Inhibitory Effect of Magnosalin Derived from *Flos magnoliae* on Tube Formation of Rat Vascular Endothelial Cells during the Angiogenic Process

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An aqueous water extract of *Flos magnoliae*, a Japanese Sino-medicine, inhibits angiogenesis in adjuvant-induced mouse pouch granuloma. Magnosalin (MSA) and magnoshinin (MSI), neolignans isolated from magnolia, have a crucial role in the anti-angiogenic effect of magnolia (Kimura et al., *Int. Arch. Allergy Appl. Immunol.*, 93, 365 (1990); *Phytother. Res.*, 6, 209 (1992)). We investigated the effects of these neolignans on tube formation of endothelial cells (EC) cultured in type I collagen gel during the angiogenic process. MSA (0.1—10 μM), MSI (0.23—7 μM) and corticosterone (CS: 0.3—30 μM) inhibited fetal bovine serum (FBS)-stimulated tube formation in a concentration-dependent manner. Their 30% inhibitory concentration (IC₃₀: 95% confidence limits) values were 0.51 (0.20—1.27) for MSA, 8.14 (2.48—26.7) for MSI and 3.65 μM (2.47—5.40) for CS, respectively. MSA and MSI (1—3 μM) also inhibited interleukin (IL)-1β-stimulated tube formation in a concentration-dependent manner. Their IC₃₀ values (95% confidence limits) were 1.22 (1.01—1.47) for MSA and 0.74 μM (0.24—2.31) for MSI against a submaximal concentration (69 μM) of IL-1β-stimulated tube formation. Their inhibitory effects on the action of IL-1β were non-competitive. These results demonstrate that MSA inhibited FBS-stimulated tube formation with a greater potency than MSI. The inhibitory effect of MSA on the action of FBS differed from that on the action of IL-1β.

Key words: magnosalin; magnoshinin; anti-tube formation; anti-IL-1β activity; anti-angiogenesis

*Flos magnoliae*, Japanese Sino-medicines, are combined in a traditional prescription, “Kakkon-to-ka-senkyushin’i” (KSS). The prescription has been used clinically for treating inflammatory nasal diseases. KSS inhibits four inflammatory parameters: fluid exudation, migration and infiltration of inflammatory cells, angiogenesis and granuloma formation in the mouse adjuvant-induced chronic inflammation model. The anti-angiogenic effect is determined by measuring the carmine content in newly formed blood vessels. The aqueous extract of magnolia was found to be the main fraction responsible for the anti-angiogenic effect of KSS. Magnosalin and magnoshinin, neolignans derived from magnolia, inhibit the angiogenesis and granuloma formation, but not the pouch fluid exudation following oral and intra-pouch administration.

Magnosalin administered intraperitoneally also inhibits the angiogenesis to a greater extent than the granuloma formation and pouch fluid exudation. The anti-angiogenic effect of magnosalin is greater than that of magnoshinin. However, hydrocortisone and corticosterone inhibit more potently the three inflammatory parameters. Thus, it seems that these inhibitory patterns of neolignans for the three parameters differ from those of hydrocortisone and corticosterone.

Many sites of action for drugs are possible in angiogenic events, involving degradation of vascular basement membranes, migration and proliferation of rat vascular endothelial cells (EC) and tube formation. We have described assay models for the proliferation of vascular EC and tube formation by culturing EC on, and in, type I collagen gel. Magnosalin and magnoshinin inhibit fetal bovine serum (FBS)-stimulated proliferation of EC in a concentration-dependent manner on the collagen gel. The inhibitory effect of magnoshinin on the proliferation of EC is greater than that of magnosalin. These results demonstrate that the anti-angiogenic action of magnosalin is different from its inhibitory action on EC proliferation. Macrophages are activated by various factors and, in turn, release many factors. Interleukin (IL)-1β is released to a greater extent than basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF; BB-homodimer) from interferon (IFN)-γ-activated intraperitoneal macrophages of rat. The activity of IL-1β on the tube formation of EC is greater than that of bFGF and PDGF-BB. However, IL-1β inhibits bFGF-stimulated proliferation of EC. These results demonstrate that IL-1β has opposite actions in different phenotypes of EC, namely tube formation and proliferation.

