 2003), and hepatocyte growth factor (HGF) (Wang and Keiser, 2000). VEGF is a specific mitogen for endothelial cells, and is responsible for induction of vascular leakage in tumors. It is generally considered that VEGF primarily stimulates the p44/p42 mitogen-activated protein kinase (MAPK, ERK) pathway, and that the ERK activation regulates the cell proliferation and angiogenesis activity (D'Angelo et al., 1995; Veikkola and Alitalo, 1999; Eriksson et al., 2003).

In order to substantiate the assumption of direct anti-angiogenic action of evodiamine, we investigated the effect of evodiamine on differentiation of endothelial cells in an in vitro model using human umbilical vein endothelial cells (HUVECs) on matrigel. Lung cancer malignancy easily occurs via angiogenesis and metastasis, and is a major cause of cancer death in Taiwan. Therefore, we examined the effect of evodiamine on lung adenocarcinoma cells, CL1, induced angiogenesis. We also investigated the effect on VEGF and its subsequent molecule, ERK, in the signal transduction of angiogenesis suppressed by evodiamine. These data suggested that evodiamine is a potent inhibitor of angiogenesis induced by tumor. Our results concluded that evodiamine inhibited angiogenic activity might be attributable to the mechanisms of inhibition of VEGF expression, probably through the repression of ERK phosphorylation.

Materials and methods

Cell culture and treatment

The human lung adenocarcinoma cell line CL1 was established from a 64-year-old man with a poorly differentiated adenocarcinoma (Yang et al., 1992). The cells were maintained in RPMI 1640 medium (Gibco-BRL, Buffalo, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. The human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical cords by treatment with collagenase as previously described (Chang et al., 2003), and maintained in endothelial cell basal media (EBM; Clonetics, Cambrex Bio Science Walkersville, Inc., Walkersville, MD, USA), supplemented with EGM (Clonetics #CC-4133). Both CL1 and HUVECs were cultured in a humidified atmosphere containing 5% CO_2 at 37 °C.

Cell viability assay and growth analysis

The colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was modified and done to quantify the effect of evodiamine on cell proliferation (Kan et al., 2004; Shyu et al., 2004). Briefly, in the continuous treatment procedure, 1000 cells/well, were seeded in 96-well microplate (Falcon, Franklin Lakes, New Jersey, USA) in a final volume of 100 μl. After seeding for 2 days, cells were treated with various doses of evodiamine ($0-2\times10^{-6}$ M) for 12, 24, 48, 72 or 96 h. After completion of the treatment, MTT solution (0.25%) (Sigma, St. Louis, MO, USA) was added to the cells and incubated for 3 h at 37 °C. The plates were centrifuged at $400\times g$ for 5 min, then MTT solution was removed and replaced by 50 μl DMSO, and the plates were shaken for 3 min. The optical density of each condition was determined using a microplate reader (Dynatech Laboratories, Chantilly, Virginia, USA) at a wavelength of 570 nm with a reference wavelength of 620 nm. Each experimental condition was replicated by three times and the percentage of cell viability was calculated against untreated cells.

Endothelial cells were seeded with 3×10^5 in a 60 mm dish for 2 days. The growth medium was changed with fresh medium with or without evodiamine. After 24 h of treatments, the cells were washed with PBS and trypsinized with trypsin-EDTA (Gibco, Invitrogen Corporation, Grand Island, NY, USA). Equal volumes of resuspended cells and 0.4% trypan blue (Sigma) were combined, incubated at room temperature for 10 min, and counted using a standard hemocytometer (Fisher Scientific, Itasca, IL). Viable cells (unstained) and nonviable cells (stained) were counted, and the percent cell viability was calculated as follows: [% cell viability = total viable cells (unstained) / total cells (stained and unstained) × 100].

In vitro capillary tube formation model

We used a modification of the matrigel assay to evaluate in vitro angiogenesis activity by quantifying the tube formation of HUVECs as previously described (Chang et al., 2003; Shyu et al., 2004). HUVECs (1.5×10^4 cells) were suspended in 150 μl of fresh whole serum EGM media (2% fetal bovine serum) containing various concentrations of evodiamine and then seeded on the polymerized matrigel. After incubation at 37 °C for 8 h, each culture was photographed at a magnification of 100× with a microscope video system. Tube formation was quantified from four randomly selected fields per experiment by measuring the total additive length of all cellular structures including all branches, using a scale ruler.

To avoid evodiamine induced toxicity of HUVECs, the conditioned media from CL1 cells incubation were used in capillary tube formation. CL1 cells were cultured with RPMI 1640 medium (supplemented with 1% FBS) alone or RPMI 1640 medium containing evodiamine (1 μM) for 8 h. These

cells were then cultured in fresh RPMI 1640 medium but free of evodiamine for another 24 h, and then the conditioned media was collected and subjected to in vitro capillary tube formation assay. The remaining CL1 cells were subjected to Western blot for measuring expression of VEGF. In another experiment, 10 ng/ml recombinant human $VEGF_{165}$ ($rhVEGF_{165}$) were added to verify the relationship of tube formation and evodiamine depressed VEGF expression.

