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The molecular mechanism of anti-angiogenic compounds isolated from *Flos magnoliae*

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Abstract

The pharmacological studies of Kakkon-to-ka-senkyu-shin'i (葛根湯加川芎辛夷), a traditional Sino-Japanese medicine, component crude fractions and their compounds were investigated on angiogenesis in an adjuvant-induced chronic inflammation model of mouse. Magnolia in the prescription was crucial roles for the inhibition of angiogenesis, inflammatory cell migration and fluid exudation, but not granuloma formation in the inflammation model. Magnosalin isolated from magnolia inhibited angiogenesis to a greater extent than granuloma formation and fluid exudation. This inhibitory pattern of magnosalin differed from that of hydrocortisone. The anti-angiogenic effect of magnosalin was greater than that of magnoshinin, another compound from magnolia. The inhibitory effects of magnosalin on fetal bovine serum- and interleukin (IL)-1 α-stimulated tube formation of endothelial cells (EC) was also greater than those of magnoshinin. Their inhibitory effects on the action of IL-1 α were in a non-competitive manner. For the EC proliferation, the inhibitory effect of magnosalin was weaker than that of magnoshinin. However, magnosalin inhibited the abnormal proliferation of synovial cells of a rheumatoid arthritis (RA) model, MRL/lpr mouse, and RA patients with a greater potency than magnoshinin. The inhibitory effect was greater for the action of IL-1 α than those of basic fibroblast growth factor and platelet-derived growth factor. These results demonstrate that the anti-angiogenic action of magnosalin depends on its inhibition of tube formation rather than that of EC proliferation. The anti-angiogenesis is associated with the inhibitory action of IL-1 α in the differential phenotype of EC. Magnosalin becomes the lead compound of an new type of anti-RA drug with the anti-angiogenic action.

Key words Kakkon-to-ka-senkyu-shin'i (葛根湯加川芎辛夷), magnolia, magnosalin, anti-chronic inflammation, anti-angiogenesis, anti-rube formation, anti-rheumatoid arthritis drug.

Kakkon-to-ka-senkyu-shin'i (KSS; Ge-Gen-Tang-Jia-Chuan-Xiong-Xin-Yi, 葛根湯加川芎辛夷), a traditional Sino-Japanese medicine, has long been used clinically in the treatment of nasal inflammation. KSS consists of nine crude fractions: pueraria root, ephedra stem, cnidium rhizome, magnolia bud, cassia cortex, peony root, licorice root, ginger root and jujube fruit. According to the traditional composition, KSS includes some related combinations: Kakkon-to (Ge-Gen-Tang, 葛根湯), consisting of seven crude fractions; pueraria, ephedra, cassia, peony, licorice, ginger and jujube: and Keishi-to (Gui-Zhi-Tang, 桂枝湯), consisting of five crude fractions; cassia, peony, licorice, ginger and jujube. Kakkon-to is used to improve some syndromes that accompany cold infection. Keishi-to has an anti-inflammatory action.[1] In the present study, (1) inhibitory effects of KSS, its related combinations and component crude fractions were investigated on granuloma angiogenesis, granuloma formation, migration of inflammatory cells and fluid

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exudation in the mouse adjuvant - induced chronic inflammation model to determine combined effects of these fractions in KSS.[2] (2) Inhibitory effects of compounds derived from an effective crude fraction, magnolia, on the angiogenesis were compared with those on the granuloma formation in the chronic inflammation model.[3,4] (3) To study an inhibitory mechanism of these compounds on the angiogenesis, their immunopharmacological effects were investigated on tube formation of rat vascular endothelial cells (EC) stimulated by macrophage (Mϕ)-derived cytokines. (4) Effects of these compounds on EC proliferation were also examined in different phenotypes of EC, competence and progression phases.[5] (5) The mechanism of these compounds was also examined on the abnormal proliferation of synovial cells (SC) in MRL/lpr mouse, a rheumatoid arthritis (RA) model.[6]

1. Anti-inflammatory effects of Kakkon-to-ka-sen-kyu-shin'i and its related combinations on mouse adjuvant-induced chronic inflammation model

