Inhibitory Effect of Isoliquiritin, a Compound in Licorice Root, on Angiogenesis in Vivo and Tube Formation in Vitro

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A water extract of licorice root inhibits granuloma angiogenesis in adjuvant-induced chronic inflammation (Phytther Res., 5, 195, 1991). The present study has investigated the effects of licorice-derived compounds on granuloma angiogenesis. Isoliquiritin (0.31—3.1 mg/kg), a licorice-derived flavonoid, inhibited the carmine content of granuloma tissue 50-fold greater than licorice extract. Glycyrrhizin (20—80 mg/kg), a licorice-derived saponin, inhibited carmine content with a weak potency. The licorice extract (0.01—1 mg/ml) also inhibited tube formation from vascular endothelial cells in a concentration-dependent manner. From the chemical structure—activities of used licorice-derived flavonoids (0.1—100 µM), their potencies for anti-tube formation were in the order isoliquiritigenin > isoliquiritin > liquiritigenin > isoliquiritin-apioside. Glycyrrhizin (0.1—100 µM) and glycyrrhetinic acid (0.1—10 µM) increased tube formation. A glycyrrhizin (82 µg/ml)-induced increase in tube formation was inhibited by isoliquiritin. The combined effect of a mixture of 82 µg/ml glycyrrhizin and 4.2 µg/ml isoliquiritin, a similar concentration ratio to their yield ratio in the licorice extract, corresponded to the effect of 100 µg/ml extract. In conclusion, the anti-angiogenic effect of licorice extract depended on the anti-tube formation effect of isoliquiritin.

**Key words** licorice-derived flavonoid; anti-angiogenesis; anti-tube formation; isoliquiritin; glycyrrhizin; vascular endothelial cell

Licorice root, a Japanese Sino-medicine, is included in traditional Chinese prescriptions containing Keishika-jutsu-ku and Kakkon-to-ka-senkyu-shin 1,2) It has been used clinically in the treatment of inflammatory diseases. The inflammatory process generally involves fluid exudation, migration and infiltration of inflammatory cells, angiogenesis and granuloma formation in this order. We have reported a quantitative method for the determination of granuloma angiogenesis in adjuvant-induced chronic inflammation in mice 3—5) This method is useful for the simultaneous measurement of angiogenesis, granuloma formation and fluid exudation in the chronic inflammation. These inflammatory parameters were determined by measuring the carmine content in newly formed blood vessels, granuloma tissue weight and pouched fluid weight, respectively. By using this method, the water-extract of licorice inhibits angiogenesis, granuloma formation and pouched fluid exudation in chronic inflammation. 1) Licorice-derived saponins containing glycyrrhizin and glycyrrhetinic acid have been reported to inhibit acute inflammation models 6,7) A licorice-derived flavonoid, isoliquiritigenin, inhibits histamine release from mast cells in the type I allergy. 8) But little is known about anti-angiogenic effects of these saponins and flavonoids in chronic inflammation.

Many sites of action of drugs are involved in angiogenic events which include degradation of vascular basement membranes, migration and proliferation of vascular endothelial cells (EC) and the organization of capillaries, including tube formation of EC. 9) We have reported an assay model for in vitro tube formation from vascular EC by culturing them in type I collagen gel with 2% fetal bovine serum (FBS). 10) Tube formation is enhanced by cytokines released from interferon-γ-activated macrophages.

The aim of the present study is to determine the effects of licorice-derived saponins and flavonoids on granuloma angiogenesis in adjuvant-induced chronic inflammation. We further investigated the chemical structure—activity of these compounds on tube formation to study the anti-angiogenic effects of licorice.

**MATERIALS AND METHODS**

**Animals** Male ddY mice (6—7 weeks of age) and male Wistar rats (9 weeks of age) were purchased from Japan Shizuoka Laboratory Center (Hamamatsu) and maintained under a constant temperature (23 ± 1°C) with lights on from 8 a.m. to 6 p.m., and were fed the usual laboratory diet (CA-1, Clea Japan, Tokyo) and tap water freely.

