Chemical Structure-Activity of Cnidium Rhizome-Derived Phthalides for the Competence Inhibition of Proliferation in Primary Cultures of Mouse Aorta Smooth Muscle Cells

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ABSTRACT—Inhibitory effects of cnidium rhizome-derived phthalides on competence and progression phases of fetal bovine serum (10%)-induced proliferation were compared in primary cultures of mouse aorta smooth muscle cells (SMC). Their potencies for the competence inhibition were in the order of senkyunolide L ((*Z*)-6-hydroxy-7-chloro-6,7-dihydroligustilide) > senkyunolide H ((*Z*)-6,7-dihydroxy-6,7-dihydroligustilide) > senkyunolide J ((3*S*)-(*E*)-6,7-dihydroxy-3,6,7,8-tetrahydroligustilide) > senkyunolide I ((*E*)-6,7-dihydroxy-6,7-dihydroligustilide) > ligustilide = senkyunolide A ((3*S*)-3,8-dihydroligustilide) > butylidenephthalide. The order of their potencies for the progression inhibition was parallel with that for the competence inhibition. Senkyunolide L is considered to have been formed during the extraction of cnidium. These results demonstrate that the (*Z*)-6,7-dihydroxy isomer of the dihydroligustilide derivatives is essential for the anti-competent effect on proliferation of the SMC in primary culture. Senkyunolide H in cnidium rhizome may be a prototype for a new anti-atherosclerotic drug.

Keywords: Cnidium rhizome-derived phthalide, Senkyunolide H, Primary culture, Smooth muscle cell (aorta)

Cnidium rhizome, a Japanese-Sino medicine is combined in traditional prescriptions including Chinese medicine "*Shimotsu-to*". It has long been used clinically in the treatment of "*Oketsu*" syndrome (stagnant blood), which included female diseases, atherosclerosis and chronic inflammation (1). The formation of atherosclerotic plaque is triggered by an abnormal proliferation of vascular smooth muscle cells (SMC) (2). We have previously reported that the anti-proliferative effect of *Shimotsu-to* on a primary culture of mouse aorta SMC may depend on cnidium rhizome-derived phthalides (3). These phthalides, containing senkyunolide H, senkyunolide A, ligustilide and butylidenephthalide, have anti-proliferative effects on the SMC. These anti-proliferative effects are selective on SMC, reversible and not due to cell damage and/or cell toxicity.

Vascular SMC migrate from the media into the intima and proliferate with the formation of atherosclerotic plaques (2). Both proliferating and non-proliferating SMC in the media can proliferate after they migrate into the intima of an injured artery (4). In vitro primary cultures of arterial SMC reversibly go through a transition from a contractile to a synthetic state before they become able to proliferate (2). The cell proliferation is regulated by specific cellular phases, including stages of competence and progression. Competence factors initiate the proliferative response of cells and induce competent cells to respond to the progression factors required for cell proliferation (5). Since several growth regulatory peptides have been implicated in the proliferation response, specific inhibition of these growth factors is required for potential therapeutic use. Search for an anti-competence activity against SMC proliferation is one of the approaches for finding a new type of anti-atherosclerotic drug.

In the present study, effects of cnidium rhizome-derived phthalides on the competence phase were investigated by measuring the cell number of 10% fetal bovine serum (FBS)-proliferated SMC in primary cultures of mouse aorta as a function of incubation time and compared with their progression inhibition.

