Note

In Vivo Anti-tumor Effects of the Ethanol Extract of *Gleditsia sinensis* Thorns and Its Active Constituent, Cytochalasin H

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Angiogenesis is the process of new vessel formation from pre-existing blood vasculature and is critical for continuous tumor growth. We previously reported that an ethanolic extract of *Gleditsia sinensis* thorns (EEGS) and its active constituent, cytochalasin H, have anti-angiogenic activity *in vitro* and *in vivo* via suppression of endothelial cell functions. In the present study, EEGS and cytochalasin H were observed to efficiently inhibit tumor growth in an *in ovo* xenograft model without significant toxicity. We repeatedly observed the anti-tumor and anti-metastatic effects of EEGS in representative animal models. These results suggest that EEGS and its active constituent, cytochalasin H, are potential candidates for the development of anti-angiogenic cancer drugs.

Key words *Gleditsia sinensis* thorn; cytochalasin H; anti-angiogenesis; anti-tumor

Angiogenesis is the complex regulatory process of blood vessel formation.¹⁾ It is considered a hallmark of tumors and is essential for their progression.²⁾ Many compounds, including phytochemicals, are extensively studied for anti-angiogenic potential. For example, *Salvia officinalis*, cinnamon extract and koetjiapic acid from *Sandoricum koetjaoe* MERR. possess anti-angiogenic activities.³⁾ Anti-cancer agents that target angiogenesis in clinical trials, such as blockers of vascular growth factors and inhibitors of tyrosine kinase receptors, have limited efficacies and exert some adverse side effects.⁴⁾ For example, bevacizumab, which targets vascular endothelial growth factor (VEGF), was approved by the U.S. Food and Drug Administration (FDA) for the treatment of certain types of cancer, including colon, lung, kidney and brain cancers.^{5,6)} However, the FDA removed the breast cancer indication from bevacizumab in 2010 due to its lack of efficacy and side effects, such as hypertension. Therefore, new strategies are required to develop more efficacious and safer anti-angiogenic cancer drugs.

The decoction of *Gleditsia sinensis* LAM. (Leguminosae) has been used in traditional oriental medicine to treat diverse diseases, such as suppuration, carbuncle, obesity, and thrombosis.^{7–10)} The crude extract of *G. sinensis* thorns has multiple pharmacological effects, including anti-mutagenic, anti-microbial, anti-allergic, anti-inflammatory, and anti-angiogenic effects.^{11,12)} Several previous studies reported anti-tumor effects of *G. sinensis* thorns in cancer cells and animal models,^{7,13,14)} but its active constituent is not clear. Our previous studies demonstrated that the ethanolic extract of *G. sinensis* thorns (EEGS) efficiently inhibited the angiogenic functions of endothelial cells and reduced pro-angiogenic factor-derived vessel formation *in vivo*.¹⁵⁾ In addition, we identified cytocha-

lasin H as an active anti-angiogenic constituent of EEGS.¹²⁾ This study used various tumor models to evaluate whether the anti-angiogenic potential of EEGS and cytochalasin H was directly responsible for the anti-tumor effects.

MATERIALS AND METHODS

Preparation of EEGS and Cytochalasin H Dried *G. sinensis* thorns were purchased from the Kwangmyungdang Medicinal Herbs Co. (Batch no. K136201010, Ulsan, Republic of Korea). A voucher specimen (KIOM-CRC-1) was deposited at the Cancer Research Center, Korea Institute of Oriental Medicine (KIOM). EEGS and cytochalasin H were prepared as previously described.^{12,15)}

Chemicals All chemicals, except those described elsewhere, were purchased from the Sigma-Aldrich Co. (St. Louis, MO, U.S.A.).

Cell Culture A549 human lung carcinoma cells and B16-F10 mouse melanoma cells were obtained from ATCC (Manassas, VA, U.S.A.) and maintained in RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM), respectively, supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin, and 100 µg/mL streptomycin (Invitrogen, Carlsbad, CA, U.S.A.) at 37°C in a humidified atmosphere containing 5% CO₂.

Chick Embryo Lethality Assay Drug toxicity was determined using chick embryos as previously described.¹⁶⁾ Eggs were randomly divided into 10 groups (*n*=15) on embryonic day (ED) 10, and drugs were injected into the chorioallantoic membrane (CAM) cavity. Egg viabilities were checked daily for 3 d.

***In Ovo* Xenograft Study** *In ovo* xenograft assay was performed as previously described.¹⁷⁾ A plastic ring was placed on the CAM surface on ED10 and loaded with A549 cells (5×10⁵) in a 1:1 mixture of serum-free medium and Matrigel

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(BD Biosciences, Bedford, MA, U.S.A.). On ED12, drugs were daily injected into the CAM cavity for 3 d. On ED16, tumors were dissected and their weights were measured.