**Adjuvant-Induced Pouchn Granuloma Angiogenesis** Air pouch granuloma angiogenesis was induced by Freund’s complete adjuvant (FCA) with croton oil as reported. 4,5) The FCA emulsion was prepared by a 2 mg heat-killed *M. tuberculosis* (from Professor I. Azuma, Hokkaido University) per ml of Freund’s incomplete adjuvant. Three ml of air was injected subcutaneously into the dorsum under ether anesthesia to produce a regular ellipsoid air sac. After 24 h, 0.5 ml of FCA emulsion containing 0.1% croton oil (Nacalai Tesque, Kyoto) was injected into the air pouch under ether anesthesia. Mice were killed after injection with 10% carmine solution (Merck, Darmstadt, Germany) containing 5% gelatin (Nacalai Tesque), which had been kept warm at 40°C, into the tail vein on day 5 after FCA injection. The cadavers were cooled at a temperature below 4°C for several hours. The weights of granuloma tissues and pouch fluid isolated from the pouch were measured. The granuloma tissues were cut, suspended, solubilized and acidified to measure the carmine content. After centrifugation, the supernatant was filtered. The carmine content in the filtrate was determined by measuring the optical density at 490 nm. The carmine content is an index of newly formed blood vessels in the pouch granuloma. 4,5)

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Tube Formation by Cultured Aortic Endothelial Cells
Primary cultured EC from the thoracic aorta of Wistar rats were prepared as reported.\textsuperscript{1,12} EC were cloned from 100–200 primary cells in Dulbecco’s modified eagle medium (DMEM, Nissui, Tokyo) supplemented with 10% heat-inactivated FBS (Bioproduct, Walkersville, MD, U.S.A.), 160 U/ml benzyl penicillin potassium (Banyu Seiyaku, Tokyo) and 0.1 mg/ml streptomycin sulfate (Meiji Seika, Tokyo) on a 0.03% type I collagen (1-AC, Koken, Tokyo)-coated 16-mm dish (Corning, Corning, NY, U.S.A.). EC (the 5th-14th passages) were cultured 2–11 weeks after confluence in 10% FBS-DMEM without antibiotics under 5% CO\textsubscript{2} and 95% air. The post confluent cells were washed with Ca\textsuperscript{2+}, Mg\textsuperscript{2+}-free phosphate-buffered saline (PBS) and 0.02% EDTA in PBS, detached by 0.25% trypsin-0.02% EDTA in PBS and harvested in 10% FBS-DMEM without antibiotics. The EC ([2.6 \pm 0.1] \times 10^4/well) were cultured in 10% FBS-DMEM (0.5 ml) at 37°C for 20 to 24 h on a collagen gel that was prepared by solidifying 0.3 ml of 0.15% type I collagen solution in a 16-mm dish. The EC-cultured medium was aspirated and the same volume of collagen solution was overlaid and solidified. The EC were cultured with 2% FBS-DMEM in the presence or absence of drugs for 4 d. The medium was changed every other day.

Measurement of Tube Formation
Tubes that developed from EC were photographed with a Leitz Diavert camera equipped with a Wild photometr automat MPS45 (Leitz, Germany). Four fields selected randomly from each 16-mm dish were photographed at \times 36 for day 4 after overlying collagen gel. Typical photographs of tubes were presented in our previous paper.\textsuperscript{10} These experiments were repeated at least 3 times. The lengths of all tubes on a photograph (\times 36) were measured and tabulated with Graphic Software MEAS I (Graphtec Corp., Tokyo) to provide total tubular length, which is an index of tube formation.\textsuperscript{10}

Licorice Extract and Its Derived Compounds
A water extract of licorice root (Glycyrrhiza uralensis FISHER, Neimenggu, China) was prepared at 50°C as reported.\textsuperscript{13} Licorice-derived saponins such as glycyrrhizin and glycyrhydrinic acid (Minophagen Co., Tokyo), and its derived flavonoids such as isoliquiritin, isoliquiritin-apioside, isoliquiritigenin and liquiritigenin (Tsumura, Tokyo) were used. Chemical structures of these compounds are presented in Fig. 1.\textsuperscript{14–17} The licorice extract, glycyrrhizin and isoliquiritin were suspended homogeneously in saline containing 1% Avicel (Asahi Chemical Industry, Tokyo), and injected intraperitoneally into mice 2 h after FCA injection and then subsequently once a day for 4 d. For the experiment involving tube formation, these drugs were dissolved in less than 0.1% ethanol (a final concentration), diluted with 2% FBS-DMEM and administered into the EC culture medium. The medium was changed every other day.

