Arch Pharm Res Vol 22, No 2, 108-112, 1999

Inhibition of Stem Cell Factor- and Nerve Growth Factor-Induced Morphological Change by Wortmannin in Mast Cells

Hyung-Min Kim, Young-Hoe Moon[1] and Nyun-Hyung An

College of Pharmacy, [1]School of Medicine, Wonkwang University, Iksan, Chonbuk 570-749, Korea

(Received November 17, 1998)

Recombinant murine stem cell factor (rmSCF) or recombinant murine nerve growth factor (rmNGF) induced the morphological change of large numbers of rat peritoneal mast cells (RPMC). We investigated the role of phosphatidylinositol 3'-kinase (PI3-kinase) in receptors-mediated morphological change in RPMC. Exposure of RPMC to PI3-kinase inhibitor, wortmannin, before the addition of rmSCF and rmNGF antagonized those factors-induced morphological change. These results suggest that the PI3-kinase is involved in the signal transduction pathway responsible for morphological change following stimulation of rmSCF and rmNGF and that wortmannin blocks these responses.

Key words : Phosphatidylinositol 3'-kinase, Stem cell factor, Nerve growth factor, Morphological change, Rat peritoneal mast cells

INTRODUCTION

Phosphatidylinositol 3'-kinase (PI3-kinase) is associated with and activated by a number of proteins containing intrinsic or associated tyrosine kinase activities (Crawley *et al.*, 1996). The availability of a specific inhibitor of PI3-kinase would be the most useful adjunct in any effort to clarify the roles of PI3-kinase. Connective tissue-type mast cells, such as those present in the peritoneal cavity and skin, represent a major mast cell population. Stem cell factor (SCF), the ligand for the receptor encoded by *c-kit*, is essential for the development of mast cells (Huang *et al.*, 1990; Martrin *et al.*, 1990; Zsebo *et al.*, 1990; Arakawa *et al.*, 1991; Williams *et al.*, 1992 Lemura *et al.*, 1994; Galli *et al.*, 1994). Analysis of mutant mice have shown that the interaction between the *c-kit* receptor and SCF is indispensable for proper development of mast cells (Kitamura *et al.*, 1978; Kitamura and Go, 1979). SCF has been reported to induce mast cell degranulation and function as a chemotactic factor for mast cells (Columbo *et al.*, 1992; Meininger *et al.*, 1992). Dastych and Metcalfe (1994) recently reported that SCF stimulates mast cell adhesion and, because SCF is produced normally in tissues, it may be a major factor responsible for the adhesion of mast cells to connective tissue matrix. Therefore, we address the question of whether SCF actually acts on connective tissue-type mast cells to induce their adhesion. One factor that has been shown to exert effects on mast cells, although not normally viewed as a cytokine, is nerve growth factor (NGF). NGF is the prototype of a group of factors known as neurotrophic factors, which are involved in mediating the survival of and enhancing the function of developing and mature neurons (Snider and Johnson, 1989). In addition to its effects on the nervous system, NGF can exert profound effects on mast cells. Exposure of isolated peritoneal mast cells to NGF in the presence of lysophosphatidylserine results in the release of histamine and other mediators (Bruni *et al.*, 1982; Pearce and Thompson, 1986). In this paper, we examine the ability of SCF and NGF on the morphological change of primary cultures of rat peritoneal mast cells (RPMC). We show that SCF and NGF treatment of RPMC induces a morphological change. In addition, we report the inhibitory effect of PI3-kinase inhibitor in the morphological change induced by SCF and NGF in RPMC.

