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# RESPONSES OF SMOOTH MUSCLE CELLS TO PLATELET-DERIVED GROWTH FACTOR ARE INHIBITED BY HERBIMYCIN-A TYROSINE KINASE INHIBITOR+

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**Platelet-derived growth factor (PDGF) plays important roles in proliferation of smooth muscle cells (SMCs). PDGF is also known to induce the cytoskeletal reorganization as a chemoattractant. In this communication, we demonstrate that the tyrosine kinase inhibitor herbimycin-A (HM-A) inhibited the effects of PDGF on SMCs in proliferation, phenotypic modulation, and reorganization of F-actin. Another tyrosine kinase inhibitor genistein also inhibited reorganization of F-actin. Since both tyrosine kinase inhibitors reduced PDGF-dependent tyrosine phosphorylation, tyrosine kinase activities may be involved not only in proliferation but also in reorganization of F-actin.** 

PDGF is one of the most potent growth factors on proliferation of SMCs (1). PDGF binds to the receptor, which stimulates tyrosine kinase (TK) activity (2,3), and activates signal transducing proteins such as phospholipase C (4) and GTPase-activating protein(5). It has been proposed that the activation of TKs is an essential step for the actions of PDGF as a growth factor. However it is not yet known whether or not PDGF expresses its function as a chemoattractant through TKs.

Herbimycin-A (HM-A) is well known to inhibit wide broad of TKs. HM-A normalizes the rat kidney cells transformed by Rous-Sarcoma virus (6). By using this compound, we investigated effects of PDGF on rat pulmonary artery smooth muscle cell (PAC-1) (7). PDGF promoted proliferation and phenotypic modulation of SMCs during 3 to 4 days incubation, and reorganized actin filaments immediately after addition of the growth factor to culture medium. These effects were antagonized by HM-A. The data

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Abbreviations---DMEM, Dulbecco's Modified Eagle's medium; FCS, fetal calf serum; DMSO, dimethyl sulfoxide.

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presented here suggest that TKs regulate not only proliferation but also reorganization of actin filaments.

## Materials and Methods

Cell culture-----Smooth muscle cell line, PAC-1 was established from rat pulmonary artery smooth muscle cells (7). SMCs were maintained in DMEM containing 10% FCS, under 5% $CO_2$ at 37°C. Before assays, SMCs were cultured in DMEM containing 0.2% FCS, in which SMCs kept contractile form. All assays were performed in DMEM containing 0.2% FCS. In the assays for analysis on the effects of HM-A or genistein, cells were pretreated by the TK inhibitor one day prior to addition of PDGF. In control, DMSO was added to the medium at 0.1%.

Chemical materials----- Human recombinant PDGF-BB was purchased from Gibco. HM-A was obtained from Wako Pure Chemicals (Japan) and solved and stored in DMSO at final concentration of 1 mg/ml. Phalloidin-rhodamine was from Molecular Probe Co. Genistein was purchased from Extrasynthese.

Antibodies-----Anti-α-actin monoclonal antibody and anti-phosphotyrosine monoclonal antibody were purchased from BioMakor and Sigma, respectively.

Cell proliferation assay-----SMCs were cultured in 96-well plate (Corning) under various conditions. Cells were trypsinized for 10 min, and counted with Hemocytometer. All assays were triplicated.

Western-blotting ----- Cells cultured on dishes (10 cm diameter), were extracted by 2% SDS sample solution saturated with urea. All samples were preliminarily electrophoresed on SDS polyacrylamide gel, diluted to make total actin concentrations equal and then immunoblotted with anti-α-actin or anti-phosphotyrosine antibody.

Cell staining----- Cells, cultured on coverslips, were treated with PDGF in the presence or absence of the tyrosine kinase inhibitor, and then fixed with 3.7% formaldehyde in phosphate-buffered saline (PBS). Fixed cells were permeabilized with 0.2% TritonX-100 and incubated in 1% phalloidin-rhodamine in PBS.

## Results and Discussion

The proliferative effects of PDGF on smooth muscle cells were investigated. Cells were counted in number 3 days after treatment with PDGF. PDGF promoted proliferation of SMCs in a dose dependent manner (Fig 1). Half maximum concentration was 2.5 ng/ml. PDGF induced phenotypic modulation in this process. α-Actin decreased as concentrations of PDGF increased (Fig 1, inset), as reported in (8). At 10 ng/ml of PDGF, α-actin was about one-third of control.

In order to clarify the involvement of TK activities in proliferative effects of PDGF, the TK inhibitor HM-A was added to the medium in the presence or absence of PDGF. The cells were cultured for 3 days and counted in number (Fig 2). HM-A inhibited the proliferation of SMCs induced by PDGF. This inhibitory effect was not caused by the cytotoxicity of HM-A, because the proportion of α-actin content to total actin increased as the concentration of HM-A increased (Fig 2, inset). HM-A inhibited phenotypic modulation of SMCs induced by PDGF as well as cell proliferation.