The effects of KSS on inflammatory parameters : granuloma angiogenesis, granuloma formation, migration of inflammatory cells and pouch fluid exudation in mouse adjuvant - induced chronic inflammation model were compared with those of Kakkon - to, Keishi - to and component crude fractions.[2] The granuloma in mouse air pouch was formed after injection of Freund's complete adjuvant with 0.1 % croton oil. The granuloma angiogenesis was determined by measuring carmine content in newly formed blood vessels in granuloma after intravenous injection of 10 % carmine in 5 % gelatin solution.[7] Granuloma formation, inflammatory cell migration and pouch fluid exudation were simultaneously determined by measuring granuloma weight, number of inflammatory cells and fluid weight in the pouch, respectively.[2,4]

KSS (50-400 mg/kg) by the intraperitoneal administration inhibited the angiogenesis, migration of inflammatory cells and pouch fluid exudation with the same potency, but not granuloma formation in the chronic inflammatory model. Both Kakkon-to and a fraction of cnidium and magnolia had similar potencies to KSS for the inhibitions of angiogenesis and fluid exudation, and had weaker potencies for the inhibition of cell migration than KSS (Fig. 1). These results indicate that Kakkon-to and the fraction of cnidium and magnolia in KSS show the additive

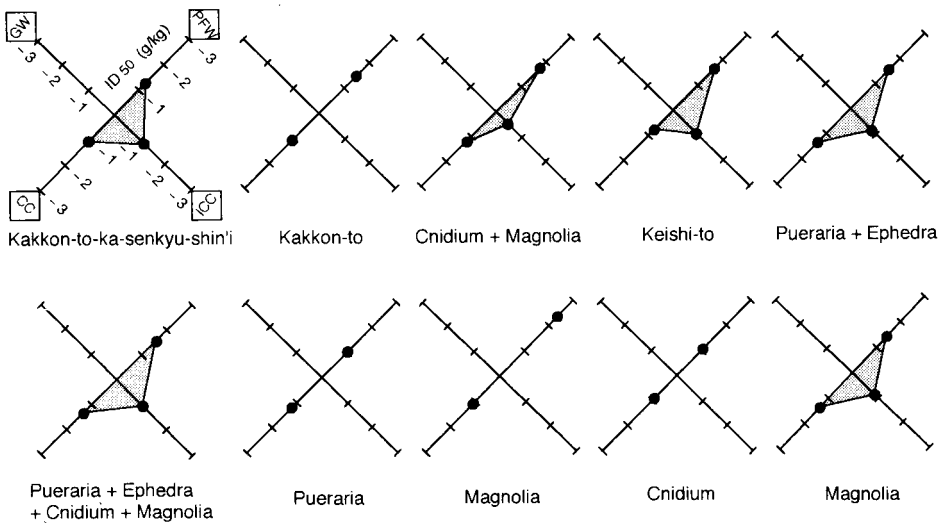

Fig. 1 Intersected axis expressing log 50 % inhibitory dose (ID50, g/kg) of Kakkon-to-ka-senkyu-shin'i, its related combinations and respective component crude fractions for granuloma weight (GW), carmine content (CC), inflammatory cell count (ICC), and pouch fluid weight (PFW). (Reproduced from Ref. 2).

effects for the inhibition of angiogenesis and fluid exudation, and the potentiative effect for the inhibition of cell migration in the chronic inflammation model.

Keishi-to had a weaker potency than KSS, and a fraction of pueraria, ephedra, cnidium and magnolia had a greater potency than KSS for the inhibition of angiogenesis. For the inhibition of inflammatory cell migration, Keishi-to had a weaker potency than KSS, and the fraction of four crude drugs had a similar potency to KSS. Keishi-to and the fraction of other four crude drugs in KSS show the additive inhibitory effect on the angiogenesis and the potentiative effect on the cell migration.

For the angiogenesis, individual fractions of magnolia and pueraria inhibited with a similar potency to the total fraction of pueraria, ephedra, cnidium and magnolia, and individual fractions of ephedra and cnidium had weaker potencies than the total fraction of four crude drugs (Fig. 1). For the inhibition of cell migration, all four individual fractions had weaker potencies than the total fraction of four crude drugs. The magnolia fraction and ephedra fraction inhibited fluid exudation with the same potency to the total fraction of four crude drugs. These results indicate that the anti-inflammatory effects of KSS depend on magnolia and pueraria for the angiogenesis; on pueraria, ephedra, cnidium and magnolia for the inflammatory cell migration; and on magnolia and ephedra for pouch fluid exudation, respectively. These results mean that the fraction of magnolia has crucial roles for the inhibitory effects of KSS on three inflammatory parameters.

