**Orally Administered Mucolytic Drug L-Carbocisteine Inhibits Angiogenesis and Tumor Growth in Mice**

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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 i.p. twice daily) significantly inhibited vascular endothelial growth factor (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 (PLC) γ, protein kinase C (PKC) μ, and extracellular signal-related kinases (ERK) 1/2, which have 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.

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**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 (Nagy et al., 2007; Takahashi, 2011; 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 biologic activities of L-carbocisteine have been reported in the context of inhibition of inflammation associated with influenza virus infection and chronic obstructive pulmonary disease (Yasuda et al., 2006; Zheng et al., 2008; Yamaya et al., 2010; Asada et al., 2012). Another report showed that L-carbocisteine possessed free radical-scavenging properties in vitro (Nogawa et al., 2009). Various inflammatory cells, including neutrophils, mast cells, natural killer cells, macrophages, and dendritic cells, are involved in the 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 (Reuter et al., 2010; Grote et al., 2011; Kim et al., 2013). However, the effects of L-carbocisteine on angiogenesis have not been reported.

We hypothesized that L-carbocisteine produces antiangiogenic 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 VEGF165 and epidermal growth factor (EGF) were purchased from PeproTech (Rocky Hill, NJ). Anti-phospho-Akt (Ser473), anti-Akt, anti-phospho ERK1/2 (Thr202/Tyr204), anti-ERK1/2, anti-phospho-stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) (Thr183/Tyr185), anti-SAPK/JNK, anti-MEK1/2, anti-phospho-PLCγ (Tyr207), anti-PLCγ, anti-phospho-PKCα/β/PKD (Ser467/494), anti-PKCα/β/PKD, anti-phospho-VEGFR2 (Tyr1175), anti-VEGFR2, and horseradish peroxidase–conjugated anti-rabbit/mouse IgG antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-CD31 antibodies were purchased from eBioscience (San Diego, CA). Anti–phospho-p38 MAP kinase (Thr180/Tyr182) antibodies, anti-p38 MAP kinase antibodies, anti-ERK1 antibodies, and growth factor-reduced Matrigel basement membrane matrix were obtained from BD Biosciences (Lexington, KY). Protein G Sepharose was obtained from GE Healthcare (Pittsburgh, PA). 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 supplemented with EGM-2 BulletKit (Lonza). HeLa human cervical cancer cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; Cell Culture Biosciences/ 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/Jms 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-hour light-dark cycle under specific pathogen-free conditions. All animals were allowed to aclimatize 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 antiangiogenic 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 volume of vehicle 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 hours of incubation, total tubule length was assayed using a phase-contrast microscope (100× magnification). Values are mean ± S.E.M. *P < 0.05; **P < 0.01 versus the VEGF-treated group. Similar results were obtained from three independent experiments.
bolus into the flanks of 6-week-old male C57BL/6Jmns mice. Injected mice were treated twice daily with or without l-carnosine (2.5 mg/kg i.p.). On day 14, mice were injected with 50 μl of 1% Evans blue solution via the orbital vein. After 1 hour, 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 hours, cells were starved overnight and treated with vehicle or the indicated agent. After 48 hours of incubation, 10 μl of WST-8 solution was added to each well, and cells were incubated for 40 minutes 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-carnosine for 30 minutes. 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 hours 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 et al., 2010). Two volumes of Cellmatrix Type I-A were mixed with 5 volumes of 0.1% acetic acid, 2 volumes of 5 mM 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 minutes to allow formation of a bottom gel layer. HUVECs were seeded into each gel-containing well at a density of 1.0 × 10⁵ cells/well and incubated for 6 hours 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 minutes (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 hours. The vehicle and test agents were allowed to diffuse into the gel matrix for 1 hour, thereby diluting their concentrations 2-fold. Formation of tubular networks was observed via phase-contrast microscopy: four 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 phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml 1-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 minutes, insoluble materials were removed by centrifugation, and supernatants were subjected to SDS-PAGE, followed by transfer to Immobilon-P membranes (Millipore, Bedford, MA) 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 Laemmli 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-carnosine (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 × 10⁶ cells in 100 μl 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 millimeters³) was calculated using the following formula:

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

(1)

Tumor tissues were fixed in 4% neutral buffered paraformaldehyde for 48 hours, embedded in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA), and cut into 9-μm sections with a cryotome (CM1900; Leica, Nussloch, Germany). Sections were dried at room temperature for 1 hour, washed with PBS, and treated with an anti-mouse CD31 antibody. After washing in PBS, sections were stained with fluorescein isothiocyanate–conjugated IgG (Invitrogen, Carlsbad, CA) and 4’,6-diamidino-2-phenylindole. After being washed in PBS, the sections 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) 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 two groups. A P value of <0.05 was regarded as significant.

**Results**

**l-Carnosine Inhibits VEGF-Induced Proliferation, Migration, and Formation of Tubular Structures of Endothelial Cells.** To assess the antiangiogenic properties of l-carnosine 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 by Western blotting the phosphorylation of key proteins downstream of VEGFR2 activation: Akt, ERK1/2, JNK, and p38 MAP kinase (Wu et al., 2006; Dellinger and Brekken, 2011; Song et al., 2012). 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 (Fig. 3, A–D).

