Orally administered mucolytic drug L-carbocisteine inhibits angiogenesis and tumor growth in mice

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Nonstandard abbreviations: EGF, epidermal growth factor; ERK, extracellular signal-related kinase; HUVECs, human umbilical vein endothelial cells; MAP kinase, mitogen activated protein kinase; MEK, mitogen extracellular kinase; PKC, protein kinase C; PLC, phospholipase C; ROS, reactive oxygen species; VEGF, vascular endothelial growth factor

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Abstract

Angiogenesis, the formation of new blood vessels from pre-existing vessels, is essential for the growth and metastasis of tumors. In this study, we found that L-carbocisteine, a widely used expectorant, potently inhibits angiogenesis in vitro and in vivo. An in vivo Matrigel plug assay revealed that L-carbocisteine (2.5 mg/kg administered intraperitoneally twice daily) significantly inhibited VEGF-induced angiogenesis. L-Carbocisteine also suppressed VEGF-stimulated proliferation, migration, and formation of capillary-like structures of human umbilical vein endothelial cells (HUVECs). We examined the signaling pathways affected in VEGF-stimulated HUVECs, and found that L-carbocisteine significantly inhibited VEGF-induced phosphorylation of phospholipase C gamma (PLCγ), protein kinase C mu (PKCμ), and extracellular signal-related kinase (ERK) 1/2, which has been shown to be essential for angiogenesis. However, these inhibitory effects of L-carbocisteine were not observed in the HeLa human cervical cancer cell line. An in vivo study of Colon-26 tumor-bearing mice found that tumor volumes were significantly smaller in mice treated with L-carbocisteine (150 mg/kg administered orally twice daily) in comparison with vehicle-treated mice. However, L-carbocisteine had no direct effect on Colon-26 cell proliferation or ERK activation. Collectively, our results suggest that L-carbocisteine inhibits tumor angiogenesis by suppressing PLCγ/PKC/ERK signaling.
Introduction

Angiogenesis plays an important role in tumor growth (Thairu et al., 2011) because blood vessels generated via this pathophysiological process supply oxygen and nutrients to cancer cells and subsequently remove carbon dioxide and metabolites, both of which are indispensable to the proliferation and survival of cells (McMahon, 2000; Bhat and Singh, 2008; Claesson-Welsh, 2012). Considerable evidence shows that appropriate suppression of tumor angiogenesis can attenuate tumor growth (Bhat and Singh, 2008; Claesson-Welsh, 2012). Vascular endothelial growth factor (VEGF)-A is a key regulator of angiogenesis. Angiogenesis-related VEGF signaling is mediated primarily by VEGF receptor 2 (VEGFR2/KDR) activation (Takahashi, 2011; Nagy et al., 2007; Shibuya, 2014), which activates various cell-signaling molecules, such as phosphoinositide 3-kinase/Akt, Cdc42/p38 mitogen-activated protein (MAP) kinase, focal adhesion kinase (FAK), Src family kinase, phospholipase C (PLC)/protein kinase C (PKC), and mitogen extracellular kinase (MEK)/extracellular signal-related kinase (ERK) (Zachary and Gliki, 2001).

L-Carbocisteine (S-carboxymethylcysteine) is used widely as an expectorant (Rhinathiol®, Mucodyne®) because it normalizes sialic acid and fucose contents in mucins through the regulation of glycosyltransferase activity, and its use is not associated with serious side effects. L-Carbocisteine removes phlegm and indications for its use include inflammation of the upper respiratory tract, acute bronchitis, bronchial asthma, chronic bronchitis,
bronchiectasis, pulmonary tuberculosis, and chronic sinusitis (Hooper and Calvert, 2008). In recent years, novel biological activities of L-carbocisteine have been reported in the context of inhibition of inflammation associated with influenza virus infection and chronic obstructive pulmonary disease (COPD) (Yamaya et al., 2010; Asada et al., 2012; Yasuda et al., 2006; Zheng et al., 2008). Another report showed that L-carbocisteine possessed free radical-scavenging properties in vitro (Nogawa, 2009). Various inflammatory cells, including neutrophils, mast cells, natural killer cells, macrophages, and dendritic cells, are involved in induction and promotion of angiogenesis (Noonan et al., 2008; Kim et al., 2013). Moreover, generation of reactive oxygen species (ROS) is a primary function of activated inflammatory cells, which serve as important stimuli for angiogenic signaling (Kim et al., 2013; Reuter et al., 2010; Grote et al., 2011). However, the effects of L-carbocisteine on angiogenesis have not been reported.

