Anti-competence Effects of Synthetic Phthalide Derivatives on Platelet-Derived Growth Factor-Induced DNA Synthesis in Primary Cultures of Rat Aorta Smooth Muscle Cells

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The inhibitory effect of 3-butyldiene-4,5-dihydroxyphthalide (BP-42) on platelet derived growth factor (PDGF)-BB-induced DNA synthesis was investigated in synchronized smooth muscle cells (SMC) in primary culture of rat aorta. BP-42 (0.3—3 μ g/ml) inhibited the PDGF-BB (30 ng/ml)-stimulated [³H]thymidine incorporation of the SMC in a concentration-dependent manner. BP-42 inhibited both the competence and progression phases of [³H]thymidine incorporation induced by PDGF-BB. Using the competence assay, BP-42 (0.3—10 μ g/ml) delayed PDGF-BB (30 ng/ml)-accelerated starting time of [³H]thymidine incorporation in a concentration-dependent manner, confirming that BP-42 inhibited PDGF-BB-induced competence phase of DNA synthesis of SMC. BP-42 (1—10 μ g/ml) also delayed basic fibroblast growth factor (bFGF: 30 ng/ml)-accelerated starting time of [³H]thymidine incorporation. The inhibitory potency of BP-42 for the competence action of PDGF-BB was similar to that for the action of bFGF. BP-421 (3-heptyldiene-4,5-dihydroxyphthalide) and BP-422 (3-benzylidene-4,5-dihydroxyphthalide) had 3-fold greater inhibitory potencies than BP-42 for the PDGF-BB-induced competence activity. These results demonstrated that BP-42 inhibited PDGF-BB-induced competence activity of DNA synthesis in primary cultured SMC of rat aorta *via* a common signal transduction mechanism with bFGF. BP-421 and BP-422 were more potent inhibitors for the competence activity, suggesting that they may become a prototype of new anti-atherosclerotic drugs.

Key words anti-competence activity; synthetic phthalide derivative; anti-atherosclerosis; platelet-derived growth factor; primary cultured smooth muscle cell; rat aorta

An abnormal proliferation of vascular smooth muscle cells (SMC) is a critical event in advanced plaque of atherosclerosis.¹⁾ The proliferation of BALB/c 3T3 cells is suggested to be regulated by specific cellular phases which involve those of both competence and progression.²⁾ Competence factors such as platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF) initiate the proliferative response of the cells.³⁾ These factors induce competent cells to respond to the progression factors required for cell proliferation. A specific inhibitor for the competence phase in SMC may be advantageous in improving atherosclerosis. The search for an anti-competence activity in primary cultured SMC is one of the approaches for finding a new type of anti-atherosclerotic drug.

We have developed a convenient assay to determine relative indices of competence and progression by measuring the starting time and rate of cell proliferation in SMC and endothelial cells.^{4–8)} With this assay, PDGF-BB and basic FGF (bFGF) are suggested to induce dual actions of competence and progression in the DNA synthesis of primary cultured SMC.^{4–6)} Butyldiene-phthalide (BP), a compound derived from cnidium rhizome, is shown to be a specific inhibitor of fetal bovine serum (FBS)-induced competence activity.^{8,9)} BP-42 (3-butyldiene-4,5-dihydroxyphthalide) potentially inhibited the competence phase as well as the progression phase in FBS-induced proliferation in the primary cultured SMC. BP-42 is thought to have two independent sites of action in both these phases.¹⁰⁾

The purpose of the present study was to examine the

inhibitory effects of BP-42 on PDGF-BB-induced competence in DNA synthesis in the primary cultured aortic SMC. The inhibitory effects of BP-42-related compounds on the PDGF-induced competence activity were also investigated.

