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Morphological alterations in rat peritoneal mast cells by stem cell factor

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SUMMARY

Stem cell factor (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. We found that the morphology of rat peritoneal mast cells (RPMC) altered after the addition of recombinant murine SCF (rmSCF) *in vitro*. The ability of rmSCF to enhance morphological alteration was dose dependent and completely abolished by anti-*c-kit* ACK2 monoclonal antibody. Exposure of RPMC to transforming growth factor-β_1, wortmannin, genistein, herbimycin A, staurosporine, indomethacin and cytochalasin D before the addition of rmSCF antagonized rmSCF-induced morphological alteration. However, nordihydroguiaretic acid had no effect. Many RPMC appeared to respond also to nerve growth factor (NGF) but the total number of cells with altered morphology was much greater when the culture was stimulated by rmSCF than by NGF. We suggest that morphological alterations of mast cells by rmSCF is an important step for the participation in adhesion to tissue under resident physiological conditions.

INTRODUCTION

Mast cell development is a complex process that results in the appearance of phenotypically distinct populations of mast cells in different anatomical sites.[1] 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.[2–8] Analysis of mutant mice has shown that the interaction between the *c-kit* receptor and SCF is indispensible for proper development of mast cells.[9,10] SCF has been reported to induce mast cell degranulation and to function as a chemotactic factor for mast cells.[11,12] Dastych & Metcalfe[13] 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 or not SCF actually does act on connective tissue-type mast cells to induce their adhesion. Transforming growth factor-β_1 (TGF-β_1) is known to be a factor affecting growth, differentiation and morphogenesis, depending on the cell type being targeted.[14] TGF-β_1 has potent positive and negative regulatory properties on haematopoiesis.[14] TGF-β_1 is associated with transmodulation of the surface receptor for SCF.[15] 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 developing and mature neurons and enhancing their function.[16] 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.[17,18] In this paper, we examine the ability of SCF to cause morphological alterations in primary cultures of rat peritoneal mast cells (RPMC). We show that SCF treatment of RPMC induces morphological changes. In addition, we report the inhibitory effects of various kinds of inhibitors on the morphological alterations induced by SCF. Furthermore, we show that NGF is also capable of inducing morphological changes in mast cells.

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Abbreviations: NDGA, nordihydroguiaretic acid; NGF, nerve growth factor; PI3-kinase, posphatidylinositol 3-kinase; rmSCF, recombinant murine stem cell factor; RPMC, rat peritoneal mast cells; SCF, stem cell factor.

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MATERIALS AND METHODS

Materials

Recombinant murine SCF (rmSCF) and TGF-β_1 were purchased from R & D Systems, Inc. (Minneapolis, MN). Anti-*c-kit* ACK2 monoclonal antibody (mAb) and genistein were purchased from Life Technologies (Gaithersburg, MD). The α-minimal essential medium (α-MEM) was purchased from ICN Biomedicals (Costa Mesa, CA). Wortmannin and nordihydroguiaretic acid (NDGA) were purchased from Sigma Chemical Co. (St. Louis, MD). Recombinant murine

interleukin-3 (rmIL-3) and recombinant murine IL-4 (rmIL-4) were purchased from Genzyme (Munich, Germany). 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 Chemical Co. The original stock of male Wistar rats (180–260 g weight) were purchased from the Korean Research Institute of Chemical Technology (Taejeon, Chungnam), and the animals were maintained at the College of Pharmacy, Wonkwang University. The animals were housed five to ten per cage in a room with laminar air flow, maintained at $22 \pm 1°$ and with a relative humidity of $55 \pm 10\%$ throughout the study.

RPMC preparation

RPMC were isolated as previously described.[19] In brief, rats were anaesthetized with 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 (Sigma Chemical Co.), into the peritoneal cavity. The abdomen was gently massaged for about 90 seconds. 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 resuspended 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.*[20] 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, Sigma Chemical Co.) 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 in a haemocytometer and their viability was assessed by Trypan blue dye exclusion. To assess the percentage of cells showing characteristic morphological features, the cells were examined by phase contrast microscopy. Photomicrography was done using Fujifilm at ×200 magnification.

Cell culture

Purified RPMC were maintained in α-MEM, 10% FCS, rmIL-3, and rmIL-4. Cells were immediately placed in four-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. They were cultured for 10 days and the culture medium was replaced with fresh medium every 2 days.