We hypothesized that L-carbocisteine produces anti-angiogenic activity, and tested this hypothesis in vitro and in vivo, because an understanding of the molecular mechanisms and targets of established drugs is essential for safe drug use and the development of novel indications.
Materials and Methods

Antibodies and reagents

L-Carbocisteine was a gift from Kyorin Pharmaceutical Co. (Tokyo, Japan). L-2-Aminoadipic acid was obtained from TCI (Tokyo, Japan). Human recombinant VEGF_{165} and epidermal growth factor (EGF) were purchased from PeproTech (Rocky Hill, NJ, USA). Anti-phospho-Akt (Ser^{473}), anti-Akt, anti-phospho ERK1/2 (Thr^{202}/Tyr^{204}), anti-ERK1/2, anti-phospho-stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) (Thr^{183}/Tyr^{185}), anti-SAPK/JNK, anti-MEK1/2, anti-phospho-PLC_{\gamma}^{\mathrm{783}}), anti-PLC_{\gamma}, anti-phospho-PKC_{\mu}/PKD (Ser^{744/748}), anti-PKC_{\mu}/PKD, anti-phospho-VEGFR2 (Tyr^{1175}), anti-VEGFR2, and horseradish peroxidase (HRP)-conjugated anti-rabbit/mouse IgG antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Anti-CD31 antibodies were purchased from eBioscience (San Diego, CA, USA). Anti-phospho-p38 MAP kinase (Thr^{180}/Tyr^{182}) antibodies, anti-p38 MAP kinase antibodies, anti-ERK1 antibodies, and growth factor-reduced Matrigel basement membrane matrix were obtained from BD Biosciences (Lexington, KY, USA). Protein G Sepharose was obtained from GE Healthcare (Pittsburgh, PA, USA). Cellmatrix types I-A and I-C and reconstitution buffer were obtained from Nitta Gelatin, Inc. (Osaka, Japan). Dulbecco’s modified Eagle’s medium (DMEM) and RPMI-1640 medium were obtained from Nissui Pharmaceutical Co., Ltd. (Tokyo, Japan).

Cell culture
Human umbilical vein endothelial cells (HUVECs) were obtained from Lonza (Basel, Switzerland) and maintained in endothelial basement medium-2 (EBM-2) supplemented with EGM-2 BulletKit™ (Lonza). HeLa human cervical cancer cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Cell Culture Bioscience/Nichirei Biosciences, Inc., Tokyo, Japan). Colon-26 murine colon carcinoma cells were obtained from Riken BioResource Center (Ibaraki, Japan) and maintained in RPMI-1640 medium supplemented with 10% FBS. Cells were cultured in a humidified atmosphere of 5% CO₂ at 37 °C.

Animals

Specific pathogen-free inbred C57BL6/JJms mice (weighing 19–21 g) and BALB/cCr mice (weighing 20–22 g) for use in this study were obtained from Japan SLC, Inc. (Shizuoka, Japan) and housed in a laminar airflow room with a 12-h light–dark cycle under specific pathogen-free conditions. All animals were allowed to acclimatize to their new environment for 1 week before experimentation. The animal experiments were performed according to the guidelines of the Kyushu University of Health and Welfare (Nobeoka, Japan), which complied with the “Law Concerning the Protection and Control of Animals” and “Standards relating to the care and management, etc. of experimental animals” (Office of the Prime Minister of Japan; http://law.e-gov.go.jp).

In vivo angiogenesis assay
The in vivo anti-angiogenic activity of L-carbocisteine was assessed with a Matrigel plug assay as described elsewhere (Suehiro et al., 2010). Matrigel was mixed with vehicle or 30 ng/mL of VEGF and injected subcutaneously in a 500-μL bolus into the flank of a 6-week-old male C57BL/6Jms mouse. Injected mice were treated twice daily with or without L-carbocisteine (2.5 mg/kg administered intraperitoneally). On day 14, mice were injected with 50 μL of 1% Evans blue solution via the orbital vein. After 1 h, mice were perfused with phosphate-buffered saline (PBS) containing 2 mM EDTA by intravenous injection into the left ventricle of the heart. Matrigel pellets were harvested and incubated with formamide for 2 days to elute Evans blue dye. Neovascular densities were determined by measuring the absorbance of pellets at 620 nm.

Cell viability assay

Cell viability was assessed with the Cell Counting Kit-8 (Dojindo, Kumamoto, Japan), according to the manufacturer’s instructions. Cells (200 μL) were seeded onto 96-well plates at a density of 2500 cells/well. After 24 h, cells were starved overnight and treated with vehicle or the indicated agent. After 48 h of incubation, 10 μL of WST-8 solution was added to each well, and cells were incubated for 40 min at 37 °C. After incubation, absorbance was measured at 450 nm.

Migration assay

HUVECs were seeded on 35-mm plates and allowed to form confluent monolayers. Cells
were starved overnight in VEGF and basic fibroblast growth factor (bFGF)-free EGM-2 medium and pretreated with L-carbocisteine for 30 min. Monolayers were subjected to scratch wounding with a sterile 200-μL pipette tip in the presence or absence of VEGF. Cells were incubated for 18 h before observation using a phase-contrast microscope. Four randomly selected fields were photographed and the number of migrated cells was determined manually.

