Effects of *Cordyceps militaris* extract on angiogenesis and tumor growth[1]

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KEY WORDS *Cordyceps militaris*; angiogenesis; human umbilical vein endothelial cells; HT1080 cells; B16-F10 cells; matrix metalloproteinases; basic fibroblast growth factor

ABSTRACT

AIM: To evaluate the effects of *Cordyceps militaris* extract (*CM*E) on angiogenesis and tumor growth. **METHODS:** Human umbilical vein endothelial cells (HUVEC), HT1080, and B16-F10 cells were used. DNA fragment, angiogenic related gene expressions (MMPs, bFGF, VEGF, *etc*), capillary tube formation, wound healing *in vitro*, tumor growth *in vivo* were measured. **RESULTS:** *CM*E inhibited growth of HUVECs and HT1080 ($P<0.01$). *CM*E 100 and 200 mg/L reduced MMP-2 gene expression in HT1080 cells by 6.0 % and 22.9 % after 3-h and 14.9 % and 32.8 % after 6-h treatment. *CM*E did not affect MMP-9 gene expression in B16-F10 melanoma cells. *CM*E 100 and 200 mg/L also reduced bFGF gene expression in HUVECs by 22.2 % and 41.3 %. *CM*E inhibited tube formation of endothelial cells *in vitro* and *in vivo*. *CM*E repressed the growth of B16-F10 melanoma cells in mice compared with control group ($P<0.05$). **CONCLUSION:** *CM*E has antiangiogenetic properties.

INTRODUCTION

Angiogenesis is the process of new blood vessel formation from existing blood vessels and a natural response of tissues to ischemia[1]. The steps of angiogenesis involve proteolytic degradation of extracelluar matrix, endothelial cell-matrix adhesion, migration, proliferation, and differentiation[2]. This biological reaction is often associated with some diseases, such as solid tumor[3], diabetic retinopathy[4], and rheumatoid arthritis[5].

Tumor formation requires the development of a blood supply to allow growth larger than 2-mm in diameter, and thus inhibition of angiogenesis could prevent tumor growth and metastasis[6]. To grow further, a tumor must induce the formation of a capillary network that invades the tumor mass[7].

A variety of growth factors can stimulate angiogenesis both *in vitro* and *in vivo*[8]. Of the known angiogenic factors, bFGF and VEGF are most commonly expressed[9,10].

Nowadays, many antiangiogenic strategies are being evaluated in clinical trials. These approaches offer new hope for the successful treatment of cancer. However, there are a number of potential problems that warrant caution in clinical trials on human[11,12]. So

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herbal medicine has some advantages because it has been used for humans for a long time.

Cordyceps militaris is one of the herbal ingredients, which has been used for patients suffering from cancer in oriental medicine and also used in East-West Cancer Center of Daejeon University Oriental Hospital, Korea since 2001. Some immunomodulatory and antitumor activities of *Cordyceps militaris* have been known[13,14], but antiangiogenic effects have not been reported yet. This present study aimed to elucidate the effects of *Cordyceps militaris* on angiogenesis and tumor growth.

MATERIALS AND METHODS

Materials *Cordyceps militaris* was purchased from Daejeon University Oriental Hospital. The fruitbody and host-insect were identified and authenticated by Prof SI RIM. Voucher specimens (#CM-2002-03-Fh) have been deposited at the Institute of Traditional Medicine and Bioscience in Daejeon University. One hundred gram of *Cordyceps militaris* was boiled in 3 L water for 2 h. Extract solution was filtered and freeze dried. The pale-dark extract was dissolved in media and filtered with 0.45-μm syringe filter for *in vitro* assay. Matrigel was obtained from BD (Los Angeles, USA). M-MLV RT, *Taq* polymerase, dNTP, and 5 X TBE buffer were obtained from Bioneer (Cheongwon, South Korea). Other chemicals were purchased from Sigma (Maryland, USA).

Animals C57BL/6 mice were obtained from commercial animal breeder (Daehan Biorink, South Korea) and used at 5-6 weeks of age after one week of acclimation. The mice were housed in an environmentally controlled room at 22±2 ℃, in relative humidity of 55 %±10 %, and 12-h light/dark cycle. They were fed with commercial pellets (Samyang Feed, Korea) and tap water *ad libitum*.

Cell culture B16-F10 melanoma cells and HT1080 cells were obtained from Korea Research Institute of Bioscience and Biothechnology (KRIBB) and cultured in DMEM (Sigma, USA) supplemented with 10 % fetal bovine serum (FBS), 100 U streptomycin, and 100 U benzylpenicillin. Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (Berkshire, England). The cells were cultured in EGM-2 (Clonetics, MD, USA).

