Cepharanthine inhibits angiogenesis and tumorigenicity of human oral squamous cell carcinoma cells by suppressing expression of vascular endothelial growth factor and interleukin-8

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Abstract. Cepharanthine is a bisoclaurine alkaloid extracted from Stephania cepharantha Hayata, which is widely used for the treatment of many acute and chronic diseases, and can exert antitumor effects on several human cancer cell lines. However, little is known about the detailed mechanisms of the antitumor activity of Cepharanthine. In the present study, we determined whether Cepharanthine could suppress angiogenesis and growth of human oral squamous cell carcinoma (OSCC) cells in vitro and in vivo. Cepharanthine significantly inhibited expression of two major pro-angiogenic molecules, vascular endothelial growth factor (VEGF) and interleukin-8 (IL-8), in cultured cells and in cells implanted into the subcutaneous tissue of nude mice. Also, Cepharanthine inhibited the nuclear factor-κB (NF-κB) activity in human OSCC cells in vitro and in vivo. The decreased expression of VEGF and IL-8 correlated with decreased tumor cell growth and decreased vascularization in vitro and in vivo. These findings suggest that Cepharanthine can suppress angiogenesis and growth of OSCC cells by inhibiting expression of VEGF and IL-8 involved in the blockade of NF-κB activity.

Introduction

Cancers of the oral cavity accounted for 274,000 cases in 2002, with almost two-thirds of them in men. Also, they are the 11th most common cancer in males and 13th in females in developed countries, and 6th in males and 10th in females in developing countries (1). Oral squamous cell carcinoma (OSCC) occupies the majority of oral cancers. OSCC is a significant public health problem throughout the world because oral function is very important for breathing, eating and conversation. In spite of recent advances in surgery, radiotherapy, chemotherapy and immunotherapy, the survival rate of OSCC is <50% in the advanced stage (2,3). In fact, we often detected local invasiveness and high rate of metastasis to cervical lymph nodes in patients with OSCC. In addition, we sometimes found distant metastasis or localized recurrence even after radical excision. Although surgery is the first choice of treatment, patients with unresectable cancers including recurrent cancers are not candidate for this approach. Chemotherapy is often used in these cases. Therefore, the development of a more effective therapeutic method for OSCC has continued.

Cepharanthine is one of the bisoclaurine alkaloids extracted from Stephania cepharantha Hayata (4), which has been widely used in Japan for treatment of many acute and chronic diseases; bronchial asthma, alopecia areata, leukopenia during radiation therapy or anticancer treatment (5). It has been reported that Cepharanthine exerts antitumor effects by increasing the immunological competence of the host (6,7). It has also been reported that Cepharanthine increases the intracellular accumulation of the anticancer drug adriamycin through inhibiting its efflux from tumor tissue (8), while reducing its intracellular accumulation in normal tissue (9). Cepharanthine might be able to circumvent doxorubicin-resistance (10,11). Therefore, it is thought that Cepharanthine may be useful in cancer chemotherapy. In addition, Cepharanthine exerted antitumor effect by inducing apoptosis in a mouse leukemia cell line (12), an adenosquamous cell carcinoma cell line (13), and an oral squamous cell carcinoma cell line (14). However, little is known about the detailed mechanisms of antitumor activity of Cepharanthine in solid tumors, including OSCC.

The progression of OSCC is dependent in part on the formation of adequate vascular support, therefore we examined...
anti-angiogenicity for OSCC treatment. It has already been reported that angiogenesis showed a correlation with short survival and regional recurrence in oral cancer (15). Vascular endothelial growth factor (VEGF) has been shown to induce the proliferation of endothelial cells, increase vascular permeability, and prolong their survival (16,17). In addition, since it has been reported that VEGF positivity is a useful predictor of poor prognosis in oral cancer, VEGF status may prove to be an important prognostic factor in head and neck cancer (18,19). Interleukin-8 (IL-8), a chemo-attractant cytokine, has been shown to attract and activate neutrophils in inflammatory regions, and it is thought to be an angiogenic factor (20,21).

Recent studies have demonstrated that inhibition of NF-κB (NF-xB) activity in ovarian cancer cells can suppress angiogenesis and progressive growth by suppressing expression of VEGF and IL-8 (22). Interestingly, it was reported that Cepharanthine might inhibit activation of NF-xB in monocytic cells (23).

In this study, we tried to determine whether Cepharanthine could suppress angiogenesis and growth of OSCC cells by suppressing expression of VEGF and IL-8 involved in blockage of NF-xB activity in vitro and in vivo.

