Rapid Communication

J. Biochem. **128**, 719–722 (2000)

Inhibitory Effects of ML-9, Wortmannin, and Y-27632 on the Chemotaxis of Vascular Smooth Muscle Cells in Response to Platelet-Derived Growth Factor-BB[1]

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Received July 24, 2000; accepted August 31, 2000

The chemotactic migration toward platelet-derived growth factor-BB of SM3, a cell line established from rabbit aorta smooth muscle, was examined by the Boyden chamber method. Myosin light-chain (MLC) kinase inhibitors ML-9 and wortmannin, and the Rho kinase inhibitor Y-27632 effectively reduced the migration. However, neither membrane ruffling nor the phosphorylation of MLC was inhibited concomitantly. The reduction is discussed with reference to a novel property of MLC kinase, which stimulates myosin ATPase activity without phosphorylating MLC [Ye *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96, 6666–6671].

Key words: chemotaxis, kinase inhibitors, myosin light-chain kinase, Rho kinase, vascular smooth muscle cells.

Arterial plaque, one of the morphological features of arteriosclerosis, is formed by the increase in the motile activity of vascular smooth muscle cells (VSMCs) when they are exposed to cytokines (*1*). The motile activity of VSMCs is generated by the interaction of actin filaments with myosin, which is converted from an inactive form to an active form by the phosphorylation of 20-kDa myosin light-chain (MLC20) (*2*). We are interested in the role of myosin light chain kinase (MLCK), because it is expected to phosphorylate MLC20 when the motile activity of VSMCs is enhanced by cytokines (*1*).

Recently, we established several variants of VSMCs lacking MLCK by targeting their expression in SM3, a cell line of VSMCs (*3*), and found that cell migration as examined by the chemotaxis in response to platelet-derived growth factor-BB (PDGF-BB) was impaired in the MLCK-deficient cells (*4*). Unexpectedly, however, phosphorylation of MLC20 in the variants did not appear to be affected. To confirm this result, we treated SM3 cells with the MLCK inhibitors ML-9 (*5*) and wortmannin (*6*) and examined whether they influence both the chemotaxis and MLC20 phosphorylation. We also examined the effect of Y-27632, an inhibitor of Rho-kinase (*7*), because this enzyme is present in SM3 cells and the MLCK-deficient cells (*4*) and might alter the level of MLC20 phosphorylation (*8, 9*). We report here that ML-9, wortmannin, and Y-27632 inhibit chemotaxis of SM3 cells for PDGF-BB without influencing the phosphorylation level of MLC20.

We analyzed the effects of ML-9, wortmannin, and Y-27632 on the motile activity of SM3 cells in response to the attractant PDGF-BB by the Boyden chamber method (*10*). As shown in Fig. 1, in the absence of PDGF-BB, 137.5 ± 19.9 cells (mean ± SD, $n = 6$) migrated from the upper to the lower chamber. When 10 ng/ml of PDGF-BB was present in the lower chamber, migration increased to 288.8 ± 72.9 cells (mean ± SD, $n = 5$) due to chemotaxis toward PDGF-BB. However, in the presence of 10 μM ML-9, 1 μM wortmannin, or 10 μM Y-27632, the numbers of migrating cells were reduced to 135.7 ± 9.8 (mean ± SD, $n = 6$), 56.3 ± 25.3 (mean ± SD, $n = 6$), and 160.0 ± 39.2 (mean ± SD, $n = 5$), respectively, indicating that all of these inhibitors suppressed the chemotaxis of SM3 cells. These findings are consistent with the inhibitory effects of ML-9, wortmannin, and Y-27632 on the chemotaxis of neutrophils (*11*), eosinophils (*12*), hepatic stellate cells (*13*), and cancer cells (*14*).