Proliferation assay[15] Cultured HUVEC, HT1080, and B16-F10 cells (2×10^4) were added into 96-well plate and treated with *CM*E for 48 h, respectively. Plates were washed twice with PBS and SRB 100 μL was added to each well. After 30 min plates were washed 5 times with 0.1 % acetic acid. Stained SRB was redissolved with 100 μL of Tris buffer 10 mmol/L. Absorbance was determined at 540 nm.

DNA fragment assay HUVECs were transferred to 6-well plate. Cells were incubated until 80 % confluence. Various concentrations of *CM*E were added to wells respectively. After 24-h incubation, DNA was extracted using G-DEX™ (Intron, Seoul, Korea), genomic DNA extraction kit, by manufacture's recommendation. Equal volume of DNA was loaded onto 2 % agarose gel (W/V) with ethidium bromide (Sigma, St Louse, USA) and electrophoresed at 50 V for 2 h.

mRNA expression for MMP-2 in HT1080 cells and MMP-9 in B16-F10 melanoma cells B16-F10 melanoma cells and HT1080 cells were added to the 6-well culture plates. After overnight culture, medium was refreshed by DMEM containing 2 % FBS. The cells were treated with *CM*E. The final volume was 2 mL/well. Total RNA was extracted by TRIzol reagents (Invitrogen, USA). RNA (1 μg) was reverse-transcribed (RT) into first strand cDNA in a RT mixture containing 2 μL dNTPs mix 10 mmol/L, 1 μL oligo-dT primer (20 nmol/L), 2 mL DTT 100 mmol/L, 4 μL 5×RT buffer (Tris-HCl 250 mmol/L, pH 8.3, KCl 375 mmol/L, $MgCl_2$ 15 mmol/L, RNase inhibitor 20 U), 1 μL M-MLV RT (200 kU/L; Promega, USA), and 2 μL DDW. RT mixture was incubated at 42 ℃ for 60 min, heated to 72 ℃ for 10 min to inactivate the reverse transcriptase activity, and chilled at 4 ℃ for 5 min. A portion of the RT product (1 μL) was then subjected to the polymerase chain reaction (PCR) in a DNA thermal cycler (TaKaRa, Tokyo, Japan).

The gene expressions of MMP-2 mRNA in HT1080 cells and MMP-9 mRNA in B16-F10 melanoma cells were detected by RT-PCR method after 3-h and 6-h treatment. Thirty-five cycles of cDNA amplification were performed by PCR. The DNA sequence of primers for MMP-2 and MMP-9 cDNA amplification were as follows. For β-actin, sense: 5'-GTG GGG CGC CCC AGG CAC CA-3'; antisense: 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3' 539. For MMP-2, sense: 5'-TTT TCT CGA ATC CAT GAT GG-3'; antisense: 5'-CTG GTG CAG CTC TCA TAT TT-3' 348. For MMP-9, sense: 5'-GGC TCA CAG GTC TGT TCG TT-3'; antisense: 5'-TCG TGA ACA CTG CTG AAA GTG-3' 332.

mRNA expression of angiogenic gene in HUVEC HUVECs were added to the 6-well culture

plates. After overnight culture, medium was refreshed by EGM-2. The cells were treated with *CM*E. Total RNA was extracted using TRIzol reagent (Invitrogen, USA). The gene expressions of vascular endothelial growth factor (VEGF), VEGF receptor (VEGFr), basic fibroblast growth factor (bFGF), bFGF receptor (bFGFr), endothelial nitric oxide (eNOS), and granulocyte-colony stimulate factor (G-CSF) were detected by RT-PCR after 6-h treatment. Thirty-five cycles of cDNA amplification were performed by PCR. The DNA sequence of primers for VEGF, VEGFr, bFGF, bFGFr, eNOS, and G-CSF were as follows. For β-Actin, sense: 5'-GTG GGG CGC CCC AGG CAC CA-3'; antisense: 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3' 539. For VEGF, sense: 5'-GGG CAG AAT CAT CAC GAA GT-3'; antisense: 5'-AAA TGC TTT CTC CGC TCT GA-3' 359. For VEGFr, sense: 5'-GTA TGT CCC ACC CCA GAT TG-3'; antisense: 5'-ACA TTT GCC GCT TGG ATA AC-3' 321. For bFGF, sense: 5'-CTC CAG AGT ATG CGG GCG CA-3'; antisense: 5'-GTC AGA GGG AAA CAC CAG GA-3' 392. For bFGFr, sense: 5'-ATG GTT GAC CGT TCT GGA AG-3'; antisense: 5'-CAT GGA TGC ACT GGA GTC AG-3' 250. For eNOS, sense: 5'-AGA TCA CCG AGC TCT GCA TT-3'; antisense: 5'-GCT CAT GTA CCA GCC ACT GA-3' 272. For G-CSF, sense: 5'-ACA TGG TTT GAC TCC CGA AC-3'; antisense: 5'-TTC ACA CAC AGG CCT GAC AT-3' 200.