MATERIALS AND METHODS

Cell culture

Primary cultured SMC of mouse aorta were prepared by the method of Chamley et al. (6) with some modifications (3, 7). The thoracic aorta of a ddY strain male mouse (6–7-week-old, Japan Shizuoka Laboratory Center, Hamamatsu) was isolated in Hanks' solution (136.8 mM NaCl, 5.4 mM KCl, 0.8 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 4.2 mM $NaHCO_3$ and 5.6 mM glucose, pH 7.3). The aorta was incubated with 1.3 mg/ml type I collagenase and 0.3 U/ml type I elastase (Sigma, St. Louis, MO, USA) in Hanks' solution for 40 min at 37℃, gently removed of adventitia, and then incubated with 2 mg/ml collagenase (Wako, Osaka) and 3.3 U/ml type I elastase in Hanks' solution for 1 hr at 37℃. Isolated cells were collected by centrifugation at 150 × g for 10 min at 4℃ and dispersed in 2 ml Dulbecco's modified eagle medium (DMEM; Nissui, Tokyo) that was supplemented with 10% heat-inactivated FBS (Whittaker Bioproduct, Walkersville, MD, USA), penicillin G potassium (160 U/ml; Banyu Seiyaku, Tokyo) and streptomycin sulfate (0.1 mg/ml; Meiji Seika, Tokyo). The cells at a density of 3.0×10^4 cells/well were plated and cultured in a 24-well plate (Corning, Corning, NY, USA) with 10% FBS-DMEM for consecutive days at 37℃ under a humidified atmosphere of 5% CO_2 and 95% air in the presence or absence of drug. The culture medium was changed every other day. The primary cultured cells were identified as SMC, because they exhibited the characteristic "hill and valley" pattern at confluence under an optical microscope (8).

Assay of SMC proliferation

The primary cultured SMC were detached in 0.25% Trypsin (Difco, Detroit, MI, USA) and 0.2% EDTA in Ca^{2+}, Mg^{2+}-free Hanks' solution and then collected by centrifugation. The number of cultured SMC was counted with a hemocytometer every 12 hr from the first to the 11th day in culture.

Agents

Heparin (Sigma) was dissolved in Ca^{2+}, Mg^{2+}-free phosphate-buffered saline and added to 10% FBS-DMEM (final concentration of solvent $<0.1\%$). Used phthalides derived from cnidium rhizome were provided by Tsumura Research Institute for Pharmacology (Tokyo). The chemical structures of senkyunolides H ((*Z*)-6,7-dihydroxy-6,7-dihydroligustilide), L ((*Z*)-6-hydroxy-7-chloro-6,7-dihydroligustilide), I ((*E*)-6,7-dihydroxy-6,7-dihydroligustilide) and J ((3*S*)-(*E*)-6,7-dihydroxy-3,6,7,8-tetrahydroligustilide); ligustilide; senkyunolide A ((3*S*)-3,8-dihydroligustilide); butylidenephthalide; butylphthalide; and senkyunolides D, E, F and G are shown in Table 1 (3, 9, 10). All phthalides were dissolved in 100% ethanol and then diluted with 10% FBS-DMEM to the final concentration (0.05% ethanol). The culture media with drugs were changed every other day. These phthalides did not damage the SMC during the 11-day treatment (data not shown), because no uptake of trypan blue is observed during these treatments (11).

Analysis of indices of competence and progression inhibitions

We analyzed the inhibitory effects of cnidium rhizome-derived phthalides on competence and progression phases of SMC proliferation by using the convenient assay reported previously (7, 12). Proliferation lines of the SMC number from 3×10^4 to 6×10^4 cells/well as a function of culturing time were fitted to the individual data of SMC number by the least-squares method. These indices of the competence and progression inhibitions were estimated as relative difference of the starting time between with and without drug, ((Ct−Cc)/Cc), and relative difference of the doubling time from the starting time, which was calculated by the slope of the proliferation line, between with and without drug, ((Pt−Pc)/Pc), respectively (7, 12).

Statistical analyses

The data are expressed as the means±S.E.M. and analyzed by one-way analysis of variance (ANOVA). The significance of the difference between the data with and without drugs was judged by the multiple range test of Scheffe or Tukey at the level of $P=0.05$ or 0.01.