Correspondence to: Hyung-Min Kim, Ph. D., College of Pharmacy, Wonkwang University, Iksan, Chonbuk 570-749, Korea

MATERIALS AND METHODS

Materials

Recombinant murine SCF (rmSCF) was purchased from R & D Systems, Inc. (Minneapolis, MN). Anti-*c-kit* ACK2 monoclonal antibody (mAb) was purchased from Life Technologies (Gaithersburg, MD). The α-minimal essential medium (α-MEM) was purchased from Flow Laboratories (Irvine). Wortmannin was purchased from Sigma (St. Louis, MO). Recombinant murine IL-3 (rmIL-3) and recombinant murine IL-4 (rmIL-4) were purchased from Genzyme (München). NGF 2.5S and

anti-murine NGF mAb were purchased from Boehringer Mannheim (Mannheim, Germany). Fetal calf serum (FCS) and other tissue culture reagents were purchased from Life Technologies. All other chemicals were obtained from Sigma. All the other chemicals were purchased from Sigma, unless otherwise indicated. The original stock of male Wistar rats (180~260 g weight) purchased from Dae Han Experimental Animal Center (Eumsung, Chungbuk) were maintained at the College of Pharmacy, Wonkwang University. The animals were housed five to ten per cage in a laminar air flow room maintained under the temperature of 22±1°C and relative humidity of 55±10% throughout the study.

RPMC preparation

RPMC were isolated as previously described (Kim *et al.*, 1998). In brief, rats were anesthetized by ether, and injected with 20 ml of Tyrode buffer B (137 mM NaCl, 5.6 mM glucose, 12 mM $NaHCO_3$, 2.7 mM KCl, 0.3 mM NaH_2PO_4) containing 0.1% gelatin, into the peritoneal cavity, and the abdomen was gently massaged for about 90 s. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells was aspirated by a pasteur pipette. Thereafter, the peritoneal cells were sedimented at 150×g for 10 min at room temperature and resus- pended in Tyrode buffer B. Mast cells were separated from the major components of rat peritoneal cells, i.e. macrophages and small lymphocytes, according to the method described by Yurt *et al.* (1977). In brief, peritoneal cells suspended in 1 ml Tyrode buffer B were layered on 2 ml of 22.5% w/v metrizamide (density, 1.120 g/ml) and centrifuged at room temperature for 15 min at 400×g. The cells remaining at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 ml Tyrode buffer B containing calcium (0.9 mM $CaCl_2$). Mast cell preparations were about 95% pure as assessed by toluidine blue staining. More than 97% of the cells were viable as judged by trypan blue uptake.

Assessment of cell viability and altered morphology

At time zero and at subsequent time points as indicated, cells were counted on a hemocytometer and their viability was assessed by trypan blue dye exclusion. To assess the percentage of cells showing the characteristic morphologic features, the cells were examined by phase-contrast microscopy. Morphological change assays were performed based upon the elongated and spindle-shaped cells.

Cell culture

Purified RPMC were maintained in α-MEM supplemented with. 10% FCS. Cells were immediately placed in 4-well plates at 3×10^4 cells/ml in 0.5 ml of medium containing various concentrations of rmSCF, or in 0.5 ml of medium containing rmIL-3 and rmIL-4, for 10 days. The culture medium was replaced with fresh medium every 2 days.

RESULTS AND DISCUSSION

To investigate the biological relevance of the PI3-kinase on differentiated phenotype of mast cells, we initially examined that rmSCF and rmNGF can induce morphological change of RPMC. As shown in Fig. 1, about 64% morphological change induced by rmSCF was attained by 6 days. After 10 days, the percentage of morphological change had dropped to 55%. However, the number of morphologically changed RPMC stimulated by NGF treatment was about 2 times lower than that stimulated by rmSCF. In Fig. 2, morphological change of RPMC was observed at 6 days after treatment with rmSCF and rmNGF at differing concentrations. The magnitude of morphological change was dependent on the concentration of rmSCF and rmNGF. More than 98% of the cells morphologically changing in response to rmSCF and rmNGF were identified as mast cells by cytoplasmic granule staining with alcian blue. After treatment with rmSCF and