Capillary tube formation assay on Matrigel[16] In brief, 48-well cell culture plates were coated with 150 μL of Matrigel and allowed to solidification at 37 ℃ for 1 h. Then HUVECs (5×10^4) were seeded to each well, then *CM*E 100, 150, and 200 mg/L were added. After 18 h, tube formation was observed periodically under a phase contrast microscope (Leica, Germany). Representative photomicrographs were taken at 18 h.

Wound healing assay HUVECs were cultured in 12-well plate. A confluent monolayer of HUVECs was wounded with a yellow pipet tip and the media was replaced by fresh medium. Closure of the wound was monitored after PBS or *CM*E treatment. Representative photomicrographs were taken at 8 h.

Mouse matrigel plug assay[17] bFGF and heparin was dissolved in PBS and mixed with liquid matrigel in proportions not exceeding 1 % of the total volume of matrigel. A mixture of 0.5 mL matrigel containing bFGF 80 μg/L and heparin 18 kU/L, and *CM*E (200 mg/L) or PBS. After 6 d, the mice were sacrificed and excised matrigel plugs were photographed.

Tumor growth assay B16-F10 melanoma cells (5×10^5) were injected into foot fed of C57BL/6 mouse. After mice were administered with DW, and *CM*E 200 or 600 mg/L for 20 d, foot fed were removed and weighted.

Statistical analysis Results were expressed as the mean±SD. Statistical analysis of the data was carried out by *t*-test.

RESULTS

Inhibtion of cell proliferation *CM*E inhibited the growth of HUVECs in dose-dependent manner. *CM*E inhibited the growth of HT1080 cells but had no effects on B16-F10 cells (Fig 1).

DNA fragment assay After HUVECs were treated with various concentrations of *CM*E for 24 h, no typical ladder pattern was observed (Fig 2). This indicated that apoptosis did not occur after *CM*E treatment.

mRNA expression for MMP-2 in HT1080 and MMP-9 in B16-F10 melanoma cells CME reduced MMP-2 gene expression in HT1080 cells by 6.0 % (100 mg/L) and 22.9 % (200 mg/L) after 3-h treatment and by 14.9 % (100 mg/L) and 32.8 % (200 mg/L) after 6-h treatment. *CM*E did not affect MMP-9 gene expression in B16-F10 melanoma cells (Fig 3).

mRNA expression for angiogenic gene in HUVECs *CM*E reduced bFGF gene expression in HUVEC by 22.2 % (25 mg/L) and 41.3 % (50 and 100 mg/L) after 6-h treatment. eNOS gene expression was downregulated by 9 % after *CM*E 100 mg/L treatment. *CM*E did not affect VEGF, VEGFr, bFGFr, and G-CSF gene expression in HUVECs (Fig 4).

Capillary tube formation assay on Matrigel In control group, endothelial cells formed network and tube. *CM*E 200 mg/L inhibited tube formation of endothelial cells at 18 h (Fig 5).

Wound healing assay In control group, wound was healed almost at 8 h. *CM*E 100 and 200 mg/L delayed wound healing (Fig 6).

Mouse Matrigel plug assay In control group many vessels were observed in dark red from the surrounding tissue into the matrigel because of filled blood, but in matrigels of *CM*E group only pale red and yellowish color were observed (Fig 7).

Tumor growth assay *CM*E 200 and 600 mg/kg inhibited B16-F10 melanoma growth compared with control group (Fig 8).