**Assay to measure formation of HUVEC tubular networks**

HUVEC tubular networks were formed according to a published method (Uchiyama, 2010). Two volumes of Cellmatrix Type I-A were mixed with 5 volumes of 0.1% acetic acid, 2 volumes of 5× NaHCO₃-free DMEM, and 1 volume of reconstitution buffer, and the resulting solution was placed on ice. This mixture (800 μL) was added to each well of a 12-well plate, which was incubated at 37 °C for 30 min to allow formation of a bottom gel layer. HUVECs were seeded into each gel-containing well at a density of 1.0 × 10⁴ cells/well and incubated for 6 h to allow adherence to the collagen gel. The cultured medium was removed gently, and 500 μL of the collagen mixture was added atop the bottom layer, followed by solidification at 37 °C for 30 min (top layer). After addition of 1 mL VEGF and bFGF-free EGM-2 medium containing the vehicle or the indicated agents above the top layer, cells were incubated at 37 °C for 18 h. The vehicle and test agents were allowed diffused into the gel matrix for 1 h, thereby diluting their concentrations 2-fold. Formation of tubular networks was observed via
phase-contrast microscopy: 4 randomly selected fields were photographed and total tubule lengths were measured.

Immunoblotting

Preparation of cell lysates and immunoblotting were conducted as described previously (Takeuchi et al., 2009). Briefly, cells were lysed with lysis buffer (20 mM Tris-HCl (pH 7.4) containing 137 mM NaCl, 2 mM EGTA, 5 mM EDTA, 1% Nonidet™ P-40, 1% Triton X-100, 100 μg/mL phenylmethanesulfonyl fluoride, 1 μg/mL pepstatin A, 1 μg/mL p-toluenesulfonyl-L-arginine methyl ester, 2 μg/mL leupeptin, 1 mM sodium orthovanadate, 50 mM sodium fluoride, and 30 mM sodium diphosphate). Lysates were incubated on ice for 30 min, insoluble materials were removed by centrifugation, and supernatants were subjected to SDS-PAGE, followed by transfer to Immobilon-P membranes (Millipore, Bedford, MA, USA) for immunoblotting with antibodies.

Immunoprecipitation

Cells were cultured exactly as described in the methods for immunoblotting and extracted in lysis buffer. Protein concentrations were measured and approximately 200 μg of cell extract from each sample was immunoprecipitated with antibodies against VEGFR2 or PLCγ that had been conjugated to 20 μL of Protein G-Sepharose. Immunoprecipitates were recovered by adding 2 volumes of Laemmlı sample buffer to the immunoprecipitated samples. Samples were analyzed by western blotting.
Colon-26 tumor-bearing mice

A Colon-26 tumor-bearing mouse model assay was set up as described previously, with some modifications (Acharyya et al., 2004). Six-week-old male BALB/cCr mice were given vehicle or L-carbocisteine (15, 75, or 150 mg/kg) via the oral route twice daily from 2 days before tumor injection. On the day of inoculation, cultured Colon-26 cells were harvested and washed with PBS. Next, $1 \times 10^6$ cells in 100 $\mu$L of serum-free RPMI 1640 culture medium was injected subcutaneously and dorsally into mice. From 6 days after injection, tumors were measured with calipers once every other day, and tumor growth (in mm$^3$) was calculated using the following formula:

$$V = (\text{narrow side})^2 \times (\text{long side})/2.$$  

Tumor tissues were fixed in 4% neutral buffered paraformaldehyde for 48 h, embedded in Tissue-Tek® OCT™ compound (Sakura Finetek, Torrance, CA, USA), and cut into 9-$\mu$m sections with a cryotome (CM1900; Leica, Nußloch, Germany). Sections were dried at room temperature for 1 h, washed with PBS, and treated with an anti-mouse CD31 antibody. After washing in PBS, sections were stained with fluorescein isothiocyanate (FITC)-conjugated IgG (Invitrogen, Carlsbad, CA, USA) and 4',6-diamidino-2-phenylindole (DAPI). After washing the sections in PBS, they were mounted and observed using a fluorescence microscope. Areas of positive staining were measured using ImageJ (National Institutes of Health; available at http://imagej.nih.gov/ij/).
Statistical analysis

SPSS version 20 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Data are presented as mean ± S.E.M. Statistical differences in the dose-response study were evaluated by applying Dunnett’s multiple comparison test. Student’s t-test was used for comparisons of 2 groups. A p-value <0.05 was regarded as significant.
**Results**

*L-Carbocisteine inhibits VEGF-induced proliferation, migration, and formation of tubular structures of endothelial cells*

To assess the anti-angiogenic properties of L-carbocisteine *in vitro*, we examined the inhibitory effects of L-carbocisteine on HUVEC proliferation. L-Carbocisteine attenuated VEGF-induced proliferation in a concentration-dependent manner and exerted a significant inhibitory effect at concentrations greater than 100 μM (Fig. 1A). The effects of L-carbocisteine on chemotactic motility were examined in a wound-healing migration assay. Treatment with L-carbocisteine (100 μM) significantly inhibited VEGF-induced HUVEC migration (Fig. 1B). We examined the potential effects of L-carbocisteine on the formation of tubular structures using a collagen gel matrix assay, and found that HUVECs formed an extended network of tubular structures in response to VEGF. Treatment with L-carbocisteine significantly abrogated VEGF-stimulated formation of tubular networks in endothelial cells (Fig. 1C).