2. Anti-angiogenic effects of magnosalin and magnoshinin isolated from magnolia in mouse adjuvant-in-

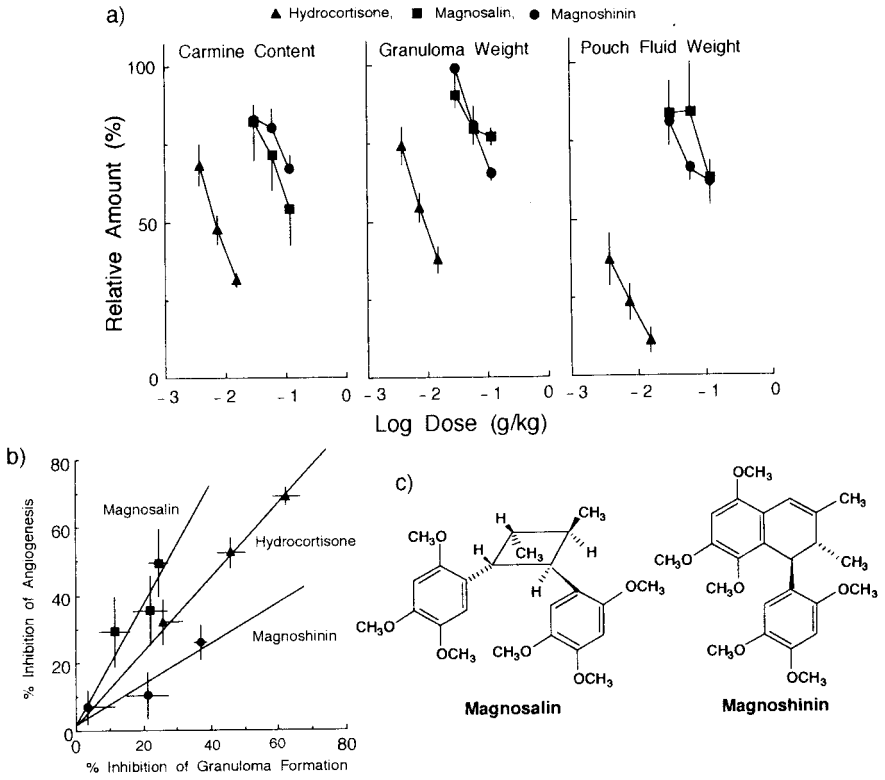

Fig. 2 a) Decrease in % relative amount ±S.E.M. of carmine content, granuloma weight and pouch fluid weight by magnosalin, magnoshinin and hydrocortisone. b) Relation between the inhibition of angiogenesis and granuloma formation by magnosalin (30-120 mg/kg, *i.p.*), magnoshinin (30-120 mg/kg, *i.p.*) and hydrocortisone (3.8-15 mg/kg, *i.p.*). c) Chemical structures of magnosalin and magnoshinin. (Reproduced from Ref. 4).

duced chronic inflammation model

The inhibitory effects of compounds isolated from magnolia were investigated in the chronic inflammation model.[3, 4, 8] Neolignans, magnosalin and magnoshinin, in magnolia compounds inhibited the angiogenesis and the granuloma formation, but not the pouch fluid exudation by their oral and intra-pouch administrations.[3, 4] Magnosalin by the intraperitoneal administration also inhibited the angiogenesis to a greater extent than the granuloma formation and pouch fluid exudation, respectively (Fig. 2a). These inhibitory patterns of neolignan differed from that of hydrocortisone which showed the inhibition for all three inflammatory parameters with the similar potency (Fig. 2a). The inhibitory effects of magnosalin, magnoshinin and hydrocortisone were compared between the angiogenesis and granuloma formation (Fig. 2b). The regression coefficient of the anti-angiogenesis and the anti-granuloma formation was 1.79 for magnosalin, 1.11 for hydrocortisone and 0.61 for magnoshinin. These results indicate that magnosalin is an inhibitor of angiogenesis rather than granuloma formation, suggesting that it inhibits the granuloma-independent action of angiogenesis. The inhibitory pattern of magnosalin is different from those of magnoshinin and hydrocortisone.