In vivo CAM assay

The chick chorioallantoic membrane (CAM) assay used was a modified procedure based on Sharma et al. (2001) to test neovascularization. Briefly, the surfaces of 10-day-old post-fertilization chick eggs (White Leghorn, Animal Drugs Inspection, Animal Health Research Institute, Council of Agriculture, Executive Yuan, ROC) were sterilized and the CAM exposed by cutting a window (1 cm^2) on one side of the egg using the false air sac technique (Ausprunk et al., 1975). An 8 mm diameter straw was placed on the exposed CAM. Aliquots (100 μl) of solution with or without evodiamine (10^{-6} M) were added into the straw and incubated for 8 h. The windows were sealed with transparent tape and the eggs incubated in a humidified incubator at 100 °F. Eggs were examined at 48 h post-inoculation intervals by using a stereoscopic dissection microscope, and positive angiogenesis was recorded if new microvessels delineating the added straw had developed.

Cell invasion assay

The invasion activity of HUVECs was tested using the transwell plates (Corning Incorporated, Corning, NY, USA) following the protocol provided by the manufacturer. The transwell inserted with polymerized matrigel was lodged into a well of a 24-well plate containing 600 μl medium. HUVECs (3×10^4 cells) were suspended in 50 μl of whole serum EGM media containing various concentrations of evodiamine and then seeded on the polymerized upper chamber. After incubation at 37 °C for 8 h, the residual cells in the upper side of the filter were screwed with cotton swabs. And then the membrane was fixed with 100% methanol for 10 min. Cells that migrated to the lower side of the membranes were stained with Liu stain (Handsel Technologies, Inc., Taipei, Taiwan). The stained cells from four high power (×200) fields (HPF) were counted. Photographs were taken by a microscope video system.

VEGF quantification by ELISA

CL1 cells were cultured with 1% FBS-RPMI 1640 medium alone or RPMI 1640 medium containing evodiamine (0.1, 0.4, or 1 μM) for 8 h. These cells were then cultured in fresh RPMI 1640 medium but free of evodiamine for another 24 h and then the conditioned media was collected. The concentration of VEGF released into the conditioned media of CL1 cells was measured by ELI SA kit (Quantikine, R&D System, Minneapolis, MN, USA) following the manufacturer's instructions. In this kit, the sensitivity was 15.6 pg/ml, the intra- and interassay coefficients of variability were 3.5–6.5% ($n=8$) and 6.7–8.5% ($n=5$). The assay was run in duplicate and repeated three times with similar results; data of the three experiments were pooled for statistical analysis.

Western blot analysis

After culture under the indicated conditions, cells were harvested and washed twice in ice-cold PBS. The cell pellets were dissolved in RIPA lysis buffer (50 mM Tris–HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml aprotinin), and the protein content of the lysate was determined using the Coomassie blue protein assay (Bio-Rad Laboratories, Inc. Richmond, CA). Aliquots (40 μg) of cell lysate were size-fractionated by SDS-polyacrylamide gels and electrotransferred onto polyvinylidene membrane (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK). The membranes were blocked with 5% nonfat milk in TBST (20 mM Tris–HCl [pH 7.6], 135 mM NaCl; 0.1% Tween 20). The blots were incubated with indicated antibody and the signals were visualized by enhanced chemiluminescence detection (ECL, Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK). Signal intensities of Western blots were determined using a Photoshop 6.0 software and used to calculate fold inhibitions after normalization to GAPDH.

Northern blot analysis

The HUVECs were incubated with or without evodiamine for 8 h. At the end of incubation, the cells were washed twice and total RNA was isolated using Trizol (Life Technologies). Aliquots containing 20 μg RNA were loaded and run in 1% agarose/formaldehyde gel electrophoresis, and then transferred overnight onto the nylon membranes (Hybond-N+, Amersham). DNA probes were prepared as previously described (Shyu et al., 2004) and labeled by Redi Prime II random priming system following the manufacturer's protocol (Amersham Pharmacia Biotech, Little Chalfont, Bucks, UK). Blots were hybridized overnight at 65 °C with 2×10^6 cpm/ml of indicated random primed cDNA probes. Blots were washed once with 5 × SSC-0.1% sodium dodecyl sulfate (SDS) at 65 °C for 20 min, and twice with 0.1 × SSC-0.1% SDS at 65 °C for 20 min. Finally, the membrane was exposed to radiographic film. Signal intensities of Northern blots were determined by a Photoshop 6.0 software and used to calculate fold inhibitions after normalization to GAPDH.

Materials

The composition of EGM (Clonetics #CC-4133, Cambrex Bio Science Walkersville, Inc. Walkersville, MD, USA) was 2% FBS, bovine brain extract, hydrocortisone (1 mg/ml), human epidermal growth factor (10 μg/ml), ampicillin (100 IU/ml), and gentamicin (50 μg/ml). The human VEGF ELISA kit and

rhVEGF$_{165}$ were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). The antibodies: anti-VEGF, and anti-GAPDH were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A). Anti-phospho-ERK, and anti-total ERK antibodies were purchased from Cell Signaling Technology (Cell Signaling Technology, Inc., Beverly, MA, USA). Redi Prime II random priming system and the ECL kit were obtained from Amersham Pharmacia Biotech (UK). Anti-mouse and anti-rabbit IgG peroxidase-conjugated secondary antibodies were bought from Calbiochem (San Diego, CA, USA). Matrigel was obtained from Chemicon International, Inc. (Temecula, CA, USA). Dimethyl sulfoxide (DMSO) was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Evodiamine was kindly supplied by Dr. L.-C. Lin at National Research Institute of Chinese Medicine (Taipei, Taiwan). In each treatment, evodiamine was diluted with DMSO, and then the solution was added into medium to be in a final concentration of 0.1% DMSO. No precipitation of evodiamine was observed under a microscope at final concentrations of less than 100 μM.

