Low c-kit Expression of Cultured Mast Cells of mi/mi Genotype May Be Involved in Their Defective Responses to Fibroblasts That Express the Ligand for c-kit

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Mutant mice of mi/mi genotype are osteopetrotic and deficient in tissue mast cells due to a defect in osteoclasts and mast cells. In an effort to further understand the mechanisms behind why mi/mi mouse-derived cultured mast cells (mi/mi-CMC) responded to interleukin-3 (IL-3), but not to the proliferative stimuli presented by fibroblasts, mi/mi-CMC and congenic normal (+/+ ) mouse-derived CMC (+/+ +CMC), both of which expressed the phenotypic characteristics of immature mast cells, were cocultured with Swiss albino/3T3 fibroblasts in a medium containing IL-3. In the in vitro CMC/fibroblast coculture, mi/mi-CMC did not acquire the phenotypes of connective tissue-type mast cells (CTMC), while +/+ +CMC did. In addition, attachment of mi/mi-CMC to the fibroblasts was found to be significantly lower than that of +/+ +CMC. Because the interaction of c-kit product with its ligand (stem cell factor [SCF]) is known to play an important role not only in proliferation and differentiation of mast cells but also in attachment of CMC to fibroblasts, the expression and function of c-kit were investigated in mi/mi-CMC and +/+ +CMC. Recombinant rat SCF (rrSCF) induced a dose-dependent proliferation of +/+ +CMC. Also, rrSCF induced +/+ +CMC to acquire the phenotypes of CTMC in the medium containing IL-3. By contrast, rrSCF did not stimulate the proliferation of mi/mi-CMC nor induce a phenotypic change of the cells from immature mast cells to mature, CTMC-like mast cells. Immunoblotting with antiphosphotyrosine antibody showed that rrSCF induced considerable tyrosine phosphorylation of 145- to 165-Kd protein, the product of c-kit, in +/+ +CMC, whereas tyrosine phosphorylation of the protein was barely detectable in mi/mi-CMC. Northern blot and flow cytometry analyses showed that mi/mi-CMC expressed much less c-kit at both protein and message levels than +/+ +CMC. Further, mi/mi-CMC were found to differ from +/+ +CMC in the expression of mouse mast cell protease-6 (MMCP-6) and MMCP-2 messenger RNA transcripts. These results suggest that the gene product of the mi locus may be important in regulating the expression of gene products such as c-kit, and that mast cell deficiency of mi/mi mice appears to be due, at least in part, to impaired signaling through the c-kit receptor because of the low c-kit expression.

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MATERIALS AND METHODS

Mice and cells. The original stock of C57BL/6-mi/+ (mi+/+) mice was purchased from the Jackson Laboratory (Bar Harbor, ME), and the mice were maintained in the Department of Pathology, Osaka University Medical School. Female mi/+ mice were crossed with male mi/+ mice, and the resulting mi/mi mice were selected by their white coat color. To obtain CMC, mice of mi/mi genotype and their normal (+/+) littermates were used at 2 to 3 weeks of age. Mice were killed by decapitation after ether anesthesia. Spleens were removed, and cell suspensions were prepared as described previously.\(^9\) The Swiss albino/3T3 fibroblast and WEHI-3 cell lines were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). These two cell lines were adapted to grow in a-minimal essential medium (a-MEM; Flow Laboratories, Irvine, UK) containing 10% fetal calf serum (FCS; Hyclone, Logan, UT), 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin.

Reagents. Recombinant rat SCF\(^1\)(4ng/ml; rrSCF\(^1\)) was a gift from Dr Kristina M. Zsebo (Asgen Inc, Thousand Oaks, CA).\(^7\) The preparation of the ACK2 rat monoclonal antibody (MoAb) and its specificity against the extracellular domain of the c-kit receptor product have been described in detail.\(^8,9\) The antiphosphotyrosine antibody is murine MoAb generated against phosphotyrosine as the immunogen\(^10\) and was generously supplied by Dr Brian Druker (Dana-Farber Cancer Institute, Boston, MA). The specificity of the antiphosphotyrosine MoAb was described previously.\(^20\) Chemically defined serum-free medium (ASF-102) was purchased from Ajinomoto (Tokyo, Japan); it contains human transferrin, insulin, and bovine serum albumin.