RESULTS

Effect of heparin on competence and progression phases of the proliferation of primary cultured aortic smooth muscle cells

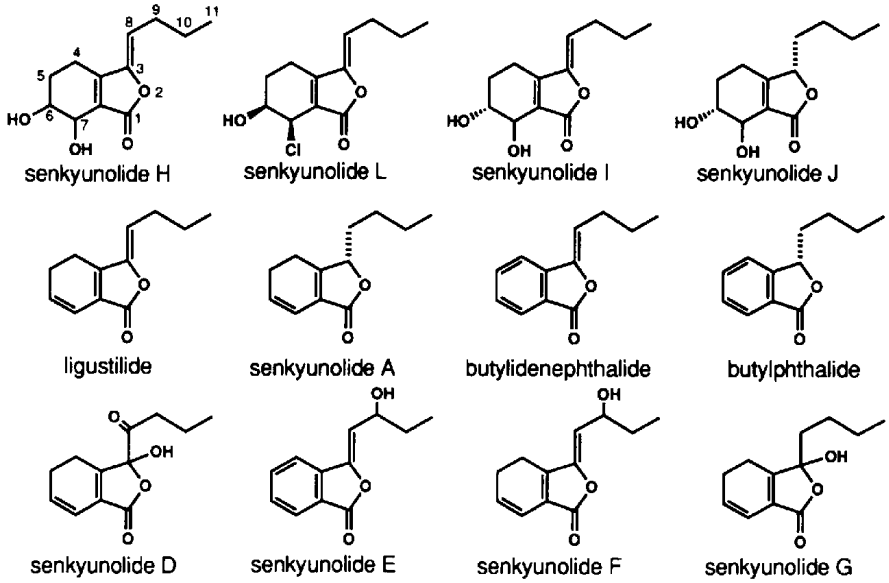
We determined the starting and doubling times of 10% FBS-stimulated proliferation of mouse aortic SMC in primary culture. When the number of SMC was plotted during a 12-hr interval, the total time (TTc) required for SMC number doubling (from 3×10^4 to 6×10^4 cells/well) from the day when the SMC were plated (day 0) was 110.9±4.3 hr (n=8). The total time was divided into the starting time of SMC proliferation, 81.0±5.0 hr (n=8, Cc), and the time, from the starting time, required for the number of proliferating SMC to double, 29.8±2.4 hr (n=8, Pc). These starting and doubling times in SMC proliferation were used as indices of 10% FBS-induced competence and progression phases, respectively.

Heparin (1 μg/ml), a positive control, significantly decreased the number of SMC proliferated by 10% FBS

Table 1. Inhibitory effects of cnidium rhizome-derived phthalides on competence and progression phases of proliferation in primary cultured smooth muscle cells of mouse aorta

	Concentration (μg/ml)	TIT_2	(Ct−Cc)/Cc	(Pt−Pc)/Pc	n
Senkyunolide H	0.1	1.74	0.50	0.64	4
	1.0	2.88	1.63	3.00	2
Senkyunolide L	0.1	1.86	0.83	0.93	4
Senkyunolide I	0.1	1.25	0.17	0.43	4
	1.0	2.37	1.13	2.48	2
Senkyunolide J	0.1	1.39	0.33	0.50	4
Ligustilide	1.0	1.95	1.00	0.86	4
Senkyunolide A	1.0	1.90	1.00	0.71	4
Butylidenephthalide	4.0	1.11	0.13	0.03	3
	8.0	1.23	0.25	0.14	3
Heparin	1.0	1.60	0.38	1.35	4

TIT_2: ratio of total time required for SMC doubling from the day SMC were plated with drug to that without it; (Ct−Cc)/Cc: relative difference between starting time of SMC proliferation with drug and that without it (competence index), (Pt−Pc)/Pc: relative difference between proliferation rate of SMC with drug and that without it (progression index), n: number of experiments. Chemical structures of cnidium-derived phthalides used are shown below:



from the fourth day to the 11th day. It prolonged the starting time of the SMC proliferation and decreased the slope of the SMC proliferation curve (Fig. 1). The value of total inhibitory time (TIT_2, TTt/TTc), i.e., the ratio of total time from day 0 required for cell number doubling in the presence of heparin (TTt) to that without it (TTc), was 1.6 (Table 1). The heparin-prolonged TIT_2 was taken into account when determining the indices of both competence inhibition, (Ct−Cc)/Cc, and progression inhibition, (Pt−Pc)/Pc, (Table 1). The progression index of heparin was 3.6-fold greater than the competence index. These results indicated that the anti-proliferative effect of heparin on SMC can be mainly attributed to inhibition of the progression phase.