Statistics

All data are presented as mean ± standard deviation (S.D.). The treatment means were tested for homogeneity by a one-way analysis of variance (one-way ANOVA), and the difference between specific means was tested for significance by Duncan's multiple range *t*-test. The difference between two means was considered statistically significant when $P<0.05$.

Results

Effect of evodiamine on cell viability of HUVECs

Evodiamine has been reported as a potent anti-metastasis agent, but no report has described evodiamine to correlate with angiogenesis. We used HUVECs to investigate evodiamine's anti-angiogenic potential. First, we performed MTT and cell viability assays to determine proper evodiamine dose and treatment time. We treated HUVECs with various dosages of evodiamine for 12–96 h, and with 0.1% DMSO in control. The subsequent MTT assay showed that the significant and dose-dependent inhibitory effect on HUVECs growth caused by evodiamine was observed at 24 h and persisted for 96 h (Fig. 1A).

We further used trypan blue exclusion assay to investigate the effect of evodiamine (0.1, 0.4, 1 μM) on the viability o f HUVECs. Data showed that in the initial 12 h, at least, there was no difference in cell viability compared with untreated control. When HUVECs were cultured with 1 μM evodiamine for 12, 24 or 48 h, the viability of HUVECs was about 97.0%, 80%, and 55% (Fig. 1B). To avoid the putative cytotoxicity of evodiamine, we chose 8 h as the treatment time for the following experiments.

Evodiamine reduced capillary tube formation

To determine whether evodiamine could block the angiogenic effect directly, whole-serum EGM alone or EGM

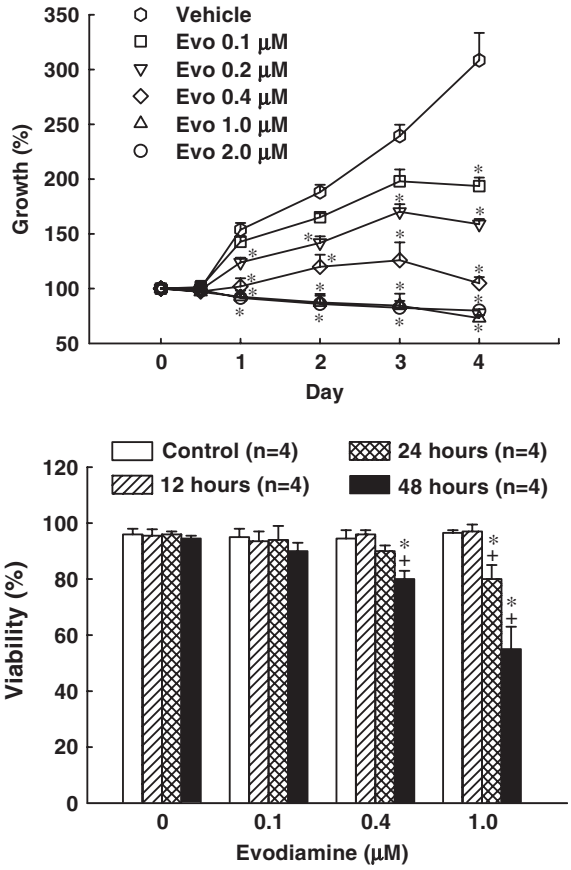

Fig. 1. The proliferation and viability of HUVECs treated with evodiamine: (A) MTT assay for the proliferation of HUVECs treated with various doses of evodiamine for 12, 24, 48, 72, or 96 h. Evo: evodiamine. *$P<0.05$ versus vehicle. (B) The cell viability analysis was measured by means of the trypan blue dye exclusion test. *$P<0.05$ compared with evodiamine at 0 M. $^{+}P<0.05$ compared with control. All data were represented as mean ± S.D. of 3 replicates from four separate experiments.

containing evodiamine (0.1, 0.4, 1 μM) were added at the time of HUVECs plating on matrigel. After 8 h, the total length of capillaries in each well was calculated. The data showed that evodiamine resulted in a dose-dependent inhibition of capillary tube formation (Fig. 2A–D). In the presence of EGM, the matrigel assay condition supported differentiation of untreated HUVECs into an extensive and complete network of capillary-like structures (Fig. 2A). The capillary tube like network was slightly inhibited at a lower dose of evodiamine (0.1 μM); however, treatment with higher doses of evodiamine resulted in some fragments of unconnected tubes (evodiamine at 0.4 μM, Fig. 2C, arrow indicated) and only fragments of unconnected tubes (evodiamine at 1 μM, Fig. 2D) as compared to the controls. Evodiamine significantly reduced average tube length as compared to the control without evodiamine (2.4 ± 0.09 mm at 0.4 μM, $n=3$, and 0.68 ± 0.08 mm at 1 μM, $n=3$ versus 6.260.26 ± 0 μM, $n=3$, $P<0.05$) and approximately 89% inhibition at 1 μM (Fig. 2E). Thus, evodiamine inhibited differentiation of HUVECs into capillary tubes.