3. Effects of compounds derived from magnolia on immunopharmacological activity ; Inhibitory effect of magnosalin on the tube formation of rat vascular endothelial cells

Angiogenesis progresses in the following four steps in this order: the degradation of basement membranes of preexisting vessels, the migration of vascular EC, the EC proliferation and the organization of capillaries, including the tube formation of EC.[9] The tube formation was investigated by measuring total length of tubes developed from rat vascular EC cultuted in 0.15 % type I collagen gel with 2 % fetal bovine serum (FBS)-contained Dulbecco's modified Eagle's medium (DMEM).[10] The total length of tubes in photographs increased in a incubation time-dependent manner for 2 days and showed a plateau by day 8. The conditioned medium (CM) derived from Mϕ which were treated with interferon-γ (IFN-γ, 0.46 nM), a typical Mϕ activator, enhanced the tube formation in a time-dependent manner from days 2 to 4 in the pre-culture of Mϕ (Fig. 3a). The effect of CM from IFN-γ-treated Mϕ for days 4 and 6 was significantly greater than that of Mϕ-CM without IFN-γ. The tube forming effect of IFN-γ-activated Mϕ-CM on the EC paralleled with the effect of co-cultured Mϕ with the EC in the presence of IFN-γ (data not shown). These results demonstrate that IFN-γ releases cytokines from the Mϕ to enhance the tube formation.

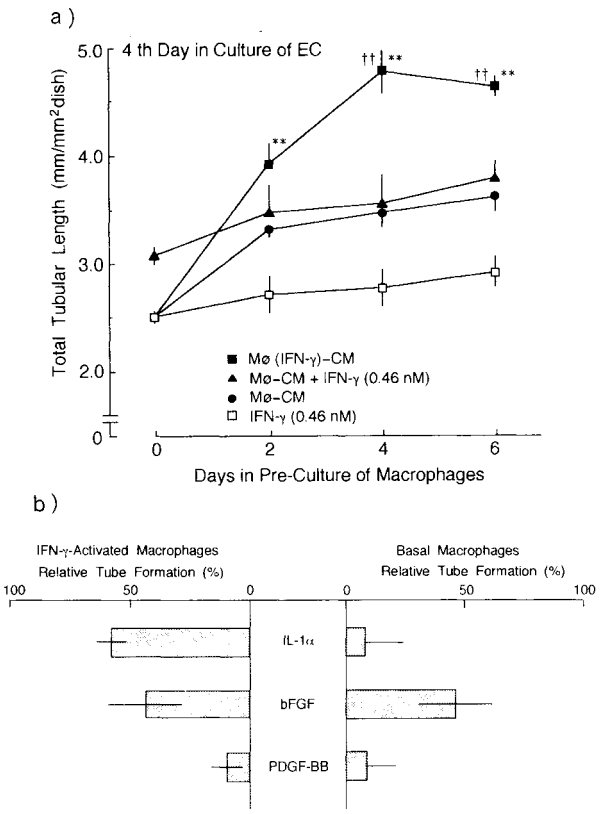

Fig. 3 a) Effect of medium conditioned with IFN-γ-treated macrophage preparation (Mϕ) on the tube formation. Conditioned medium (50 μl) derived from IFN-γ (0.46 nM)-treated Mϕ, untreated Mϕ, and freshly prepared 0.46 nM IFN-γ in the presence or absence of conditioned medium of Mϕ. b) Activities of IL-1 α, bFGF and PDGF-BB released from IFN-γ-activated and basal Mϕ. Relative tube formation was estimated from the inhibitory activity (%) of maximal concentrations of anti-IL-1 α, anti-bFGF and anti-PDGF-BB on the tube formation (100 %) induced by conditioned medium from IFN-γ (0.46 nM) activated and basal Mϕ. (Reproduced from Ref. 10 and 11).

To determine these cytokines enhancing tube formation, neutralizing effects of antibodies against

interleukin (IL)-1 α, basic fibroblast growth factor (bFGF) and platelet-derived growth factor (PDGF)-BB were investigated on the actions of CM from the IFN-γ-treated and basal Mϕ.[11] Anti-IL-1 α inhibited the tube-forming action of the IFN-γ-activated Mϕ to a greater extent than the action of the basal Mϕ, indicating that the release of IL-1 α depended on the action of IFN-γ (Fig. 3b). Anti-bFGF inhibited both actions of IFN-γ-activated and basal Mϕ with the similar extent, demonstrating that bFGF was released in a IFN-γ-independent manner. Anti-PDGF-BB did not inhibit the actions of both Mϕ significantly. These results demonstrate that IL-1 α is selectively released from the IFN-γ-activated Mϕ.

