

Inhibitory Effects of Magnoshinin and Magnosalin, Compounds from “*Shin-i*” (*Flos Magnoliae*), on the Competence and Progression Phases in Proliferation of Subcultured Rat Aortic Endothelial Cells

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ABSTRACT—Anti-proliferative effects of magnoshinin and magnosalin derived from “*Shin-i*” (*Flos magnoliae*) were investigated using subcultured endothelial cells (EC) of rat aorta. The inhibitory effects of magnoshinin were 2-fold greater at 10 $\mu\text{g/ml}$ than that of magnosalin on the increase in cell number when EC were stimulated by 5% fetal bovine serum. In the ^3H -thymidine incorporation monitored at 3 hr-intervals, magnoshinin (0.1–3 $\mu\text{g/ml}$) prolonged the starting time of DNA synthesis and reduced the rate of incorporation into EC. Magnosalin (0.3–3 $\mu\text{g/ml}$) reduced only the incorporation rate. These results suggest that magnoshinin inhibits both the competence phase and progression phase, but magnosalin preferentially inhibits the progression phase in EC proliferation.

Keywords: Neolignan-derivatives (magnoshinin and magnosalin), Endothelial cell proliferation

“*Shin-i*” (*Flos magnoliae*) is composed of dried flower buds of *Magnolia salicifolia* MAXIM and has been used traditionally to cure chronic rhinitis and nasosinusitis in Sino-Japanese medicine. Magnoshinin and magnosalin are neolignan-derivatives isolated from *Shin-i* (1). Both compounds significantly inhibit adjuvant-induced granuloma formation and angiogenesis in the mouse air pouch (2, 3). Magnoshinin inhibited granuloma formation to a greater extent than angiogenesis, having half the inhibitory effect of hydrocortisone, when it was injected into an air pouch or orally administered (2, 3). Magnosalin inhibits angiogenesis more greatly than granuloma formation (3). Angiogenesis requires proliferation of vascular endothelial cells (EC) (4). In this study, we investigated the anti-proliferative effects of magnoshinin and magnosalin on subcultured EC of rat aorta. The inhibitory effects were analyzed in two phases of the cell cycle, competence and progression (5).

Rat aortic EC were prepared by the method of Schwartz (6), with the following modifications: The

thoracic aorta of male Wistar rat (9 weeks old, weighing 250–330 g) was isolated and the periadventitial connective tissue was carefully stripped-off. The vessel was cannulated with a needle, and the other end of the vessel lumen was closed with a vascular clamp. Using the needle, Hank's balanced salt solution containing 2 mg/ml collagenase (Wako, Osaka, Japan) was injected into the vessel, and the vessel was incubated for 30 min at 37°C. The EC which were suspended in the vessel lumen were centrifuged at $100 \times g$ for 10 min. The pellet was resuspended in Dulbecco's modified Eagle medium (DMEM, Nissui, Tokyo, Japan) containing 160 U/ml penicillin G potassium (Banyu Seiyaku, Tokyo), 100 $\mu\text{g/ml}$ streptomycin sulfate (Meiji Seika, Tokyo), 4.2 mM NaHCO_3 and 20% heat-inactivated fetal bovine serum (FBS, Whittaker Bioproducts, Walkersville, MD, U.S.A.). EC were seeded at a density of 100–200 cells/0.5 ml/16-mm well into a Type I collagen (Koken, Tokyo)-coated, 24-well plate (Corning, Corning, NY, U.S.A.). The cells were cultured in a humidified atmosphere of 5% CO_2 in air at 37°C. The contaminating non-endothelial cells such as smooth muscle cells were removed with a rubber cleaner

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(Ikemoto Scientific Technology, Tokyo) under an optical microscope. The cloned EC were subcultured at a split ratio of 1:2 or 1:4 by treatment with Ca^{2+} - and Mg^{2+} -free phosphate-buffered saline containing 0.5 mg/ml trypsin (Difco, Detroit, MN, U.S.A.) and 0.54 mM EDTA-2Na. EC were identified by their typical cobblestone-like morphology and the ability of tube-like formation in vitro (7).