*L-Carbocisteine inhibits VEGF-induced angiogenesis in vivo*

To ascertain the effects of L-carbocisteine on angiogenesis *in vivo*, we conducted a Matrigel plug assay. VEGF-loaded Matrigel (30 ng/mL) was stained positively with Evans blue, suggesting that new blood vessels formed within the Matrigel via VEGF-induced angiogenesis (Fig. 2A). In contrast, treatment with 2.5 mg/kg L-carbocisteine almost
completely abolished angiogenesis, as evidenced by the remarkably reduced level of Evans blue staining in the L-carbocisteine-treated group (Fig. 2B), suggesting that L-carbocisteine effectively inhibited angiogenesis in vivo.

**L-Carbocisteine inhibits VEGF-induced phosphorylation of ERK1/2 in HUVECs**

To evaluate the molecular mechanisms associated with L-carbocisteine-induced inhibition of VEGF-dependent angiogenesis, we measured phosphorylation of key proteins downstream of VEGFR2 activation: Akt, ERK1/2, JNK, and p38 MAP kinase by western blotting (Dellinger and Brekken, 2011; Song et al., 2012; Wu et al., 2006). L-Carbocisteine (100 μM) potently suppressed VEGF-induced ERK1/2 activation in HUVECs, but had no effect on activation of Akt, JNK, or p38 MAP kinase (Figs. 3A-D).

To determine whether L-carbocisteine inhibits ERK1/2 phosphorylation in non-endothelial cells, we examined the effect of L-carbocisteine on ERK1/2 activation induced by 100 ng/mL EGF in HeLa cells. However, L-carbocisteine did not affect ERK1/2 activation in epidermal cells (Fig. 4).

**L-Carbocisteine inhibits activation of VEGFR2/PLCγ/PKC/MEK signaling in endothelial cells**

To clarify the mechanisms underlying L-carbocisteine-mediated inhibition of the activation of ERK, we examined the effects of L-carbocisteine on phosphorylation of VEGFR2, PLCγ, PKC, and MEK. VEGFR2 phosphorylation in VEGF-stimulated HUVECs was not
suppressed by L-carbocisteine. In contrast, pretreatment with L-carbocisteine significantly suppressed the phosphorylation of PLCγ and PKCμ (Figs. 5A-C). In addition, L-carbocisteine inhibited MEK1/2 phosphorylation after VEGF treatment (Fig. 5D).

L-Carbocisteine attenuates the association of PLCγ with VEGFR2

To determine whether L-carbocisteine suppresses the formation of the PLCγ/VEGFR2 complex, cell lysates were immunoprecipitated with antibodies against VEGFR2 or PLCγ and immunoblotted with reciprocal antibodies. VEGF stimulated complex formation in HUVECs, whereas pretreatment with L-carbocisteine prevented complex formation (Fig. 6). L-Carbocisteine suppressed signals for angiogenesis by inhibiting VEGF-induced formation of the PLCγ/VEGFR2 complex (Fig. 7).

L-Carbocisteine suppresses the tumor growth and angiogenesis

To determine the effects of L-carbocisteine on tumor growth and angiogenesis in vivo, we evaluated the effect of L-carbocisteine in Colon-26 tumor-bearing mice. For this purpose, we injected Colon-26 tumor cells into male BALB/c mice, following which they were orally administered various concentration of L-carbocisteine (experimental group) or vehicle (control group) daily for 26 days (Fig. 8A). On day 22, mice treated orally twice daily with 150 mg/kg L-carbocisteine presented with considerably smaller tumors than those observed in control mice (Fig. 8B). At 15 mg/kg and 75 mg/kg doses, L-carbocisteine was associated with slight retardation of tumor growth in comparison with the control treatment. From 10 days
after inoculation with tumor cells, tumor volumes were significantly smaller in mice treated with 150 mg/kg L-carbocisteine in comparison with the control group (Fig. 8C). No apparent toxic effects were observed in any of the treatment groups. Capillary density in the peritumoral region was determined by staining sections with anti-CD31 antibodies. Treatment with 150 mg/kg L-carbocisteine significantly reduced the number of capillary microvessels (Fig. 8D). From 12 days after the injection of tumor cells, tumor volume was significantly smaller in mice treated intraperitoneally twice daily with 10 mg/kg L-carbocisteine in comparison with the control group (Figs. S1A and S1B).