Materials and methods

**Chemicals.** Cepharanthine was obtained from Kaken Shoyaku Co., Ltd. (Tokyo, Japan). Cepharanthine was easily dissolved in complete culture medium and the antiproliferative activity of Cepharanthine stored in the medium was stable for at least 6 months at 4˚C (data not shown).

**Cells and culture.** B88 cells were isolated from a patient with OSCC in our laboratory (24). HSC3 cells (OSCC) were obtained from American Type Culture Collection (Manassas, VA, USA). Bovine aortic endothelial cells (BAEC) were cloned from bovine aortas, cultured and characterised by Schor et al. (25). B88 and HSC3 cells were cultured in DMEM supplemented with medium supplemented with 10% fetal bovine serum (FBS), 100 μg/ml streptomycin and 100 U/ml penicillin. BAEC were maintained on gelatinised dishes in DMEM supplemented with 10% FBS, 1 mM sodium pyruvate, non-essential amino acids (81.4 μg/l), 2 mM glutamine, 50 mg/ml ascorbic acid, penicillin (100 U/ml) and streptomycin (100 μg/ml). All cells were incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37˚C.

**Animals.** Female athymic nude mice with CAnN.Cg-Foxn1nu/CrlCrlj genetic background (CLEA Japan, Inc. Tokyo, Japan) were purchased at 4 weeks of age and kept under sterile conditions in a pathogen-free environment. The mice were provided with sterile water and food ad libum.

**Enzyme-linked immunosorbent assay (ELISA) for quantitative determination of VEGF and IL-8.** VEGF and IL-8 contained in CM either from untreated control or from Cepharanthine-treated cells were measured by a microtitre-based sandwich enzyme immunoassay system, which is commercially available and specifically estimates the total amount of both factors. According to the protocol of the ELISA kit, concentrated CM was subjected to the ELISA using immunoassay kits for VEGF (BioSource International Inc., CA, USA) and IL-8 (BioSource International).

**Relative real-time mRNA quantitation.** Total RNA was isolated from cells with Isogen (Nippon Gene Co., Toyama, Japan). Total RNA (1 μg) was reversely transcribed into complementary DNA (cDNA) by Moloney murine leukemia virus reverse transcriptase, using random hexamer primers ( Gibco BRL, Rockville, MD, USA). Real-time RT-PCR measurement of VEGF and IL-8 cDNA was performed in the Taqman detection protocol in an ABI PRISM 7000 sequence detector (Applied Biosystems, Foster City, CA, USA). The results for VEGF and IL-8 relative real-time RT-PCR assay were normalized to those obtained for the corresponding β-actin mRNA, providing a relative quantitation value. Taqman probes were obtained from Applied Biosystems and the following sequences were used: VEGF, 5'-AATTCACCACACTTGTGATGATCTTGGCCTCC-3'; 5'-CTTGGCAATACTGCACTTCACACAGA-3'; β-actin, 5'-CAGAGCTCACACCCAGAGTACTT-3'; PCR was performed in a total volume of 50 μl
of each amplification mixture containing 1 μl of each RT product, 25 μl of Universal master mix (Applied Biosystems), 200 nM VEGF or IL-8 forward and reverse primers, 100 nM fluorogenic probe. Each assay included a standard curve, a no-template control and cDNA samples in triplicate. The standard curve was generated by 5-fold dilutions of pooled cDNA obtained from cells. The relative expression level of VEGF or IL-8 was expressed as the ratio of VEGF/β-actin or IL-8/β-actin in arbitrary units.

Western blot analysis. Cytosolic protein was isolated from control and Cepharanthine-treated B88 cells. The soluble protein was separated on 10% SDS-PAGE by electrophoresis and electrothermally transferred onto PVDF membrane (Bio-Rad, Hercules, CA, USA), and the membrane was incubated with a 1:500 dilution of the rabbit polyclonal antibody against VEGF (A-20: Santa Cruz Biotechnology, Inc., CA, USA) and the rabbit polyclonal antibody against IL-8 (Santa Cruz Biotechnology) as the primary antibody, and an Amersham ECL kit (Amersham Pharmacia Biotech). Also, anti-α-tubulin monoclonal antibody (Zymed Laboratories, San Francisco, CA, USA) was used for normalization of Western blot analysis.

