Antiangiogenic Activity of the Lipophilic Antimicrobial Peptides from an Endophytic Bacterial Strain Isolated from Red Pepper Leaf

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The induction of angiogenesis is a crucial step in tumor progression, and therefore, efficient inhibition of angiogenesis is considered a powerful strategy for the treatment of cancer. In the present study, we report that the lipophilic antimicrobial peptides from EML-CAP3, a new endophytic bacterial strain isolated from red pepper leaf (Capsicum annum L.), exhibit potent antiangiogenic activity both in vitro and in vivo. The newly obtained antimicrobial peptides effectively inhibited the proliferation of human umbilical vein endothelial cells at subtoxic doses. Furthermore, the peptides suppressed the in vitro characteristics of angiogenesis such as endothelial cell invasion and tube formation stimulated by vascular endothelial growth factor, as well as neovascularization of the chorioallantoic membrane of growing chick embryos in vivo without showing cytotoxicity. Notably, the angiostatic peptides blocked tumor cell-induced angiogenesis by suppressing the expression levels of hypoxia-inducible factor-1α and its target gene, vascular endothelial growth factor (VEGF). To our knowledge, our findings demonstrate for the first time that the antimicrobial peptides from EML-CAP3 possess antiangiogenic potential and may thus be used for the treatment of hypervascularized tumors.

INTRODUCTION

Angiogenesis is a multi-step process of new blood vessel formation from pre-existing vessels by endothelial cells (Bussolino et al., 1997; Carmeliet, 2003; Folkman, 1995). Though angiogenesis is essential for normal and vital physiological processes such as embryonic development, wound healing, and tissue or organ regeneration; it is also a hallmark of tumorgenesis and various ischemic and inflammatory diseases (Carmeliet and Jain, 2000; Folkman, 2001). Particularly, angiogenesis is recognized as a crucial step in the transition of tumors from a dormant condition to a malignant state by inducing tumor growth and metastasis (Andre et al., 1998; Hanahan and Folkman, 1996). Accordingly, efficient inhibition of angiogenesis is considered a promising strategy for the treatment of human cancers (Battegay, 1995).

A number of therapeutic agents with antiangiogenic potential have been developed for this purpose (Carmeliet and Jain, 2011; Jung and Kwon, 2013). Several angiogenesis inhibitors, including the anti-vascular endothelial growth factor (VEGF) monoclonal antibody bevacizumab (Avastin®), are approved by the U.S. Food and Drug Administration to treat a variety of cancers (Cardones and Banez, 2006; Zakaria and Soft, 2005). However, recent clinical studies have revealed that the current antiangiogenic drugs are not sufficient to block the complex biological processes involved in angiogenesis and tumor development, owing to the diverse genetic heterogeneity in cancer and alternative pathways leading to drug resistance (Giles et al., 2003; Miller et al., 2007). In addition, there are several concerns regarding the toxic side effects of angiogenesis inhibitors such as bleeding, cardiotoxicity, hypertension, gastrointestinal perforation, and birth defects (Cook and Figg, 2010; Verheul and Pinedo, 2007). Therefore, continuing efforts to discover new angiogenesis inhibitors are required to reduce the clinical failure rate and to increase the development of improved anti-angiogenic therapeutics.

In recent years, a variety of antimicrobial peptides (AMPs) from different natural sources has been reported, including plants, animals, and microorganisms (Chen and Chen, 2010; Cytryńska et al., 2007; Gálvez et al., 1993; Wu et al., 2005; Zhang et al., 2008). AMPs were initially discovered because of their role in the host defense mechanism by exerting cytotoxicity against the invading pathogenic microorganisms (Brogden, 2005). However, their unique structural properties and specific modes of action also serve a broad range of bioactivities such as immune modulators, mitogens, signaling molecules, and antitumor agents (Gaspar et al., 2013; Pushpanathan et al., 2013; Thundimadathil, 2012). AMPs could thus be considered...
interesting and promising therapeutic lead molecules with elevated pharmacological action, lower cytotoxicity, and decreased resistance in a wide range of infectious and malignant diseases. Recently, we isolated the fraction containing AMPs from the culture supernatant of EML-CAP3 which is the novel endophytic bacterial strain isolated from red pepper seed (Capsicum annum L.). In the current study, we report not only on the in vitro and in vivo antiangiogenic activity of these lipophilic AMPs (called SC39 peptides), but also on their potent inhibition of tumor-induced angiogenesis.