The aim of the present study is to determine the effects of magnosalin and magnoshinin on the tube formation of vascular EC to clarify their anti-angiogenic activity. The effect of magnosalin on the action of IL-1β was further investigated.

MATERIALS AND METHODS

Tube Formation by Cultured Vascular Endothelial Cells

Primary cultured EC from the thoracic aorta of Male Wistar rats (9—10 weeks of age, Japan Shizuoka Laboratory Center, Hamamatsu) were prepared as reported. The EC were cloned from 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.). The EC (the 5—14th passages) were cultured 2—11 weeks after confluence in 10% FBS—DMEM without antibiotics under 5% CO₂ and 95% air. The post confluent cells were washed with Ca²⁺-, Mg²⁺-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 \([2.6 \pm 0.1] \times 10^4/\text{well}\) 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 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 1% or 2% FBS–DMEM for 4 d and 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 photoautomat MPS54 (Leitz, Germany). Four fields selected randomly from each 16-mm dish were photographed at \(\times 36\) magnification on day 4 after overlaying collagen gel. Typical photographs of tubes were presented in an earlier paper. 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 (Graphitec Corp., Tokyo) to provide the total tubular length, which is an index of tube formation.

Agents: Magnosalin and magnoshin were isolated from magnolia by Professor Kikuchi in the Institute for Wakan-yaku (Oriental Medicines) of our university. These compounds and corticosterone 21-acetate (Sigma, St. Louis, MO, U.S.A.) were dissolved in ethanol, diluted more than 1000-fold with 1% or 2% FBS–DMEM (a final concentration of less than 0.1% ethanol) and introduced into the medium of EC cultured in type I collagen gel in the presence or absence of various concentrations of recombinant mouse IL-1α (Genzyme, Cambridge, MA, U.S.A.). The EC were cultured for 4 d. The medium was changed every other day.

Statistical Analysis: Data were expressed as means \(\pm\) S.E. and tested by one-way analysis of variance. The significance of any differences was assessed by Scheffe’s and Tukey’s tests at \(p = 0.05\) or 0.01, respectively.

RESULTS

Inhibitory Effects of Magnosalin and Magnoshin on the Tube Formation of Vascular Endothelial Cells: To investigate inhibitory modes of action of magnosalin and magnoshin in the angiogenic process, the effects of these neolignans were compared with that of corticosterone on the tube formation of rat vascular EC cultured in type I collagen gel with 2% FBS–DMEM for 4 d. Magnosalin \((0.1–10 \mu M)\), magnoshin \((0.23–7 \mu M)\) and corticosterone \((0.3–30 \mu M)\) inhibited 2% FBS-stimulated tube formation in a concentration-dependent manner (Fig. 1). However, their effects did not exceed 50% inhibition of the control without drug. Their 30% inhibitory concentration \((IC_{30})\) values with 95% confidence limits were 0.51 \(\mu M\) \((0.20–1.27)\) for magnosalin, 8.14 \(\mu M\) \((2.48–26.7)\) for magnoshin in 3.65 \(\mu M\) \((2.47–5.40)\) for corticosterone, respectively. These results demonstrate that the potency of magnosalin was significantly greater than those of magnoshin and corticosterone.

Inhibitory Effects of Magnosalin and Magnoshin on Interleukin-1α-Stimulated Tube Formation: Murine recombinant IL-1α \((6.9–690 \mu M)\) stimulated tube formation in the presence of 1% FBS–DMEM in a concentration-dependent manner (Fig. 2). FBS \((2%)\) also significantly increased tube formation, compared with 1% FBS alone (Figs. 1, 2). Magnosalin and magnoshin \((1–3 \mu M)\) inhibited IL-1α-stimulated tube formation in a concentration-dependent manner (Fig. 2). The \(IC_{50}\) values with 95% confidence limits of magnosalin and magnoshin on tube formation induced by a submaximal concentration \((69 \mu M)\) of IL-1α was 1.22 \(\mu M\) \((1.01–1.47)\) and 0.74 \(\mu M\) \((0.24–2.31)\), respectively. These values were not significantly different from each other. The inhibitory effects of these neolignans on IL-1α-stimulated tube formation...
formation were non-competitive. Since magnosalin inhibited FBS-stimulated tube formation to the greater degree than magnoshinin, the inhibitory effects of these neolignans on the action of IL-1x were different from those on the action of FBS.