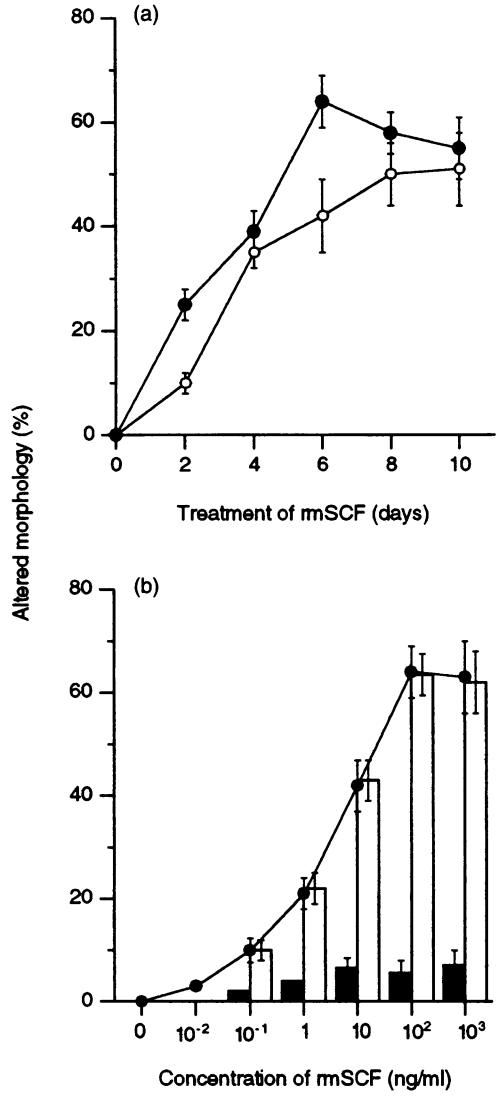

Figure 1. Morphological alteration of RPMC is induced by rmSCF treatment. (a) Kinectics of induction of morphological alteration. RPMC were plated at low density (3×10^4 cells/ml) and rmSCF (100 ng/ml) was added. Cells were treated with rmSCF for the times indicated and were assessed for appearance of altered morphology. Morphological alteration is presented as percentage of cells per six fields 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 alteration. (●) Cultures with IL-3 and IL-4, (○) cultures without IL-3 and IL-4. (b) Dose–response of rmSCF for morphological alteration and the effects of anti-*c-kit* ACK2 mAb (20 μg/ml) and anti-NGF mAb (200 μg/ml). RPMC (3×10^4 cells/ml) were treated for 6 days with the indicated concentrations of rmSCF and then assessed for the altered morphology. (■) Cultures with anti-*c-kit* ACK2 mAb, (□) cultures with anti-NGF mAb. Each experiment was carried out with duplicate wells, and values are the means ± SEM of nine (a) and seven (b) independent experiments.

RESULTS

Induction of morphological changes after stimulation of RPMC by SCF

We first demonstrated that rmSCF can induce morphological alteration of RPMC. As shown in Fig. 1(a), rmSCF induced morphological alterations in about 64% of RPMC after 6 days of culture. After 10 days, the percentage of RPMC with morphological alteration had dropped to 55%. Figure 1(b) shows the morphological alterations observed in RPMC after 6 days of treatment with rmSCF at differing concentrations.

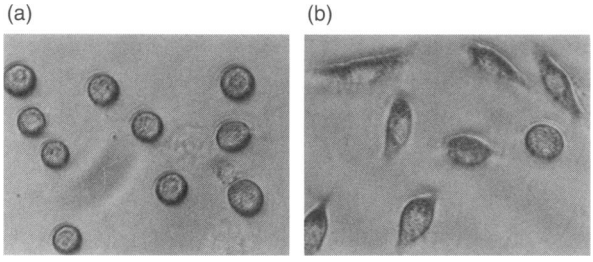

Figure 2. Phenotype of RPMC treated with rmSCF (100 ng/ml). RPMC were plated at low density on four-well plates. RPMC were cultured in the absence (a) or in the presence (b) of rmSCF. Photomicrographs were taken at 6 days. Magnification ×200.

The proportion of cells showing morphological alterations was dependent on the concentration of rmSCF. The effect of rmSCF was observed at 1 ng/ml and reached maximal levels at 100 ng/ml. These values are comparable with the effective concentrations of rmSCF for the proliferation of mast cells[21] and for the survival of primordial germ cells.[22–24] This implies that the effect of SCF on RPMC is physiological. The effect of rmSCF was completely abolished by ACK2 mAb, but not by anti-murine NGF mAb (Fig. 1b).

As shown in Fig. 2(a), we confirmed that purified RPMC did not change morphologically in response to medium containing rmIL-3 and rmIL-4. When used as the exogenous cytokine, rmSCF can stimulate morphological alteration of the RPMC at a later period of its development than that influenced by the effects of rmIL-3 plus rmIL-4 (Fig. 2b). The cells in Fig. 2(b) show the response to rmSCF. More than 98% of the cells that changed morphologically in response to rmSCF were identified as mast cells by staining of the cytoplasmic granules with Alcian blue. We found that many RPMC maintained *in vitro* with rmSCF altered their shape. However, our results indicate that some RPMC maintained in rmSCF do not change shape detectably.