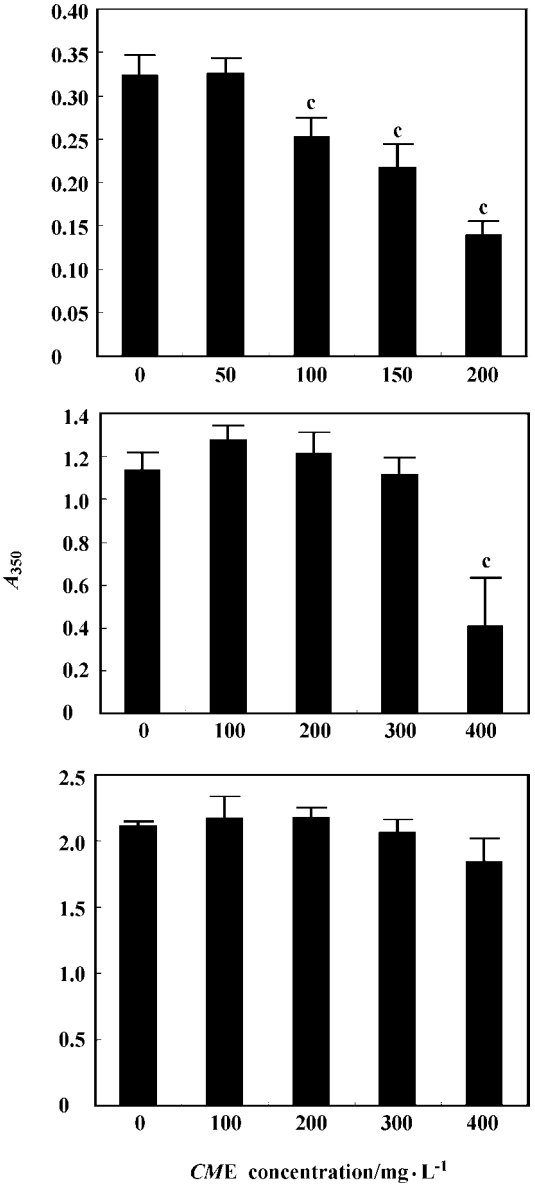

Fig 1. Effects of *CM*E on HUVECs (A), HT1080 (B), and B16F10 (C) proliferation for 48 h. *n*=4. Mean±SD. ᶜ*P*<0.01 *vs* PBS.

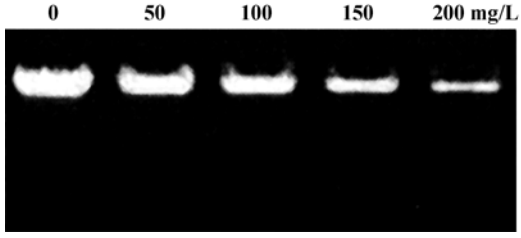

Fig 2. DNA fragment assay after *CM*E treatment for 24 h.

DISCUSSION

Mammalian cells require oxygen and nutrients for their survival and are therefore located within 100 to 200 of blood vessels - the diffusion limit for oxygen. For multicellular organisms to grow beyond this size, they must recruit new blood vessels by vasculogenesis and angiogenesis[12].

Blood capillaries are primarily composed of endothelial cells, which are usually quiescent in the adult mammal under physiological conditions[18]. In living animals, endothelial cells form new capillaries wherever they are required. It is presumed that when cells in tissues are deprived of oxygen, they release angiogenic factors that induce new capillary growth[7]. Of the known angiogenic factors, FGF and VEGF are most commonly expressed[9,10]. For the response of this stimulation, lining collagens and extracellular matrix are degraded by protease such as MMP, and endothelial cells migrate and proliferate from the existing vessels

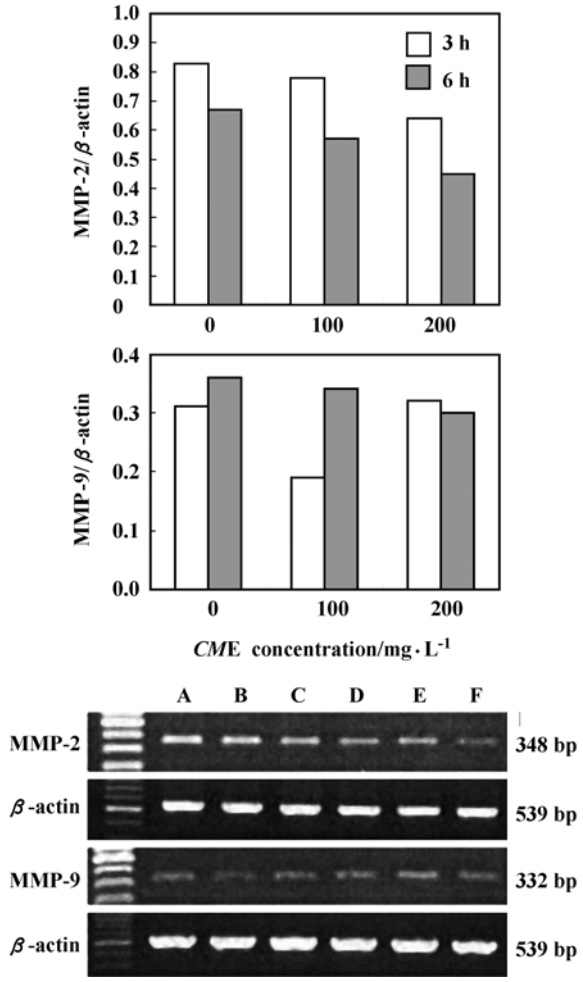

Fig 3. MMP-2 in B16-F10 and MMP-9 gene expression in HT1080 after *CM*E treatment for 3 h (A-C) or 6 h (D-F). (A, D) Control; (B, E) *CM*E 100 mg/L for 3 h; (C, F) *CM*E 200 mg/L.