In the CAM assay, the straw was placed on top of the CAM, a highly vascularized membrane in a growing chicken egg. After administration of evodiamine for 8 h and inoculation for 48 h, the change in blood vessel density was assessed in the

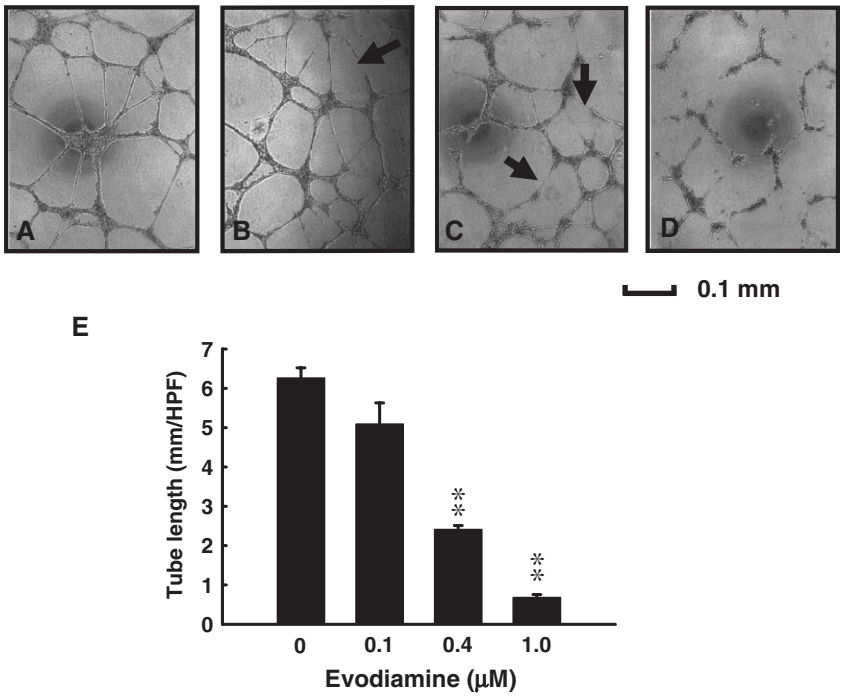

Fig. 2. The effect of evodiamine on capillary tube formation by HUVECs. The procedure of treatment and the measurement of tube length are as described in Materials and methods. Photographs showed the tube formation by HUVECs cultured with evodiamine at (A) 0, (B) 0.1 μM, (C) 0.4 μM, and (D) 1 μM. The arrows indicated the fragmented capillary tubes. As shown in (E), the total length of capillary tubes was measured. Each bar represents mean ± S.D. ($n=3$). **$P<0.01$ compared with evodiamine at 0 M. HPF, high power field.

area surrounding straw placement. Fig. 3 represents digitized images of representative CAMs treated for 8 h with vehicle (0.1% DMSO), or Evo (1 μM evodiamine). The amount of vessels, particularly smaller vessels, was significantly decreased with evodiamine treatment.

Evodiamine inhibited endothelial cell invasion

Based on the results described above that evodiamine inhibited HUVECs capillary tube formation, we further examined its activity endothelial cell invasion through matrigel (Fig. 4A–D). We counted the number of invaded cells after 8 h of cells seeded and found that evodiamine efficiently suppressed the HUVECs invasion through the matrigel-coated filter (88.7 ± 15.6 cells/HPF 200× at 0.1 μM, $n=3$, 54.0 ± 4.8 cells/HPF 200× at 0.4 μM, $n=3$, and 24 ± 2.1 cells/HPF 200× at 1 μM, $n=3$ versus 153.0 ± 9.6 cells/HPF 200× at 0 M, $n=3$, $P<0.01$) (Fig. 4E). The finding suggested that evodiamine resulted in a concentration-dependent inhibition manner and achieved 84% inhibition at 1 μM.

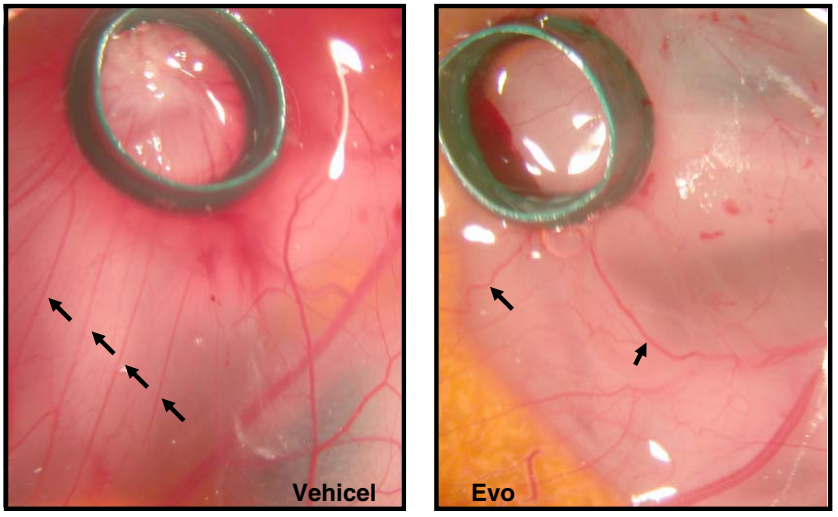

Fig. 3. Effect of evodiamine on blood vessel density in the CAM assay. Digitized images of vehicle (0.1% DMSO) treated for 48 h, small blood vessels (arrows indicated) continuous with those of the CAM have surrounded the implanted straw. The amount of vessels, particularly smaller vessels, was significantly decreased with evodiamine treatment.