The content of phosphotyrosine was determined by immunoblotting with anti-phosphotyrosine antibody (Fig 3). In the absence of HM-A, phosphotyrosine was increased 15 min after addition of PDGF and then decreased. The most remarkable proteins phosphorylated on tyrosine residues were the two proteins with apparent molecular weights around 180 kDa, most probably PDGF receptor (9). In the presence of HM-A, phosphotyrosine was increased to less extent, suggesting that HM-A antagonized the effects of PDGF on SMCs by inhibiting PDGF-dependent TK activity.

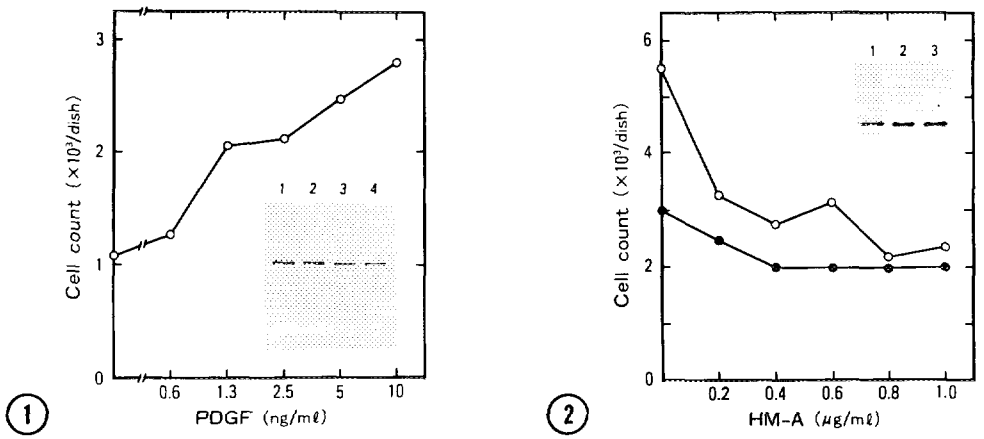


Fig. 1. PDGF-dependent proliferation of SMCs. Triplicate cultures of SMCs were maintained in the assay medium containing various concentrations of PDGF for 3 days. Cells were trypsinized and counted in number. Inset; Western blotting with anti-α-actin monoclonal antibody. Before blotting, content of total actin was adjusted equally. Concentration of PDGF was 0 (lane 1), 2.5 (lane 2), 5.0 (lane 3), and 10 (lane 4) ng/ml, respectively.

Fig. 2. HM-A inhibited PDGF-dependent proliferation of SMCs. SMCs were cultured in presence (open circles) or absence (closed circles) of 10 ng/ml of PDGF with various concentrations of HM-A. Triplicate assays were performed for each condition. Proliferation of SMCs was measured as described in Fig 1. Inset; Cells were cultured in the presence of 10 ng/ml of PDGF with 0 (lane 1), 0.2 (lane 2), and 1.0 (lane 3) μg/ml of HM-A. Cell extracts were immunoblotted with anti-α-actin antibody.

Several growth factors and hormones, such as epidermal growth factor (EGF) and insulin, are known to induce cytoskeletal reorganization in early phase as well as cell proliferation in late phase. As a chemoattractant, PDGF induced reorganization of F-actin in SMCs (Fig 4). Before PDGF treatment , stress fibers were stained with phalloidin-rhodamine. In the absence of HM-A, 15 min after addition of PDGF, stress fibers disappeared, as reported in fibroblast (10). Three hours later, stress fibers reappeared (data not shown). Similar time course of tyrosine phosphorylation and reorganization of actin filaments suggests that reorganization of F-actin is caused by tyrosine

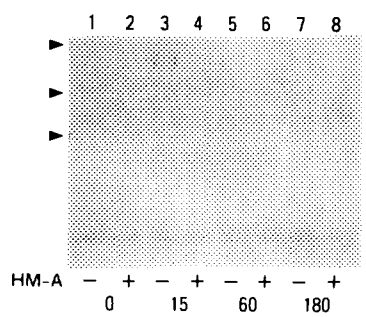


Fig. 3. Time course of tyrosine phosphorylation induced by PDGF. SMCs were treated with 10 ng/ml of PDGF for 0 (lanes 1 and 2), 15 (lanes 3 and 4), 60 (lanes 5 and 6), and 180 (lanes 7 and 8) min in the presence ( lanes 2, 4, 6, and 8) or absence (lanes 1, 3, 5, and 7) of HM-A (1.0 μg/ml). Cell extracts were immunoblotted with anti-phosphotyrosine antibody. Arrowheads indicate molecular weights of 232k, 140k, and 67k.