To determine whether L-carbocisteine directly induces apoptosis in tumor cells, we tested the effect of L-carbocisteine on Colon-26 cell viability. We found that treatment with a high concentration (approximately 500 µM) of L-carbocisteine had no effect on Colon-26 cell proliferation (Fig. S2A). Subsequently, we used western blot analysis to examine the effect of L-carbocisteine on growth factor-induced phosphorylation of ERK1/2 in tumor cells, and found that L-carbocisteine did not suppress EGF-induced activation of ERK1/2 in Colon-26 cells (Fig. S2B).

*L-2-Aminoadipic acid inhibits VEGF-induced proliferation and activation of ERK1/2 in endothelial cells*

L-2-Aminoadipic acid is a substitution product of L-carbocisteine (sulfur to carbon). To confirm whether sulfur is important for the anti-angiogenic effect of L-carbocisteine, we
evaluated the effects of L-2-aminoadipic acid in VEGF-stimulated endothelial cells. L-2-aminoadipic acid suppressed VEGF-induced proliferation in a concentration-dependent manner and suppressed VEGF-induced ERK1/2 activation in HUVECs (Figs. 9A and 7B).
Discussion

Angiogenesis plays a crucial role in the tumor growth and metastasis (Fischer et al., 2008; Zetter, 2008). Therefore, inhibition of tumor angiogenesis has become an important strategy for cancer treatment. Several inhibitors of tumor angiogenesis have been shown to prevent the growth and metastasis of solid tumors (Argyriou, 2009), and such findings have spurred efforts to discover novel angiogenic inhibitors. Human umbilical endothelial cells (HUVECs) are derived from the endothelium of large veins in the umbilical cord and are used as a model system for angiogenesis studies (Wang et al., 2015; Mu et al., 2011). L-Carbocisteine was synthesized in the 1930s and was first used as a mucoregulatory agent (Rhinathiol®, Mucodyne®) in the treatment of respiratory diseases in the 1960s (Hooper and Calvert, 2008). In recent years, novel biological activities of L-carbocisteine have been reported. L-Carbocisteine inhibits inflammation associated with influenza virus infection and COPD (Yamaya et al., 2010; Asada et al., 2012; Yasuda et al., 2006; Zheng et al., 2008), suppresses oxaliplatin-induced hepatocyte toxicity by inhibiting oxaliplatin-induced decreases in the Bcl2/Bim ratio, and inhibits oxaliplatin-induced apoptosis in vitro (Zhai et al., 2012). Moreover, L-carbocisteine possesses free radical-scavenging and anti-inflammatory properties in vitro (Zheng et al., 2008; Nogawa et al., 2009).

Based on recent studies showing that L-carbocisteine inhibits multiple steps of VEGF-induced angiogenesis, we hypothesized that it is a promising novel anti-cancer agent.
This is the first report to demonstrate comprehensively that L-carbocisteine inhibits angiogenesis and tumor growth. Unlike conventional anti-cancer agents, the uses of which are complicated by various side effects and/or severe cytotoxicity, L-carbocisteine produces exceptional anti-angiogenic activity without cytotoxicity or side effects.

Angiogenesis is a complex, multistep process that involves the proliferation, migration, and tubular-network formation of endothelial cells (Patan, 2004), and inhibition of any step of this process has been shown to prevent formation of new blood vessels (Tournaire et al., 2004). In this study, we showed that L-carbocisteine significantly inhibits endothelial cell proliferation in a concentration-dependent manner (Fig. 1A). Moreover, L-carbocisteine inhibits VEGF-induced angiogenic responses such as cell migration and formation of capillary-like structures (Figs. 1B and C). Furthermore, L-carbocisteine inhibited angiogenesis in a Matrigel plug assay in mice (Fig. 2), showing that L-carbocisteine inhibits angiogenesis in vitro and in vivo.

VEGFR2-mediated activation of Akt, ERK, JNK, and p38 MAP kinase contributes to VEGF-induced survival, proliferation, migration, and tubular-network formation of endothelial cells (Zachary and Gliki, 2001; Dellinger and Brekken, 2011; Song et al., 2012; Wu et al., 2006). Our data showed that L-carbocisteine significantly abrogated ERK activation specifically in endothelial cells; no effect was observed in epidermal cells (Figs. 3B and 4). Reports have noted that, unlike other representative growth factor receptor
tyrosine kinases, VEGFR2 forms a complex with and subsequently phosphorylates PLCγ, which is critical for ERK activation (Takahashi et al., 2001; Takahashi and Shibuya 1997; Wu, 2000). In contrast, Ras is weakly activated by VEGF (Takahashi et al., 1999). VEGF stimulates activation of PKCµ (PKD) via the VEGFR2/PLCγ/PKC pathway. PKCµ in endothelial cells is rapidly phosphorylated at Ser744/Ser748 in response to VEGF, and PKCµ is involved in VEGF-induced ERK signaling and endothelial cell proliferation (Wong et al., 2005). In the present study, l-carnosine had no effect on VEGFR2 phosphorylation. However, l-carnosine significantly attenuated VEGF-induced phosphorylation of ERK and PLCγ, as well as upstream formation of VEGFR2/PLCγ complexes (Figs. 5 and 6).