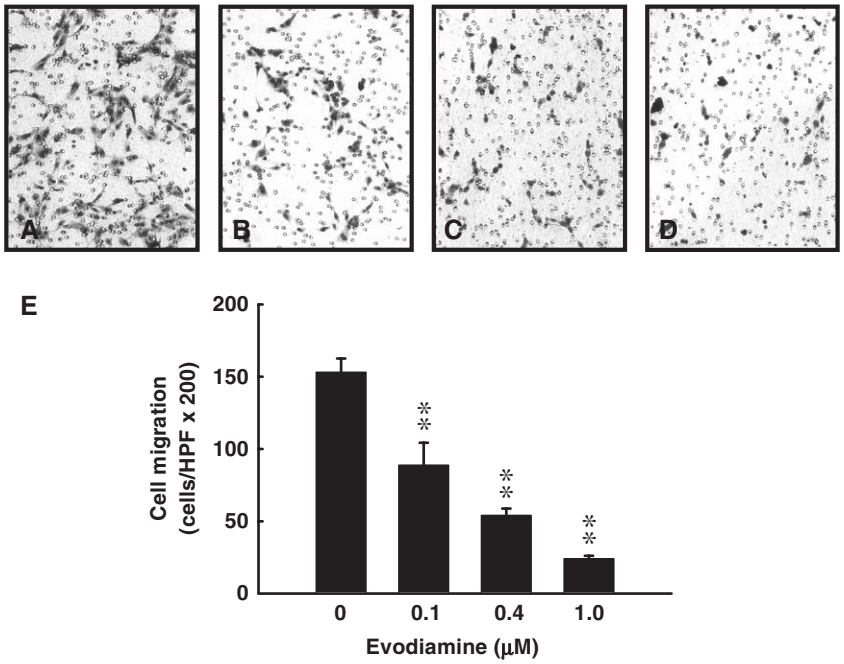

Fig. 4. The effect of evodiamine on invasion of HUVECs through the matrigel-coated filter. The procedure of treatment and the count of in vitro HUVECs invasion are as described in Materials and methods. As shown in (A)–(D), the invasion cells of HUVECs after evodiamine treatment were stained and photographed. (E) The number of invasion cells was counted after 8 h of cell seeding on matrigel and simultaneously treated with evodiamine. Each bar represents mean ± S.D. ($n=3$). **$P<0.01$ compared with evodiamine at 0 M. HPF, high power field.

Evodiamine decreased VEGF expression and phospho-ERK in endothelial cells

For the HUVECs growth, we analyzed the expression of VEGF that is the major involved in angiogenesis (Kanda et al., 2002). Western blot showed that the expression levels of VEGF were markedly decreased by evodiamine, and achieved 37% inhibition for VEGF (Fig. 5A, $n=3$). Since the reciprocal regulation between VEGF and ERK phosphorylation has been found, we detected the phospho-ERK, the active form of ERK. We found the phosphor-ERK was inhibited by evodiamine and achieved 64% inhibition (Fig. 5A, $n=3$).

Northern blot showed that mRNA expression of VEGF was inhibited by treatment of HUVECs with 1 μM evodiamine (Fig. 5B), and decreased by 66% ($P<0.01$, $n=3$).

Evodiamine inhibited CL1 cell-induced capillary tube formation of endothelial cells and VEGF expression of CL1 cells

To investigate if evodiamine affected tumor-induced angiogenesis, the conditioned medium as described in Materials and methods were used. Using a sensitive ELISA system, we observed that CL1 cells released 466.75 ± 18.94 pg of VEGF into the culture medium after a 24-h incubation. Evodiamine treatment of CL1 cells for 8 h strongly decreased the level of VEGF release into the followed 24 h conditioned medium in a dose-dependent manner (Fig. 6A). VEGF levels were reduced by 11% (to 89% of control) by 0.1 μM ($P<0.05$), by 26% (to 76% of control) by 0.4 μM ($P<0.01$), and by 40% (to 60% of control) by 1 μM evodiamine ($P<0.01$).

We also demonstrated that the serum-reduced conditioned medium produced from untreated CL1 cells was able to induce tube formation (Fig. 6B). Compared with that of untreated CL1 cells, the conditioned medium produced from evodiamine-pretreated CL1 cells resulted in losing the ability of capillary tube formation (Fig. 6C). Evodiamine significantly reduced average tube length as compared to the control without evodiamine (0.94 ± 0.12 mm at 1 μM, $n=3$ versus 2.06 ± 0.19 at 0 μM, $n=3$, $P<0.01$) (Fig. 6D).

After collection the conditioned medium, the remaining CL1 cells were subjected to Western blot analysis for their cellular levels of VEGF (Fig. 6E). The VEGF protein expression of evodiamine-pretreated CL1 cells was significantly lower than that of untreated CL1 cells (Fig. 6F). Evodiamine reduced CL1 cells-induced capillary tube formation of HUVECs by approximately 54%, and decreased the VEGF expression of CL1 cells by 37%. The results shown in Fig. 6 indicated that evodiamine decreased the VEGF expression and release in CL1 cells, and then inhibited capillary tube formation of HUVECs in vitro.