All of Mϕ-derived cytokines and related substances used increased the tube formation in a concentration-dependent manner.[11] IL-1 α had the greatest effect on the tube formation, and followed by PDGF-BB and bFGF (Fig. 4). The results confirm that the released IL-1 α has a predominant role for the tube-forming action of the IFN-γ-activated Mϕ.

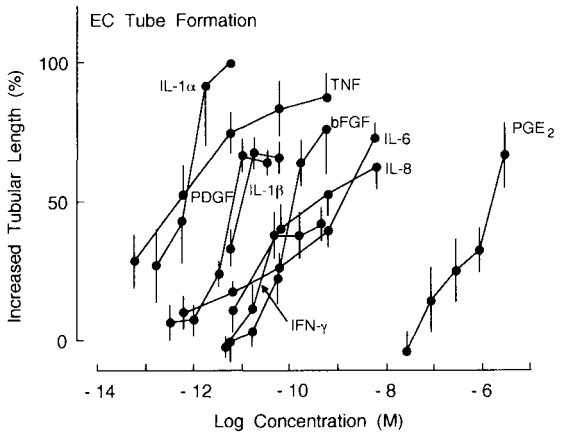

Fig. 4 Effects on the tube formation of macrophage-derived cytokines and related substances. (Reproduced from Ref. 11).

Magnosalin (1-10 μM) inhibited 2% fetal bovine serum (FBS)-induced tube formation more potently than magnoshinin and corticosterone (Fig. 5a). Magnosalin and magnoshinin (1-3 μM) also inhibited IL-1 α-stimulated tube formation in a concentration-dependent manner (Fig. 5b). The inhibitory pattern of

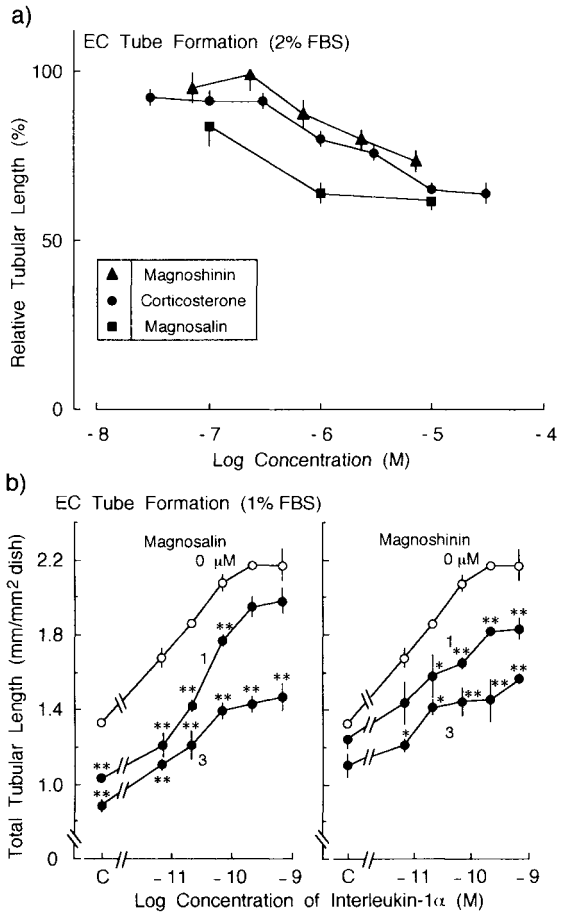

Fig. 5 a) Inhibitory effects of magnosalin, magnoshinin and corticosterone on 2% fetal bovine serum-stimulated tube formation. b) Inhibitory effects of magnosalin and magnoshinin on interleukin-1 α-induced tube formation.

these neolignans on the action of IL-1 α was in a non-competitive fashion. The effect of magnosalin was greater than that of magnoshinin. The potency order of these neolignans for the anti-tube formation was indentical with the order for their anti-angiogenesis. The results demonstrate that the anti-angiogenic action of magnosalin is associated with the anti-tube formation of EC. The action of magnosalin is associated with the inhibitory action of IL-1 α in the vascular EC differentiated by type I collagen gel.