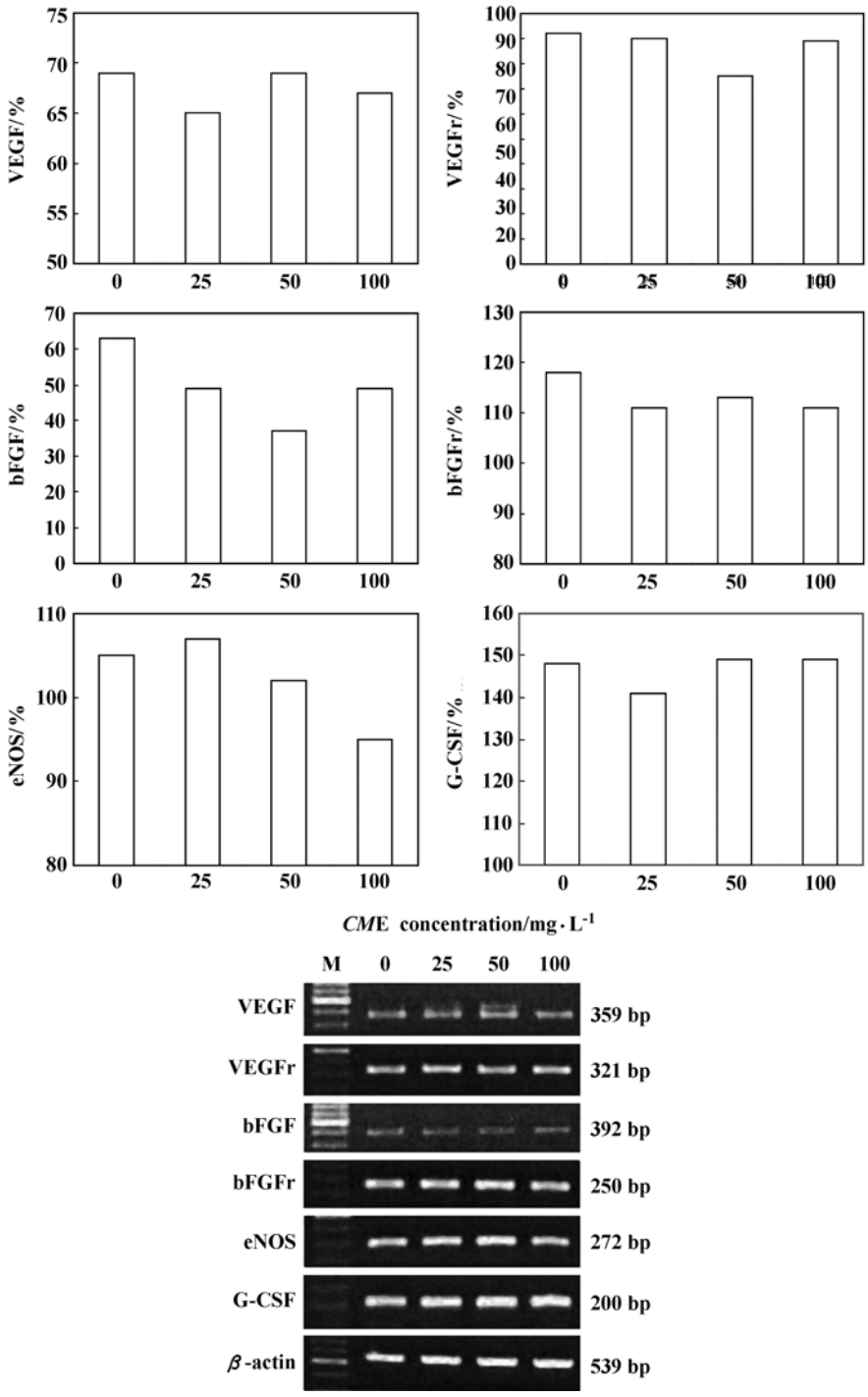

Fig 4. Effect of 6-h *CM*E 25-100 mg/L treatment on VEGF, VEGFr, bFGF, bFGFr, eNOS, and G-CSF gene expression in HUVEC. Gene expression was shown as percentage to β-actin.

to the area of angiogenesis stimulation[19].