Evodiamine inhibited VEGF induced angiogenesis and ERK phosphorylation in endothelial cells

The results shown in Fig. 6 indicated that the possibility of evodiamine reduced VEGF production required for angiogenesis in conditioned medium. Therefore, we examined if VEGF reversed the evodiamine down-regulated angiogenesis and ERK phosphorylation of HUVECs. As shown in Fig. 7, the angiogenic response of HUVECs was inhibited by evodiamine (Fig. 7B) and stimulated by rhVEGF$_{165}$ (Fig. 7C). In contrast,

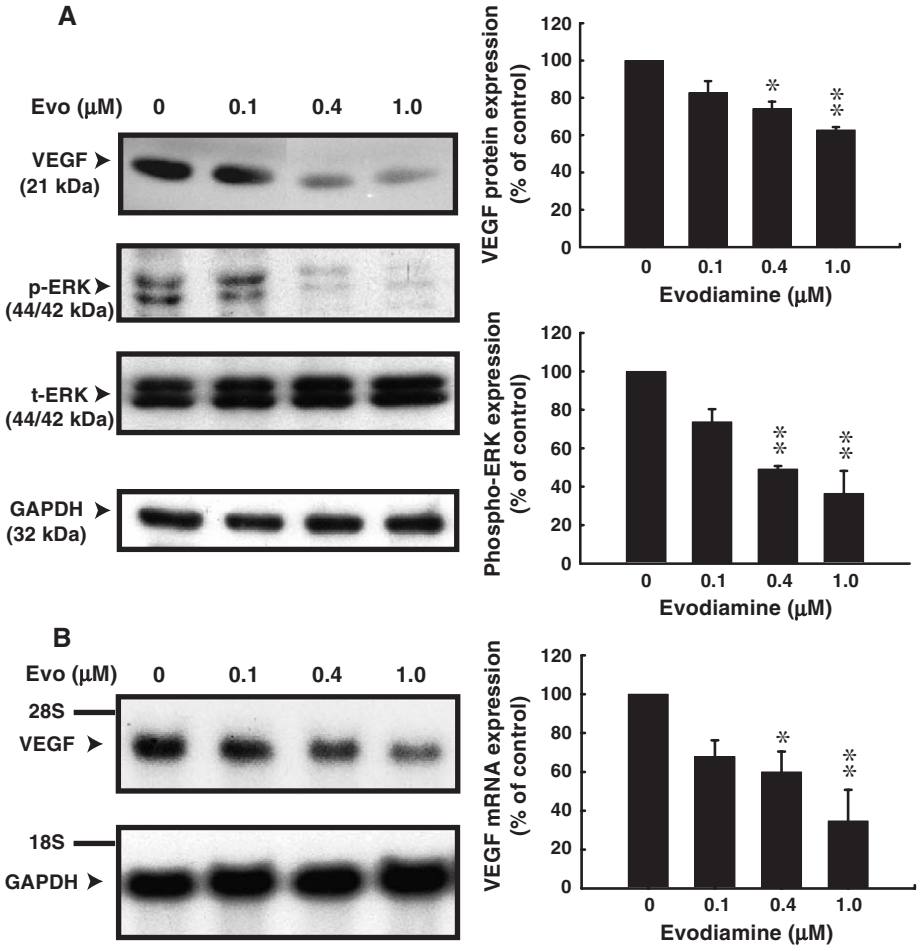

Fig. 5. The effects of evodiamine on VEGF protein and mRNA expression in HUVECs. (A) Western blot for VEGF and phospho-ERK, (B) Northern blot for VEGF after different doses of evodiamine treated HUVECs. The protein and mRNA expression were quantitated by densitometer and normalized with GAPDH. The ratios of VEGF/GAPDH at 0 μM evodiamine were assigned to be 100%. Data represent mean ± S.D. of three separate experiments. Evo: evodiamine. $^{**}P<0.01$ compared with evodiamine = 0 μM.

the angiogenic patterns stimulated by rhVEGF$_{165}$ were not abolished by addition of evodiamine (Fig. 7D). Evodiamine caused approximately 70% inhibition in capillary tube formation, but in the presence of rhVEGF$_{165}$, evodiamine only caused 20% inhibition in tube formation (Fig. 7E). Addition of rhVEGF induced phosphorylation of ERK, but basal- and rhVEGF$_{165}$-induced phospho-ERK were decreased significantly by evodiamine (Fig. 7F). Administration of rhVEGF$_{165}$ attenuated evodiamine inhibited phospho-ERK expression (54% inhibition in the absence of rhVEGF$_{165}$, and 26% inhibition in the presence of rhVEGF$_{165}$, $n=3$) (Fig. 7G). Taken together, our data suggested that evodiamine down-regulated angiogenesis and ERK phosphorylation induced by VEGF.

Discussion

Evodiamine is one of the major bioactive compounds isolated and purified from a longstanding Chinese herbal named Wu-Chu-Yu. Evodiamine has been reported to inhibit metastasis of tumor and growth of cultured cell lines (Ogasawara et al., 2001a,b; Fei et al., 2003; Kan et al., 2004; Huang et al., 2005). Our study demonstrated that evodiamine inhibited the capillary tube formation, VEGF and phospho-ERK expression of endothelial cells in vitro. The CAM assay was performed to confirm the data obtained in vitro. We also found evodiamine inhibited lung cancer induced capillary tube formation of endothelial cells and VEGF release and expression of lung cancer cells. We also demonstrated that VEGF attenuated evodiamine-inhibited tube formation and ERK phosphorylation in vitro. Taken together, our data indicated that evodiamine treatment deprived the ability of angiogenesis. Down-regulation of VEGF and phospho-ERK is an important pharmacological property of evodiamine.