Chick CAM Assay Anti-angiogenic potential of EEGS and cytochalasin H was evaluated using a chick CAM assay as previously described.¹⁸⁾

Animal Studies For *in vivo* tumor xenograft study, A549 cells (5×10^6) were subcutaneously injected into the left flanks of six-week-old male Balb/c^{nu/nu} mice (SLC, Hamamatsu, Japan). On the following day, mice were randomly divided into 3 groups ($n=6$), and receive daily an oral dose of 0.5% carboxymethyl cellulose (CMC) (group 1) or 250 mg/kg/d EEGS (group 2). Group 3 received weekly intraperitoneal injections of cisplatin (5 mg/kg/week). After 6 weeks, the tumor volumes were measured using the formula, $w^2 \times l \times 0.52$, where w is the width and l is the length of the tumor. For lung metastasis study, B16-F10 cells (1×10^5) were administered into six-week-old male C57BL6 mice (Orientbio, Seongnam, Republic of Korea) through tail veins. On the following day, mice were randomly divided into 2 groups ($n=5$) and received daily an oral dose of 0.5% CMC or 250 mg/kg/d EEGS. Non-tumor bearing mice received no treatment. After 3 weeks, lungs were excised from the euthanized mice and their weights were determined. All animal studies were performed according to protocols approved by the Institutional Animal Care and Use Committee at KIOM.

Statistics Differences of the categorical variables were compared by χ^2 -test followed by Bonferroni correction. Differences of the continuous variables were compared by ANOVA followed by *post-hoc* Dunnett test.

RESULTS AND DISCUSSION

We previously demonstrated that EEGS and its active constituent, cytochalasin H, inhibit the angiogenic process *in vitro* and *in vivo* via suppression of pro-angiogenic proteins, such as endothelin-1 (ET-1) and metalloproteinase 2 (MMP2).^{12,15)} We also demonstrated that cytochalasin H may originate from cytochalasin-producing endophytic fungi, such as *Chaetomium globosum*.¹²⁾ In the present study we determined whether EEGS and cytochalasin H with anti-angiogenic potential can inhibit tumor growth *in vivo*. We first determined the *in vivo* toxicity of EEGS and cytochalasin H using chicken embryos to determine a non-toxic dose range because toxicity issues may complicate experiments of the anti-tumor

effect of test drugs. All embryos developed normally when treated with $\leq 250 \mu\text{g}$ of EEGS or $\leq 1.25 \mu\text{g}$ of cytochalasin H per egg on ED10 (Table 1). The calculated LD₅₀ for EEGS and cytochalasin H was 828.0 and 6.2 μg per egg, respectively. The maximum non-toxic doses of cytochalasin H (1.25 μg per egg) and EEGS (250 μg per egg) were 8-fold lower and 25-fold higher than that of cisplatin (10 μg per egg), respectively.

Next, we determined the anti-tumor potential of EEGS and cytochalasin H using an *in ovo* tumor xenograft model with doses showing no toxicity. This assay implicating a human tumor growth on the chick CAM surface is known as a fast and reproducible avian anti-tumor model,¹⁷⁾ and can be used as an alternative *in vivo* model to test anti-tumor potential.⁴⁾ Each egg with a solid tumor was daily treated with drugs for 3 d, and the weights of embryos and tumors were mea-

Fig. 1. Evaluation of the Anti-tumor Potential of EEGS and Cytochalasin H *in Ovo*

A549 cells were xenografted onto the chick CAM surface. Eggs were daily treated with EEGS (200 μg) or cytochalasin H (Cyth, 1 μg) for 3 d. Vehicle (Veh) and cisplatin (CDDP) were included in parallel as negative and positive control drugs, respectively. Anti-tumor effects and toxicities of drugs were determined by measurement of the weights of tumors (gray bars) and embryo bodies (closed circle), respectively. The representative tumors of each group were shown in the lower panel. Data are represented as means $\pm$ S.E. of 10 eggs. * $p < 0.05$.

Table 1. Toxicities of EEGS and Cytochalasin H on Chicken Embryo Development

Drugs	Dose (μg per egg)	Total tested embryos	Dead embryos	Toxicity (%)	Bonferroni
Vehicle	5% DMSO	15	0	0	—
Cisplatin	10	15	0	0	n.s.*
EEGS	125	15	0	0	n.s.
	250	15	0	0	n.s.
	500	15	3	20	n.s.
	1000	15	15	100	$p < 0.0001$
Cytochalasin H	1.25	15	0	0	n.s.
	2.5	15	1	7	n.s.
	5	15	6	40	n.s.
	10	15	12	80	$p < 0.0001$

*n.s., not significant after Bonferroni correction.

sured 2 d after final drug injections. Tumor weights of EEGS (200 μ g)- and cytochalasin H (1 μ g)-treated groups significantly decreased by 28% and 40%, respectively, compared to the vehicle group, which was comparable to the cisplatin (10 μ g)-treated group (Fig. 1). All embryos survived the study, and no significant changes in embryo body weights were observed in any group. Collectively, these data suggest that EEGS and cytochalasin H inhibited tumor growth at non-toxic doses.