Effects of cnidium rhizome-derived non-polar alkyl phthalides on competence and progression phases in smooth muscle cell proliferation

Modes of the anti-proliferative effects of cnidium rhizome-derived non-polar alkyl phthalides containing ligustilide, senkyunolide A, butylidenephthalide and butylphthalide were compared with that of heparin in the primary cultured SMC. Both ligustilide (1 μg/ml) and senkyunolide A (1 μg/ml) significantly inhibited 10% FBS-induced proliferation of SMC from the fourth to the 11th day in culture. They also prolonged the starting time

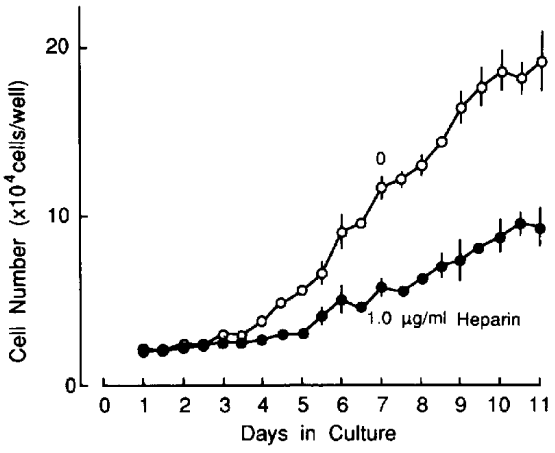


Fig. 1. Time-dependent inhibitory effect of heparin on 10% FBS-induced proliferation of primary cultured smooth muscle cells (SMC) of mouse aorta. SMC were plated at a density of 3.0×10^4 cells/well in 10% FBS-DMEM with (●) or without (○) 1 μg/ml heparin. The values represent the means±S.E.M. of 4 experiments. The values with heparin were significantly different from those without it from day 4 to day 11 ($P<0.05$: day 4, $P<0.01$: days 4.5–11).

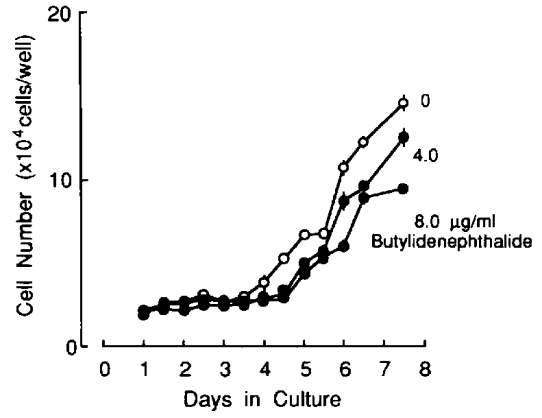


Fig. 3. Time-dependent inhibitory effect of butylidenephthalide (BP) on 10% FBS-induced proliferation of primary cultured SMC. SMC (3×10^4 cells/well) were incubated with (●) or without (○) BP (4 and 8 μg/ml). Values represent the means±S.E.M. of 3 experiments. The values with BP were significantly different from those without it from day 4.5 to day 7.5 ($P<0.05$: BP (4 μg/ml), days 5.5–6 and day 7.5; BP (8 μg/ml), day 5.5; $P<0.01$: BP (4 μg/ml), days 4.5–5 and day 6.5; BP (8 μg/ml), days 4.5–5 and days 6–7.5).

of the SMC proliferation and decreased the slope of the proliferation curve (Fig. 2). Butylidenephthalide (4 and 8 μg/ml) significantly inhibited SMC proliferation from days 4.5 to 7.5 and prolonged the starting time but did not decrease the slope of the proliferation curve (Fig. 3). Values of TIT_2 increased by ligustilide and senkyunolide A were 1.95 and 1.90, respectively; and the competence indices of both phthalides were 1.2- and 1.4-fold greater than the corresponding progression index, respectively (Table 1). Values of TIT_2 and competence index increased by butylidenephthalide (4 and 8 μg/ml) were smaller than those of ligustilide and senkyunolide A, but the ratios of the competence to the progression indices for butylidenephthalide were greater than those of ligustilide and senkyunolide A. Butylphthalide (4 μg/ml) showed no effect on the SMC proliferation (data not shown). These results indicated that ligustilide, senkyunolide A and butylidenephthalide among the non-polar alkyl phthalides had relatively greater inhibitory effects on the competence than progression, being different from heparin.