As described previously, PDGF-BB induces membrane ruffling of SM3 cells (*4*), which is characterized by the assembly of actin filaments that are associated with myosin (*15–19*). To determine whether these inhibitors affect the induction of membrane ruffling, we added 10 ng/ml PDGF-BB to the culture medium of SM3 cells. As shown in Fig. 2, the proportion of cells with membrane ruffling in the control culture was 11.6 ± 12.4% (mean ± SD, $n = 20$ fields). This increased to 57.7 ± 25.9% (mean ± SD, $n = 20$ fields) when 10 ng/ml PDGF-BB was added. In the presence of 10 μM ML-9, the proportion was also elevated, from 3.3 ± 6.7% (in the absence of PDGF-BB, mean ± SD, $n = 20$ fields) to 34.0 ± 26.2% (in the presence of PDGF-BB, mean ± SD, $n = 20$ fields). Thus, ML-9 does not affect the development of membrane ruffling by PDGF-BB. Wortman-

[1] This work was supported by grants from the Mitsubishi Foundation, the Smoking Research Foundation, and the Special Coordination Funds from the Science and Technology Agency of the Japanese Government, and by Grants-in-Aids for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

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Abbreviations: MEM, minimum essential medium; MLCK, myosin light-chain kinase; MLC20, 20-kDa myosin light-chain; PBS, phosphate-buffered saline; PDGF, platelet-derived growth factor; VSMCs, vascular smooth muscle cells.

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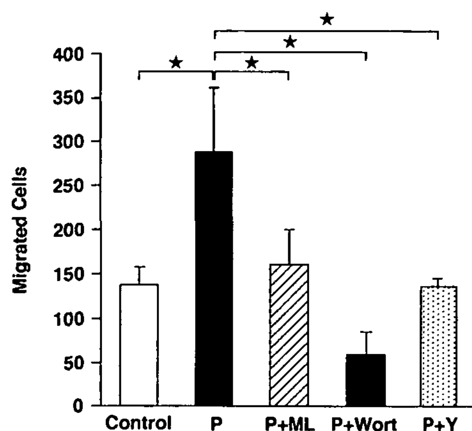

Fig. 1. **Effect of ML-9, wortmannin, and Y-27632 on chemotaxis of SM3 cells.** SM3 cells, which were established from rabbit aorta smooth muscle cell (*3*), were grown in the proliferative form in minimum essential medium (MEM, Life Technologies), supplemented with 10% fetal bovine serum (JRH Biosciences) at 37°C in a 5% CO_2 incubator. The cultured cells were trypsinized and suspended in MEM. Chemotaxis in response to PDGF-BB was examined with a modified Boyden chamber (Neuroprobe), where upper and lower chambers were separated by polycarbonate filters with 8-μm pore (*4*). Twenty thousand cells suspended in 50 μl of MEM were applied to the upper chambers. In the lower chambers, 10 ng/ml PDGF-BB dissolved in MEM was applied in a volume of 29 μl/chamber. The cells were then incubated for 3 h at 37°C in a 5% CO_2 incubator. After unmigrated cells were removed, the migrated cells were stained with Giemsa and counted with a light microscope in 5 fields at ×400. Control, P, P+ML, P+Wort, and P+Y denote that lower chambers contained vehicle, 10 ng/ml PDGF-BB, 10 ng/ml PDGF-BB plus 10 μM ML-9, PDGF-BB plus 1 μM wortmannin, or PDGF-BB plus 10 μM Y-27632, respectively. Each bar represents the mean ± SD of five or six individual wells. ★, $p < 0.05$.