Establishment of CMC. WEHI-3 is a mouse myelomonocytic leukemia cell line that constitutively produces IL-3.\(^3,21\) WEHI-3-conditioned medium was prepared as described previously.\(^21\) WEHI-3 cells (2 \(\times\) 10\(^7\)/mL) were incubated for 5 days in \(\alpha\)-MEM containing 10% FCS. The conditioned medium was centrifuged, filtered through a 0.22-\(\mu\)m filter (Millipore Corp, Bedford, MA), and stored at \(-80^\circ\)C.

Culture flasks (Nunc, Roskilde, Denmark) containing 4 \(\times\) 10\(^7\) spleen cells and 5 mL \(\alpha\)-MEM supplemented with 10% FCS and 50% WEHI-3-conditioned medium were incubated at 37°C in a humidified atmosphere of 5% \(\text{CO}_2\) in \(\text{air}\).\(^4,12\) Half of the medium was replaced every 7 days, and more than 95% of cells were CMC at 4 weeks after the initiation of the culture.\(^4,12\)

Coculture with fibroblasts. Swiss albino/3T3 fibroblasts (1 \(\times\) 10\(^6\) cells) were suspended in 2 mL \(\alpha\)-MEM supplemented with 10% FCS, and seeded in 35-mm culture dishes. The culture medium was aspirated and replaced with 2 mL of fresh medium every 3 days. After 7 days, the fibroblasts formed a confluent monolayer in each dish containing 0.5 to 1.0 \(\times\) 10\(^6\) fibroblasts. The medium of the confluent culture was aspirated, and 5 \(\times\) 10\(^5\) CMC were suspended in 2 mL \(\alpha\)-MEM supplemented with 10% FCS was added with or without WEHI-3-conditioned medium (50% vol/vol). The medium of CMC/fibroblast coculture was changed every 2 days by two different methods. First, it was changed according to the original method described by Levi-Schaffer et al.\(^15\) The medium was aspirated, and the fibroblast layer was washed once with 2 mL of fresh medium to remove nonattaching CMC. The aspirated medium and the medium used for washing were discarded. Second, the aspirated medium and the medium used for washing were centrifuged at 12,000g for 5 minutes and resuspended in 2 mL of fresh medium, and then returned to the coculture. CMC that did not attach to fibroblasts were not lost in this method.

At various times after the initiation of coculture, the numbers of mast cells were estimated from the total hemocytometer count and the proportion of mast cells. The proportion of mast cells was determined by staining the cytospin preparations (Shandon Southern, Elliott, IL) with alcian blue.

Proliferation assay of CMC. To quantitate the proliferation of CMC, we used 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT; Sigma Chemical, St Louis, MO) rapid colorimetric assay as described previously,\(^22,23\) but with a minor modification.\(^22,23\) Briefly, triplicate aliquots of CMC (3 \(\times\) 10\(^6\) cells suspended in 100 \(\mu\)L of \(\alpha\)-MEM containing 10% FCS and rrSCF\(^1\)) were cultured in 96-well flat-bottom microtiter plates for 72 hours at 37°C. For the MTT assay, MTT (10 \(\mu\)L of a 5 mg/mL solution of MTT in phosphate-buffered saline (PBS)) was added to all wells, and plates were incubated for 4 hours. Acid isopropanol (100 \(\mu\)L of 0.04N HCl in isopropanol) was added to all wells, and mixed thoroughly to dissolve the dark blue crystals. The optical density (OD) was then measured on a Microplate reader (Corona Electric, Ibaragi, Japan) with a test wavelength of 550 nm and a reference wavelength of 630 nm. This assay was found to be more reproducible and reliable than \(^3\)-H-thymidine incorporation or cell enumeration, as described previously,\(^20,24\) although equivalent results were obtained with all three assays.

