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Brief Communication

Licochalcone A, a Major Phenolic Constituent of *Glycyrrhiza inflata*, Suppresses Angiogenin Expression in Colon Cancer Cells

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Tumor angiogenesis, which is essential for tumor growth and tumor metastasis, depends on angiogenic factors produced by tumor cells and/or infiltrating cells such as endothelial cells and immune cells in tumor tissue. Previously, we reported that licochalcone A (LicA), an important bioactive compound of *Glycyrrhiza inflate*, suppresses angiogenesis, tumor growth and metastasis. In this study, we evaluated the effect of LicA on angiogenin production in colon cancer cells because angiogenin is an essential factor to regulate angiogenesis and tumor progression. When we examined the angiogenin levels in three human colon cancer cells, HT-29, SW480 and Caco-2, LicA treatment significantly reduced the amounts of angiogenin among three cancer cell lines. In an *in vivo* study in which mice were implanted with HT-29 cells, oral administration of LicA reduced angiogenin in tumor tissues when compared with vehicle-administered mice. These results suggest that reduced angiogenin in response to LicA treatment may play essential role to inhibit tumor growth, angiogenesis as well as metastasis.

Key Words: Angiogenin, Colon cancer cell, Angiogenesis, Licochalcone A

Angiogenin is a 14 kDa protein that belongs to the pancreatic ribonuclease A (RNase) superfamily and is the first human tumor cell derived protein with *in vivo* angiogenic activity. Although the angiogenin was first isolated from conditioned medium from the human colon adenocarcinoma cell line HT-29 (Fett et al., 1985), various studies have demonstrated that angiogenin is expressed in human cancers or in malignant cell lines and in gastro-intestinal malignancies (Shimoyama et al., 1996; Montero et al., 1998; Tello-Montoliu et al., 2006). In addition, inhibition of angiogenin function through protein-protein interactions blocks the establishment, progression and metastasis in mice (Olson et al., 2001; Kao et al., 2002). Angiogenin may function as a tRNA-specific ribonuclease that binds to actin on the surface of endothelial cells. Once bound, angiogenin is translocated to the nucleus, promoting the endothelial invasiveness necessary for blood vessel formation.

Tumor angiogenesis, which is believed to play a crucial role in the biological behavior of malignant cells, is mediated by several angiogenic factors. Angiogenin is a powerful one of these factors. Etoh et al. (2000) demonstrated that high expression of angiogenin had a significant relationship with vascular involvement, lymph node metastasis, liver metastasis, and advanced clinical stage. These findings suggest that angiogenin contributes to tumor aggressiveness and metastatic potential in colon cancer patients.

Anti-tumor and anti-angiogenic activities of licochalcone A (LicA), a major phenolic constituent of *Glycyrrhiza inflate*, were shown in our previous studies (Kim et al., 2010a; Kim et al., 2010b). Since angiogenin is an essential factor for regulation of angiogenesis, tumor progression and metastasis, we investigated the effects of LicA on angiogenin expression in various colon cancer cells and cancer tissues isolated from a xenografted mouse model in the present study.

HT-29, SW-480 and Caco-2 human colorectal carcinoma cell lines were obtained from American Tissue Culture Collection (ATCC, Manassas, VA, USA). All tumor cell lines were maintained in Dulbecco's minimal essential

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medium (DMEM, Hyclone, Logan, UT) supplemented with 5% fetal bovine serum (Hyclone), 2 mM L-Glutamine, 100 U/ml penicillin and 100 mg/ml streptomycin (Hyclone). Cells were maintained at 37℃ in a 5% CO_2 incubator in monolayer culture to 75% to 90% confluence, at which point they were detached using 0.05% trypsin-EDTA (Hyclone). Cells were counted using trypan blue and adjusted to the desired concentration for plating.

Three colon cancer cell lines were seeded in 96-well plates. After 1 day, various concentrations of LicA (Calbiochem, San Diego, CA) were added to the wells. The effects of LicA on the cell proliferation and cytotoxicity were tested using the CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI) and CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega), respectively.