The anti-angiogenic activities of EEGS and cytochalasin H were also confirmed using a chick CAM assay with comparable doses used in the *in ovo* xenograft model. On ED4.5, Thermanox coverslips containing various amounts of EEGS, cytochalasin H, or retinoic acid as a positive control, were applied to the CAM surface. After 48 h, inhibition zones of angiogenesis were determined. EEGS at 100 μ g per egg and cytochalasin H at 125 ng per egg effectively inhibited the angiogenesis by 80% and 50%, respectively, without any toxicity (Supplementary materials). Discrepancy in drug toxicities between *in ovo* xenograft and CAM assays is possibly due to (1) the different developmental stages of embryos when test drugs were applied (ED12 for *in ovo* xenograft assay and ED4.5 for CAM assay) and (2) the sites of drug application (CAM cavity for *in ovo* xenograft assay and CAM surface for CAM assay).

Moreover, the effect of EEGS on tumor growth was examined in a tumor xenograft model using immunocompromised

mice. Figure 2A shows that EEGS (250 mg/kg/d) moderately inhibited A549-xenografted tumor growth but it was less effective than cisplatin (5 mg/kg/d). However, EEGS-treated animals appeared healthy and gained weight appropriately throughout the duration of the study period, unlike the cisplatin-treated group, which exhibited significant weight loss after 6 weeks (Fig. 2B). This data confirms that EEGS contains substances that attenuate tumor growth *in vivo*. Our unpublished data showed that intraperitoneal injections of cytochalasin H at 2.5 mg/kg/d (3 injections/week) retarded A549-xenografted tumor growth without any signs of toxicity. The effective dose of cytochalasin H (2.5 mg/kg) was much lower than the reported LD₅₀ of cytochalasin H (12.5 mg/kg in day-old cockerels).¹⁹ However, the anti-tumor effect of cytochalasin H at 2.5 mg/kg/d, 3 injections/week, was smaller than EEGS at 500 mg/kg/d, 5 injections/week (unpublished data).

Our previous studies revealed that EEGS and cytochalasin H down-regulated ET-1 and MMP2 in human umbilical vein endothelial cells (HUVECs).^{12,15} ET-1 was known to up-regulate MMP2 and MMP9 whose activities are required for intra- and extravasation of tumor cells during metastasis by inducing their expression as well as by repressing their endogenous inhibitors, such as tissue inhibitor of metalloproteinase-1 (TIMP1) and TIMP2.²⁰ These previous findings led us to hypothesize that EEGS is able to inhibit tumor metastasis. To prove this hypothesis, we adopted a tail-vein injection of B16-F10 melanoma cells into mice that is a well-known lung metastasis animal model. C57BL/6 mice were injected with B16-F10 cells through tail veins and treated daily with EEGS for 3 weeks. Figure 3 shows that the lungs of mice with metastatic tumors were heavier than normal control. The average lung weight of the EEGS-treated group was 358.5 mg, which is approximately 35% less than the vehicle control group (551.0 mg). However, this difference did not reach statistical

Fig. 2. Evaluation of the Anti-tumor Potential of EEGS *in Vivo*

A549 human lung cancer cells were xenografted onto the flanks of male Balb/c^{nu/nu} mice. EEGS and cisplatin were administered as described in Materials and Methods. Anti-tumor effects and toxicities were determined by measurement of the tumor size (A) and body weight (B). Non-tumor bearing mice were included as a normal control group.

Fig. 3. The Effect of EEGS on the Metastasis of Cancer Cells to Lungs

B16-F10 cells (1×10^5) were injected into the tail veins of animals. EEGS or vehicle was administered as described in Materials and Methods. After 3 weeks, metastasis of B16-F10 cells to lungs was determined by measuring lung weights. Non-tumor bearing mice were included as a normal control group. The representative lungs of each group were shown in the lower panel.

significance.

Taken together, our work demonstrates that EEGS and its active anti-angiogenic compound, cytochalasin H, are effective inhibitors of tumor growth and metastasis *in vivo*. These results reinforce the importance of traditionally used medicinal herbs as important resources for the development of novel anti-cancer agents.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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