Microwell colorimetric NF-κB assay. The assay was performed by TransAM™ NF-κB p65/NF-κB p50 transcription factor assay kits (Active Motif, Carlsbad, CA, USA) according to the manufacturer’s instructions. Briefly, nuclear extracts were prepared using Nuclear and Cytoplasmic Extraction Reagents (NE-PER, Pierce Biotechnology, IL, USA) and placed on ice. Step 1: binding of NF-κB to its consensus sequence. p65 (20 μl) diluted in lysis buffer or 20 μl of cell extract were incubated with 30 μl of binding buffer [4 mM HEPES pH 7.5, 100 mM KCl, 8% glycerol, 5 mM DTT, 0.2% BSA, 0.016% poly (dIC)] in microwells coated with the probes containing the NF-κB binding consensus. After 1 h incubation at room temperature with a mild agitation (200 rpm on an IKA MS2 vortex), microwells were washed 3 times with PBS (+)/+ 0.1% Tween-20. Step 2: binding of primary antibody. Rabbit anti-NF-κB antibodies (100 μl), diluted 1000 times in a 10 mM phosphate buffer (pH 7.4) containing 50 mM NaCl and 1% non-fat dried milk, were incubated in each well for 1 h at room temperature. Microwells were then washed 3 times with 200 μl PBS (+)/+ 0.1% Tween-20. Step 3: binding of secondary antibody. Peroxidase-conjugated goat anti-rabbit IgG (100 μl) and 1% non-fat dried milk, were incubated in each well for 1 h at room temperature. Microwells were then washed 4 times with 200 μl PBS (+)/+ 0.1% Tween-20. Step 4: colorimetric reaction. Tetramethylbenzidine (100 μl) was incubated for 10 min at room temperature before adding 100 μl of stopping solution. Optical density was then read at 450 nm, using a 650-nm reference wavelength, with a Bio-Rad microplate reader (Bio-Rad) (27).

In vitro angiogenesis assay. An in vitro angiogenesis assay kit (Kurarbo, Osaka, Japan) was used according to manufacturer instructions. Briefly, the basal medium including VEGF (final concentration 10 ng/ml) was used for the human umbilical vein endothelial cell (HUVEC)/fibroblast co-culture. The medium including Cepharanthine was changed every 3 days. After 11 days, cells were fixed at room temperature in 70% ethanol and stained with anti-CD31 antibody according to the manufacturer’s protocol.

Tumor measurements and data analysis. Cultured tumor cells (1x10⁶) were suspended in 0.1 ml of serum-free medium and injected into the subcuta neous tissue of 5-week-old nude mice using a 27-gauge needle. The size of the tumors were determined by first measuring length (L) and width (W) and then calculating the volume (0.5 x L x W²) every 2 days. The body weight of the mice was also measured every 2 days. Experiments were started when tumors reached an average volume of about 100 mm³. The mice were sacrificed at 21 days by cervical dislocation.

Immunohistochemistry. Subcutaneous tumors harvested at autopsy were processed for immunostaining using a rabbit polyclonal antibody against VEGF, IL-8 and p65 (Santa Cruz Biotechnology), and a mouse monoclonal antibody against CD34 (Santa Cruz Biotechnology), and appropriate peroxidase-conjugated anti-mouse or rabbit IgG second antibody. The slides were examined in a bright-field microscope. A positive reaction was indicated by a reddish-brown precipitate in the cytoplasm. Negative controls were done using nonspecific IgG.

Vascularity. Vascularity was determined by the stereological method of point counting, which estimates the volume occupied by the vasculature (28). An eyepiece graticule that contained 100 points was used. CD34-positive vessels intersecting the points were counted in 15 random fields across each section (i.e. 1500 points examined), and the results are expressed as the mean percentage ± SD. Vascularity was quantified in the stroma close to the epithelium, up to ~750 μm from the basal lamina.

Statistics. The significance of the in vitro and in vivo results was determined by the Mann-Whitney U test.

Results

Effect of Cepharanthine on in vitro cell growth. We tested the effect of Cepharanthine at seven concentrations on in vitro cell growth of B88 and HSC3 cells. A low concentration of Cepharanthine (0.1-1.0 μg/ml) did not suppress the cell growth of B88 and HSC3 cells. However, high concentrations of Cepharanthine (2.0-20.0 μg/ml) significantly suppressed the growth of B88 and HSC3 when compared with that of control (Cepharanthine 0 μg/ml) (Fig. 1). Moreover, the growth of BAEC was not suppressed in a low concentration of Cepharanthine (0.1-2.0 μg/ml), but was suppressed in a high concentration of Cepharanthine (5.0-20.0 μg/ml) (Fig. 2).