Angiogenesis is also important for tumor growth. The growth of a solid tumor is limited by its blood supply: if it were not invaded by capillaries, a tumor would be dependent on the diffusion of nutrients from its surroundings and could not enlarge beyond a diameter of a few millimeters. To grow further, a tumor must induce formation of a capillary network that invades the tumor mass[7]. Tumor cells overexpress one or more of these angiogenic factors that may function synergistically in promoting tumor growth. Also, expression of the angiogenic inhibitor must be simultaneously down-

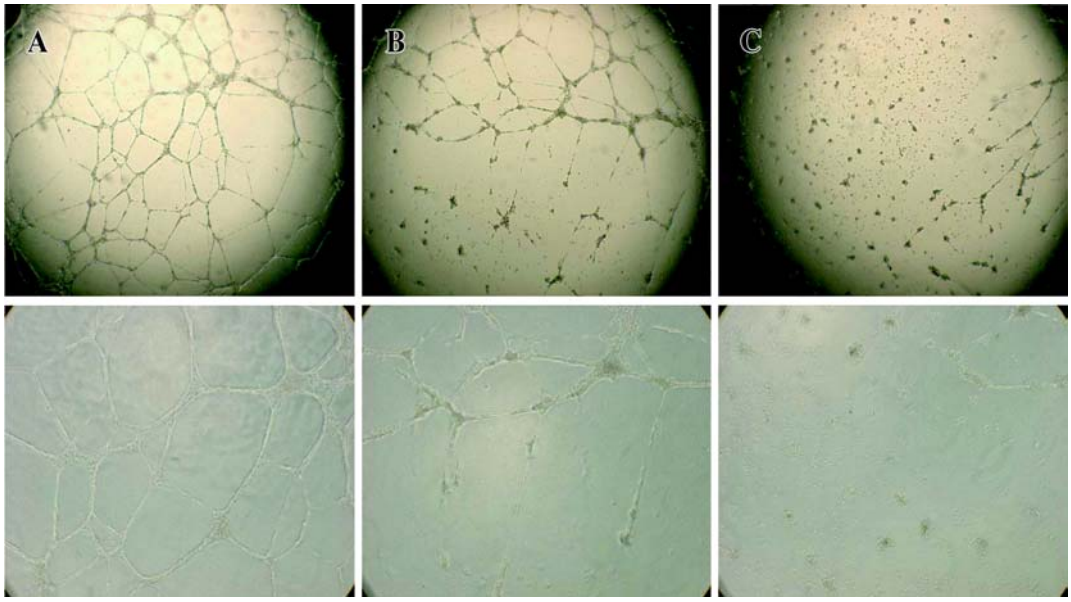

Fig 5. Effect of *CM*E on tube formation of HUVECs in matrigel. HUVECs were treated with PBS (A), *CM*E 150 mg/L (B), and *CM*E 200 mg/L (C) relatively for 18 h. ×40 (up). ×100 (below).