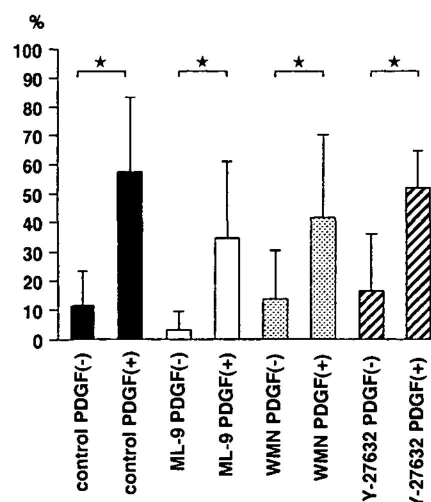

Fig. 2. **Effects of ML-9, wortmannin, and Y-27632 on the proportion of SM3 cells with membrane ruffling induced by PDGF-BB.** SM3 cells were harvested by trypsinization (see the legend to Fig. 1), plated on a glass coverslip at a density of 1×10^5 cells in a 60-mm dish, and cultured overnight. The cells were washed with phosphate-buffered saline (PBS) and in MEM containing vehicle (Control), 10 μM ML-9 (ML-9), 1 μM wortmannin (WMN), and 10 μM Y-27632 (Y-27632) for 30 min. To the culture shown by PDGF (+), concentrated PDGF-BB was added in the final concentration of 10 ng/ml, and to the culture shown by PDGF (−), only vehicle was added. Cells were then cultured for 10 min to develop membrane ruffling, fixed with 1% formaldehyde in PBS for 10 min, permeabilized with 0.1% Triton-X-100 in PBS for 2 min, and stained with rhodamine-labeled phalloidin (Molecular Probes). After removing the excess phalloidin by washing with PBS, the cells on the coverslips were observed with a fluorescence microscope (see Fig. 3). Cells that developed the rhodamine-stained actin filaments in the cellular periphery were considered as membrane-ruffling positive (*4*). About 170 cells each were scored in 20 fields. The percentage of cells showing membrane ruffling was calculated. Each bar represents the mean ± SD ★, $p < 0.05$.

nin also failed to suppress the PDGF-BB-induced membrane ruffling: in the presence of 1 μM wortmannin, PDGF-BB increased the numbers of SM3 cells with membrane ruffling from 14.0 ± 16.7 to 41.6 ± 29.1% (mean ± SD, n = 20 fields). However, the shape of membrane ruffling as examined by the accumulation of rhodamine-stained actin filaments was altered by ML-9: the membrane ruffling showed apparent shrinkage of the cellular edge (compare Fig. 3B with Fig. 3F). In the presence of wortmannin, membrane ruffling was condensed to pinpoints at the cellular edges (Fig. 3C, arrows). In the presence of Y-27632, PDGF-BB increased the proportion of cells with membrane ruffling from 17.0 ± 19.5 to 52.1 ± 13.1% (mean ± SD, n = 20 fields), but no changes were noted in the cellular shape (Fig. 3D).

Thus, inhibition of the chemotaxis of SM3 does not appear to be attributable to inhibited development of the membrane ruffling. This observation conforms with the idea that the development of membrane ruffling at the leading edge is necessary but not sufficient for migration such as chemotaxis, suggesting that coordinated development of membrane ruffling is required for migration (*20*). We speculate that the pathway of the signal transduction for the coordination is quite distinct from the pathway for developing membrane ruffling. In this connection, Walker *et al.* have reported that blocking MLCK with peptide inhibitors leads to inhibition of ameboid cell locomotion of eosinophils without affecting membrane ruffling (*21*).

We next examined whether the inhibitors alter MLC20 phosphorylation. PDGF-BB was added to the culture medium of SM3 cells in the presence and absence of the inhibitors, then the cells were fixed, harvested, and subjected to glycerol-PAGE followed by blotting. As shown in Fig. 4, we stained the blot with an antibody that recognizes both phosphorylated and unphosphorylated MLC20 (*3*) and quantified the percentage of phosphorylated MLC20 in total MLC20 by densitometry. The percentages in control culture with and without PDGF-BB were similar, *i.e.*, 39.8 and 36.2%, respectively, confirming our previous report (*4*). In the presence of the inhibitors, the percentages were also similar. The respective percentages with and without PDGF-BB were: 35.4 and 32.0% in the presence of 10 μM ML-9, 33.6 and 29.7% in the presence of 1 μM wortmannin, and 32.9 and 32.5% in the presence of the Y-27632. Thus, the inhibitors for MLCK and Rho-kinase did not affect the extent of MLC20 phosphorylation in SM3 cells.