Since evodiamine also inhibited growth of colon 26-L5 (Ogasawara et al., 2001a,b; Ogasawara and Suzuki, 2004), B16-F10 melanoma (Ogasawara and Suzuki, 2004), and LNCaP (Kan et al., 2004), our data showed that in the initial 12 h, at least, there was no difference in cell growth and viability compared with untreated control (Fig. 1). In the incubation with HUVECs for 18 h, evodiamine (1 μM) had only a marginal anti-proliferative effect (data not shown). When cells were continuously exposed to evodiamine for longer periods of time (24 h to 96 h), the inhibition of

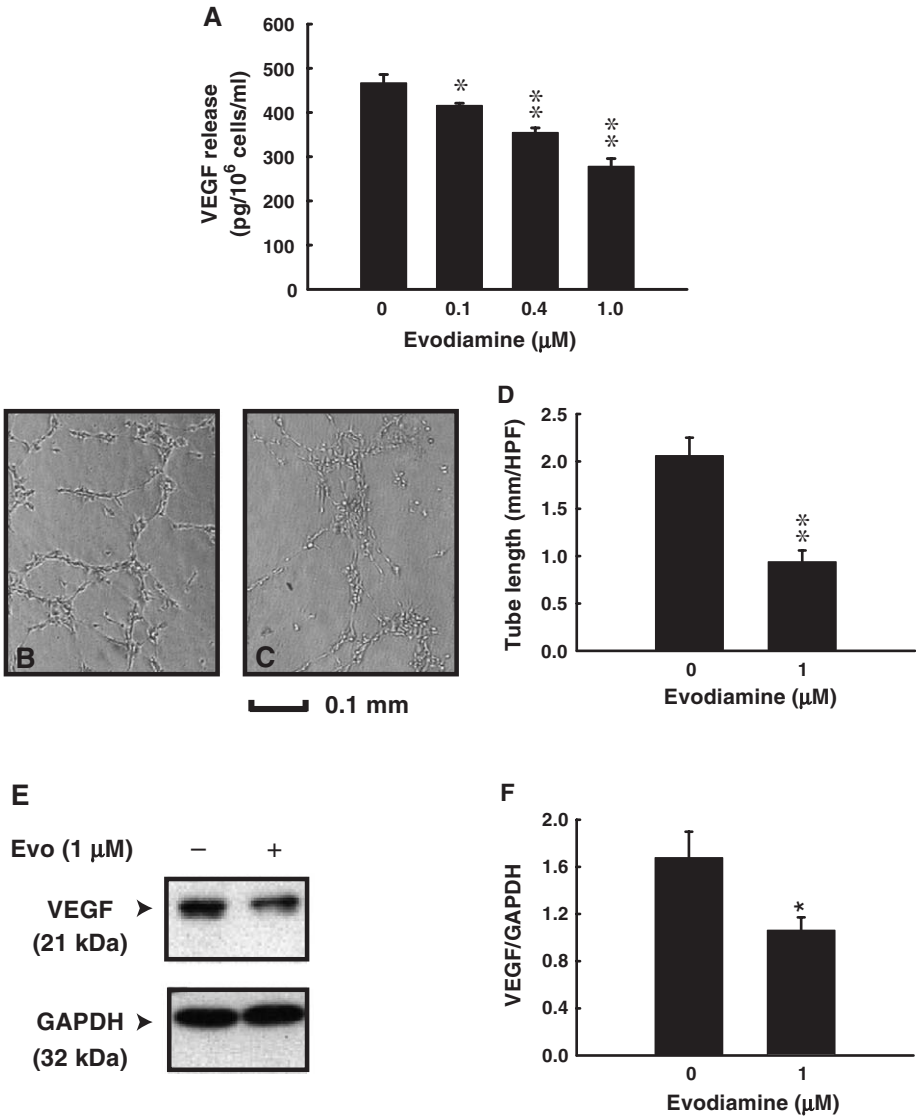

Fig. 6. Evodiamine inhibited the CL1-induced tube formation of HUVECs and VEGF expression of CL1 cells. (A) The CL1 cells were left untreated or pretreated with evodiamine for 8 h, and then incubated with fresh media without evodiamine for 24 h. The collection of conditioned media and the measurement of VEGF concentration by an ELISA kit were as described in Materials and methods. *$P<0.05$, and **$P<0.01$ compared with evodiamine=0 μM. The tube formation assay of HUVECs were performed using conditioned media prepared from CL1 cells that were either left untreated (B) or pretreated with 1 μM evodiamine (C) and the measurement of total length of tube formation (D) were as described in Materials and methods. **$P<0.01$ compared with evodiamine=0 μM. As shown in (E), after the collection of conditioned media, the remaining CL1 cells were then subjected to Western blot analysis for VEGF and normalized with GAPDH (F). Values were shown as the mean±S.D. from three independently performed experiments. Evo: evodiamine. *$P<0.05$ compared with evodiamine=0 μM.

proliferation was prominently observed (Fig. 1). We did not exclude that evodiamine is an unselective cytostatic agent, but the anti-angiogenic action of evodiamine is independent from a direct cytotoxic effect toward endothelial cells.

For the endothelial cell proliferation, VEGF is a potent endothelial cell mitogen and suppression of VEGF was consistent with reduced capillary tube formation (Jung et al., 1999; Plate et al., 1992; Veikkola and Alitalo, 1999; Xu et al., 2001). We demonstrated that evodiamine reducing the expression of phosphor-ERK and VEGF (Fig. 5) was the mechanism involved in evodiamine inhibited endothelial cells growth (Fig. 1), and angiogenesis in vitro and in vivo (Figs. 2 and 3). On $rhVEGF_{165}$ stimulation (10 ng/ml), ERK became phosphorylated and induced capillary tube formation; evodiamine attenuated rhVEGF-induced tube formation and phospho-ERK activation (Fig. 7). Taken together, our data suggested that evodiamine down-regulated angiogenesis and ERK phosphorylation induced by VEGF. We know of no other published report on the regulation of capillary tube formation, and VEGF expression by evodiamine.