DISCUSSION

To study the sites of action of anti-angiogenic agents, bioassays have been developed to systematically investigate not only the entire process of angiogenesis in vivo but also the individual steps, including capillary tube formation and proliferation of vascular EC.\textsuperscript{10,13} The present study focused on capillary tube formation. The inhibitory effects of magnosalin were compared with those of magnoshinin and corticosterone on FBS-stimulated tube formation developed by EC cultured for 4 d in type I collagen gel. The potency of magnosalin was 0.5\(\mu\)M (IC\(_{30}\)) and this was 16-fold and 7.2-fold greater than those of magnoshinin and corticosterone, respectively. The potency order of magnosalin and magnoshinin corresponded to the order for their anti-angiogenic activities in vivo.\textsuperscript{4} However, magnolol (7\(\mu\)M) does not significantly inhibit the number of proliferative EC cultured in 5% FBS for 4 d.\textsuperscript{9} These results demonstrate that magnosalin inhibits tube formation more effectively than EC proliferation. The inhibitory effect of magnosalin on tube formation was compared with its inhibitory effect on thymidine incorporation in different phenotypes of EC. The inhibitory action of magnosalin on FBS-stimulated thymidine incorporation has been sub-divided into the inhibitory action on the competence phase in the differentiated EC and that on the progression phase in the proliferative EC.\textsuperscript{9} Magnosalin inhibits the progression phase rather than the competence phase.\textsuperscript{9} These results suggest that the anti-tube forming action of magnosalin may not be associated with the inhibition of the competition phase in the differentiated EC.

The anti-tube forming action of magnosalin was compared with that of corticosterone. Hydrocortisone inhibits angiogenesis in vivo to an extent 21-fold greater than magnosalin.\textsuperscript{41} Corticosterone has a similar potency for anti-angiogenesis in vivo.\textsuperscript{7} However, corticosterone had a weaker potency in terms of anti-tube formation than magnosalin. These results suggest that these glucocorticoids may have a minor role in anti-tube formation, and their actions are different from that of magnosalin.

IL-1x is suggested to have opposite actions in different phenotypes of EC, increasing tube formation and decreasing EC proliferation.\textsuperscript{10,13} IL-1x stimulates tube formation more effectively than PDGF-BB and bFGF.\textsuperscript{11} In addition, IFN-\(\gamma\)-activated rat peritoneal macrophages release IL-1x rather than bFGF and PDGF-BB,\textsuperscript{11} suggesting that IL-1x has a predominant role in the tube formation of vascular EC during chronic inflammation. Anti-IL-1x antibody inhibits the tube-forming action of IL-1x, but does not inhibit the action of FBS.\textsuperscript{11} In addition, IL-1x inhibits bFGF-induced proliferation of EC,\textsuperscript{13} but FBS stimulates EC proliferation. These results demonstrate that IL-1x and FBS bind to different receptors for tube formation and EC proliferation. Magnosalin inhibited FBS-stimulated tube formation more effectively than magnoshinin, but inhibited IL-1x-stimulated tube formation to the same degree as magnoshinin. The inhibitory effect on the IL-1x-action were non-competitive. These results demonstrate that magnosalin inhibits the actions of FBS and IL-1x by different mechanisms. Magnosalin and magnoshinin may have a similar inhibitory mechanism for their action on IL-1x.

In conclusion, magnosalin inhibited FBS-stimulated tube formation to a greater extent than magnoshinin in the angiogenic process. The inhibitory effect of magnosalin on the action of FBS differs from that on the action of IL-1x.

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REFERENCES AND NOTES

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