Subcultured EC (6–9th passage) were seeded at a density of 3.0×10^4 cells/16-mm well, and they were stimulated by 5% FBS with or without magnoshinin or magnosalin (gifts from Prof. T. Kikuchi, Research Institute for Wakan-Yaku of our university). The medium containing the compound was exchanged with a fresh one every other day. Cell number was daily measured using a hemocytometer (Nitrin, Tokyo). Cell viability

was confirmed by non-staining with trypan blue (Wako). Tritium-thymidine incorporation was measured as previously described (8). Subconfluent EC (cultured for 6 days) were synchronized in the G_0 phase by serum starvation for 2 days, and then they were stimulated by 5% FBS and 0.037 MBq [methyl- ^3H]-thymidine (925 GBq/mmol, Amersham Japan, Tokyo) in the presence or absence of magnoshinin or magnosalin. The radioactivity of labeled cells was counted at 3 hr-intervals by a scintillation spectrometer (LS3801, Beckman, Clifton, U.S.A.).

Magnoshinin (10 or 30 $\mu\text{g}/\text{ml}$, 23.4 or 70.3 μM) and magnosalin (10 $\mu\text{g}/\text{ml}$, 23.9 μM) significantly inhibited EC proliferation stimulated by 5% FBS, as monitored by cell number (Fig. 1, a and b). At 10 $\mu\text{g}/\text{ml}$, the anti-proliferative effects of magnoshinin were two-fold