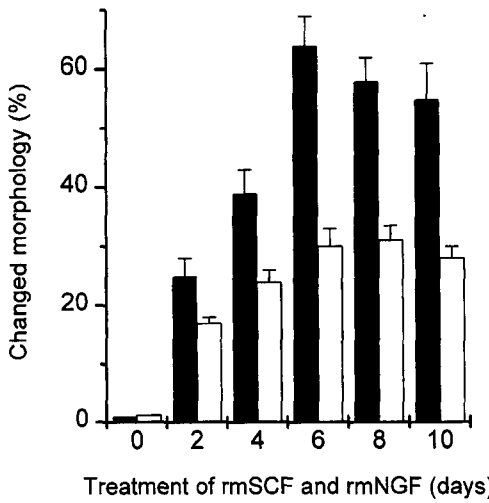

Fig. 1. Morphological change of RPMC is induced by rmSCF and rmNGF treatment. RPMC were plated at low density (3×10^4 cells/ml) and rmSCF (100 ng/ml) and rmNGF (200 ng/ml) were added. Cells were treated with factors for the indicated times and assessed for appearance of changed morphology. Morphological change is presented as percentage of cells per 4 field undergoing shape change. The ability of the cells to exclude the vital dye trypan blue was examined. All cells remained viable by this criterion, including those that had undergone morphological change. (solid bars) Cultures with rmSCF, (open bars) cultures with rmNGF. Each experiment was carried out with duplicate wells, and values are the means±SEM of 4 independent experiments.

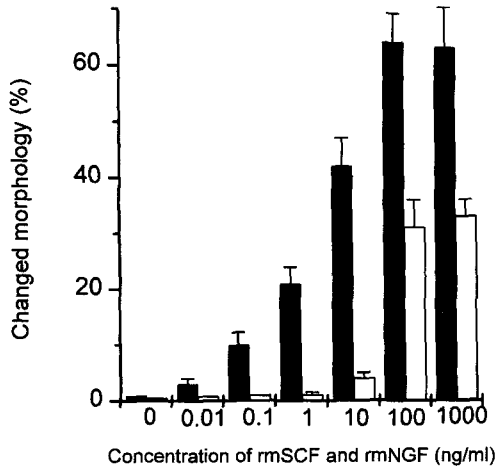

Fig. 2. Dose response of rmSCF and rmNGF for morphological change. RPMC (3×10^4 cells/ml) were treated for 6 days with the indicated concentrations of factors and then assessed for the changed morphology. (solid bars) Cutures with rmSCF, (open bars) cultures with rmNGF. Each experiment was carried out with duplicate wells, and values are the means±SEM of 3 independent experiments.

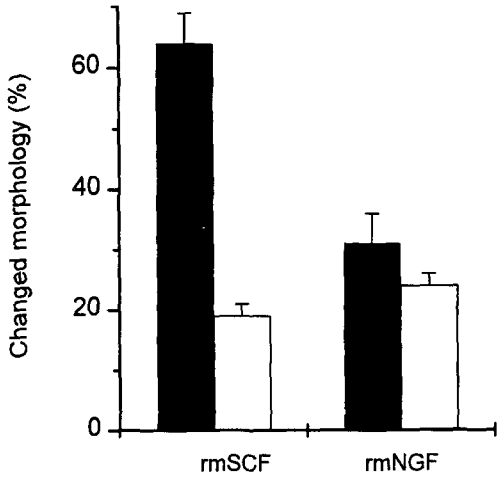

Fig. 3. Inhibitory effect of morphological change by wortmannin treatment. RPMC (3×10^4 cells/ml) were treated with rmSCF and rmNGF alone or in combination with wortmannin (5 M). At day 6, the morphologically changed cells were counted as described in Materials and Methods. (solid bars) Cutures without wortmannin, (open bars) cultures with wortmannin. Each experiment was carried out with duplicate wells, and values are the means±SEM of 4 independent experiments.

rmNGF in the media, the RPMC gradually acquired morphological changes, indicating further development of the differentiated phenotype of RPMC in response to rmSCF.