Differentiation assay of CMC. CMC were cocultured with Swiss albino/3T3 fibroblasts as described above, or maintained in culture medium supplemented with rrSCF\(^1\) (100 ng/mL) and WEHI-3-conditioned medium (50% vol/vol) as a source of IL-3. In the in vitro culture with IL-3 and rrSCF\(^1\), half of the culture medium was replaced twice a week. At various times after the initiation of the culture, the number of mast cells was estimated from the hemocytometer count, and the cytospin preparations were fixed in Carnoy’s solution for staining with alcian blue and safranin. Staining methods for alcian blue and safranin have been described.\(^12,25\) Alcian blue stains all types of mast cells such as CTMC and immature mast cells, whereas safranin stains only CTMC.\(^15,26\)

In addition, CMC or a CMC/fibroblast coculture were centrifuged, and 2% perchorlic acid was added to the pellet. CMC dissolved in 2% perchorlic acid were applied to a column of high

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performance liquid chromatography coupled with fluorometry to measure histamine concentration, as described previously.27 The histamine content per 10^6 CMC was calculated. The histamine content is known to be higher in CTMC than in MMC or immature mast cells.26

Proportion of CMC attaching to fibroblasts. To analyze the attachment of CMC to fibroblasts, 1.0 x 10^3 CMC were cocultured with Swiss albino/3T3 fibroblasts as described above. At various times after the coculture of +/-CMC and mi/mi-CMC with fibroblasts in the presence or absence of WEHI-3-conditioned medium, culture dishes were washed four times with Alpha MEM to remove nonattaching CMC as described.17 Fibroblasts and CMC attaching to the fibroblasts were harvested after the trypsin treatment and fixed with Carnoy's solution. Cytocentrifuge specimens were first stained with alcian blue and then with nuclear fast red. The number of remaining mast cells per fibroblast was calculated as described.17

Stimulation with rrSCFlM and cell lysis. Exponentially growing CMC were washed three times with α-MEM, and incubated in serum-free ASF-102 medium for 6 hours at 37°C to factor-deprive the cells. The cells (10^6 cells suspended in 1 mL of ASF-102 medium) were then exposed to 100 ng/mL of rrSCF for 15 minutes. After stimulation, cells were washed with cold PBS and lysed in 100 μL of lysis buffer containing protease and phosphatase inhibitors, as described previously. Insoluble material was removed by centrifugation at 10,000g for 15 minutes at 4°C. The cell lysates were frozen at -80°C until use.

Gel electrophoresis and immunoblotting. Cell lysates (150 μg) were mixed 2:1 with 3X sodium dodecyl sulfate (SDS) sample buffer with 2-mercaptoethanol (Sigma), heated at 100°C for 5 minutes, and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 7.5% polyacrylamide. After electrophoresis, proteins were electrotransferred from the gel onto a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA) in a buffer containing 25 mmol/L Tris, 192 mmol/L glycin, and 20% methanol at 0.4 A for 4 hours at 4°C. After blocking residual binding sites on the membrane with gelatin, immunoblotting was performed with an antiphosphotyrosine MoAb as described previously.20

Flow cytometry. CMC were incubated first with ACK 2 rat MoAb against the c-kit product at 4°C for 30 minutes, rinsed, and developed with fluorescein isothiocyanate-conjugated MoAb prepared at Kumamoto University Medical School.18,19 CMC were rinsed and analyzed on FACScan (Becton Dickinson, Mountain View, CA).

Detection of c-kit, mouse mast cell protease-6 (MMCP-6), MMCP-2, and mast cell carboxypeptidase A (MC-CPA) mRNA transcripts by means of Northern blot analysis. Total cellular RNA was isolated with a guanidine isocyanate method in combination with a cesium chloride modification, and equal amounts of RNA (15 or 20 μg) were size-fractionated by electrophoresis through 1.0% agarose gels containing 2.2 mmol/L formaldehyde. Before transfer to nitrocellulose papers, the ethidium-stained gels were visualized under ultraviolet illumination to determine the position of the ribosomal RNA bands, and to verify that equal amounts of RNA had been loaded. The c-kit probe consisting of a 1.1-kb fragment was prepared in our laboratory, and encoded part of the extracellular domain (nucleotide number 1 to 1090) of c-kit. The full-length β-actin probe was kindly provided by Dr S. Sakiyama (Chiba Cancer Center Institute, Japan). and used to verify that an equal amount of mRNA was loaded in each lane.