Effect of Cepharanthine on in vitro cell migration. Cell migration is an essential process involved in tumor angiogenesis as well as tumor invasion and metastasis. A low concentration of Cepharanthine (0.1-2.0 μg/ml) did not suppress the cell migration of B88, HSC3 and BAEC. However, higher concentrations of Cepharanthine (5.0-20.0 μg/ml) significantly suppressed the migration of B88, HSC3 and BAEC when compared with that of control (Cepharanthine 0 μg/ml) (Fig. 3).
Effect of Cepharanthine on VEGF and IL-8 production. We next investigated whether the Cepharanthine-treated B88 or HSC3 cells would result in a reduced expression of VEGF and IL-8. Figs. 4 and 5 show the quantities of VEGF and IL-8 secreted by cells as determined by ELISA. Secretion of VEGF and IL-8 was significantly reduced in the Cepharanthine-treated B88 or HSC3 cells relative to untreated B88 or HSC3 cells on 48-96 h treatments. In addition, as shown in Fig. 6, Western blot analysis demonstrated that the expression of VEGF and IL-8 by Cepharanthine decreased in a time-dependent manner after 72 h of treatment. This effect of Cepharanthine on decreasing VEGF and IL-8 levels was coincidental with Cepharanthine-induced suppression of VEGF and IL-8 mRNA by real-time RT-PCR analysis (Fig. 7).

Effect of Cepharanthine on NF-κB activation. It is thought that head and neck SCC cells show significantly higher NF-κB binding activity than normal oral epithelial and salivary gland cells (29). We therefore investigated whether Cepharanthine could decrease the NF-κB binding activity on B88 cells. In the case of untreated B88 cells, NF-κB activation was not detected. When B88 cells were treated with TNF-α (50 ng/ml), NF-κB binding activity was markedly increased at 0.5 h after treatment. Moreover, incubation with 20 μg/ml of Cepharanthine fully suppressed the TNF-α-induced NF-κB activation (Fig. 8).

Effect of Cepharanthine on angiogenesis in vitro. To investigate the effect of Cepharanthine on induced anti-angiogenesis in oral tumors, we examined the effects of Cepharanthine on HUVECs. In vitro angiogenesis assays showed that the basal medium including VEGF (final concentration 10 ng/ml) promoted tubule formation in HUVECs, 11 days after treatment, which was ~30% inhibited by the addition of Cepharanthine (1.0 μg/ml), ~90% inhibited by the
addition of Cepharanthine (5.0 μg/ml), ~95% inhibited by the addition of Cepharanthine (10.0 μg/ml), and ~98% inhibited by the addition of Cepharanthine (20.0 μg/ml) (Fig. 9).

Effect of Cepharanthine on nude mice tumor growth. The therapeutic effect of Cepharanthine on B88 tumor-bearing nude mice is shown in Fig. 10. When the Cepharanthine

Figure 3. Migration inhibitory effect of Cepharanthine on B88, HSC3 and BAEC cells. Cell migration was evaluated by using 48-well modified Boyden chamber. After 24 h (B88 and HSC3) or 4 h (BAEC) of incubation, the migrated cells in each treatment were counted in 9 different fields under a light microscope.

Figure 4. Quantitative determination of VEGF secreted by Cepharanthine-treated B88 cells with ELISA. For detection of secretion of VEGF, cells were seeded into six-well plate, and after 24 h, medium was replaced. B88 cells were then cultured with standard medium of cancer cells for 24, 48, 72 and 96 h in the absence or presence of 20 μg/ml Cepharanthine. Supernatants were then cleared and subjected to a commercial VEGF or IL-8 immunoassay. Data represent the means ± SD from three independent experiments. *P<0.01 when compared with that of control by Mann-Whitney U test.
was administered daily at the dose of 40 mg/kg for 21 days, the tumor growth was significantly suppressed when compared with that of control (saline alone). During the experimental period, no loss of body weight was observed in the Cepharanthine treated group and control group (data not shown).