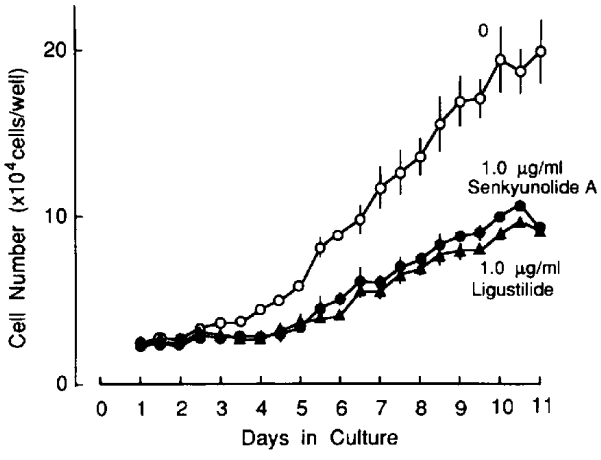


Fig. 2. Time-dependent inhibitory effects of ligustilide and senkyunolide A on 10% FBS-induced proliferation of primary cultured SMC. SMC (3.0×10^4 cells/well) were incubated with ligustilide (1 μg/ml, ▲) and senkyunolide A (1 μg/ml, ●) or without them (○). Values represent the means±S.E.M. of 4 experiments. The values with ligustilide or senkyunolide A were significantly different from those without them from day 4 to day 11 ($P<0.01$: ligustilide, days 4–11; senkyunolide A, days 4–11).

Effects of cnidium rhizome-derived hydroxylated phthalides on competence and progression phases in smooth muscle cell proliferation

Anti-proliferative modes induced by hydroxylated phthalides in cnidium rhizome on the primary culture of SMC were compared with those of ligustilide and senkyunolide A. Senkyunolide H (0.1 and 1 μg/ml) and senkyunolide L (0.1 μg/ml) potently prolonged the starting time of the proliferation and decreased the slope of the proliferation curve (Fig. 4). At 1 μg/ml, senkyunolide H-induced increases in TIT_2 and competence index were greater than those induced by ligustilide and senkyunolide A at the same dose (Table 1). Its progression index was also greater than those induced by these non-polar alkyl phthalides. The anti-progression effects of senkyunolide H (0.1 and 1 μg/ml) was 1.3-fold and 1.8-fold greater than the corresponding anti-com-

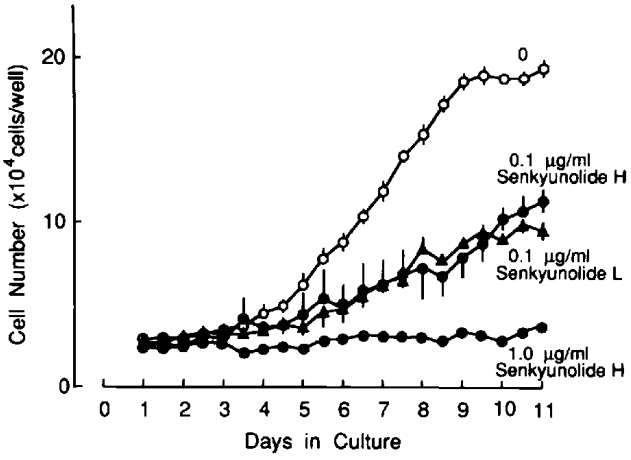


Fig. 4. Time-dependent inhibitory effects of senkyunolide H and senkyunolide L on 10% FBS-induced proliferation of primary cultured SMC. SMC (3.0×10^4 cells/well) were incubated with senkyunolide H (0.1 and 1 μg/ml, ●) and senkyunolide L (0.1 μg /ml, ▲) or without them (○). Values represent the means±S.E.M. of 2–8 experiments. The values with senkyunolide H or L (0.1 μg/ml) were significantly different from those without them from day 6 to day 11 ($P<0.05$: senkyunolide H, day 6.5; $P<0.01$: senkyunolide H, day 6 and days 7–11; senkyunolide L, days 6–11).