4. Selective inhibition of magnosalin and magnoshinin on different phenotypes, competence and progression phases of rat vascular endothelial cells

Growth-arrested EC in the Go phase transit

through at least two phases, first competence and then progression, to start to proliferate.[12] The effects of the competence phase and the progression phase are reflected in the starting time and the rate of DNA synthesis, respectively.[12, 13] Effects of magnosalin and magnoshinin on the competence and progression phases in DNA synthesis during the proliferation of rat vascular EC were compared with those on the tube formation. FBS-stimulated [^{3}H]-thymidine incorporation was measured at 3 hr-intervals for 36 hrs into subconfluent EC in the Go phase synchronized by the serum starvation for 2 days. Magnosalin (0.1-3 μg/ml, 0.24-7.17 μM) and magnoshinin (0.1-3 μg/ml, 0.23-7.03 μM) inhibited [^{3}H]-thymidine incorporation in a concentration-dependent manner (Fig. 6a).[5] The anti proliferative effect of magnoshinin was greater than that of magnosalin. Magnoshinin prolonged the starting time of [^{3}H]-thymidine incorporation at 0.3 μg/ml (0.70 μM) and reduced the rate of thymidine incorporation at 3 μg/ml (7.03 μM). However, magnosalin reduced only the rate of the thymidine incorporation at 1-3 μg/ml (2.39-7.17 μM). The potency order of magnosalin and magnoshinin on the EC proliferation differed from those of the tube formation and angiogenesis. The anti-proliferative effects were analyzed by the inhibition of an index of competence, (Ct-Cc)/Cc, and the inhibition of an index of progression, (Pt-Pc)/Pc (Fig. 6b). Ct and Cc were the starting time of proliferation with and without drug. Pt and Pc were doubling time of cell number from the starting time of cell proliferation with and without drug. The contribution of FBS to the EC proliferation was also plotted as a standard proliferation line. The effect of FBS on the EC proliferation was due to the progression phase rather than the competence phase. The anti-proliferative effect of magnosalin was attributed to the inhibition of the progression phase rather than that of the competence phase (Fig. 6b). The anti - proliferative effect of magnoshinin showed the same extent of contribution to the competence phase and the progression phase. These results demonstrate that the inhibitiory effect of magnosalin on the progression phase does not depend on its anti-angiogenic effect.

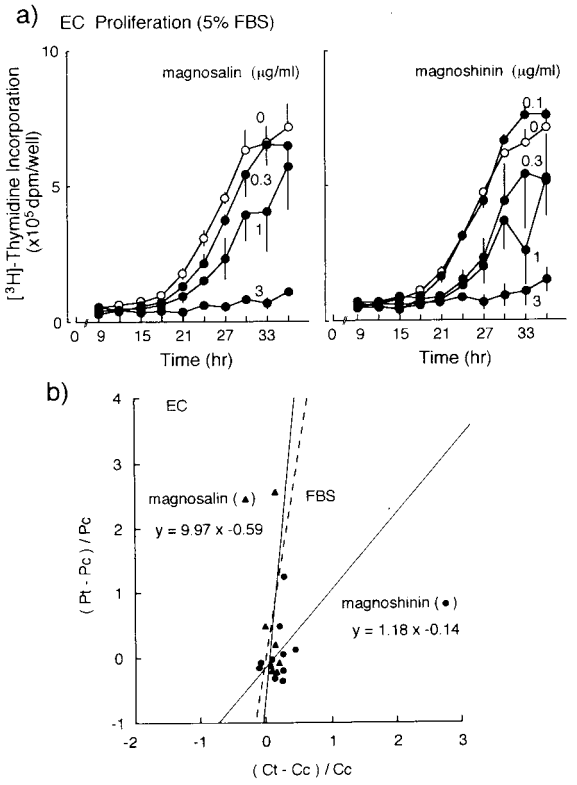

Fig. 6 a) Inhibitory effects of magnosalin and magnoshinin on 5 % FBS-stimulated [^{3}H] thymidine incorporation into synchronized vascular endothelial cells (EC) at Go phase. b) The inhibition by magnosalin and magnoshinin of the competence phase and the progression phase in the EC proliferation of rat aorta. Data were calculated from experiments of a). The proliferation effects of FBS on EC proliferation are plotted as the standard line. The regression lines of magnosalin, magnoshinin and FBS were obtained by the least-squares method. (Reproduced from Ref. 5).

