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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.

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