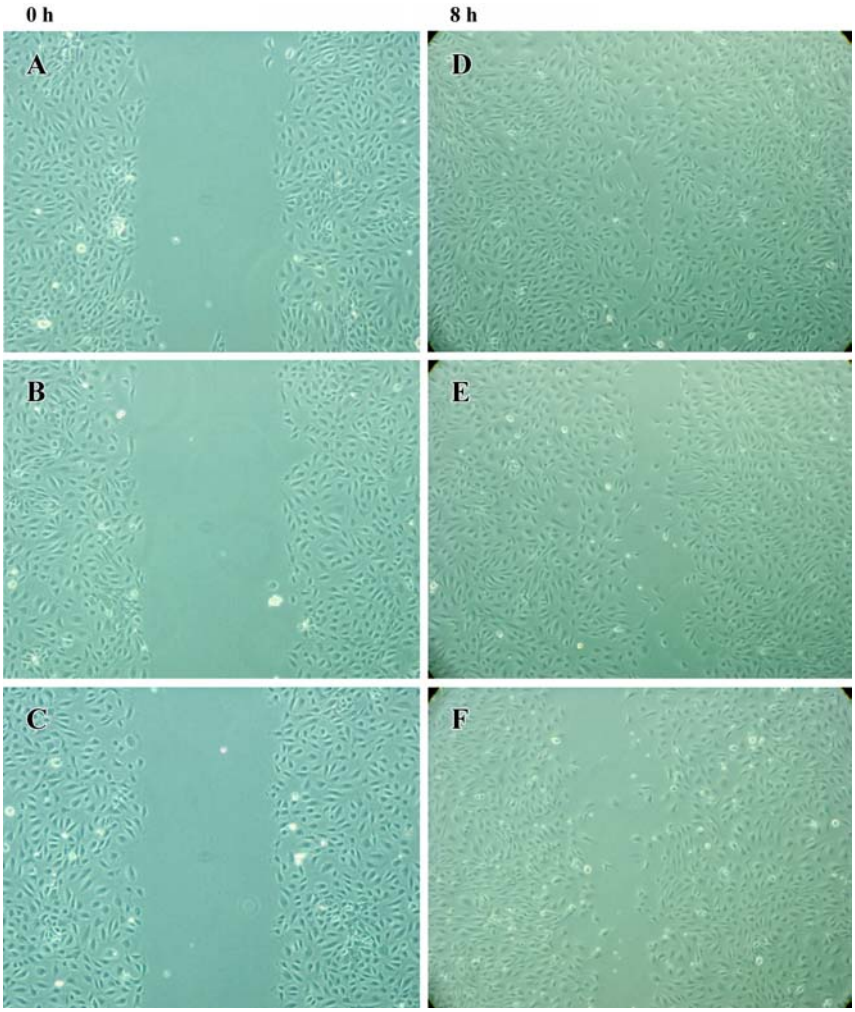

Fig 6. Effect of *CM*E on wound healing of HUVECs. HUVECs were treated with yellow tip (A, B, and C) and treated with PBS (D), *CM*E 100 mg/L (E), and *CM*E 200 mg/L (F) for 8 h.

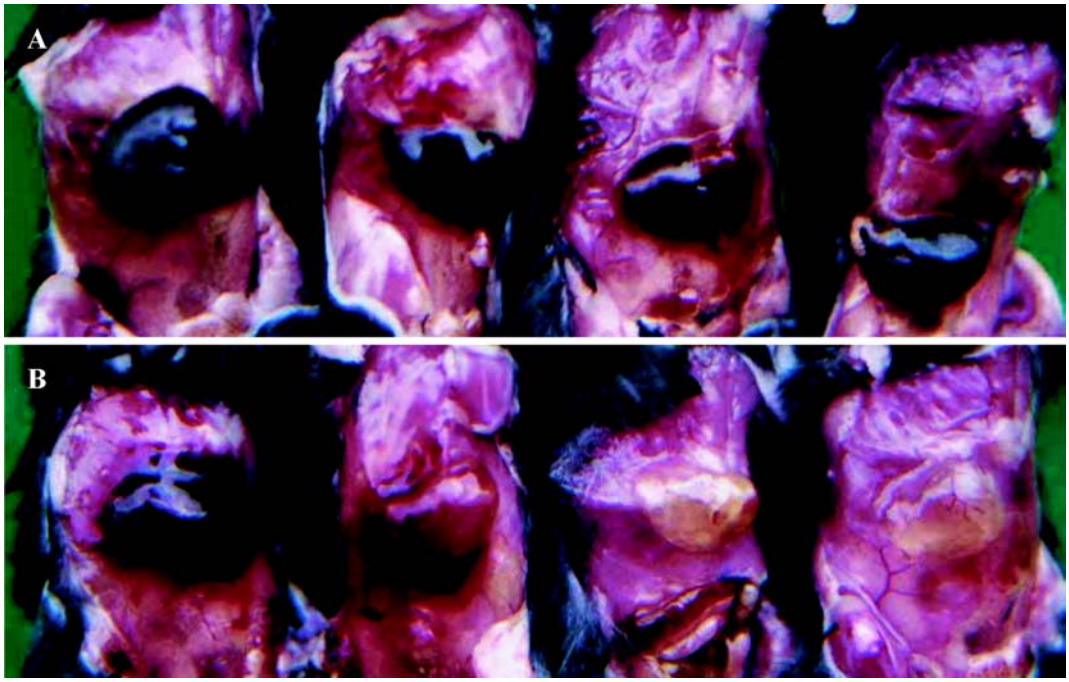

Fig 7. ***In vivo*** **angiogenesis assay using Matrigel plug. C57BL/6 mice were implanted 0.5-mL matrigel containing bFGF 80 μg/L, heparin 18 kU/L, and *CM*E 200 mg/L or PBS for 6 d. (A) Control; (B) *CM*E 200 mg/L.**