Statistical Analysis
Data were expressed as means ± S.E. and tested by one-way analysis of variance. The significant difference was assessed by Scheffe’s or Tukey’s test at \( p = 0.05 \) or 0.01.

RESULTS

Anti-angiogenic Effects of Licorice-Derived Compounds in Adjuvant-Induced Pouch Granuloma
The effects of isoliquiritin and glycyrrhizin on granuloma angiogenesis were compared with that of licorice extract in adjuvant-induced chronic inflammation. Isoliquiritin (0.31–1.3 mg/kg) inhibited the carmine content in granuloma tissues in a dose-dependent manner (Fig. 2). Its 50% inhibitory dose value (ID\textsubscript{50}; 95% confidence limits) was assumed to be 1.46 mg/kg (0.82–2.58). The potency of isoliquiritin was 50-fold greater than that of the licorice extract. Glycyrrhizin (20–80 mg/kg) inhibited the carmine content, but its effect was less than 50% of the control without glycyrrhizin and weaker than that of the licorice extract. These three drugs simultaneously inhibited granuloma weight, but their effects were less than 50%. Isoliquiritin also inhibited the weight of pouch fluid and its ID\textsubscript{50} was 0.771 mg/kg (0.513–1.18). The potency of isoliquiritin was 18-fold greater than that of the licorice extract, but the potency of glycyrrhizin was smaller. These results demonstrated that the potency ratio of isoliquiritin to that of the licorice extract for angiogenesis was greater than...
Fig. 2. Effects of Isoliquiritin, Licorice Extract and Glycyrrhizin on Carmine Content, Granuloma Weight and Pouch Fluid Weight in Adjuvant-Induced Chronic Inflammation Model

Isoliquiritin (●, 0.31–1.3 mg/kg), licorice extract (○, 12.5–100 mg/kg) and glycyrrhizin (■, 20–80 mg/kg) were suspended in 1% Avicel-saline and injected intraperitoneally once a day for 5 d. Absolute values of carmine content, granuloma weight and pouch fluid weight without drug were 0.256 ± 0.017 mg (n = 22), 353 ± 15 mg (n = 23) and 144 ± 26 mg (n = 21), respectively. The values are expressed as the means ± S.E. of % relative amount to the value without drug.

Fig. 3. Inhibitory Effect of Licorice Extract on Tube Formation from Cultured Vascular Endothelial Cells (EC)

EC were cultured in type I collagen gel with 2% FBS-DMEM in the presence of 1–1000 μg/ml licorice extract for 4 d. Values of % relative tubular length to value without drug (2.62 ± 0.08 mm/mm² dish, n = 21) are expressed as the means ± S.E. of data of 4 analyses in 3 dishes.

for fluid exudation.

Effects of Licorice-Derived Compounds on Tube Formation from Cultured Aortic Endothelial Cells Licorice extract (0.01–1 mg/ml) inhibited tube formation from EC cultured in type I collagen gel with 2% FBS-DMEM in a concentration-dependent manner (Fig. 3). Its value of 50% inhibitory concentration (IC₅₀) was 0.518 mg/ml (0.352–0.764). Isoliquiritin (1–100 μM) also inhibited tube formation in a concentration-dependent manner (Fig. 4). Its IC₅₀ value was 28.3 μM (20–40). The potency of isoliquiritin for anti-tube formation was 44-fold greater than that of licorice extract. Its potency ratio was similar to the ratio for anti-angiogenesis.