Effect of Cepharanthine on VEGF and IL-8 production, angiogenesis and NF-κB activation in vivo. We determined whether the production of VEGF and IL-8 was decreased, the angiogenesis was suppressed, and NF-κB activity was suppressed by the Cepharanthine administration in a nude mouse model. B88 tumors were resected and processed for immunohistochemical analyses of VEGF and IL-8 expression, vascular formation and NF-κB activation using anti-VEGF, IL-8, CD34 and p65 antibodies respectively. As shown in Fig. 11 and Table I, higher expression of VEGF and IL-8 was found in untreated control tumor lesions than in Cepharanthine treated tumors. In addition, expression of VEGF and IL-8 was markedly decreased in the tumors given Cepharanthine (40 mg/kg). Consistent with the alteration of VEGF and IL-8 expression, untreated control tumors were highly vascularized, whereas tumors given Cepharanthine had a significant decrease in microvessel density (Table I). Moreover, expression of p65 was observed in the cytoplasm of Cepharanthine-treated tumors though expression of p65 was seen in the nuclei of untreated control tumor.

Discussion
We investigated whether Cepharanthine could suppress angiogenesis and growth of OSCC cells, and confirmed that...
it does so by inhibiting the expression of VEGF and IL-8 involved in the blockade of NF-κB activity. We previously reported that Cepharanthine can exert a direct antitumor effect on solid tumors including adenosquamous cell carcinoma and OSCC. Briefly, we have clarified that Cepharanthine can exert antitumor effects by inducing G1...
arrest via expression of cell cycle regulators such as p21WAF1 or p27Kip1 (13,14). However, little is known about the other detailed mechanisms of antitumor activity of Cepharanthine in solid tumors.

Angiogenesis is thought to have important roles in metabolic function and solid tumor growth, facilitating tumor invasion and metastasis (30,31), and to be regulated by a number of growth factors with angiogenic activity. Tumor
angiogenesis is controlled by angiogenic factors directly induced from tumor cells, as well as by factors indirectly induced from surrounding stromal tissues. Among several growth factors, VEGF seemed to play an important role in tumor angiogenesis of OSCC (32). Also, IL-8 has been shown to contribute to cancer progression by acting as a mitogenic and angiogenic factor (33). The role of IL-8 in OSCC was observed in several studies in which its expression has been strongly correlated with metastatic phenotype (34,35). Briefly, IL-8 as well as VEGF must play an important role in tumor angiogenesis of OSCC.

Our results show that Cepharanthine treatment in cultured OSCC cells induced a decrease in the levels of VEGF mRNA and protein, followed by the suppression of the cell growth. Also, we found that mRNA and protein levels of IL-8 decreased following Cepharanthine treatment. Briefly, Cepharanthine was able to suppress the VEGF and IL-8 expression levels in OSCC cells. Moreover, anti-angiogenic effect of Cepharanthine was reconfirmed by an in vivo tumorigenicity assay in mice. As expected from its stronger growth inhibition, a significant suppression of tumor growth was detected in Cepharanthine-treated tumors when compared with that of control in vivo animal tumor model.

Interestingly, Okamoto et al reported that Cepharanthine might inhibit activation of NF-κB in monocytic cells (23). In addition, several previous studies have implicated that NF-κB can upregulate VEGF and IL-8 transcription (36,37). Huang et al demonstrated that inhibition of NF-κB activity by the expression of the inhibitor of nuclear factor-κB (IκB) super repressor suppressed the induction of IL-8 and VEGF in prostate, ovarian, and melanoma cancer cells (22,38-39). This inhibition also reduced tumorigenicity, angiogenesis, and metastasis of tumors formed from these cells. Bancroft et al reported very similar results for a human head and neck squamous cell carcinoma cell line (36). However, a later study from this same group showed that expression of IκBα blocked the induction of IL-8 but not VEGF in head and neck squamous cell carcinoma cells treated with epidermal growth factor (40). There are conflicting studies on the correlation between inhibition of NF-κB activity and suppression of VEGF induction. Also, the inhibition of tumorigenicity by suppression of NF-κB activity had to occur by other mechanisms, such as inhibition of cell adhesion (41), inhibition of pro-inflammatory cytokine production (42), or inhibition of plasminogen activator and matrix metalloproteinase (43,44), which contribute to neoplastic angiogenesis, growth, and metastasis. Briefly, suppression of NF-κB activity may induce anti-angiogenesis without down-regulation of VEGF and IL-8. In this study, we detected that Cepharanthine inhibited TNF-α induced activation of NF-κB in OSCC cells, suggesting that Cepharanthine has a potent inhibitor of NF-κB activation induced by TNF-α. In conclusion, we showed that Cepharanthine could decrease angiogenesis, retarded tumor growth, and reduced formation of malignant ascites, in part.
through down-regulation of the angiogenic molecules VEGF and IL-8.

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References