MATERIALS AND METHODS

Cell Culture The thoracic aorta of male Wistar rats (9—11 weeks old, Japan Shizuoka Laboratory Center, Hamamatsu) were used. Primary cultured SMC of the rat aorta were prepared by the procedure of Chamley *et al.*¹¹⁾ with the following modification.⁴⁾ The aorta was isolated in Hanks' solution (136.8 mM NaCl, 5.4 mM KCl, 0.8 mM MgSO₄, 1.3 mM CaCl₂, 0.4 mM KH₂PO₄, 0.3 mM Na₂HPO₄, 4.2 mM NaHCO₃ and 5.6 mM glucose, pH 7.3) and was incubated with 1 mg/ml type I collagenase and 0.1—0.8 U/ml type I elastase (Sigma, St. Louis, MO, U.S.A.) in Hanks' solution for 30 min at 37 °C. After removal of the adventitia, the remaining tissue was incubated with 6 mg/ml collagenase (Wako, Osaka) and 0.25—2.5 U/ml type I elastase in Hanks' solution for 1.5 h at 37 °C with gentle shaking. Isolated cells were collected by centrifugation at 150 × *g* for 5 min at 4 °C and dispersed in Dulbecco's modified Eagle's medium (DMEM, Nissui, Tokyo) that was supplemented with 5% heat-inactivated FBS (Whittaker Bioproduct, Walkersville, MD, U.S.A.), 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 × 10⁴ cells/well were plated and cultured in a 24-well plate (Corning, Corning, NY,

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U.S.A.) at 37 °C under a humidified atmosphere of 5% CO₂ and 95% air. The primary cultured cells grew in a "hills and valleys" pattern at confluence¹²⁾ and were bound positively with anti- α -smooth muscle actin-monoclonal antibody.⁴⁾

Thymidine Incorporation into Synchronized Smooth Muscle Cells DNA synthesis of SMC was determined by measuring [³H]thymidine incorporation of SMC by the method of Stiles *et al.*³⁾ with some modification.^{4,5)} After being cultured for 6 d, the subconfluent SMC were synchronized at the G₀ phase by serum starvation for 2 d.¹³⁾ The synchronized cells were incubated in serum-free DMEM containing recombinant human PDGF-BB (30 ng/ml, Amersham Japan, Tokyo) and recombinant human bFGF (30 ng/ml, expressed in *Escherichia coli*, Kaken Seiyaku, Tokyo) with BP-42, BP-421 (3-heptylidine-4,5-dihydroxyphthalidel) and BP-422 (3-benzylidene-4,5-dihydroxyphthalide) for 3 h at 37 °C in DMEM. These drugs were provided by Tsumura Research Institute for Pharmacology (Tokyo). After washing out those factors and drugs, 0.037 MBq [methyl-³H]thymidine (925 GBq/mmol, Amersham Japan, Tokyo) was incubated in the culture medium (1% FBS-DMEM) of SMC with the same factors and drugs at 37 °C for the indicated period. For measurement of the competence activity alone (the competence assay), the SMC were pre-incubated with the factors and drugs for 3 h and then incubated without them and with 1% FBS-DMEM containing [³H]thymidine for the indicated period. Tritium-thymidine incorporation into the SMC was stopped every 3 h by removing the culture medium and adding 0.5 ml 5% trichloroacetic acid. After incubation for 30 min at 4 °C, [³H]-labeled cells were washed 3 times with 5% trichloroacetic acid and lysed with 0.5 ml 1 N NaOH. The cell lysate was neutralized and the radioactivity of the sample was measured with a scintillation spectrometer (LS-3801, Beckman, Clifton, U.S.A.). These drugs did not damage and/or have a toxic effect on the SMC during their treatments, because no uptake of trypan blue was observed thereafter.¹⁴⁾

Estimation of Relative Indices for Competence and Progression Phases in DNA Synthesis The values of [³H]thymidine incorporation were plotted against incubation time and a most-likely regression line was estimated by the least-squares method. C_c and C_i were the starting time of thymidine incorporation with or without drug. P_c and P_i were the doubling time of thymidine incorporation with or without drug. The index of competence inhibition was estimated as the relative difference $((C_i - C_c)/C_c)$ of the starting time of DNA synthesis. The index of progression inhibition was estimated as the relative difference $((P_i - P_c)/P_c)$ of the doubling time.^{5,6)}

Statistical Analysis Data were expressed as means $\pm$ S.E. and tested by one-way analysis of variance (ANOVA). The significance of difference was judged using Scheffe's or Tukey's test at $p = 0.05$ or 0.01 .

RESULTS

Inhibitory Effect of BP-42 on PDGF-BB-Induced DNA Synthesis of Synchronized Smooth Muscle Cells in Primary

Fig. 1. Time-Dependent Inhibitory Effects of BP-42 on PDGF-BB-Induced [³H]Thymidine Incorporation in Primary Cultured and Synchronized Smooth Muscle Cells of Rat Aorta

The cells were administered for 3 h with PDGF-BB (30 ng/ml) plus BP-42 (●: 0, 0.3, 1, 1.5 and 3 μ g/ml), and 10% FBS alone (○). After washing out the PDGF-BB and BP-42, the cells were incubated with [³H]thymidine in the presence of 1% FBS, 30 ng/ml PDGF-BB and BP-42 (●: same concentration as preadministration), and 10% FBS alone (○). The values represent the means $\pm$ S.E. of 3–6 experiments. The significance of difference from the control without drug was determined by one-way ANOVA and Scheffe's test: a) $p < 0.05$, b) $p < 0.01$.