To determine whether L-carbocisteine inhibits ERK1/2 phosphorylation in nonendothelial 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).

1. 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 PKCβ (Fig. 5, A–C). In addition, L-carbocisteine inhibited MEK1/2 phosphorylation after VEGF treatment (Fig. 5D).

2. 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).

3. L-Carbocisteine Suppresses 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 concentrations 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 (Supplemental Fig. 1, A and B).

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 (Supplemental Fig. 2A). 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 (Supplemental Fig. 2B).

1.2-Aminoadipic Acid Inhibits VEGF-Induced Proliferation and Activation of ERK1/2 in Endothelial Cells. 1.2-Aminoadipic acid is a substitution product of L-carbocisteine (sulfur to carbon). To confirm whether sulfur is important for the angiogenic effect of L-carbocisteine, we evaluated the effects of 1.2-aminoadipic acid in VEGF-stimulated endothelial cells. 1.2-Aminoadipic acid suppressed VEGF-induced proliferation in a concentration-dependent manner and suppressed VEGF-induced ERK1/2 activation in HUVECs (Fig. 9, A and B).

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 et al., 2009), and such findings have spurred efforts to discover novel angiogenic inhibitors. 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). L-Carbocisteine was synthesized in the 1930s and was first used as a mucocutaneous agent (Rhinanthiul, Mucodyne) in the treatment of respiratory diseases in the 1960s (Hooper and Calvert, 2008). In recent years, novel biologic activities of L-carbocisteine have been reported. L-Carbocisteine inhibits inflammation associated with influenza virus infection and chronic obstructive pulmonary disease (Yasuda et al., 2006; Zheng et al., 2008; Yamaya et al., 2010; Asada et al., 2012), 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).

On the basis of recent studies showing that L-carbocisteine inhibits multiple steps of VEGF-induced angiogenesis, we hypothesized that it is a promising novel anticancer agent. This is the first report to demonstrate comprehensively that
L-carbocisteine inhibits angiogenesis and tumor growth. Unlike conventional anticancer agents, the uses of which are complicated by various side effects and/or severe cytotoxicity, L-carbocisteine produces exceptional antiangiogenic 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 (Fig. 1, B 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; Wu et al., 2006; Dellinger and Brekken, 2011; Song et al., 2012). 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 and Shibuya, 1997; Wu et al., 2000; Takahashi et al., 2001). In contrast, Ras is weakly activated by VEGF (Takahashi et al., 1999). VEGF stimulates activation of PKCζ (PKD) via the VEGFR2/PLCγ/PKC pathway. PKCζ in endothelial cells is rapidly phosphorylated at Ser744/Ser748 in response to VEGF, and PKCζ is involved in VEGF-induced ERK signaling and endothelial cell proliferation (Wong and Jin, 2005). In the present study, L-carbocisteine had no effect on VEGFR2 phosphorylation. However, L-carbocisteine 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-carbocisteine affects formation of VEGFR2 and PLCγ complexes without inhibiting VEGFR2 phosphorylation, which subsequently affects signaling cascades in a manner that may be responsible for the antiangiogenic effects of L-carbocisteine. VEGF-induced VEGFR2/PLCγ complex formation and activation of PLCγ evoke Ca2+ mobilization, phosphatidylinositol
4,5-biphosphate breakdown, and inositol 1,4,5-triphosphate production, which are signaling events upstream of PKC (Ayada et al., 2009). Therefore, the results of our study suggest that L-carbocisteine suppressed Ca$^{2+}$ mobilization, 4,5-biphosphate breakdown, and 1,4,5-triphosphate 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 (Fig. 8, B 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 (Supplemental Fig. 2A). 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)α–induced activation of phosphatidylinositol-specific phospholipase C in NCH-H292 epithelial cells (Ishibashi et al., 2006). L-Carbocisteine has also been shown to attenuate N-formyl-Met-Leu-Phe (FMLP)–stimulated neutrophil activation by inhibiting phosphatidylinositol-specific phospholipase C–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.

As with 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 antigenotoxic 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 (Albini et al., 1995; Agarwal et al., 2004; Sceneay et al., 2013). Therefore, the antiangiogenic effects of NAC are attributable to its ability to reduce VEGF expression.
antioxidant activity and are distinct from the antiangiogenic 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 (Lu 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 the result of 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 antiangiogenic 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 antiangiogenic agents, such as bevacizumab, sunitinib, and sorafenib, which attenuate VEGFR2- and VEGFR2-mediated phosphorylation and activation of ERK, Akt, JNK, and p38 MAP kinase (Okines et al., 2011; Reddy et al., 2012). 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 (Fig. 3, A–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 biologic half-life ($t_{1/2}$) (about 2 hours; from a medical package insert of Mucodyne). However, antiangiogenic effects might be produced with lower doses of L-carbocisteine by reducing the dosing interval. Currently used antiangiogenic drugs such as the anti-VEGF antibody bevacizumab can induce transient functional normalization of tumor vasculature that can potentiate the activity of coadministered chemoradiotherapeutics (Ma 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

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**Fig. 8.** L-Carbocisteine inhibits tumor growth and angiogenesis in Colon 26-bearing mice. (A) Experimental schedule of 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 cubic millimeters. 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 4′,6-diamidino-2-phenylindole (DAPI; blue). The area of CD31-stained capillaries was measured using ImageJ software. Data are the mean ± S.E.M. of four experiments. *$P < 0.05$ versus the vehicle group.
L-Carbocisteine Is a Novel Inhibitor of Tumor Angiogenesis

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References


Dellinger MT and Brekken RA (2011) Phosphorylation of Akt and ERK1/2 is required for VEGF-A/VEGFR2-induced proliferation and migration of lymphatic endothelial cells. Int J Cancer 130:223–228.