Next, we investigated the effect of inhibitor which might be expected to be related with mast cell morphological change. PI3-kinase is an enzyme important in intracellular trafficking (Jullien *et al.*, 1993), actin polymerization (Kundra *et al.*, 1994), and growth factor signalling (Cohen *et al.*, 1990; Ruderman *et al.*, 1990). Wortmannin, a potent inhibitor of PI3-kinase, was used to evaluate rmSCF- and rmNGF-induced morphological change in RPMC. The action of wortmannin was examined in the presence of rmSCF. Wortmannin inhibited rmSCF- and rmNGF-stimulated RPMC morphological change at a 6-day (Fig. 3). Removal of the inhibitor revealed that the inhibitory effect of this agent was reversible and not due to cytotoxicity (data not shown). These properties indicate that PI3-kinase is important for the changing of morphology in RPMC.

The morphological change effect of rmSCF was compared to that of rmNGF at optimal levels. NGF is suggested to be one of the factors responsible for the differentiation of uncommitted mast cells to the connective tissue-type mast cells phenotype (Matsuda *et al.*, 1991; Valent *et al.*, 1991). Because both SCF and NGF are spontaneously produced by fibroblasts, there is a possibility that fibroblast-derived NGF modulate the morphological change of RPMC. Addition of rmNGF to rmSCF did not increase the number of morphologically altered RPMC (Fig. 4). Our results showed that NGF also might act as a key factor to promote morphological change of RPMC. However, the effect of rmNGF for the morphological change of RPMC freshly isolated showed low activity (Fig. 1). Thus, we considered that NGF, unlike SCF, may be a weak factor to change morphology of RPMC under the physiological conditions *in vivo*. A better understanding

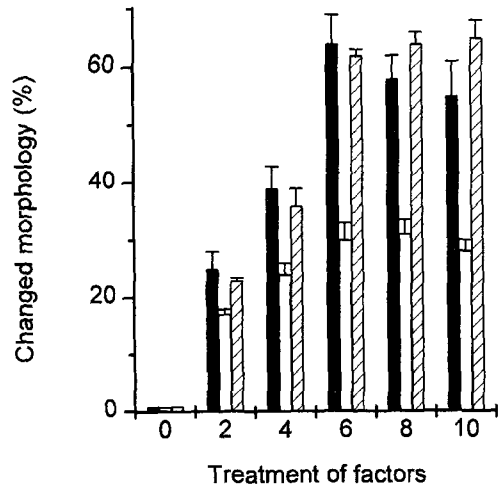

Fig. 4. Effect of rmSCF and rmNGF on morphological change of RPMC. RPMC (3×10^4 cells/ml) were plated at low density on 4-well plates and rmSCF (100 ng/ml), NGF (200 ng/ml) or their combination were added. Morphological change is presented as percentage of cells per 6 field undergoing shape change. (solid bars) Cultures with rmSCF, (open bars) cultures with rmNGF, and (diagonal bars) cultures with rmSCF plus NGF. Each experiment was carried out with duplicate wells, and values are the means±SEM of 3 independent experiments.

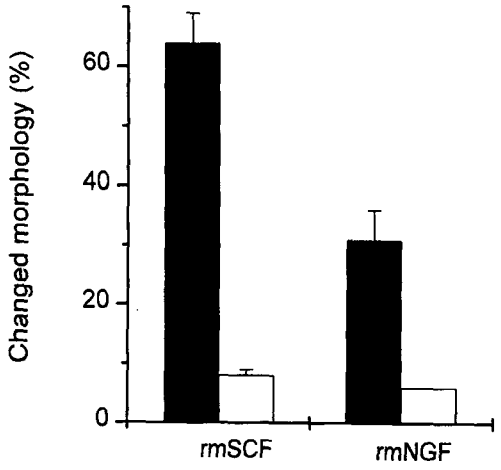

Fig. 5. Effect of anti-c-kit ACK2 mAb and anti-murine NGF mAb on morphological change of RPMC. RPMC (3×10^4 cells/ml) were treated for 6 days with the indicated concentrations of rmSCF rmNGF and then assessed for the changed morphology. (solid bars) Cutures without each neutralizing mAb, (open bars) cultures with each neutralizing mAb. Each experiment was carried out with duplicate wells, and values are the means±SEM of 3 independent experiments.