**MATERIALS AND METHODS**

**Materials**

Endothelial growth medium-2 (EGM-2) was purchased from Lonza (USA). RPMI 1640 and fetal bovine serum (FBS) were purchased from Invitrogen (USA). Recombinant human vascular endothelial growth factor (VEGF), Matrigel®, and Transwell® chamber systems were obtained from Koma Biotech (Korea), BD Biosciences (USA), and Corning Costar (USA), respectively. Anti-hypoxia-inducible factor-1 α (HIF-1 α), anti-cyclin D1, and anti-tubulin antibodies were purchased from BD Biosciences, Cell Signaling (USA), and Millipore (USA), respectively.

**Isolation of the endophytic bacterial strain**

The endophytic bacterial strain was isolated from red pepper leaf. Four segments (1 cm × 1 cm) from each leaf were surface-sterilized through immersion in 2% sodium hypochlorite (NaOCl) solution for 1 min and washed three times with distilled water. The endophytes were isolated by the direct plating and dilution plating method. In the dilution plating method, fragments of the surface-sterilized leaf were ground in sterile distilled water (1:1 g/ml). The 100 μl of a solution of pure and 10-fold diluted leaf extract were spread on a PDA (potato dextrose agar, Difco, USA) plate using a sterile spreader. Plates were kept in an incubator (HT-103-4, Hanbaek, Korea) at 23°C for 14 days. The colonies were grouped by color and morphological characteristics and transferred onto LB (Luria-Bertani) agar plates (Difco, USA). The isolated colonies were stored in 20% sterile glycerol at -80°C for further studies.

**Culture of the endophytic bacterial strain for antimicrobial peptide production**

The production of AMPs by the endophytic bacterial strain EML-CAP3 was carried out in a jar fermenter (10 L scale, LiFius GX, BIOTRON, Korea). The strain was cultured in a 3 L working volume of LB medium adjusted to pH 7.0 at 32°C. After 48 h of incubation, the culture broth was collected from the jar and used in subsequent steps.

**Isolation and purification of the antimicrobial peptides**

After liquid culture, the cells were removed by centrifugation at 15,000 rpm for 15 min. Peptides in the supernatant were heated at 65°C for 20 min and subsequently centrifuged at 15,000 rpm for 15 min to obtain heat-stable peptides. The peptide extract was passed through a C18-SPE (solid phase extraction) column (Grace, UK), and eluted with increasing amounts of acetonitrile (ACN) from 20 to 60% and from 60 to 100%. All fractions were collected, freeze-dried, and tested for antifungal activity. The fraction with the strongest antifungal activity was further purified by reverse-phase high-performance liquid chromatography (RP-HPLC). The active fraction was dissolved in solvent A containing 0.1% (v/v) trifluoroacetic acid (TFA) and 5% ACN in HPLC grade water and was applied to a C18 RP-HPLC column (Shimadzu, Japan). The column was equilibrated with 0.1% TFA. The peptide was eluted from the column with a linear gradient of solvent B (ACN containing 0.1% TFA and 5% HPLC grade water) using 50, 75, and 100% solvent B from 0-5, 5-30, and 30-36 min, respectively. The presence of peptides was detected by the absorbance at 230 nm. Fractions containing the target peptide were obtained from the elution with 75% solvent B, freeze-dried, and tested for antimicrobial activity. The resulting peptides were verified using 16.5% tricine sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (Schägger and von Jagow, 1987). A single band on the SDS-PAGE gel represented the purified peptide. For electrophoresis, peptide samples were diluted in loading buffer and heated at 95°C for 5 min. Samples containing 10 μg of peptides were loaded into each well, and electrophoresis was carried out at 25 mA per gel for approximately 3 h in running buffer composed of 0.1 M Tris HCl, 0.1 M tricine, and 0.1% (w/v) SDS. The gels were stained with 0.025% (w/v) Coomassie Blue G-250 in 40% methanol and 10% acetic acid and destained in methanol-acetic acid-water (40:10:50). The protein concentrations were determined using a bicinchoninic acid assay as described previously (Walker, 1994).