L-Carbocisteine Is a Novel Inhibitor of Tumor Angiogenesis

Fig. 9. 1-Lamininoid acid inhibited VEGF-induced proliferation and activation of ERK1/2 in endothelial cells. (A) HUVECs were pretreated with various concentrations of 1-L-amino acid and incubated with VEGF. After 2 days of incubation, cell viability was quantified using a Cell Counting Kit-8 (n = 6). Similar results were obtained from three independent experiments. (B) HUVECs were pretreated with 100 μM l-Lamino 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. Immunobots are from an experiment representative of three similar experiments. Quantitative results were obtained by densitometry. Data are presented as mean ± S.E.M. *P < 0.05.

Attemnt endothelial cell proliferation, as well as to inhibit formation of VEGF2/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 antiangiogenic therapy with few side effects. Our discovery of this novel action of l-carbocisteine supports the notion that it is a promising antiangiogenic agent and a valuable lead compound in the development of antiangiogenic therapies.

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Authorship Contributions

Participated in research design: Shinya, Takahashi, Sato.
Conducted experiments: Shinya, Yokota, Nakayama, Oki, Mutoh.

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thou-Fukai M (2007) VEGF signaling through NADPH oxidase-derived ROS.
Uchiyama T, Toda K, and Takahashi S (2010) Resveratrol inhibits angiogenic re-
Takahashi T, Yamaguchi S, Chida K, and Shibuya M (2001) A single autophos-
Takahashi T, Ueno H, and Shibuya M (1999) VEGF activates protein kinase C-
Takahashi T and Shibuya M (1997) The 230 kDa mature form of KDR/Flk-1 (VEGF
Suehiro J, Hamakubo T, Kodama T, Aird WC, and Minami T (2010) Vascular en-
to basic fibroblast growth factor.
Angiogenesis

Antioxid Redox Signal

PLC-gamma and DNA synthesis in vascular endothelial cells.

mitogen-activated protein kinase

Zachary I and Gliki G (2001) Signaling transduction mechanisms mediating biological
Zetter BR (2008) The scientific contributions of M. Judah Folkman to cancer re-
Zhao Q, Bian X, Lu SR, Zhu B, and Yu B (2012) Carbocisteine reduces the cyto-
Chen BY, et al. (2008) Effect of carbocisteine on acute exacerbation of chronic
obstructive pulmonary disease (PRACE Study): a randomised placebo-controlled

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Wong C and Jin ZG (2005) Protein kinase C-dependent protein kinase D activation
modulates ERK signal pathway and endothelial cell proliferation by vascular en-
VEGF-induced angiogenesis via PI3 and JNK pathways in vascular endothelial
Wu LW, Mayo LD, Dunbar JD, Kessler KM, Baerwald MR, Jaffe EA, Wang D,
Warren RS, and Donner DB (2000) Utilization of distinct signaling pathways by
receptors for vascular endothelial cell growth factor and other mitogens in
Yamaya M, Nishimura H, Shinya K, Hatachi Y, Sasaki T, Yasuda H, Yoshida M,
VEGF-induced angiogenesis via P38 and JNK pathways in vascular endothelial
Yasuda H, Yamaya M, Sasaki T, Inoue D, Nakayama K, Yamada M, Asada M,
Yoshida M, Suzuki T, and Nishimura H, et al. (2006) Carbocisteine inhibits rhi-
(2008) Thymoquinone inhibits tumor angiogenesis and tumor growth through
suppressing AKT and extracellular signal-regulated kinase signaling pathways.
Med Cancer Ther 7:1789–1796.
Zachary I and Gliki G (2001) Signaling transduction mechanisms mediating biological
Zetter BR (2008) The scientific contributions of M. Judah Folkman to cancer re-
Zhao Q, Bian X, Lu SR, Zhu B, and Yu B (2012) Carbocisteine reduces the cyto-
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VEGF-induced angiogenesis via PI3 and JNK pathways in vascular endothelial
Wu LW, Mayo LD, Dunbar JD, Kessler KM, Baerwald MR, Jaffe EA, Wang D,
Warren RS, and Donner DB (2000) Utilization of distinct signaling pathways by
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VEGF-induced angiogenesis via P38 and JNK pathways in vascular endothelial
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Yoshida M, Suzuki T, and Nishimura H, et al. (2006) Carbocisteine inhibits rhi-
(2008) Thymoquinone inhibits tumor angiogenesis and tumor growth through
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