The chemical structure-activities of licorice-derived flavonoids were investigated on tube formation (Fig. 4). Isoliquiritigenin (0.1–10 μM), a chalcone derivative, and liquiritigenin (0.1–100 μM), a flavanone derivative, inhibited

Fig. 4. Inhibitory Effects on Tube Formation of Isoliquiritin, Isoliquiritigenin (●), Liquiritigenin (■) and Isoliquiritin-Apiside (○)

These flavonoids (0.1–100 μM) were incubated in EC-culture for 4 d. The values of % relative tubular length to the value without drug (data shown in Fig. 3) are expressed as the means ± S.E. of 4 analyses of 3 dishes.

Fig. 5. Increased Effects of Glycyrrhizin and Glycyrrhetic Acid on Tube Formation

Glycyrrhizin (0.1–100 μM) and glycyrrhetic acid (0.1–10 μM) were incubated in EC-culture for 4 d. The values of % relative tubular length to the value without drug (data shown in Fig. 3) are expressed as the means ± S.E. of 4 analyses of 3 dishes.

tube formation in a concentration-dependent manner. These IC₅₀ values were 7.39 μM (4.78–11.4) and 39.2 μM (17.1–90.1), respectively. Isoliquiritigenin-apiside had no effect on tube formation. The potency of isoliquiritigenin was 3.6-fold greater than that of isoliquiritin. The potency of isoliquiritigenin was also 5.3-fold greater than that of liquiritigenin. These results demonstrated that increased glycosides at the C-4 position of isoliquiritigenin decreased the anti-tube formation effect. The effect of the chalcone derivative was greater than that of the flavanone derivative.

Glycyrrhizin (1–100 μM) and glycyrrhetic acid (1–10 μM), licorice-derived saponins, in contrast, increased tube formation in a concentration-dependent manner (Fig. 5). The increasing effect of glycyrrhetic acid was 10-fold greater than that of glycyrrhizin.

Combined Effect of Isoliquiritin and Glycyrrhizin on Tube Formation from Cultured Aortic Endothelial Cells The combined effects of the mixture of isoliquiritin and
glycyrrhizin were investigated on EC tube formation. The increasing action of glycyrrhizin (82 µg/ml) on tube formation was significantly inhibited by isoliquiritin (0.42—42 µg/ml) in a concentration-dependent fashion (Fig. 6). The inhibitory effect of isoliquiritin in the presence of glycyrrhizin was 0.28-fold weaker than that of isoliquiritin in its absence. The effect of the mixture of isoliquiritin and glycyrrhizin at the concentration ratio of 0.05:1 was similar to that of licorice extract (100 µg/ml), in which their yield ratio is estimated to be similar to their ratio in the mixture. These results demonstrated that the effects of these compounds on tube formation were negatively combined in the licorice extract.

DISCUSSION

It has been reported that the anti-inflammatory action of licorice depends on two main compounds, glycyrrhizin and its aglycone, glycyrrhetic acid.6 7 However, the present study demonstrated that the anti-angiogenic effect of licorice in adjuvant-induced chronic inflammation was not associated with the effect of glycyrrhizin. This result is supported by several lines of evidence. First, glycyrrhizin inhibited angiogenesis with a weaker potency than that of the licorice extract. Secondly, the licorice extract inhibited tube formation from EC in the angiogenic process, but glycyrrhizin increased it. Therefore, compounds other than glycyrrhizin in licorice were required for the inhibition of angiogenesis.

Little is known about the anti-inflammatory action of licorice-derived flavonoids containing isoliquiritin. The present study indicated that isoliquiritin potently inhibited granuloma angiogenesis and pouch fluid exudation, but not granuloma formation. These effects of isoliquiritin were similar to those of the licorice extract. The potency of isoliquiritin on angiogenesis was 50-fold greater than that of the licorice extract. Their potency ratio corresponded to their yield ratio in the licorice extract, which is 0.8—1.6%.18 These results demonstrated that the anti-angiogenic effect of licorice depended on the effect of isoliquiritin. For inhibition of the pouch fluid exudation, their potency ratio was smaller than that for antiangiogenesis, indicating that isoliquiritin mainly inhibited angiogenesis.