**Cell culture and hypoxic conditions**

Early passages (4-8 passages) of human umbilical vein endothelial cells (HUVECs) were grown in EGM-2 supplemented with 10% FBS. Human hepatocellular carcinoma (HepG2) cells were grown in RPMI 1640 medium containing 10% FBS. All cells were maintained at 37°C in a humidified 5% CO2 incubator. For hypoxic conditions, cells were incubated in a hypoxic chamber (Forma Scientific, USA) under 5% CO2 and 1% O2, balanced with N2.

**Cell viability assay**

HUVECs were seeded at a density of 1.5 × 10⁴ cells/well in gelatin-coated 24-well culture plates (SPL Life Sciences, Korea). The AMPs (0-40 μg/ml) were added to each well and the cells were incubated for up to 72 h. After 72 h, the cells were stained with Trypan blue and counted using a hemocytometer as described previously (Jung et al., 2003a; Lee et al., 2012a).

**Cell proliferation assay**

HUVECs were plated at 3 × 10⁵ cells/well in gelatin-coated 96-well plates (SPL LifeSciences, Korea). The AMPs (0-50 μg/ml) were added to each well and the cells were incubated for 72 h. Cell proliferation was measured using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay as described previously (Jung et al., 2003b).

**Chemo-invasion assay**

The invasiveness of HUVECs was determined in vitro using a Transwell® chamber system with polycarbonate filter inserts with a pore size of 8.0 μm as described previously (Jung et al., 2003). Briefly, the lower side of the filter was coated with 10 μl gelatin (1 mg/ml) and the upper side was coated with 10 μl Matrigel® (3 mg/ml). HUVECs (1 × 10⁵ cells) were placed in the upper chamber of the filter and the AMPs were added to the lower chamber in the presence of VEGF (30 ng/ml). The chamber was incubated at 37°C for 18 h, and the cells were subsequently fixed with methanol and stained with hematoxylin/eosin. The total number of cells that invaded the lower chamber of the filter was counted using an optical microscope (Olympus, USA) at a 100× magnification.
Capillary tube formation assay

Capillary tube formation by HUVECs in vitro was assessed as described previously (Jung et al., 2003). Briefly, HUVECs (1 × 10^5 cells) were inoculated on a surface containing Matrigel® and were incubated with the AMPs for 6-18 h in the presence or absence of VEGF (30 ng/ml). Morphological changes of the cells and tube formation were visualized under a microscope and photographed at a 100 × magnification (Olympus). Tube formation was quantified by counting the total number of branched tubes in randomly selected fields at a 100 × magnification.

Chorioallantoic membrane assay

Fertilized chick eggs were maintained in a humidified incubator at 37°C for 3 days. Approximately 2 ml egg albumin was removed with a hypodermic needle, allowing the chorioallantoic membrane (CAM) and yolk sac to drop away from the shell membrane. On day 3.5, the shell was punched out and removed, and the shell membrane was peeled away. Thermanox® coverslips (NUNC, USA) containing the AMPs were air-dried and applied to the CAM surface at embryonic day 4.5. Two days later, 2 ml 10% fat emulsion (Greencross Co., Korea) were injected into the chorioallantoic and the CAM was visualized under a microscope. Retinoic acid (RA), a known anti-angiogenic compound, was used as a positive control for antiangiogenic responses to the AMPs. The response was scored as positive when the AMP-treated CAM showed an avascular zone similar to that of a RA-treated CAM with very few vessels compared to that of a control coverslip. The response was calculated as the percentage of positive eggs relative to the total number of eggs tested.