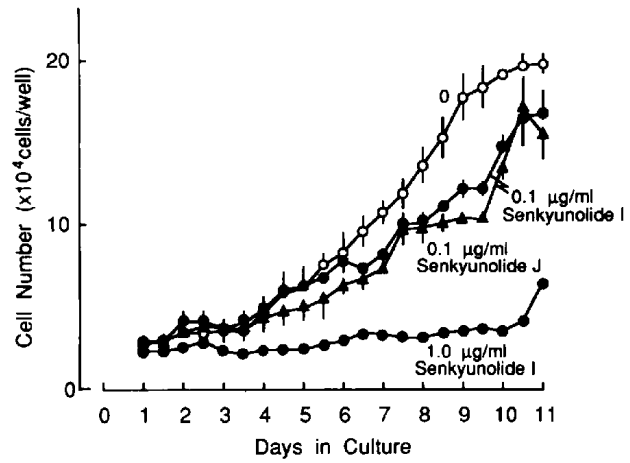


Fig. 5. Time-dependent inhibitory effects of senkyunolide I and senkyunolide J on 10% FBS-induced proliferation of primary cultured SMC. SMC (3.0×10^4 cells/well) were incubated with senkyunolide I (0.1 and 1 μg/ml, ●) and senkyunolide J (0.1 μg /ml, ▲) or without them (○). Values represent the means±S.E.M. of 2–5 experiments. The values with senkyunolides I and J (0.1 μg/ml) were significantly different from those without them on day 9.5 ($P<0.05$) and day 10 ($P<0.01$) (senkyunolide I) and on day 9 ($P<0.05$) and days 9.5–10 ($P<0.01$) (senkyunolide J).

petence ones, respectively. Senkyunolide L (0.1 μg/ml) showed greater values of TIT_2, competence and progression indices than senkyunolide H. These results indicated that (*Z*)-6,7-chlorohydrin and (*Z*)-6,7-dihydroxy isomers among the dihydroligustilide derivatives had a more potent anti-competence effect than ligustilide and senkyunolide A.

Senkyunolide I (0.1 and 1 μg/ml) and senkyunolide J (0.1 μg/ml) also prolonged the starting time of the proliferation and decreased the slope of the proliferation curve (Fig. 5). The values of TIT_2, competence and progression indices increased by these (*E*)-dihydroxy isomers were smaller than those of senkyunolide H at 0.1 and 1 μg/ml (Table 1). The progression indices increased by senkyunolide I at these concentrations were 2.5- and 2.2-fold greater than their competence indices, respectively. The ratios of progression to competence indices increased by senkyunolide I were greatest among those of the hydroxylated phthalides used. Senkyunolide J had a 1.9-fold greater competence index than senkyunolide I (Table 1). However, the value of the competence index changed by senkyunolide J was smaller than those of senkyunolide H and L. These results demonstrate that the 3-*n*-butyl side chain had only a minor action in the competence inhibition.

Other hydroxylated cnidium phthalides (0.1 μg/ml) including senkyunolides D, E, F and G had no effect on the SMC proliferation (data not shown). These results indicated that the 3- and 9-hydroxy groups in ligustilide, dihydroligustilide and butylidenephthalide derivatives did not influence the SMC proliferation, but (*Z*)-6,7-dihydroxy and (*Z*)-6,7-chlorohydrin isomers in the dihydroligustilide derivatives contributed to competence inhibition.