Taken together, our data suggest that l-carnosine affects formation of VEGFR2 and PLCγ complexes without inhibiting VEGF2 phosphorylation, which subsequently affects signaling cascades in a manner that may be responsible for the anti-angiogenic effects of l-carnosine. VEGF-induced VEGFR2/PLCγ complex formation and activation of PLCγ evoke Ca2+ mobilization, phosphatidylinositol 4,5-biphosphate (PIP2) breakdown, and inositol 1,4,5-triphosphate (IP3) production, which are signaling events upstream of PKC (Ayada et al., 2009). Therefore, the results of our study suggest that l-carnosine suppressed Ca2+ mobilization, PIP2 breakdown, and IP3 production.

Inhibition of tumor angiogenesis represents a novel therapeutic modality for controlling tumor metastasis (Kruger et al., 2001; Yi et al., 2008). In this report, we elucidated some
mechanisms underlying the inhibitory effect of L-carbocisteine on VEGF-induced angiogenesis by using matrigel containing VEGF. However, we also studied the effects of L-carbocisteine in a tumor-bearing mouse model, because malignant cells release a wide range of growth factors in addition to VEGF. In our in vivo Colon-26 tumor-bearing mouse model, we demonstrated the effectiveness of oral administration of 150 mg/kg L-carbocisteine as a tumor suppressor (Figs. 8B and C). Related immunohistochemical analyses further revealed that expression of the endothelial marker CD31 was reduced markedly in tumor sections from L-carbocisteine-treated mice (Fig. 8D). Furthermore, we determined that 500 μM L-carbocisteine did not directly induce apoptosis or inhibit proliferation of Colon-26 cells (Fig. S2A). These results suggest that L-carbocisteine inhibits tumor growth indirectly by inhibiting tumor angiogenesis.

It has been reported that L-carbocisteine suppresses tumor necrosis factor (TNF)-alpha-induced activation of phosphatidyl inositol-specific phospholipase C (PI-PLC) in NCH-H292 epithelial cells (Ishibashi et al., 2006; Ishibashi et al., 2012). L-Carbocisteine has also been shown to attenuate N-formyl-Met-Leu-Phe (FMLP)-stimulated neutrophil activation by inhibiting PI-PLC-mediated signal transduction (Ishii et al., 2002). In this study, we demonstrated for the first time that L-carbocisteine directly inhibits formation of VEGFR2 and PLCγ complexes in endothelial cells.

Similar to L-carbocisteine, N-acetylcysteine (NAC) is a cysteine-derivative mucolytic drug
that acts by breaking disulfide bridges between macromolecules (Mallet et al., 2011). At the cellular level, NAC inhibits endothelial cell invasion and angiogenesis, probably by inhibiting metalloproteinase activities (Albini et al., 1995). NAC has also been shown to exert direct cytoprotective and anti-genotoxic effects on endothelial cells (Aluigi et al., 2000). Given the possible association between NAC treatment and reduced tumor-dependent angiogenesis, a reported and potentially important aspect of the effectiveness of NAC is its ability to limit VEGF expression (Albini et al., 2001; Agarwal et al., 2004), and this effect may be related to its suppression of ROS and hypoxia-induced transcription via hypoxia inducible factor-1 (Agarwal et al., 2004; Albini et al., 1995; Sceneay et al., 2013). Therefore, the anti-angiogenic effects of NAC are due to its anti-oxidant activity and are distinct from the anti-angiogenic effects of L-carbocisteine reported in the present study.

VEGF stimulates ROS production (Ushio-Fukai, 2007) and ROS play a critical role in stimulation of angiogenic signaling, including ERK and JNK signaling (Lee et al., 2014). Because sulfur compounds have strong anti-ROS activity, we considered whether the inhibitory effect of L-carbocisteine on VEGF-induced ERK activation was based on anti-ROS activity. We showed that L-2-aminoadipic acid inhibited proliferation and activation of ERK1/2 in VEGF-stimulated endothelial cells (Fig. 9), indicating that the anti-angiogenic effect of L-carbocisteine is not conferred by its constituent sulfur. We believe that steric effects associated with L-carbocisteine and L-2-aminoadipic acid are important to their
inhibitory effects. Additionally, we expect that addition of another carboxymethyl or amino
group to L-carbocisteine could enhance its interference with VEGFR2 and suppression of
ERK activation in endothelial cells. The effect of L-carbocisteine does not seem to be
stronger than other available anti-angiogenic agents, such as bevacizumab, sunitinib, and
sorafenib, which attenuate VEGFR2- and VEGFR2-mediated phosphorylation and activation
of ERK, Akt, JNK, and p38 MAP kinase (Reddy et al., 2012; Okines et al., 2011). In the
present study, we found that L-carbocisteine suppressed VEGF-induced ERK1/2 activation
but had no effect on activation of Akt, JNK, or p38 MAP kinase (Figs. 3A-D). Furthermore,
the usual oral dose of L-carbocisteine prescribed to adults is 500 mg of L-carbocisteine (3
times daily). In our study, L-carbocisteine inhibited angiogenesis, but did so at a dose about
10 times greater than the normally prescribed dose. One of the reasons why a higher
concentration of L-carbocisteine was required is its short biological half-life (t_{1/2}) (about 2 h;
from a medical package insert of Mucodyne®). However, anti-angiogenic effects might be
produced with lower doses of L-carbocisteine by reducing the dosing interval. Currently used
anti-angiogenic drugs such as the anti-VEGF antibody bevacizumab can induce transient
functional normalization of tumor vasculature that can potentiate the activity of
coadministered chemoradiotherapeutics (Jie et al., 2008). We believe that the combination of
L-carbocisteine with conventional chemotherapeutic agents might increase their efficacy.