5. Effects of compounds derived from magnolia on immunopharmacological activity ; Inhibitory effects of magnosalin on the abnormal proliferation of synovial cells of a MRL/lpr mouse

The initial pathological change in the focus of RA was the infiltration of lymphocytes and Mϕ, the proliferation of SC and angiogenesis, followed by the pannus formation and joint destruction.[14] Inhibitory effects of magnosalin and magnoshinin on the abnormal proliferation of SC in the RA model, a MRL/lpr mouse, were compared with those of typical anti-RA drugs ; hydrocortisone, corticosterone and bucillamine (Fig. 7).[6] The inhibitory effect of magnosalin was greater than those of hydrocortisone and bucillamine, but smaller than that of corticosterone. The effect of magnosalin was greater than that of magnoshinin.

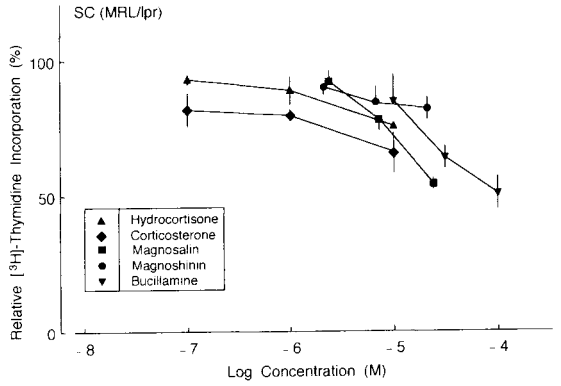

Fig. 7 Inhibitory effects of magnosalin, magnoshinin, hydrocortisone, corticosterone and bucillamine on abnormal proliferation of synovial cells of MRL/lpr mouse. (Reproduced from Ref. 6).

The potency order of their neolignans for the SC proliferation was identical with those for the angiogenesis and tube formation.

In the Mϕ-derived cytokines, IL-1 α increased the proliferation of SC in C57BL/6J mouse to the greater extent than PDGF-BB and bFGF (Fig. 8 a). The results demonstrate that IL-1 α has a crucial role for the abnormal proliferation of SC in the initial pathological change of RA. Magnosalin inhibited the action of IL-1 α with a greater potency than those of PDGF-BB, bFGF and FBS (Fig. 8b). The results demonstrate that the inhibitory effect of magnosalin on the abnormal proliferation of SC in the RA model depends on the inhibition of the action of Mϕ-derived IL-1 α.

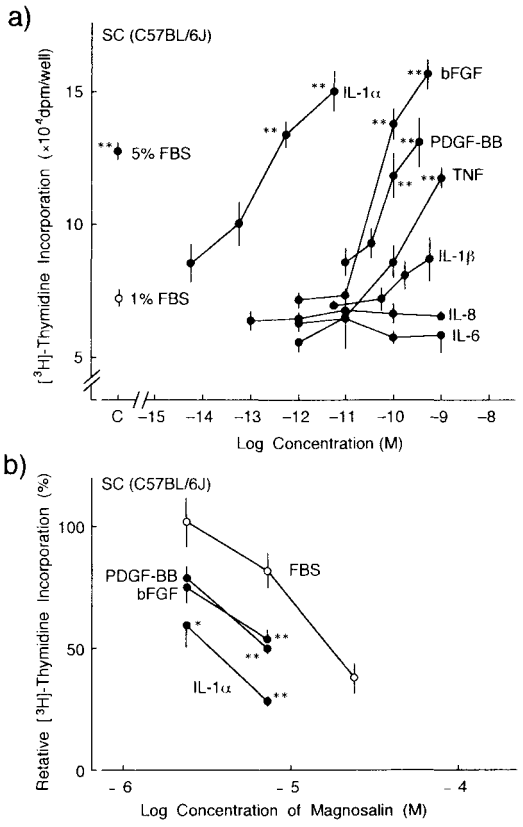

Fig. 8 a) Effects of macrophage-derived cytokines on proliferation of synovial cells of normal C57BL/6J mouse. b) Inhibitory effects of magnosalin on proliferation of synovial cells of C57BL/6J mouse stimulated by IL-1 α, bFGF, PDGF-BB and FBS.