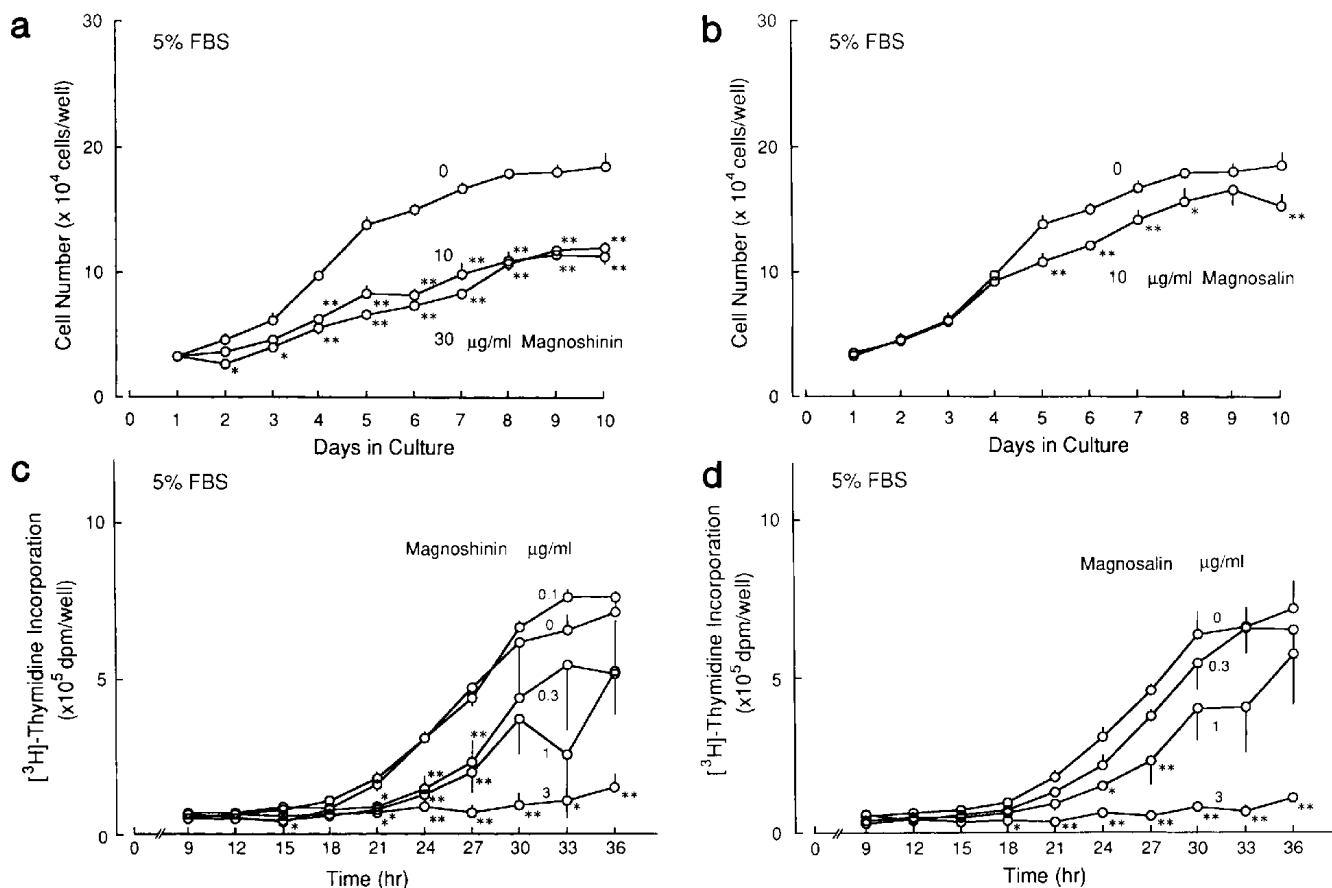


Fig. 1. Effects of magnoshinin (a, c) and magnosalin (b, d) on proliferation of subcultured rat aortic endothelial cells (EC) stimulated by 5% fetal bovine serum (FBS). a, b: EC (6–9th passage) were seeded at a density of 3.0×10^4 cells/16-mm well in the presence or absence of magnoshinin (10 or 30 $\mu\text{g}/\text{ml}$) or magnosalin (10 $\mu\text{g}/\text{ml}$). Cell number was counted every day to the 10th day of culture. The values represent the means \pm S.E.M. of 3 experiments. * $P < 0.05$, ** $P < 0.01$: compared to the control (5% FBS alone), as determined by two-way analysis of variance (ANOVA) and Tuckey's test. c, d: Subconfluent EC (cultured for 6 days) were synchronized by serum starvation for 2 days, and stimulated with 5% FBS and ^3H -thymidine in the presence or absence of the above compounds (0.1, 0.3, 1 or 3 $\mu\text{g}/\text{ml}$). Tritium-thymidine incorporation was measured at 3-hr intervals in culture. The values represent the means \pm S.E.M. of 3–9 experiments. * $P < 0.05$, ** $P < 0.01$: compared to the control (5% FBS alone), as determined by one-way ANOVA and Scheffe's test.

greater than that of magnosalin; the inhibitory percentage of both compounds at 10 day-culture were 39.2% (magnoshinin) and 17.7% (magnosalin) for the control with 5% FBS alone. Magnoshinin and magnosalin at the same concentration as above, however, did not inhibit EC proliferation stimulated by 10% or 20% FBS (data not shown).

To elucidate the effects of magnoshinin and magnosalin on the cell cycle from the G_0 to S phase, we measured ^3H -thymidine incorporation every 3 hr in culture (Fig. 1, c and d). EC which were arrested at the G_0 phase by serum starvation initiated DNA synthesis from 18–21 hr after the stimulation of 5% FBS without the compounds. Magnoshinin and magnosalin (0.1, 0.3, 1 or 3 $\mu\text{g}/\text{ml}$) concentration-dependently inhibited ^3H -thymidine incorporation. Especially, magnoshinin prolonged the starting time of DNA synthesis and reduced the rate of ^3H -thymidine incorporation, but magnosalin reduced only the rate of ^3H -thymidine incorporation. Both compounds completely inhibited the DNA synthesis for the 36-hr culture at 10 or 30 $\mu\text{g}/\text{ml}$ (data not shown). Consequently, magnoshinin and magnosalin inhibited ^3H -thymidine incorporation into EC to a greater extent than the cell number of EC.