To measure various angiogenic factors that are affected by LicA treatment, conditioned medium that was cultured with LicA for 48 h in serum free DMEM medium at 37℃ in a 5% CO_2 incubator was collected and passed through a 0.45 μm filter membrane (Sartorius, Hannover, Germany). The amount of angiogenin was determined using human angiogenin Fluorokine MAP (R&D system, Minneapolis, MN) according to the manufacturer's instructions.

Tumor xenografts were established by subcutaneous injection of 2×10^6 HT-29 cells into the flank area of six week-old nu/nu athymic mice (n=5; OrientBio, Seoungnam, Korea). Five days after HT-29 injection, LicA (15 mg/kg body weight) in 200 μl of PBS was administered orally for 10 days. All mice were sacrificed at 15 days after HT-29 injection and tumor tissues were isolated for the measurement of angiogenic factors using a human angiogenesis assay kit (Panomics, Santa Clara, CA) according to the manufacturer's instructions. All animal procedures were approved by the Hallym University Institutional Animal Care and Use Committee, and thus within the guidelines for human care of laboratory animals.

Statistical analysis was performed using GraphPad Prism

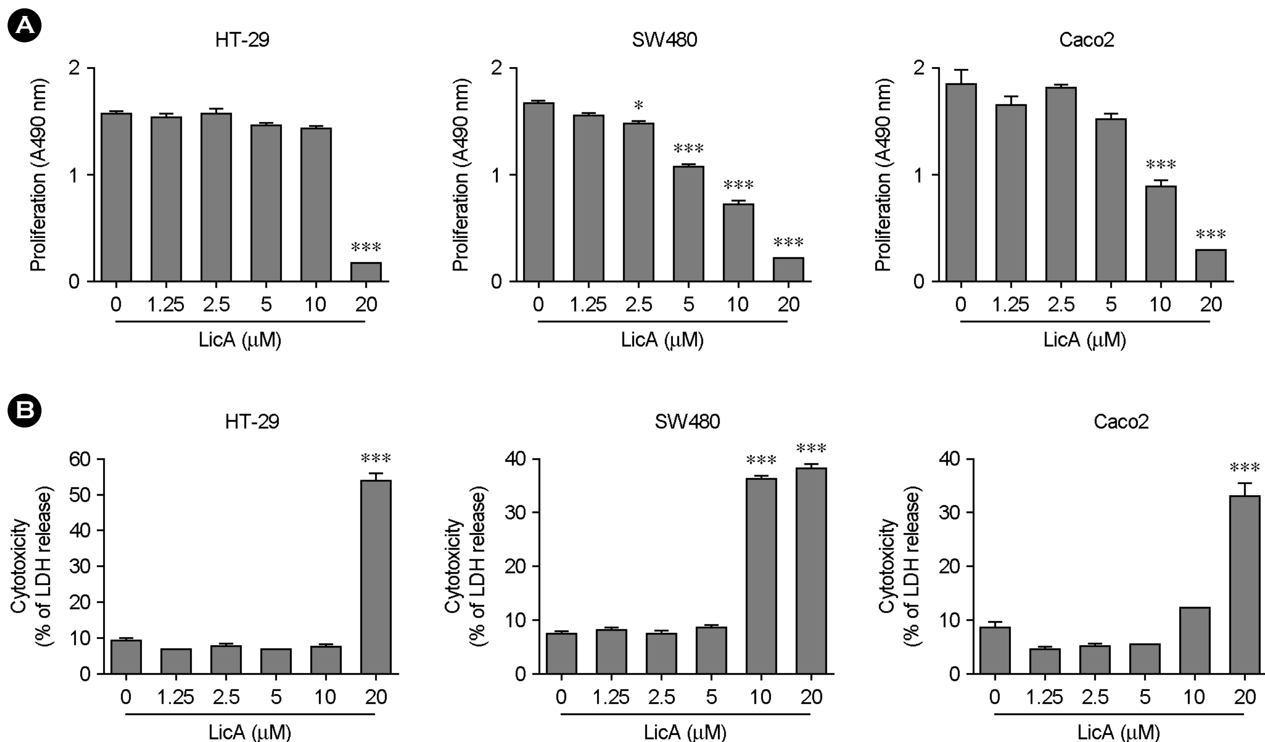


Fig. 1. Effects of LicA on colon cancer cell proliferation and cytotoxicity. (A) HT-29, SW480 and Caco-2 cells were treated with the indicated concentrations of LicA for 48 h, after which proliferation was determined. (B) HT-29, SW480 and Caco-2 cells were treated in the same manner as in Fig. 1A, and the LDH release was determined. The results are reported as mean ± S.E.M. for four independent experiments in triplicate. Statistical significance is based on the difference when compared with 0 μM LicA-treated cells ($^{*}P<0.05$, $^{***}P<0.001$).

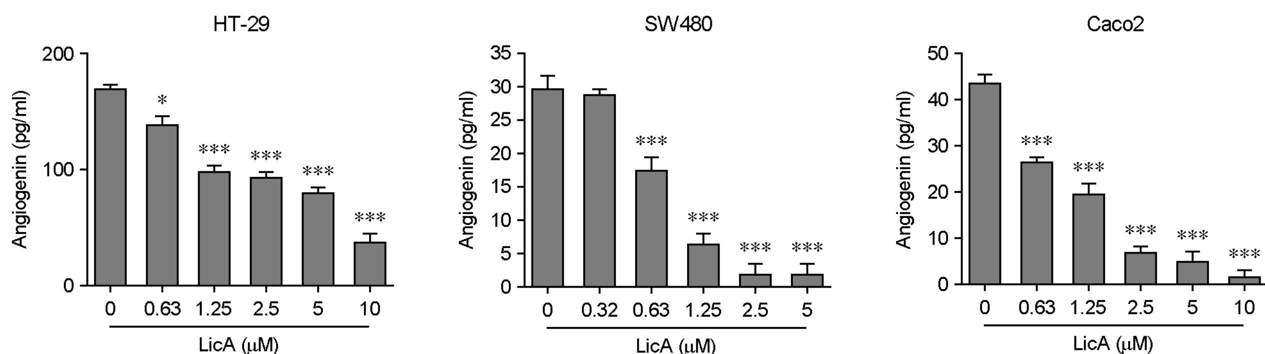


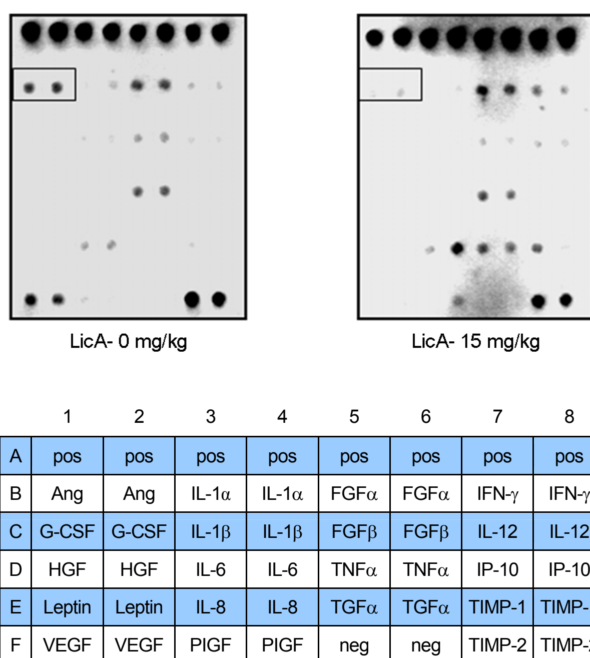
Fig. 2. Effects of LicA on angiogenic release. HT-29, SW480 and Caco-2 cells were treated with the indicated concentrations of LicA for 48 h and the angiogenic amounts were then measured. The results are reported as the mean ± S.E.M. of four independent experiments in triplicate. Statistical significance is based on the difference when compared with 0 μM LicA-treated cells ($^{*}P$<0.05, $^{***}P$<0.001).