Western blot analysis

Cell lysates were separated by 10% SDS-PAGE electrophoresis and the separated proteins were transferred to polyvinylidene difluoride membranes (Millipore, USA) using standard electroblotting procedures (Lee et al., 2012b). The blots were blocked and immunolabeled with primary antibodies against HIF-1α, cyclin D1 and tubulin overnight at 4°C. Immunolabeling was detected using an ECL kit (Amersham, UK) according to the manufacturer’s instructions.

Measurement of VEGF by enzyme-linked immunosorbent assay (ELISA)

VEGF concentration in the media from the AMP-treated cells was determined using a VEGF Immunoassay kit (R&D Systems, USA) according to the manufacturer’s instructions. The results are expressed as the concentration of VEGF relative to the total amount of VEGF from each well.

Statistical analysis

The results are expressed as the mean ± standard error (SE). Student’s t-test was used to determine statistical significance between the control and test groups. A P value of < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

The effect of SC39 peptides on the proliferation of human umbilical vein endothelial cells

To explore the antiangiogenic activity of the SC39 peptides, we first investigated the effect of the peptides on the growth of HUVECs using the MTT colorimetric assay. SC39 peptides dose-dependently inhibited the proliferation of HUVECs with an IC_{50} of 28.35 μg/ml (Fig. 1A). These data indicate that SC39 peptides may exhibit antiangiogenic activity through the inhibition of endothelial cell proliferation. To further evaluate whether the suppressed proliferation by SC39 peptides was due to cytotoxic or cytostatic activity, a viability assay was performed using the Trypan blue exclusion method. As shown in Fig. 1B, the viability of HUVECs exceeded 95% even after treatment with 40 μg/ml SC39 peptides for 72 h. These results demonstrate that the antiproliferative activity of SC39 peptides results from a cytostatic and not a cytotoxic effect by inducing cell cycle arrest.

The in vitro and in vivo antiangiogenic activity of SC39 peptides

We next investigated the effect of SC39 peptides on key angiogenic phenotypes of endothelial cells such as cell invasion and tube formation. Based on the above viability assay, in vitro angiogenesis assays were performed in an optimal dose range of 5-20 μg/ml of SC39 peptides at which no cytotoxicity was observed. VEGF was used as a specific angiogenic factor. In vitro angiogenesis assays were performed on serum-starved HUVECs stimulated by VEGF with or without SC39 peptides. As shown in Figs. 2 and 3, VEGF profoundly induced the invasion and tube formation of HUVECs, which were both inhibited by SC39 peptides in a dose-dependent manner. No cytotoxicity was observed at the indicated concentrations as shown by Trypan blue staining performed in parallel to the in vitro angiogenesis assays.

The antiangiogenic activity of SC39 peptides was further validated in vivo using a CAM assay. Thermanox® coverslips containing SC39 peptides were placed on the CAM surface and...
neovascularized zones were visualized under a microscope. RA was used as a positive control for antiangiogenic responses. The inhibition of angiogenesis by RA and on control coverslips was 67% (n = 12) and 10% (n = 10), respectively. SC39 peptides inhibited the neovascularization of the CAM in a dose-dependent manner (62% at 2 μg/egg, n = 13; 75% at 5 μg/egg, n = 16) without showing any rupture of or toxicity against pre-existing vessels (Fig. 4). These results demonstrate that SC39 peptides potently inhibited angiogenesis without exhibiting a cytotoxic effect on endothelial cells both in vitro and in vivo.

The inhibitory activity of SC39 peptides on tumor cell-induced angiogenesis

HIF-1α plays a key role in tumor angiogenesis by regulating the expression of angiogenic factors, including VEGF (Carmeliet et al., 1998; Forsythe et al., 1996). HIF-1α overexpression has been implicated in several human cancers and is associated with increased vascularization, drug resistance, and poor prognosis (Semenza, 2003; Unruh et al., 2003). We thus examined the effect of SC39 peptides on HIF-1α stabilization in the human hepatocellular carcinoma cell line HepG2, a hypervascularized tumor. As shown in Fig. 5A, SC39 peptide-treated HepG2 cells reduced the hypoxia-induced accumulation of HIF-1α protein in a dose-dependent manner without inhibiting the expression levels of cyclin D1 and tubulin that are related to the cell cycle and cytoskeleton, respectively. Hence, SC39 peptide treatment dose-