For detection of MMCP-6, MMCP-2, and MC-CPA mRNA transcripts, all of which are known as good markers for the analysis of mast cell phenotypes,31,36 sense and antisense oligonucleotide primers specific for the coding regions of those genes were synthesized by conventional technology. The following oligonucleotide primers were used: MMCP-6: forward primer, 5'-CTCTTCT-CTTCCACCCACAGT-3' (209 through 228), reverse primer, 5'-TGTAGATGGCAGCTTGTG-3' (779 through 798); MMCP-2: forward primer, 5'-ACACAGACATGATGAG-3' (688 through 707), reverse primer, 5'-TAAAGAGACTTCATGAGC-3' (1358 through 1377); MC-CPA: forward primer, 5'-GTGATGACTGCTGACACTG-3' (194 through 213), reverse primer, 5'-CTTGAAGCTCCTGACG-3' (769 through 788). The numbers represent the nucleotide numbers on the complementary strands of each cDNA sequence.31,22 Total cellular RNA (5 μg) from CMC of C57Bl/6 +/- mice was used as a template and the single-strand cDNA was synthesized with downstream antisense primers by reverse transcriptase (Takara Shuzou, Kyoto, Japan). cDNA was amplified in a 100 μL reaction mixture by a Perkin-Elmer Cetus DNA thermal cycler (Norwalk, CT) using Taq DNA polymerase in 30 cycles of 1 minute of denaturation at 94°C, 2 minutes of annealing at 55°C, and 3 minutes of synthesis at 72°C.30 Polymerase chain reaction (PCR) products were examined on 1% agarose gel. For the analysis of DNA sequence, PCR products were gel purified, treated with T4 polynucleotide kinase, and then with the Klenow fragment of DNA polymerase I. The products were subcloned into the EcoRV site of Bluescript KS (Stratagene, La Jolla, CA). DNA sequencing reaction was performed by the method of Sanger et al26 using [32P]-dCTP.

After prehybridization, the filters were hybridized with random 3P-labeled probes with specific activities of 1 x 106 cpm/μg in 10% dextran sulfate, 50% formamide, 4X standard saline citrate (SSC), 1 x Denhardt's solution, and 10 μg/mL salmon sperm DNA for 24 hours at 42°C. The filters were washed and autoradiographed at -70°C with two intensifying screens.

RESULTS

Coculture of CMC with Swiss albino/3T3 fibroblasts. CMC were prepared from spleens of either mi/mi mice or normal (+/+ ) mice in a medium containing WEHI-3-conditioned medium as a source of IL-3. The proliferative response of mi/mi-CMC to IL-3 was comparable with that of +/-CMC. To determine whether mi/mi-CMC acquired CTMC-like phenotypes when cocultured with Swiss albino/3T3 fibroblasts in the presence of IL-3, we used two coculture systems. In the first, CMC that did not attach to fibroblasts were discarded at each medium change. In the in vitro CMC/fibroblast coculture system, the number of +/-CMC was maintained for 4 weeks, but the number of mi/mi-CMC decreased to about 1% of that of +/-CMC (Fig 1). Because of the disappearance of mi/mi-CMC, it was impossible to determine whether mi/mi-CMC acquired the CTMC phenotypes on the fibroblast layer. In the second, we did not discard the culture media, but the media containing nonattaching CMC were collected and centrifuged, and then the cells recovered in the pellets were returned to the coculture. In this coculture system, the number of mi/mi-CMC was comparable to that of +/-CMC 2 weeks after the initiation of the coculture. We therefore used our modified method to examine the phenotypic change of mi/mi-CMC.

Phenotypes of CMC were examined before and 2 weeks after the initiation of the coculture. Before the coculture, there was little difference in histamine content and the proportion of safranin-positive cells between mi/mi-CMC and +/-CMC (histamine content of mi/mi-CMC and
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+/-CMC, 140 ± 20 v 130 ± 10 ng/106 CMC; the proportion of safranin-positive cells, 0% in both types of CMC). Two weeks after the initiation of the coculture, the increase in histamine content per 106 mast cells was significantly lower in mi/mi-CMC cocultured with fibroblasts than in +/+-CMC (mi/mi, 330 ± 10 ng; +/-, 1,380 ± 140 ng). Also, the proportion of safranin-positive cells was significantly lower in the former than in the latter (mi/mi, 0.2% ± 0.1%; +/-, 53.1% ± 11.2%).