Culture The inhibitory effect of BP-42 on PDGF-BB-induced DNA synthesis was investigated on synchronized SMC in primary culture. PDGF-BB (30 ng/ml) and BP-42 (0.3, 1, 1.5 and 3 μ g/ml) were continuously treated during the culture of synchronized SMC. The continuously treated PDGF-BB started the incorporation of [³H]-thymidine into the SMC from 12 h after the culture with 1% FBS and increased for up to 21 h in a time-dependent manner (Fig. 1). The time-dependent effect of PDGF-BB was greater than that of 10% FBS. BP-42 inhibited the PDGF-BB-stimulated [³H]thymidine incorporation in a concentration-dependent manner (Fig 1). BP-42 significantly inhibited the [³H]thymidine incorporation from 21 h to 24 h at 0.3 μ g/ml, from 12 h to 27 h at 1 and 1.5 μ g/ml and from 9 h to 30 h at 3 μ g/ml. Its 50% inhibitory concentration value (IC_{50} : 95% confidence limits) was 1.67 μ g/ml (1.52–1.84) at 27 h. BP-42 prolonged the starting time and decreased the rate of [³H]thymidine incorporation.

Inhibitory Effects of BP-42 on PDGF-BB and bFGF-Induced Competence Phases of DNA Synthesis in Synchronized Smooth Muscle Cells in Primary Culture The effect of BP-42 on the competence action on DNA synthesis of PDGF-BB preadministered for 3 h was compared with that on the action of continuously treated PDGF-BB. The preadministered PDGF-BB (30 ng/ml) started the incorporation of the [³H]thymidine from 15 h after culture in 1% FBS and increased for up to 27 h in a time-dependent manner (Fig. 2). The time-dependent effect of PDGF-BB was comparable to that of 10% FBS. BP-42 (0.3, 3 and 10 μ g/ml) delayed the starting time of PDGF-BB-accelerated [³H]thymidine incorporation to 18 h at 3 μ g/ml and to 27 h at 10 μ g/ml. However, the slope of [³H]thymidine incorporation curve was not changed

Fig. 2. Time-Dependent Inhibitory Effects of BP-42 on PDGF-BB-Induced Competence Phase of $[^3\text{H}]$ Thymidine Incorporation in Primary Cultured and Synchronized Smooth Muscle Cells of Rat Aorta

The cells were administered for 3 h with PDGF-BB (30 ng/ml) plus BP-42 (●: 0, 0.3, 3 and 10 $\mu\text{g}/\text{ml}$), and 10% FBS alone (○). After washing out the PDGF-BB and BP-42, the cells were incubated with $[^3\text{H}]$ thymidine in the presence of 1% FBS (●) and 10% FBS (○). The values represent the means $\pm$ S.E. of 5–8 experiments. The significance of difference from the control without drug was determined by one-way ANOVA and Scheffe's test: b) $p < 0.01$.

Fig. 3. Relative Inhibitory Effects of BP-42 on the Competence Phase and Progression Phase of PDGF-BB-Induced $[^3\text{H}]$ Thymidine Incorporation in Primary Cultured and Synchronized Smooth Muscle Cells of Rat Aorta

All points were replotted from the data in Fig. 1 (●) and Fig. 2 (▲) as described in Methods. The regression line was obtained by the least-squares method.

by 3 $\mu\text{g}/\text{ml}$ BP-42. The IC_{50} value of BP-42 for the PDGF-BB-induced thymidine incorporation on 27 h was 2.96 $\mu\text{g}/\text{ml}$ (1.29–6.80).

The effects of competence and progression indices induced by continuously treated PDGF-BB and BP-42 were compared with those induced by their preadministration (Fig. 3). These values were replotted from the data of Figs. 1 and 2. Slope of the regression line under the continuously treated experiment was 0.62 and the slope under the pretreated experiment was 0.03. These results demonstrated that BP-42 inhibited both competence and progression phases of DNA synthesis induced by PDGF-BB and only the anti-competence effect of BP-42 was measured in the competence assay.