**Fig. 2.** The effect of SC39 peptides on the invasion of HUVECs. Serum-starved human umbilical vein endothelial cells (HUVECs) were stimulated with vascular endothelial growth factor (VEGF) (30 ng/ml) in the presence or absence of SC39 peptides. The invasiveness of HUVECs induced by VEGF in serum-free (SF) media was normalized to 100%. *P < 0.01 versus the VEGF control. Each value represents the mean ± SE from three independent experiments.

**Fig. 3.** The effect of SC39 peptides on the tube-forming ability of HUVECs. Serum-starved human umbilical vein endothelial cells (HUVECs) were stimulated with vascular endothelial growth factor (VEGF) (30 ng/ml) in the presence or absence of SC39 peptides. The level of VEGF-induced capillary tube formation of HUVECs in serum-free (SF) media was normalized to 100%. *P < 0.01 versus the VEGF control. Each value represents the mean ± SE from three independent experiments.

**Fig. 4.** The effect of SC39 peptides on the in vivo angiogenesis in CAMs. Fertilized chick eggs were maintained in a humidified incubator at 37°C. At embryonic day 4.5, SC39- or RA-loaded Thermanox® coverslips were applied to the chorioallantoic membrane (CAM) surface. Two days later, the chorioallantoic was visualized under a microscope. The presence of an avascular zone (arrows) in treated CAMs was scored as a positive response. The calculations were based on the proportion of positive eggs relative to the total number of eggs tested.
dependently decreased the hypoxia-induced production of VEGF, a HIF-1α target gene (Fig. 5B).

Next, we investigated the effect of SC39 peptides on the angiogenesis-promoting potential of HepG2 cells cultured under hypoxic conditions. The conditioned media from the HepG2 cells incubated in the presence or absence of SC39 peptides during hypoxia were collected, and their effects were investigated using an in vitro angiogenesis assay. The media was found to activate the invasion of HUVECs, whereas those from SC39 peptide-treated HepG2 cells blocked the hypoxia-induced tumor angiogenesis of HUVECs in a dose-dependent manner (Fig. 6). These results demonstrate that SC39 peptides may inhibit tumor cell-induced angiogenesis through the suppression of the HIF-1α-mediated angiogenic signaling.

**CONCLUSION**

AMPs have been primarily studied and developed as potential drug candidates for combating infections and microbial drug resistance (Brogden, 2005). However, recent reports have revealed that AMPs also possess other biological activities because of their diverse structural properties and specific mechanisms of action (Pushpanathan et al., 2013). Particularly, their use as anticancer peptides has proved to be a resourceful strategy for targeted cancer therapy via efficient tissue penetration and uptake, higher selectivity, and reduced harmful effects (Gaspar et al., 2013; Thundimadathil, 2012). Accumulating evidence suggests that AMPs may display anticancer effect through varied modes of action, including the disruption of plasma/mitochondrial membranes, necrosis, apoptosis, mediated immunity, membrane receptor involvement, inhibition of DNA synthesis, and antiangiogenesis (Gaspar et al., 2013; van Zoggel et al., 2012).

We recently isolated the fraction containing lipophilic AMPs from the culture supernatant of EML-CAP3, a new endophytic bacterial strain isolated from red pepper leaf. In this study, we found that the newly obtained AMPs exhibited potent antiangiogenic activity both in vitro and in vivo with no cytotoxicity. Furthermore, these angiostatic peptides effectively inhibited tumor cell-induced angiogenesis by down-regulating HIF-1α and its...
target gene, VEGF. In conclusion, to the best of our knowledge, ours is the first study to report on the antiangiogenic potential of the lipophilic AMPs isolated from the endophytic bacterial strain EML-CAP3. Further investigations identifying their structures and specific molecular target(s) could provide new insights into their regulation of angiogenesis.

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Hye Jin Jung et al.
The Antiangiogenic Effect of AMPs from EML-CAP3

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