When the growth curve (^3H -thymidine incorporation or log cell number) is plotted against the culture time, the starting time and the rate of proliferation reflect the effects of the compounds on the competence phase and progression phase in the cell cycle, respectively (5, 8). If a growth inhibitor affects only the competence phase, the starting time of proliferation is prolonged from C_c (without) to C_t (with the inhibitor). If a growth inhibitor affects only the progression phase, the doubling time of cell number from the starting time of proliferation is prolonged from P_c (without) to P_t (with the inhibitor). Therefore, we evaluated two convenient indices, $(C_t - C_c)/C_c$ and $(P_t - P_c)/P_c$, to assess the relative effects of the competence phase and the progression phase in the cell cycle. Both indices of the inhibitory effects of magnoshinin and magnosalin on the EC cell cycle were calculated from the data of Fig. 1, c and d (Fig. 2). The anti-proliferative effects of magnoshinin showed the same extent of contribution to the competence phase and the progression phase. The anti-proliferative effects of magnosalin were attributed to the inhibition of the progression phase rather than that of the competence phase (Fig. 2).

Growth-arrested cells at the G_0 phase proceed to two phases, first to the competence phase and then to the progression phase, to start proliferation (5). However, there have been few reports on the effects of drugs on the cell cycle in EC proliferation. In the present study, magnoshinin inhibited both the competence phase and

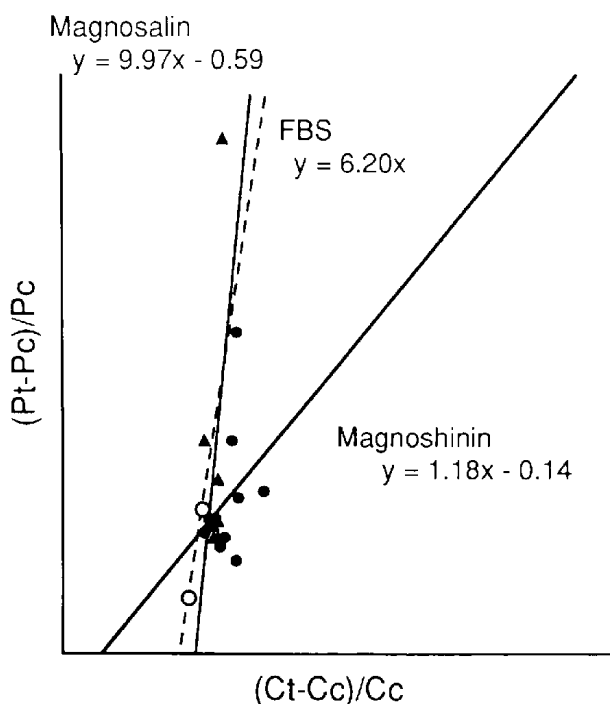


Fig. 2. The inhibition by magnoshinin (●) and magnosalin (▲) of the competence phase and the progression phase in EC proliferation of rat aorta. Data were calculated from each experiment in Fig. 1, c and d. The proliferation effects of FBS (2% and 5%, ○) on EC proliferation are plotted as the standard line (dotted line). The regression lines of magnoshinin, magnosalin and FBS were obtained by the least-squares method.

progression phase to the same extent, but magnosalin preferentially inhibited the progression phase in EC proliferation. These results suggest that the anti-angiogenic effects of both compounds *in vivo* (3) are attributed to the direct inhibition of EC proliferation. EC proliferation is an important process of angiogenesis (4). Angiogenesis aggravates many diseases including rheumatoid arthritis and diabetic vascular complications (9). Magnoshinin and magnosalin may be very effective in the treatment and prevention of angiogenic diseases.

In conclusion, we found that magnoshinin inhibited both the competence phase and the progression phase, and magnosalin inhibited preferentially the latter phase in FBS-stimulated EC proliferation of rat aorta.

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