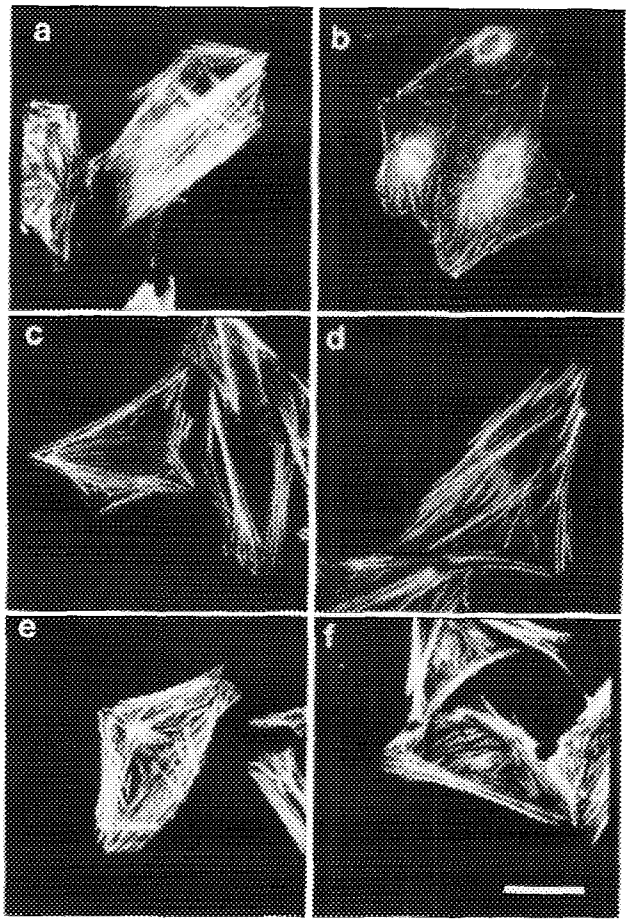


Fig.4. Distribution of actin filaments. SMCs were stained with phalloidin-rhodamine in the absence (a and b) or presence (c and d) of 1.0 μg/ml of HM-A. In e and f, cells were pretreated with 100 μg/ml of genistein. Cells were incubated with (b, d, and f) or without (a, c, and e) PDGF (10 ng/ml) for 15 min. Bar represents 5 μm.

phosphorylation induced by PDGF. Indeed, when tyrosine phosphorylation was inhibited by HM-A, stress fibers were not affected by PDGF (Fig 4,d). Another TK inhibitor genistein exhibited the inhibitory effect on PDGF-dependent tyrosine phosphorylation at half inhibitory concentration of 50 μg/ml (data not shown) in good agreement to the effective concentration of genistein to inhibit EGF-dependent tyrosine phosphorylation (11). As shown in Fig 4, e and f, in the presence of 100 μg/ml genistein, stress fibers were not disrupted by PDGF as in the case of HM-A.

Since reorganization of actin filaments is closely related to cell motility, these findings suggest that migratory ability of SMCs may also be regulated through TK activity activated by PDGF.

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## References

1. Ross,R., Raines,W.E., and Bowen-Pope,D.F. (1986) Cell 46, 155-169
2. Ek,B., Westermark,B., Wasteson,A., and Heldin,C-H. (1982) Nature 295, 419-420
3. Nishimura,J., Huang,J.S., and Deuel,T.F. (1982) Proc. Natl. Acad. Sci. USA 79, 4303-4307
4. Kumjian,D.A., Wahl,M.I., Rhee,S.G., and Daniel,T.O. (1989) Proc. Natl. Acad. USA 86,8232-8236
5. Kaplan,D.R., Morrison,D.K., Wong,G., McCormick,F., and Williams,L.T. (1990) Cell 61, 125-133
6. Uehara,Y., Hori,M., Takeuchi,T., and Umezawa,H. (1986) Mol. Cell. Biol. 6, 2198-2206
7. Rothman,A., Kulik,T.J., Taubman,M.B., Berk,B.C., Smith,C.W.J., and Nadal-Ginard,B. (1992) Circulation 86, 1977-1986
8. Corjay,M.H., Thompson,M.M., Lynch,K.R., and Owens,G.K. (1989) J. Biol. Chem. 264, 10501-10506
9. Frackelton,A.R.,Jr., Tremble,P.M., and Williams,L.T. (1984) J. Biol. Chem. 259, 7909-7915
10. Mellstrom,K., Heldin,C-H., Westermark,B. (1988) Exp. cell Res. 177, 347-359
11. Akiyama,T., Ishida,J., Nakagawa,S., Ogawara,S., Watanabe,S., Itoh,N., Shibuya,M., and Fukami,Y. (1987) J. Biol. Chem. 262, 5592-5595