Hepatocyte growth factor-induced invasion and migration of tumor cells were inhibited by evodiamine, although the underlying mechanism remained unclear (Ogasawara and Suzuki, 2004). ERKs inhibitor augmented evodiamine-induced necrosis, moreover, the expression and phosphorylation of ERKs were down-regulated in melanoma cells by evodiamine (Zhang et al., 2003, 2004). Evodiamine has recently been shown to inhibit NF-κB activation and NF-κB-regulated gene expression involved in cell invasion (e.g. COX-2, MMP-9, and ICAM-1) (Takada et al., 2005). Evodiamine also has been

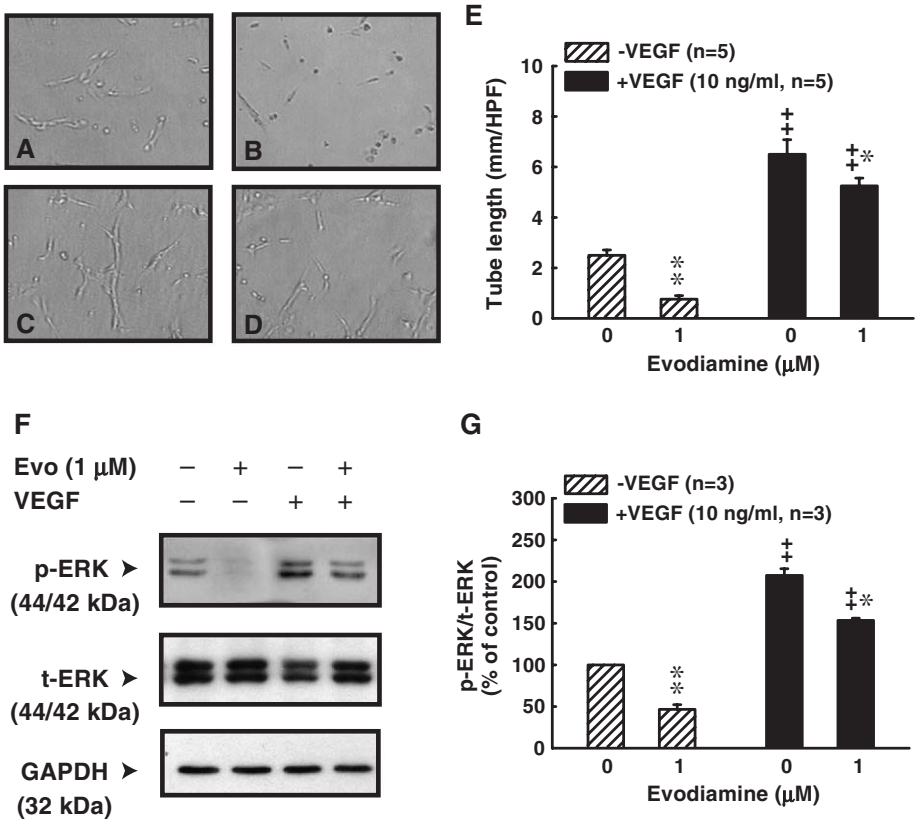

Fig. 7. Evodiamine inhibited VEGF induction in tube formation and phospho-ERK expression. (A–D) Photographs showed the tube formation. The HUVECs were treated with (A) low-serum medium, (B) evodiamine (1 μM), (C) VEGF (10 ng/ml), (D) combined evodiamine and VEGF. As shown in (E), the total length of capillary tubes was measured. Each bar represents mean ± SEM ($n=5$). HPF, high power field. (E) The HUVECs lysate were subjected to Western blot analysis for phosphor-ERK and normalized with total-ERK and evodiamine at 0 μM. Values were shown as the mean ± S.D. from three independently performed experiments. Evo: evodiamine. *$P<0.05$ or **$P<0.01$ compared with evodiamine at 0 M. $^{++}P<0.01$ compared with VEGF untreated.

shown to specifically inhibit TNF-induced Akt activation, but not TNF-induced JNK and p38 MAPK activation (Takada et al., 2005). In the present study, decreased VEGF and phospho-ERK was demonstrated to be involved in the evodiamine-suppressed tube formation, but it was unclear whether other angiogenesis related MAPKs family was involved. Evodiamine also inhibited the activity on endothelial cell invasion; the relevant angiogenic factors, like MMPs family, and the associated intracellular factors affected by evodiamine remain to be further investigated.

It has been indicated that administration of evodiamine inhibited proliferation and lung metastasis of colon 26-L5 cells and did not affect levels of GOT, GPT, CRE and cell density of leukocytes in blood of the mice (Ogasawara et al., 2001a,b). Endothelial cells are the source of new blood vessels, and they have a remarkable ability to migrate, proliferate, and differentiate. We focused on the effect of evodiamine on the endothelial cells. Our study revealed that by down-regulating phospho-ERK and VEGF expression, evodiamine could decrease the capillary tube formation in vitro. Therefore, blockade of angiogenesis and invasion of endothelial cells represent an important part of evodiamine's anti-cancer capacity. We suggested that evodiamine may be a candidate chemical for clinical treatment or prevention of cancer metastasis in human beings.

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