It might be argued that the above data represent MLC20 phosphorylation of the total lysate of SM3 cells, and that the method could not detect phosphorylation that might occur in the limited part of the cell that is responsible for chemotaxis. Therefore, we stained SM3 cells cultured in the presence of PDGF-BB with an antibody that recognizes phosphorylated MLC20 (*22*). In the control experiment

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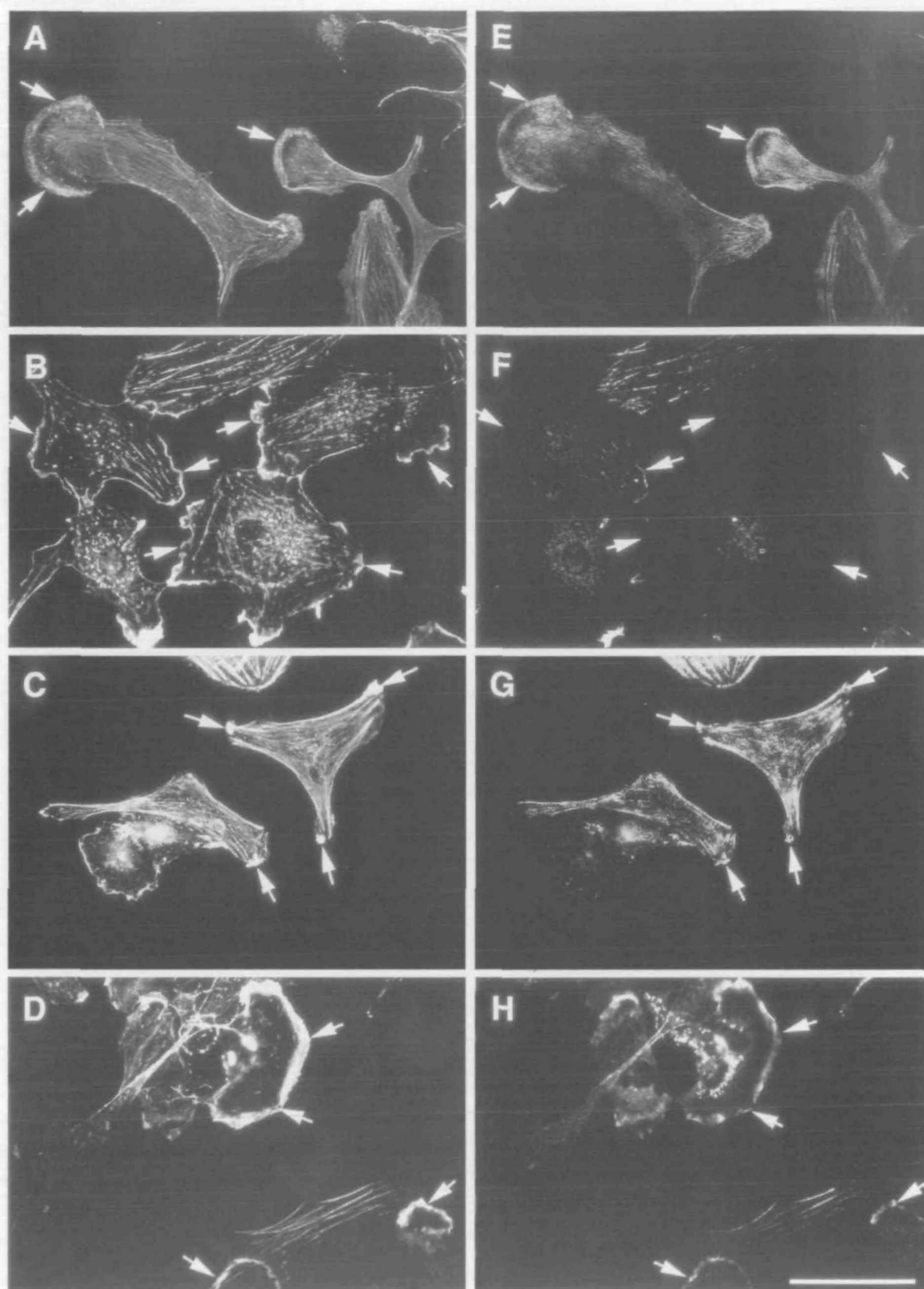