Inhibition of SCF-induced morphological alteration by TGF-β_1 or various kinds of inhibitors

The morphological response of RPMC to rmSCF was inhibited dose-dependently when TGF-β_1 was present in the culture (Fig. 3a). In addition, we investigated the effects of various kinds of inhibitors which might be expected to be related with mast cell morphological alteration. Phosphatidylinositol 3-kinase (PI3-kinase) is an enzyme important in intracellular trafficking,[25] actin polymerization[26] and growth factor signalling.[27,28] Wortmanin is a potent inhibitor of PI3-kinase. Protein tyrosine kinase inhibitors have been shown to inhibit induction of the morphological alterations associated with adhesion.[29] Genistein has been reported to partially inhibit SCF-induced adhesion of mast cells.[13] It has been shown that *c-kit* exhibits intrinsic tyrosine kinase activity, and tyrosine kinase activity is postulated to play a role in signalling through this receptor. We used, therefore, the tyrosine kinase inhibitors genistein and herbimycin A to study the role of receptor tyrosine kinases in rmSCF-induced morphological alteration of RPMC. We also examined the role of arachidonic acid metabolites. Finally, to examine the role of the cytoskeleton on morphological alteration of RPMC by rmSCF treatment, we examined the effect

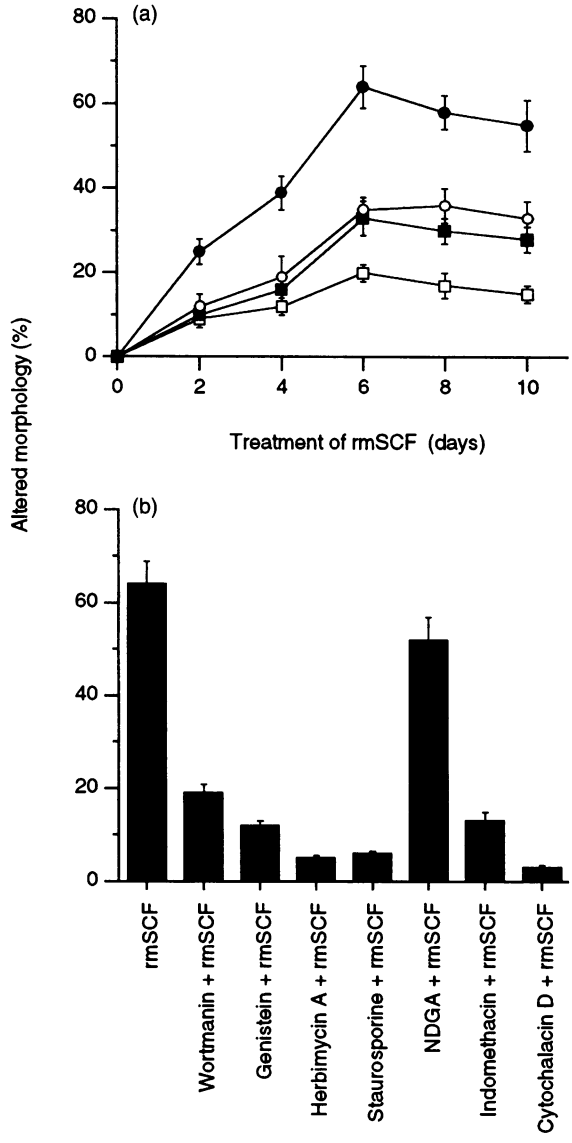

Figure 3. Inhibition of morphological alteration by TGF-β_1 or treatment with various inhibitors. (a) Morphological alteration in rmSCF-treated RPMC with and without TGF-β_1. Cells received rmSCF (100 ng/ml) with or without 0·1, 1, or 10 ng/ml TGF-β_1. Cells with morphological alteration were counted as a percentage of total cells in six independent areas of the plates. (●) Cultures with rmSCF alone, (○) cultures with rmSCF plus TGF-β_1 (0·1 ng/ml), (■) cultures with rmSCF plus TGF-β_1 (1 ng/ml), and (□) cultures with rmSCF plus TGF-β_1 (10 ng/ml). (b) Effect of various inhibitors on rmSCF-stimulated RPMC. Cells were treated with rmSCF alone or in combination with wortmanin (5 μM), genistein (10 μg/ml), herbimycin A (20 μM), staurosporine (100 nM), NDGA (10 μM), indomethacin (10 μM), or cytochalasin D (0·5 μM). At day 6, the morphologically altered cells were counted as described in the Materials and Methods. Each experiment was carried out with duplicate wells, and values are the means ± SEM of seven (a) and six (b) independent experiments.