The licorice extract and isoliquiritin also inhibited tube formation from EC. The inhibitory concentration range of licorice extract for tube formation nearly overlapped the inhibitory dose range of the extract for angiogenesis. The potency ratio of isoliquiritin to the licorice extract for anti-tube formation was parallel with their ratios for anti-angiogenesis and the yield ratio of isoliquiritin from the extract.13 18 However, glycyrrhizin increased tube formation but inhibited angiogenesis at higher dosages. These results demonstrated that the anti-angiogenic effect of isoliquiritin depended on the tube formation.

The licorice-derived flavonoids also inhibited tube formation in the angiogenic process. From the chemical structure—activity of these chalcone derivatives in flavonoids, increased glycosides at the C-4 position of an aglycone decreased the anti-tube formation effect. The potency of the aglycone of chalcone derivatives was greater than that of the flavanone derivative. The yields of isoliquiritin, isoliquiritigenin and liquiritigenin are estimated to be 0.8—1.6%, 0.04—0.08 and 0.12—0.4% from the licorice extract, respectively.13 18 From these potencies and yields of flavonoids in licorice extract, isoliquiritin is suggested to play a main role in the inhibition of licorice extract on tube formation in angiogenesis.

Licorice-derived saponins showed adverse effects on tube formation. A glycyrrhizin-induced increase in tube formation was inhibited by isoliquiritin. The concentration ratio in the mixture of glycyrrhizin (82 µg/ml) and isoliquiritin (4.2 µg/ml) was similar to the ratio of their yields in the licorice extract. The effect of their mixture was similar to that of the licorice extract (100 µg/ml). In addition, 82 µg/ml glycyrrhizin and 4.2 µg/ml isoliquiritin are speculated to be contained in 256 µg/ml of licorice extract. From the concentration-response curve of the extract, the effect of 256 µg/ml extract was calculated as 63% inhibition. This speculated effect of the extract is not different from the effect of the mixture of both drugs. These results indicated that the effects of glycyrrhizin and isoliquiritin on tube formation were negatively combined in the licorice extract. The balance of yields of these compounds in the licorice extract is important for their combined effect on tube formation.

Many modes of anti-angiogenic drug action have been suggested in the angiogenic process: the degradation of basement membranes, the migration and proliferation of EC and tube formation.9 The anti-angiogenic mode of isoliquiritin was associated with the inhibitory action on tube formation. However, glycyrrhizin inhibited angiogenesis, but increased tube formation. Glycyrrhizin has also been reported to inhibit the proliferation of tumor cells.19

Fig. 6. Combined Effects of Mixture of Isoliquiritin and Glycyrrhizin on Tube Formation

Isoliquiritin (0.42—42 µg/ml) was incubated in EC-culture in the presence (open columns) or absence (open circles) of 82 µg/ml glycyrrhizin. The data of licorice extract (closed circles) are replotted from Fig. 3. The concentration ratios of isoliquiritin to glycyrrhizin are represented at the bottom in this figure. The ratio of isoliquiritin (4.2 µg/ml) to glycyrrhizin (82 µg/ml) was set up according to their ratio in licorice extract (shadowed column; 100 µg/ml). a, glycyrrhizin 82.3 µg/ml + isoliquiritin; b, licorice extract. The values of % relative tubular length to the value without drug (data shown in Fig. 3) are expressed as the means ± S.E. of 4 analyses of 3 dishes. a) p < 0.05, b) p < 0.01: Significantly different from without glycyrrhizin.
These results suggested that glycyrrhizin may have dual actions: the activation of tube formation at a low concentration and the inhibition of EC proliferation at a higher concentration. Since the anti-angiogenic effect of glycyrrhizin was weaker, it did not play a main role in the anti-angiogenic effect of licorice.

In conclusion, the anti-angiogenic effect of licorice extract depended on the anti-tube formation of isoliquiritin.

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