The effect of BP-42 on the competence action of bFGF

Fig. 4. Time-Dependent Inhibitory Effects of BP-42 on bFGF-Induced Competence Phase of $[^3\text{H}]$ Thymidine Incorporation in Primary Cultured and Synchronized Smooth Muscle Cells of Rat Aorta

The cells were administered for 3 h with bFGF (30 ng/ml) plus BP-42 (●: 0, 1, 3 and 10 $\mu\text{g}/\text{ml}$), and 10% FBS alone (○). After washing out the bFGF and BP-42, the cells were incubated with $[^3\text{H}]$ thymidine in the presence of 1% FBS (●) and 10% FBS (○). The values represent the means $\pm$ S.E. of 5 experiments. The significance of difference from the control without drug was determined by one-way ANOVA and Tukey's test: a) $p < 0.05$, b) $p < 0.01$.

Fig. 5. Time-Dependent Inhibitory Effects of BP-421 on PDGF-BB-Induced Competence Phase of $[^3\text{H}]$ Thymidine Incorporation in Primary Cultured and Synchronized Smooth Muscle Cells of Rat Aorta

The cells were administered for 3 h with PDGF-BB (30 ng/ml) plus BP-421 (●: 0, 0.3, 1 and 3 $\mu\text{g}/\text{ml}$). After washing out the PDGF-BB and BP-421, the cells were incubated with $[^3\text{H}]$ thymidine in the presence of 1% FBS. The values represent the means $\pm$ S.E. of 5 experiments. Significance of difference from the control without drug was determined by one-way ANOVA and Tukey's test: a) $p < 0.05$, b) $p < 0.01$.

was compared with that on the competence action of PDGF-BB in the competence assay. bFGF (30 ng/ml) started the incorporation of $[^3\text{H}]$ thymidine 15 h after the SMC culture with 1% FBS and increased for up to 27 h in a time-dependent manner (Fig. 4). The time-dependent curve of bFGF was also comparable to that of 10% FBS. BP-42 (1, 3 and 10 $\mu\text{g}/\text{ml}$) delayed the bFGF-accelerated starting time of the incorporation to 18 h (1 $\mu\text{g}/\text{ml}$), to 21 h (3 $\mu\text{g}/\text{ml}$) and to 27 h (10 $\mu\text{g}/\text{ml}$), respectively. Its IC_{50}

Fig. 6. Time-Dependent Inhibitory Effects of BP-422 on PDGF-BB-Induced Competence Phase of $[^3\text{H}]$ Thymidine Incorporation in Primary Cultured and Synchronized Smooth Muscle Cells of Rat Aorta

The cells were administered for 3 h with PDGF-BB (30 ng/ml) plus BP-422 (●: 0, 0.3, 1 and 3 $\mu\text{g/ml}$). After washing out the PDGF-BB and BP-422, the cells were incubated with $[^3\text{H}]$ thymidine in the presence of 1% FBS. The values represent the means $\pm$ S.E. of 5 experiments. The significance of difference from the control without drug was determined by one-way ANOVA and Tukey's test: a) Significant $p < 0.05$, b) $p < 0.01$.

value for the bFGF-induced action on 27 h was 3.35 $\mu\text{g/ml}$ (1.82–6.15). These results demonstrated that the inhibitory effect of BP-42 on the bFGF-stimulated competence activity was similar to that on the PDGF-BB-stimulated activity.

Inhibitory Effects of BP-421 and BP-422 on PDGF-BB-Induced Competence Phase of DNA Synthesis in Synchronized Smooth Muscle Cells The inhibitory effects of BP-421 and BP-422 were examined on the PDGF-BB-induced competence activity. BP-421 delayed the PDGF-BB (30 ng/ml)-accelerated starting time of thymidine incorporation to 18 h at 1 $\mu\text{g/ml}$ and to 30 h at 3 $\mu\text{g/ml}$ (Fig. 5). The slope of thymidine incorporation curve was not changed by 1 $\mu\text{g/ml}$ BP-421. BP-422 (1 and 3 $\mu\text{g/ml}$) also delayed the starting time of $[^3\text{H}]$ thymidine incorporation to 18 h at 1 $\mu\text{g/ml}$ and to 24 h at 3 $\mu\text{g/ml}$ (Fig. 6). IC_{50} values of BP-421 and BP-422 for the PDGF-BB-induced action on 27 h were 0.88 $\mu\text{g/ml}$ (0.52–1.51) and 1.19 $\mu\text{g/ml}$ (0.87–1.62), respectively. These potencies of BP-421 and BP-422 for the inhibition of the PDGF-BB-induced competence activity were 3.4- and 2.5-fold greater than that of BP-42, respectively.