of how SCF and NGF participate in adhesion is critical to expand our knowledge about morphological changes of mast cells. Anti-c-kit ACK2 mAb and anti-murine NGF mAb prevented rmSCF- and rmNGF-induced morphological change of RPMC, establishing SCF and NGF as the active molecule. The effects of rmSCF and rmNGF were abolished completely by ACK2 mAb and anti-murine NGF mAb (Fig. 5). We conclude that under certain conditions SCF and NGF can act as a morphological change inducer for RPMC. Thus SCF and NGF appears to be a second feature of the tissue microenvironment governing mast cell distribution. Our observation supports an important role for SCF and NGF in that these factors induce the initiation of mast cell adhesiveness. In addition, our results demonstrated that PI3-kinase plays an important role in these factors-induced morphological change of mast cells. To our knowledge, our report is the first one to describe the action of SCF and NGF on the morphological change in RPMC.

ACKNOWLEDGEMENTS

This study was supported by the Academic Research Fund (GE 97-129) of the Ministry of Education, and partly by Wonkwang University in 1998, Republic of Korea.

REFERENCES CITED

Arakawa, T., Yphantis, D. A., Lary, J. W., Narhi, L. O., Lu, H. S., Prestrelski, S. J., Clogston, C. L., Zsebo, K. M., Mendiaz, E. A., Wypych, J. and Langley, K. E., Glycosylated and unglycosylated recombinant-derived cell factors are dimeric and have extensive regular secondary structure. *J. Biol. Chem.*, 266, 18942-18948 (1991).

Bruni, A., Bigon, E., Boarato, E., Mietto, L., Leon, A. and Toffano, G., Interaction between nerve growth factor and lysophosphatidylserine on rat peritoneal mast cells. *FEBS Lett.*, 138, 190-192 (1982).

Cohen, B., Liu, Y., Drucker, B., Roberts, T. M. and Schaffhauser, B. S., Characterization of pp85, a target of oncogenes and growth factor receptors. *Mol. Cell Biol.*, 10, 2909-2915 (1990).

Columbo, M., Horowitz, E. M., Botana, L. M., MacGlashan, D. W., Jr, Bochner, B.. Gillis, S., Zsebo, K. M., Galli, S. J. and Lichtenstein, L. M., The human recombinant *c-kit* receptor ligand, rhSCF, induces mediator release from human cutaneous mast cells and enhances IgE-dependent mediator release from both skin mast cells and peritoneal blood basophils. *J. Immunol.*, 149, 599-608 (1992).

Crawley, J. B., Williams, L. M., Mander, Tom, Brennan, F. M. and Foxwell, B. M., Interleukin-10 stimulation of phosphatidylinositol 3-kinase and p70 S6 kinase is required for the proliferative but not the antiinflammatory effects of the cytokine. *J. Biol. Chem.*, 271, 16357-16362 (1996).

Dastych, J. and Metcalfe, D. D., Stem cell induces mast cell adhesion to fibronectin. *J. Immunol.*, 152, 213-219 (1994).

Galli, S. J., New insights into "the riddle of the mast cells" microe nvironmental regulation of mast cell development and phenotypic heterogeneity. *Lab. Invest.* 62, 5-33 (1990).

Galli, S. J., Zsebo, K. M. and Geissler, E. N., The kit ligand, stem cell factor. *Adv. Immunol.*, 55, 1-96 (1994).

Huang, E., Nocka, K., Beier, D. R., Chu, T.-Y., Buck, J., Lahm, H.-W., Wellner, D., Leder, P. and Besmer, P., The hematopoietic growth factor KL is encoded by SI locus and is the ligand of the *c-kit* receptor, the gene product of the W locus. *Cell,* 63, 225-233 (1990).

Jullien, D., Tanti, J. F., Heydrick, S. J., Gautier, N., Gremeaux, T., Obberghen, E. Van. and Marchand-Brustel, Y. Le., Differential effects of okadaic acid on insulin-stimulated glucose and amino acid upake and phoshpatidylinositol 3-kinase activity. *J. Biol. Chem.*, 268, 15246-15251 (1993).