6. *Conclusion*

The results of the present study were summarized in Figure 9. Magnosalin derived from magnolia inhibited angiogenesis to a greater extent than the granuloma formation and fluid exudation in the adjuvant-induced chronic inflammation model. The inhibitory pattern of magnosalin differed from those of corticosterone and hydrocortisone which showed the inhibitions for angiogenesis, granuloma formation and fluid exudation with the same potency. The anti-angiogenic effect of magnosalin was greater than that of magnoshinin. The potency order of magnosalin and magnoshinin on angiogenesis was identical with the tube formation of EC but neither the competence nor progression phases of the EC proliferation. These results mean that the anti-angiogenic effect of magnosalin is associated with the EC tube formation rather than the EC proliferation. IL-1 α predominantly released by IFN-γ-activated Mϕ stimulated the tube formation. Magnosalin inhibited IL-1 α-induced tube formation in a non-competitive manner, suggesting that the action of magnosalin depends on the inhibitory action of IL-1α. The mechanism of magnosalin was associated with the inhibition of abnormal proliferation of SC in a RA model, MRL/lpr mouse, and in RA patients. These results demonstrate that magnosalin becomes the lead compound of an anti-RA drug with the anti-angiogenic action.

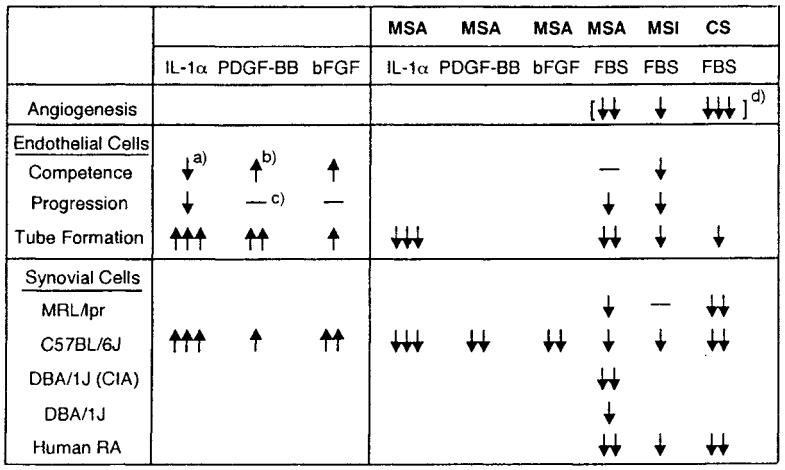

Fig. 9 Summary of the effects of magnosalin and magnoshinin on angiogenesis, competence and progression phases in abnormal proliferation of endothelial cells (EC), tube formation of EC and abnormal proliferation of synovial cells of MRL/lpr mouse, type II collagen-induced arthritis in DBA/1J mouse and RA patients.

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和文抄録

漢方方剤葛根湯加川芎辛夷の抗炎症作用を薬理学的に解析すると，辛夷が主役的役割を担った。この抗慢性炎症作用を引き起こす辛夷の成分はネオリグナン類であり，Magnosalin (MSA) と Magnoshinin (MSI) が単離同定された。両成分は血管新生と肉芽増殖をともに抑制し，前者にMSAが，後者にMSIがそれぞれ強力に奏功した。MSA は新生過程にある管腔形成作用も corticosterone や MSI より強力に抑制し，この作用は血管新生機構の制御とよく一致した。管腔形成を誘発する活性化マクロファージ (Mφ) からインターロイキン (IL)-1α が多量に放出することを確認し，更に Mφ 由来サイトカインの中で一番強力であった。IL-1α の作用も MSA が非競合的に拮抗したのでこの機構に IL-1α が主因子と成っていることを明らかにした。これらを踏まえて，慢性リウマチモデルマウス MRL/lpr の滑膜細胞への効果を検討すると，IL-1α の異常増殖作用を MSA が特異的に抑制した。この結果は血管新生や管腔形成の抑制作用と完全に平行した。以上，慢性炎症による血管新生機構は IL-1α の異常放出が主因であり，この作用を制御する MSA は慢性関節リウマチ治療薬のリード化合物に成りうると結論した。

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