To our knowledge, this is the first report to demonstrate that the mucolytic drug
L-carbocisteine inhibits angiogenesis *in vitro* and *in vivo*. Moreover, L-carbocisteine was found to attenuate endothelial cell proliferation, as well as to inhibit formation of VEGFR2/PLCγ complexes and ERK activation in endothelial cells. These findings suggest that L-carbocisteine inhibits tumor angiogenesis and growth by inhibiting cellular PLCγ/PKC/ERK activity *in vivo*; however, this specific effect of L-carbocisteine does not occur in epidermal cells, which suggests that L-carbocisteine could serve as a useful selective anti-angiogenic therapy with few side effects. Our discovery of this novel action of L-carbocisteine supports the notion that it is a promising anti-angiogenic agent and a valuable lead compound in the development of anti-cancer therapies.
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Authorship Contributions

Participated in research design: Shinya, Takahashi, and Sato

Conducted experiments: Shinya, Yokota, Nakayama, Oki, and Mutoh

Performed data analysis: Shinya, Yokota, Nakayama, and Mutoh

Wrote or contributed to the writing of the manuscript: Shinya, Takahashi, and Sato
References


Yasuda H, Yamaya M, Sasaki T, Inoue D, Nakayama K, Yamada M, Asada M, Yoshida M,


Footnotes

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Legends for Figures

**Figure 1.** L-Carbocisteine inhibits VEGF-induced changes in viability, migration, and capillary-structure formation in endothelial cells. A, HUVECs were pretreated with various concentrations of L-carbocisteine and incubated with VEGF. After 2 d of incubation, cell viability was quantified using a Cell Counting Kit-8. B, HUVECs were pretreated with 100 µM L-carbocisteine for 30 minutes and then incubated with 30 ng/mL VEGF. Migrated cells were quantified by manual counting. C, HUVECs were pretreated with 100 µM L-carbocisteine and incubated with 30 ng/mL VEGF. After 18 h of incubation, total tubule length was assayed using a phase-contrast microscope (100x magnification). Values are mean ± S.E.M. *P < 0.05; **P < 0.01 vs. the VEGF-treated group. Similar results were obtained from 3 independent experiments.

**Figure 2.** L-Carbocisteine inhibits VEGF-induced angiogenesis in an *in vivo* Matrigel model. C57BL6/J mice were injected with 0.5 mL of Matrigel mixed with vehicle or VEGF. Matrigel-bearing mice were treated with or without L-carbocisteine *via* the intraperitoneal route twice per day. After 14 d, Evans blue dye was administered and Matrigel pellets were harvested. A, Representative Matrigel plugs were photographed. B, Neovascular density was determined. Data points represent mean ± S.E.M. (n = 6). *P < 0.05 vs. VEGF alone. Similar results were obtained from 3 independent experiments.

**Figure 3.** L-Carbocisteine attenuated VEGF-induced ERK1/2 phosphorylation in endothelial
cells. A-D, HUVECs were pretreated with L-carbocisteine for 30 minutes and stimulated with 30 ng/mL VEGF for the indicated periods, and cellular lysates were analyzed by SDS-PAGE and immunoblotting with phosphorylation site-specific antibodies, after which the membranes were reprobed with antibodies against unmodified proteins. Protein levels of p-Akt (A), p-ERK (B), p-JNK (C), and p-p38 MAPK (D) were determined.

**Figure 4.** L-Carbocisteine had no effect on EGF-induced ERK activation in epithelial cells. HeLa cells were pretreated with L-carbocisteine for 30 minutes and incubated with EGF for the indicated periods. The cells were harvested and equal aliquots of protein were analyzed for anti-phospho-ERK1/2 by immunoblotting. Results are from an experiment representative of 3 independent experiments. Data are presented as mean ± S.E.M. *P < 0.05.