Kim, H. M., Lee, E. H., Cho, H. H. and Moon, Y. H., Inhibitory effect of mast cell-mediated immediate-type allergic reactions in rats by spirulina. *Biochem. Pharmacol.*, 55, 1071-1076 (1998).

Kitamura, Y., Go, S. and Hatanaka, K., Decrease of mast cells in W/Wv mice and their increase by bone marrow transplantation. *Blood,* 52, 447-452 (1978).

Kitamura, Y. and Go, S., Decreased production of

mast cells in Sl/Sld anemic mice. *Blood*, 53, 492-497 (1979).

Kundra, V., Escobedo, J. A., Kazlauskas, A., Kim, H. K., Rhee, S. G., williams, L. T. and Zetter, B. R., Regulation of chemotaxis by the platelet-derived growth factor receptor-beta. *Nature*, 367, 474-476 (1994).

Lemura, A., Tsai, M., Ando, A., Wershil, B. K. and Galli, S. J., The c-kit ligand, stem cell factor, promotes mast cell survival by suppressing apoptosis. *Am. J. Pathol.*, 144, 321-328 (1994).

Martrin, F. H., Suggs, S. V., Langley, K. E., Lu, H. S., Ting, J., Okino, K. H., Morris, C. F., McNiece, I. K., Jacobsen, F. W., Mendiaz, E. A., Birkett, N. C., Smith, K. A., Johnson, M. J., Parker, V. P., Flores, J. C., Patel, A. C., Fisher, E. F., Erjavec, H. O., Herrera, C. J., Wypych, J., Sachdev, R. J., Pope, J. A., Leslie, I., Wen, D., Lin, C. H., Cupples, R. L. and Zsebo, K. M., Primary structure and functional expression of rat and human stem cell factor DNAs. *Cell*, 63, 203-211 (1990).

Matsuda, H., Kannan, Y., Ushio, H., Kiso, Y., Kanemoto, T., Suzuki, H. and Kitamura, Y., Nerve growth factor induces development of connective tissue-type mast cells in vitro from murine bone marrow cells. *J. Exp. Med.*, 174, 7-14 (1991).

Meininger, C. J., Yano, H., Rottapel, R., Bernstein, A., Zsebo, K. M. and Zetter, B. R., The c-kit receptor ligand functions as a mast cell chemoattractant. *Blood*, 79, 958-963 (1992).

Pearce, F. L. and Thompson, H. L., Some characteristic of histamine secretion from rat peritoneal mast cell stimulated with nerve growth factor. *J. Physiol. (Lond.)*, 372, 379-383 (1986).

Ruderman, N. B., Kapeller, R., White, M. F. and Cantley, L. C., Activation of phosphatidylinositol 3-kinase by insulin. *Proc. Natl. Acad. Sci. USA*, 87, 1411-1415 (1990).

Snider, W. D. and Johnson, E. M.,Jr., Neurotrophic molecules. *Ann. Neurol.*, 26, 489-506 (1989).

Valent, P. Sillaber, C. and Bettelheim, P., The growth and differentiation of mast cells. *Prog. Growth Factor Res.* 3, 27-41 (1991).

Williams, D. E., de Vries, P., Namen, A. E., Widmer, M. B. and Lyman, S. D., The steel factor. *Dev. Biol.*, 151, 368-376 (1992).

Yurt, R. W., Leid, R. W. and Austen, K. F., Native heparin from rat peritoneal mast cells. *J. Biol. Chem.*, 252, 518-521 (1977).

Zsebo, K. M., Wypych, J., McNiece, I. K., Lu, H. S., Smith, K. A., Karkare, S. B., Sachdev, R. K., Yuschenkoff, V. N., Birkett, N. C., Williams, L. R., Satyagal, V. N. Tung, W., Bosselman, R. A., Mendiaz, E. A. and Langley, K. E., *Cell*, 63, 195-201 (1990).