DISCUSSION

The proliferation of vascular SMC is controlled by competence and progression factors.¹⁵⁾ PDGF stimulates proliferation of vascular SMC¹⁶⁾ and its action is closely related to the promotion of atherosclerosis.¹⁾ PDGF-BB- and bFGF-induced cell proliferations are suggested to be induced by their activation of competence phase (early G_0 and G_1) in SMC and fibroblast.^{2,15)} We previously reported that PDGF-BB and bFGF activate not only competence phase but also progression phase of SMC proliferation in the primary culture.^{4–6)} The following

cellular events after PDGF binding to the receptor have been reported^{17–20)}: tyrosine phosphorylation of the receptor, internalization of the receptor-ligand complex, phosphatidyl inositol turnover, protein kinase C activation, tyrosine- and serine-specific phosphorylation, mobilization of Ca^{2+} , alternation of cytoskeletal element, expression of the protooncogenes *c-fos* and *c-myc*, and cell proliferation. In the case of bFGF, the events after its binding to the receptor are elucidated as follows: tyrosine phosphorylation of the receptor, protein kinase C activation and expression of *c-fos* mRNA.^{13,21,22)} PDGF and bFGF thus possess a common signal transduction pathway in their post-receptor processes to proliferate SMC.

In the present study we showed that BP-42 inhibited both PDGF-BB-induced competence and progression phases in DNA synthesis of the SMC in the primary culture. The result was paralleled by the evidence that it potentially inhibits the competence and progression phases of FBS-induced SMC proliferation.¹⁰⁾ Using the competence assay, BP-42 was demonstrated to inhibit both PDGF-BB-induced and bFGF-induced competence activities in their DNA synthesis with the same potency. These results suggested that the site of action for BP-42 is not in the receptors for these growth factors, but in a common signal transduction pathway in their competence activities. BP-42 was selected from the viewpoint of structure-activity relationship on the competence inhibition of cnidium rhizome-derived compounds, senkyunolide H and BP which potently and specifically inhibit the competence phase of FBS-induced proliferation in primary cultured SMC, respectively.^{8,9)} The anti-proliferative effect of cnidium derived phthalide is selective for the vascular SMC but not for the vascular endothelial cells.⁹⁾ BP-42 did not damage and/or had a toxic effect on the SMC during the 11-d treatments, because no uptake of trypan blue was observed after these treatments.¹⁰⁾ IC_{50} value of BP-42 for the FBS-induced SMC proliferation was 0.17 $\mu\text{g/ml}$ (0.14–0.22) on day 10. In the present study, the IC_{50} for the PDGF-BB-induced DNA synthesis was 1.67 $\mu\text{g/ml}$ (1.52–1.84) at 27 h. Since the potency of BP-42 on PDGF-BB-induced DNA synthesis was weaker than that on FBS-induced SMC proliferation, the site of action for BP-42 was not selective for the receptor of PDGF-BB.

We previously reported that longer side chains such as 3-bromo-1-octenyl at C3 in the 6,7-dihydroxyphthalide cause the potent anti-competence effect on the SMC proliferation.⁸⁾ The present study demonstrated that 3-heptylidine (BP-421) and 3-benzylidene side chains (BP-422) at C3 in the 4,5-dihydroxyphthalide had 3-fold greater potencies for the competence inhibition of DNA synthesis than BP-42. These anti-competence drugs may thus become a new type of anti-atherosclerotic drug. Since diabetic macrophages abnormally release PDGF-BB and are suggested to have a crucial role in inducing complications of diabetes mellitus such as retinopathy,²³⁾ these anti-competence drugs may be candidates for remedies for retinopathy in diabetic complications as well as atherosclerosis.

In conclusion, BP-42 suppressed PDGF-BB-induced competence activities of DNA synthesis of primary

cultured SMC of rat aorta *via* a common mechanism of signal transduction of bFGF. BP-421 and BP-422 were more potent inhibitors for the competence activity, suggesting that they may become prototypes of new anti-atherosclerotic drugs.

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