Detection of MMCP-6, MMCP-2, and MC-CPA mRNA transcripts in +/-+CMC and mi/mi-CMC. In addition to histamine content and staining properties with alcian blue/safranin, MMCP and MC-CPA have recently been reported to be good markers for defining the phenotypes of mast cells.31-35 We therefore examined the transcription of MMCP-6, MMCP-2, and MC-CPA in +/-+CMC and mi/mi-CMC before and 2 weeks after coculture with fibroblasts (Fig 2). The 1.1-kb mRNA transcripts for MMCP-6 were detected in +/-+CMC and found to be increased after the coculture, whereas MMCP-6 mRNA was not detected in mi/mi-CMC before or even after the coculture (Fig 2). By contrast, the 1.0-kb mRNA transcripts for MC-CPA-2 were barely detectable in +/-+CMC, but easily detected in mi/mi-CMC, and coculture of CMC with fibroblasts resulted in a slight increase of MMCP-2 transcripts in +/-+CMC, whereas a decrease of the transcripts was seen in the RNA sample from mi/mi-CMC (Fig 2). The 1.5-kb mRNA transcripts for MC-CPA were readily detectable in all RNA samples of +/-+CMC and mi/mi-CMC with an equal level. No transcription of MC-CPA was seen in RNA samples from Swiss albino/3T3 fibroblasts (Fig 2).

Attachment of CMC to fibroblasts. When mi/mi-CMC were cocultured with fibroblasts even in the presence of IL-3, the number of mi/mi-CMC decreased after discarding nonattaching CMC, suggesting impaired attachment of the cells to fibroblasts. To directly show this impaired attachment of mi/mi-CMC to fibroblasts, mi/mi-CMC and +/-+CMC were cocultured with Swiss albino/3T3 fibroblasts in the presence or absence of IL-3, and CMC attaching to the fibroblasts were determined at various times after the initiation of the coculture. The number of +/-+CMC attaching to fibroblasts reached a maximum at 3 to 6 hours, and gradually decreased thereafter (Fig 3). The number of mi/mi-CMC attaching to fibroblasts was significantly lower than that of +/-+CMC (Fig 3). The addition of IL-3 did not influence the number of attaching CMC in the coculture of either +/-+CMC or mi/mi-CMC (Fig 3).

Effect of SCF on proliferation and differentiation of CMC. We next examined whether SCF induced proliferation of CMC derived from mi/mi mice. CMC were cultured in the presence of 0 to 1,000 ng/mL of rrSCF164 for 3 days, followed by measurement of cell proliferation with the MTT colorimetric method. Although rrSCF164 induced a dose-dependent proliferation of the +/-+CMC over the range of 0.1 to 1,000 ng/mL, rrSCF164 had little effect on proliferation of mi/mi-CMC, even at a concentration of 1,000 ng/mL (Fig 4). The results of the MTT assay shown in Fig 4 were confirmed by counting cells and by measuring...
shown as mean ± SEM of four dishes.

*H*-thymidine incorporation (data not shown). Because 100 ng/mL of rrSCF164 induced almost maximal proliferation of +/+ -CMC, either +/+ -CMC or mi/mi-CMC were cultured at this concentration of rrSCF164 hereafter.

Further, to determine whether mi/mi-CMC acquired the CTMC-like phenotypes in response to rrSCF164 CMC were cultured for 6 weeks with or without SCF in a medium containing IL-3. At various times after the initiation of the culture in the presence of both SCF and IL-3, changes in CMC attaching to the fibroblasts was determined at various times after the initiation of the CMC/fibroblast coculture. The results are shown as mean ± SEM of four dishes.

Tyrosine phosphorylation of cellular proteins in CMC after stimulation with rrSCF164. To better understand the impaired effect of SCF on the proliferation and differentiation of mi/mi-CMC, we investigated the effect of SCF on tyrosine kinase activity in +/+ and mi/mi-CMC. CMC were removed from growth factors for 6 hours and then treated with 100 ng/mL of rrSCF164 for 15 minutes. Changes in tyrosine phosphorylation were detected by immunoblotting with an MoAb specific for phosphotyrosine. Increased phosphotyrosine was observed in proteins, particularly at molecular weights 145 to 165 Kd, after stimulation of +/+ -CMC with SCF (Fig 5). Tyrosine phosphorylation of the protein was dependent on the dose of rrSCF164 and the degree of tyrosine phosphorylation roughly paralleled SCF-induced proliferation of +/+ -CMC (data not shown). In contrast, tyrosine phosphorylation of the 145- to 165-Kd protein was barely observed in mi/mi-CMC after exposure to rrSCF164 (Fig 5).