of cytochalasin D. The actions of all of these agents were examined in the presence of rmSCF. Wortmanin, genistein, herbimycin A, staurosporine (protein kinase C inhibitor), indomethacin (cyclo-oxygenase pathway inhibitor) and cytochalasin D (cytoskeleton-inhibiting agent) totally suppressed

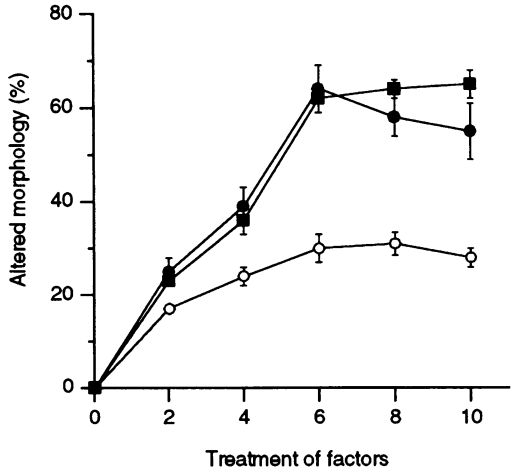

Figure 4. Effect of rmSCF and NGF on morphological alteration of RPMC. Cells were plated at low density on four-well plates and rmSCF (100 ng/ml), NGF (200 ng/ml) or their combination were added. Morphological alteration is presented as percentage of cells per six fields undergoing shape change. (●) Cultures with rmSCF, (○) cultures with NGF, and (■) cultures with rmSCF plus NGF. Each experiment was carried out with duplicate wells, and values are the means ± SEM of three independent experiments.

rmSCF-stimulated RPMC morphological alterations over a 10-day time–course (Fig. 3b). Removal of the inhibitor revealed that the inhibitory effect of these agents was reversible and not due to cytotoxicity (data not shown). However, NDGA (lipoxygenase pathway inhibitor) had no effect on rmSCF-stimulated RPMC morphological alteration (Fig. 3b).

Induction of morphological alterations after stimulation of RPMC by NGF

The effect of rmSCF on RPMC morphology was compared to that of NGF at optimal levels. The number of morphologically changed RPMC stimulated by NGF treatment was about two times lower than that stimulated by rmSCF (Fig. 4). Moreover, adding both NGF and rmSCF did not further increase the number of morphologically altered RPMC (Fig. 4).

DISCUSSION

In this study, we demonstrated that SCF induced morphological alterations in purified RPMC. After treatment with rmSCF, the RPMC gradually acquired morphological changes, indicating further development of the differentiated phenotype of RPMC in response to rmSCF. A better understanding of how SCF participates in adhesion is critical to furthering our knowledge about morphological changes of mast cells. Anti-*c-kit* ACK2 mAb prevented SCF-induced morphological changes establishing SCF as the active molecule (Fig. 1b). To our knowledge, our report is the first one to describe this action of SCF. We found that TGF-β_1 inhibited the effect of SCF on RPMC morphology. Some groups have shown that TGF-β_1 relieves *c-kit* kinase-associated tyrosine phosphorylation of target proteins. However, this requires an almost complete down-regulation of SCF surface binding sites.[15,30] TGF-β_1, also known to be an important regulator of cell growth and function, inhibits the SCF-mediated rescue from apoptosis off IL-3-deprived mast cells.[31] TGF-β_1, which is produced in high concentrations in various inflammatory conditions that are associated with mast cell proliferation, may inhibit the effect of SCF and thus, serve as a down-regulatory mechanism.[31] Our results indicate that SCF and TGF-β_1 might act together to regulate morphological alteration of RPMC in culture.

In this paper, we have shown that various kinds of specific inhibitors that can block signal transduction pathways can affect the response of RPMC to SCF. These results indicate that PI3-kinase, tyrosine kinases, protein kinase C, cyclo-oxygenase, and cytoskeletal activity are important for the response of RPMC to SCF. Under the same conditions, NDGA differed from the other inhibitors. These findings suggest differential sites of action for the various specific inhibitors and perhaps that NDGA accessed different steps in the morphological alteration. These results also suggest that some arachidonic acid metabolites are critical intermediaries in the regulation of morphological alteration.

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.[32,33] Because both SCF and NGF are spontaneously produced by fibroblasts, there is a possibility that locally produced fibroblast-derived NGF modulates morphological alterations of connective tissue mast cells. Our results showed that NGF might act as an additional factor to promote morphological alteration of RPMC (Fig. 4). However, NGF showed a lower activity in inducing morphological changes in RPMC and was not synergistic with SCF. Thus, we considered that NGF, unlike SCF, may be a weak factor in inducing morphological changes of RPMC under physiological conditions *in vivo*.

We conclude that under certain conditions, SCF can act as an inducer of morphological alterations in RPMC. Thus, SCF appears to be another factor in the tissue microenvironment that governs mast cell function. Our observation supports an important role for SCF in the initiation of mast cell adhesiveness.

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