Fig. 3. **Staining of PDGF-BB–induced membrane ruffling with an antibody against phosphorylated MLC20.** SM3 cells grown on a coverslip were pretreated with MEM containing vehicle (A, E), 10 μM ML-9 (B, F), 1 μM wortmannin (C, G), or 10 μM Y-27632 (D, H) for 30 min. Concentrated PDGF-BB was added to each culture at the final concentration of 10 ng/ml, then the cells were allowed to develop membrane ruffling by incubating for 10 min (*4*). The cells were treated as described in the legend to Fig. 2, and stained with rhodamine-phalloidin (A, B, C, D) and with an antibody that specifically recognizes phosphorylated MLC20 (*22*) (E, F, G, H). Bar = 50 μm.

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without the inhibitors, the major portion of staining by the antibody was membrane ruffling (Fig. 3, A and E, arrows). The data indicate that membrane ruffling was associated with the phosphorylated myosin, which confirms the report of Matsumura *et al.* (*19*). Similarly, membrane ruffling was associated with phosphorylated myosin even if wortmannin (Fig. 3, C and G, arrows) and Y-27632 (Fig. 3, D and H, arrows) were present. However, in the presence of ML-9, the membrane ruffling was not stained by the antibody (Fig. 3, B and F, arrows). We speculate that MLC20 phosphorylation is not necessarily required for SM3 cells to induce membrane ruffling.

While the MLCK inhibitors ML-9 and wortmannin both inhibited the chemotaxis in response to PDGF-BB, their effects on the PDGF-induced membrane ruffling are not identical, as described above (compare Fig. 3, B and F with C and G, respectively). Wortmannin has been reported to inhibit not only MLCK but also phosphatidylinositol 3-kinase (*23*). Therefore, we speculate that the dissimilarity might be attributable to a difference in the site of action.

The present study suggests the involvement of MLCK and Rho-kinase in the chemotaxis in response to PDGF-BB. And because the level of MLC20 phosphorylation was not altered, we need to identify the alternative sites of action of these kinases. Recently, we reported a novel property of MLCK, which was found to stimulate myosin ATPase activity without phosphorylating MLC20 (*24*). The stimulatory property was evident when MLCK itself was phosphorylated (unpublished observation). We speculate that such phosphorylation may be involved in the chemot-

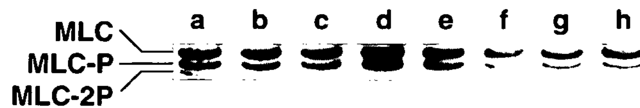

Fig. 4. **Western blots to examine MLC20 phosphorylation of SM3 cells.** SM3 cells were cultured in 10-mm dish to confluence, then pretreated with MEM-containing vehicle (a, b), 10 μM ML-9 (c, d), 1 μM wortmannin (e, f), or 10 μM of Y-27632 (g, h) for 30 min. Vehicle (a, c, e, g) or concentrated PDGF-BB (b, d, f, h) was then added at the final concentration of 10 ng/ml, and incubation was continued for 10 min to develop membrane ruffling. The culture was terminated by adding trichroloacetic acid to a final concentration of 5%, and subjected to glycerol-PAGE followed by transfer to a membrane filter (*3*). The blots were stained with an antibody that recognizes both phosphorylated and unphosphorylated MLC-20 (*3*). MLC, unphosphorylated MLC20; MLC-P, mono-phosphorylated MLC20; MLC-2P, di-phosphorylated MLC20.

axis, and its regulation might be a subject for future studies on how arterial plaques are formed in arteriosclerosis.

We thank Dr. Yasuharu Sasaki (Asahi Chemical Industry Ltd., Shizuoka) for supplying the antibodies and Yoshitomi Pharmaceutical Industries, Ltd. (Osaka) for the gift of Y-27632.

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