Low expression of c-kit in mi/mi-CMC. We have previously shown that SCF induces tyrosine phosphorylation of c-kit product at a molecular weight of 145 to 165 Kd.38,39 To determine whether the weak tyrosine phosphorylation of the 145- to 165-Kd protein in mi/mi-CMC after SCF treatment was due to low c-kit expression in mi/mi-CMC, we examined the expression of c-kit in +/+ -CMC and mi/mi-CMC. Flow cytometry analysis using an MoAb (ACK2) against the extracellular domain of c-kit product showed that expression of c-kit product on mi/mi-CMC was significantly lower (approximately 10%) than on +/+ -CMC (Fig 6A). Further, expression of c-kit gene was examined by means of Northern blot analysis. Consistently with the previous findings, c-kit transcripts gave a band of approximately 5.5 kb.40 The expression of c-kit mRNA transcripts in mi/mi-CMC was much lower (approximately 10%) than that in +/+ -CMC (Fig 6B). These results

Table 1. Changes in Histamine Content and Proportion of Safranin-Positive Cells After the Culture of Normal (+/+ ) and mi/mi Mouse-Derived CMC With rrSCF164 and IL-3

<table>
<thead>
<tr>
<th>Days</th>
<th>Histamine Content per 10^6 Mast Cells (ng)</th>
<th>Proportion of Safranin-Positive Mast Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+/+</td>
<td>mi/mi</td>
</tr>
<tr>
<td>0</td>
<td>130 ± 10</td>
<td>140 ± 20</td>
</tr>
<tr>
<td>7</td>
<td>170 ± 10</td>
<td>180 ± 30</td>
</tr>
<tr>
<td>14</td>
<td>320 ± 180</td>
<td>160 ± 70</td>
</tr>
<tr>
<td>21</td>
<td>590 ± 250</td>
<td>320 ± 30</td>
</tr>
<tr>
<td>28</td>
<td>1,400 ± 450</td>
<td>340 ± 120</td>
</tr>
<tr>
<td>42</td>
<td>1,430 ± 370</td>
<td>380 ± 60</td>
</tr>
</tbody>
</table>

WEHI-3-conditioned medium was used as a source of IL-3. Triplicate aliquots of +/+ -CMC or mi/mi-CMC were cultured for indicated days in the presence of rrSCF164 (100 ng/mL) and WEHI-3-conditioned medium (50% vol/vol).

Abbreviation: NT, not tested.

*The results are shown as mean ± SEM for one of three similar experiments.

†Results are shown as mean ± SEM of triplicate cultures.
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indicating that \textit{mi/mi}-CMC expressed a low amount of c-kit at both protein and message levels.

**DISCUSSION**

The product of \textit{c-kit} proto-oncogene is considered to be an indispensable molecule for stromal cell-dependent proliferation and differentiation of hematopoietic cells such as cells of erythroid and mast cell lineages as well as stem cells.\textsuperscript{1,20,41} The product of \textit{c-kit} is reported to be expressed on the surface of mast cells, and its ligand on stromal cells such as fibroblasts,\textsuperscript{20} and the interaction of the \textit{c-kit} receptor with its ligand is known to play an important role in mast cell growth and differentiation.\textsuperscript{26} However, the expression and function of \textit{c-kit} have not been studied in \textit{mi/mi} mast cells in any detail.

We previously showed that \textit{mi/mi}-CMC did not respond to the proliferative stimuli presented by fibroblasts that expressed \textit{c-kit} ligand on their surface.\textsuperscript{4} Furthermore, in light of data on staining properties and histamine content, the present findings show that \textit{mi/mi}-CMC do not acquire CTMC-like phenotypes, whereas \textit{+/-}-CMC do acquire them during culture with fibroblasts or SCF. It has recently been reported that, in murine rodents, two subpopulations of mast cells, CTMC and MMC, can be identified on the basis of the differential expression of MMCP and MC-CPA mRNA transcripts,\textsuperscript{31-35} and also that bone marrow-derived CMC are a progenitor population and are not MMC equivalents, as has been suggested by histochemical criteria.\textsuperscript{31-35}