4.0 software (GraphPad Software Inc., San Diego, CA). The data shown are the means ± S.E.M. of at least three independent experiments. The values were evaluated by one-way analysis of variance (ANOVA) with Bonferroni multiple comparison post tests. *P* values < 0.05 were considered statistically significant.

We began monitoring the anti-proliferative and cytotoxic effects of LicA in various colon cancer cells, HT-29, SW-480 and Caco-2. As shown in Fig. 1A, treatment with 20 μM, 2.5 μM and 10 μM LicA led to a significant reduction in the proliferation of HT-29, SW480 and Caco-2, respectively. In addition, 20 μM and 10 μM LicA treatment was cytotoxic toward HT-29, Caco-2 and SW480, respectively (Fig. 1B). These results indicate that LicA has the potential to inhibit the growth of colon cancer cells.

Since angiogenin was identified as an angiogenic substance based on its capacity to induce blood-vessel formation, we measured the amounts of angiogenin in conditioned medium that were cultured with various concentrations of LicA that showed no cytotoxic effect in various colon cancer cells. Significantly reduced amounts of angiogenin were detected in all three colon cancer cells, HT-29, SW480 and Caco-2, following LicA treatment (Fig. 2).

An earlier study demonstrated that the establishment of CT-26 tumors, which are mouse colon cancer cells, in syngenic mice was delayed significantly and prevented by the administration of LicA (Kim et al, 2010b). Since these effects were observed in response to administration of greater than 15 mg/kg LicA, we administered LicA orally for ten days and then measured the levels of angiogenin in



	1	2	3	4	5	6	7	8
A	pos	pos	pos	pos	pos	pos	pos	pos
B	Ang	Ang	IL-1α	IL-1α	FGFα	FGFα	IFN-γ	IFN-γ
C	G-CSF	G-CSF	IL-1β	IL-1β	FGFβ	FGFβ	IL-12	IL-12
D	HGF	HGF	IL-6	IL-6	TNFα	TNFα	IP-10	IP-10
E	Leptin	Leptin	IL-8	IL-8	TGFα	TGFα	TIMP-1	TIMP-1
F	VEGF	VEGF	PIGF	PIGF	neg	neg	TIMP-2	TIMP-2

Fig. 3. Effect of LicA on angiogenin production in an *in vivo* xenograft tumor model. Six week-old nu/nu athymic mice injected with HT-29 cells were randomly assigned and treated with vehicle or LicA. Tumors were collected 15 days after tumor implantation and various angiogenic factors were then measured using a human angiogenesis array.

tumor tissues isolated from mice using a human angiogenesis array. As shown in Fig. 3, dramatically decreased levels of angiogenin were observed in LicA-treated mice when compared with PBS-treated mice. In addition to angiogenin, tumor necrosis factor-α, interleukin-8, tumor growth factor-α and VEGF, which are important angiogenic factors, also reduced LicA administration (Fig. 3). These results are consistent with our previous finding that LicA

inhibited the production of various angiogenic factors in human endothelial cells (Kim et al., 2010b). Taken together, these results suggest that LicA treatment blocks the production of essential angiogenic factors to regulate angiogenesis and cancer progression in both endothelial cells and cancer cells.

In summary, the results of this study demonstrated that LicA negatively regulated angiogenin production in colon cancer cells and tissues isolated from xenografted mice. Based on our previous results together with the present data, we suggest that LicA exerts anti-angiogenesis and anti-tumor effects via a reduction of angiogenin in cancer cells, at least in part. Further investigation of the detailed mechanism involved in the downregulation of angiogenin by LicA will help provide a better understanding of the physiological activity of LicA.

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