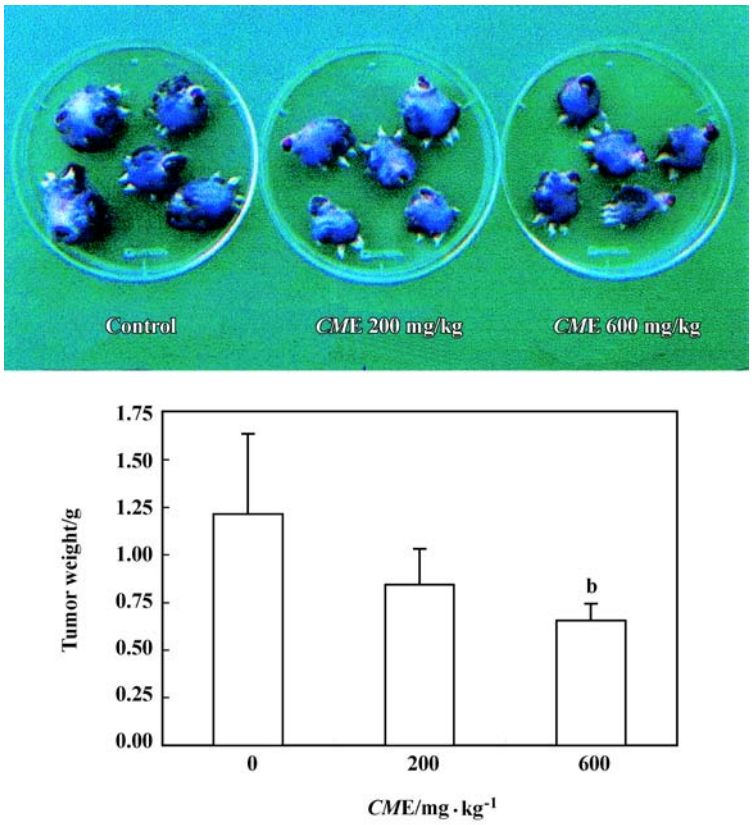

Fig 8. Effect of 20-d *CM*E treatment on B16-F10 growth in foot fed. *n*=5. Mean±SD. $^{b}P<0.05$ *vs* control.

regulated[20,21].

A number of promising molecular approaches are being introduced to suppress tumor angiogenesis. Major categories of angiogenesis antagonists include protease inhibitors, direct inhibitors of endothelial cell proliferation and migration, suppressor of angiogenic growth factors, inhibitor of endothelial-specific integrin/survival signaling, chelators of copper and inhibitors with specific other mechanisms[11]. Several compounds which inhibited angiogenesis, protamine[22], angiostatic steroids[23], retinoid[24] and cartilage factor[25] have been found, although they are not clinically useful for toxicity and potency. Therefore, it is a major task to find out new angiogenesis inhibitors.

Cordyceps militaris has been used as a medicinal stuff for a long time in oriental medicine for patients suffering from cancer. It also has been used for cancer patients in East-West Cancer Center of Daejeon University Oriental Hospital and it has been shown to have clinical efficacy. It has been known to have immunomodulatory function, antitumor and immunoenhancing activities[13,14].

This present study aimed to elucidate the effects of *CM*E on cancer by antiangiogenesis and metastasis. In this study, *CM*E has some cytotoxic potency for HUVEC and HT1080 cell *in vitro*. It shows *CM*E has antiangiogenic effects on HUVEC, and antitumor effects on HT1080. *CM*E reduced MMP-2 gene expression in HT1080 cells in proportion to concentration and time. But typical ladder pattern was not observed and this result indicates that apoptosis did not occur upon incubation with *CM*E. So anti-proliferation effects of *CM*E was not dependent on cytotoxicity.

Expression of MMP-2 is strongly correlated with tumor invasion and metastasis in a variety of cancer[26,27]. So we can suggest *CM*E has antiangiogenic and antitumor effects by reducing MMP-2. In order to investigate the antiangiogenesis mechanism of *CM*E, we studied the effects on VEGF, VEGFr, bFGF, bFGFr, eNOS, G-CSF mRNA expressions in HUVEC. *CM*E reduced bFGF gene expressions in HUVEC. *CM*E did not affect VEGF, VEGFr, bFGFr, eNOS, G-CSF gene expression in HUVEC. A variety of growth factors can stimulate angiogenesis *in vitro* and *in vivo*[8]. Of the known angiogenic factor, bFGF and VEGF are most commonly expressed[9-10]. For the response of this stimulation, lining collagens and extracellular matrix are degraded by protease such as MMP, and endothelial cells migrate and proliferate from the existing vessels to the area of angiogenesis stimulation[19]. These results suggest that *CM*E has antiangiogenic effect by reducing bFGF expressions. In experiments assessing the inhibitory effects on capillary tube formation, *CM*E inhibited tube formation of endothelial cells. These results imply that *CM*E inhibits angiogenesis by preventing tube formation of endothelial cell. In experiments assessing the inhibitory effects on wound healing, *CM*E inhibited wound healing process in dose-dependent manner. These results show that *CM*E inhibits angiogenesis by preventing HUVEC forming. In order to investigate the antitumor effects of *CM*E *in vivo*, we excise tumor growth assay. *CM*E inhibited tumor growth compared with control group. Based on these results, it could be concluded that *CM*E has significant properties on antiangiogenesis and tumor growth *in vitro* and *in vivo*. These results suggest that *CM*E is a good candidate for diseases associated with angiogenesis.

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