DISCUSSION

It is well established that vascular SMC proliferation is an early key event in the formation of atherosclerotic plaques (2, 4). The vascular SMC appear in at least two different phenotypic states in the media: one is the contractile state in which the SMC become highly differentiated muscles; the other is the synthetic state in which they have a fibroblast-like appearance and proliferate. The change in differentiated characteristics of the SMC is reversible in a primary culture. The SMC proliferation is initiated to be competent for synthesis of DNA by competence factors, which allows competent cells to progress through G_0/G_1 and then synthesize DNA by progression factors (5). The present study focused on finding new anti-atherosclerotic compounds that are effective in the primary cultured SMC and investigated the anti-competence effects of cnidium-derived phthalides on FBS-induced proliferation. These phthalides potently inhibited the competence phase, and their potencies were in the order of senkyunolides L>H>J>I>ligustilide=senkyunolide A>butylidenephthalide. These results demonstrate that selective anti-competence effects were observed by (*Z*)-6,7-chlorohydrin and (*Z*)-6,7-dihydroxy isomers among the dihy-

droligustilide derivatives. These two compounds showed more potent inhibition than the (*E*)-6,7-dihydroxy isomer. Senkyunolide L is considered to be formed during the extraction of cnidium rhizome (10).

These cnidium-derived phthalides, in addition, inhibited the progression phase; and their potencies were in the order of senkyunolides L > H > J > I > ligustilide > senkyunolide A > butylidenephthalide. The order of their progression inhibition was parallel with that for the competence inhibition. Three inhibitory patterns of these phthalides were observed: the relative competence inhibition of butylidenephthalide, the relative progression inhibition of senkyunolide I, and both inhibitions as exhibited by senkyunolides H and L. These results suggest the possibility that the cnidium-derived phthalides have two independent sites of action on both competence and progression phases of the SMC proliferation.

Senkyunolide J had a greater anti-competence effect than senkyunolide I. The 3-*n*-butyl side chain in senkyunolide J may contribute to the inhibition of the competence phase rather than the 3-*n*-butylidene chain in senkyunolide I. However, anti-competence effects of senkyunolide A and butylphthalide that have a 3-*n*-butyl chain were not greater than those of ligustilide and butylidenephthalide that each have a 3-*n*-butylidene chain. These results demonstrate that the 3-*n*-butyl chain in the ligustilide derivatives induced minor inhibition of the competence, compared with the (*Z*)-6,7-chlorohydrin and (*Z*)-6,7-dihydroxy derivatives.

The anti-proliferative effect of these cnidium-derived phthalides is selective for SMC and not for vascular endothelial cells (3). The inhibitory effect is reversible and not due to cell damage and/or toxicity. The modes of anti-proliferation were attributed to both inhibitions of the competence and progression, but these inhibitory mechanisms are not well known. Tyrphostins, which have similar chemical structures to these cnidium-derived phthalides, inhibit platelet-derived growth factor (PDGF)-dependent proliferation of cultured SMC at doses similar to those that inhibit PDGF-dependent tyrosine phosphorylation (13).

The competence phase of DNA synthesis is initiated by competence factors containing PDGF and FBS (5, 7, 14). We have previously analyzed PDGF- and FBS-stimulated competence of DNA synthesis by a convenient method (7, 12). Heparin does not inhibit the PDGF-induced competence phase, suggesting that it inhibits the progression phase in the SMC (7). The present analysis on the number of proliferative SMC supported the effect of heparin on the progression activity. These results mean that the analysis of competence and progression indices in SMC proliferation could also be performed by counting cell number. The mechanisms of heparin are suggested to suppress the expression of c-myb proto-oncogene, which induces the progression from the G_1 phase to the S phase in SMC (15), and also inhibit the induction of mRNA expression of competence genes containing c-fos and c-myc in SMC (16, 17).

In conclusion, the (*Z*)-6,7-dihydroxy isomer of the dihydroligustilide derivatives is essential for the anti-competent effect on the proliferation in primary cultures of SMC in mouse aorta. Senkyunolide H derived from cnidium rhizome may be a prototype for a new anti-atherosclerotic drug.

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