**Figure 5.** L-Carbocisteine inhibits VEGF-induced PLCγ/PKC/ERK signaling in HUVECs. A-D, HUVECs were pretreated with L-carbocisteine and stimulated with VEGF for the indicated periods. Lysates were subjected to SDS-PAGE and the membranes were hybridized with phospho-specific antibodies, after which the membranes were reprobed. Protein levels of p-VEGFR2 (A), p-PLCγ (B), p-PKCµ (C), and p-MEK1/2 (D) were determined. Quantitative results were obtained by densitometry. Data are presented as mean ± S.E.M. from 3 independent experiments. *P < 0.05.

**Figure 6.** L-Carbocisteine attenuated VEGF-induced formation of PLCγ/VEGFR2 complexes. HUVECs were pretreated with L-carbocisteine and stimulated with VEGF for the indicated
periods. The cells were harvested and equal aliquots of protein extracts were immunoprecipitated with antibodies against VEGFR2 or PLCγ. Immunoprecipitates were subjected to SDS-PAGE and blotted with antibodies against PLCγ or VEGFR2 as indicated. Total cell extracts were prepared and subjected to SDS-PAGE for detection of VEGFR2 and PLCγ. The blot was reprobed with beta-actin antibodies as a loading control. Data are presented as mean ± S.E.M. from 3 independent experiments. *, \( P < 0.05 \).

**Figure 7.** Schematic representation of the mechanism by which L-carbocisteine inhibits VEGF-stimulated angiogenesis. VEGF stimulates formation of complexes between VEGFR2 and PLCγ, and this phenomenon induces angiogenesis. Conversely, L-carbocisteine suppresses VEGFR2/PLCγ complex formation and downstream signaling.

**Figure 8.** L-Carbocisteine inhibits tumor growth and angiogenesis in Colon 26-bearing mice. A, Experimental schedule of \textit{in vivo} tumor growth (schematic). B, Typical example of tumor-bearing mice from the groups treated with vehicle or 150 mg/kg L-carbocisteine on day 6 and day 22. C, Tumor growth was measured with calipers once every other day and calculated in mm\(^3\). All data are presented as mean tumor volume ± S.E.M. (n = 8 animals per group). D, Representative photomicrographs of CD31 capillaries in tumor sections stained with antibodies against CD31 (green fluorescence), an endothelial marker. Nuclei were counterstained with DAPI (blue). The area of CD31-stained capillaries was measured using Image J software. Data are the mean ± S.E.M. of 4 experiments. \(*P < 0.05\) vs. the vehicle
Figure 9. L-2-aminoadipic acid inhibited VEGF-induced proliferation and activation of ERK1/2 in endothelial cells. A, HUVECs were pretreated with various concentrations of L-2-aminoadipic acid and incubated with VEGF. After 2 d of incubation, cell viability was quantified using a Cell Counting Kit-8 (n = 6). Similar results were obtained from 3 independent experiments. B, HUVECs were pretreated with 100 µM L-2-aminoadipic acid for 15 minutes and treated with VEGF for the indicated periods. Lysates were prepared from the treated cells, and phospho-ERK1/2 protein was measured by immunoblotting. Immunoblots are from an experiment representative of 3 similar experiments. Quantitative results were obtained by densitometry. Data are presented as mean ± S.E.M. *P < 0.05.
Figures

A

Cell viability

(% of control)

L-Carbocisteine (µM)

0 10 50 100 200

VEGF

B

Migration cell number

(% of control)

VEGF - + - +

L-Carbocisteine

C

Tube length

(% of control)

VEGF - + - +

L-Carbocisteine

Fig. 1
Fig. 2

A
control  VEGF  L-Carbocisteine  +VEGF

B

\[
\begin{align*}
\text{Vascular density (a.u.)} & \\
\text{VEGF} & - & + & - & + \\
\text{L-Carbocisteine} & & & & \\
\end{align*}
\]

*
Fig. 3
Fig. 5
Fig. 6
Fig. 7
Fig. 8

A

- colon-26 injection
- Day 0, 2, 8, 26
- measurement tumor volume

- vehicle group
- distilled water
- L-carbocisteine group
  - 15 mg/kg
  - 75 mg/kg
  - 160 mg/kg
- oral administration twice a day.

B

Day 6
Day 22

- vehicle
- 150 mg/kg L-carbocisteine

C

Tumor volume (mm$^3$)

Day after tumor injection

D

CD31$^+$, DAPI, merge

CD31$^+$-capillary vessels (% of control)

vehicle
150 mg/kg L-carbocisteine
Fig. 9

Cell viability (% of control) vs. L-2-Aminoadipic Acid (µM) and VEGF

B

L-2-Aminoadipic Acid
30 ng/mL VEGF - 5 15 - 5 15 (min)

p-Erk1/2 (Thr202/Tyr204)
Erk1/2
β-actin

p-Erk1

Fig. 9