To analyze further the phenotypes of \textit{+/-}-CMC and \textit{mi/mi}-CMC and also the alteration of those phenotypes in an in vitro culture, the transcription of MMCP-6, MMCP-2, and MC-CPA mRNA was examined in \textit{+/-}-CMC and \textit{mi/mi}-CMC before and after coculture with fibroblasts. MMCP-6 and MC-CPA mRNA transcripts were detected in \textit{+/-}-CMC, but MMCP-2 transcripts were barely expressed in the cells, indicating that \textit{+/-}-CMC have imma-

![Image of flow cytometric analysis of the surface binding of monoclonal anti-c-kit antibody (ACK-2) to \textit{+/-}-CMC and \textit{mi/mi}-CMC (A). CMC were incubated in either negative control antibody (---) or ACK-2 (--), washed, incubated with fluorescein-conjugated monoclonal antirat k antibody, and analyzed on a FACScan. For Northern blot analysis of c-kit mRNA transcripts in \textit{+/-}-CMC and \textit{mi/mi}-CMC (B), 15 \mu g of total cellular RNA was electrophoresed in 1% agarose gels. The blots were then hybridized with a \textsuperscript{32P}-labeled cDNA probe for c-kit. The \beta-actin probe was used to verify that an equal amount of mRNA was loaded in each lane.](image-url)
ture mast cell phenotypes. The transcription of MMCP-6 mRNA was increased by coculture of \(+/+-\)CMC with fibroblasts. These results, indicating the phenotypic alteration of \(+/+-\)CMC from immature mast cells to CTMC-like mast cells, coincide with the data obtained from staining properties and histamine content. The expression of MMCP-6 and MMCP-2 mRNA transcripts in \(mi/mi\)-CMC is in marked contrast to that in \(+/+-\)CMC. \(mi/mi\)-CMC did not contain detectable levels of MMCP-6 mRNA transcripts, but expressed considerable amounts of MMCP-2 and MC-CPA mRNA transcrip tions. In addition, coculture of \(mi/mi\)-CMC with fibroblasts resulted in a slight decrease in MMCP-2 transcripts. The difference in the expression of MMCP-2 and MMCP-6 mRNA transcripts between \(+/+-\)CMC and \(mi/mi\)-CMC is interesting and suggests that some mechanism must exist to regulate MMCP expression in CMC. It is possible that \(mi\) may encode a gene product that is involved in regulating the expression of MMCP-2 and MMCP-6 genes. Also, it is possible that the gene product of \(mi\) locus may have some effect on the differentiation of mast cells so that \(mi/mi\)-CMC lie at a different stage of differentiation than \(+/+-\)CMC. Regarding MMCP-2 and MMCP-6 expression, \(mi/mi\)-CMC resemble MMC that are reported to express abundant mRNA transcript of MMCP-2 and little mRNA transcript of MMCP-6 in \(+/+-\)mice. However, \(mi/mi\)-CMC differ from MMC in the expression of MC-CPA: MC-CPA is reported to be barely detectable in MMC, but it has been readily detectable in \(mi/mi\)-CMC. This is the first report regarding the expression pattern of MMCP and MC-CPA transcripts in \(mi/mi\)-CMC, and the pattern has not been described so far in any mast cell population. Therefore it is rather difficult to use the expression pattern of MMCP and MC-CPA as a marker for analyzing the phenotypic change in \(mi/mi\)-CMC.

In this study, we first cocultured \(mi/mi\)-CMC with fibroblasts according to the method originally described by Levi-Schaffer et al., in which nonattaching CMC were discarded at each time of medium change. The number of \(mi/mi\)-CMC, but not of \(+/+-\)CMC, continued to decrease in the coculture system. In contrast, \(mi/mi\)-CMC were maintained by our modified coculture system in which nonattaching CMC were collected and returned to the coculture. Because the attachment of \(mi/mi\)-CMC to fibroblasts was significantly impaired, it was suggested that the expression of the molecule necessary for attachment to fibroblasts was impaired in \(mi/mi\)-CMC.

We recently investigated the role of the c-kit product in the attachment of CMC to fibroblasts in a CMC/fibroblast coculture system.\(^{17}\) The attachment to fibroblasts of \(W/W\)-CMC, which did not express the extracellular domain of the c-kit receptor, was significantly impaired. However, \(W/W^2\), \(W/W^P\), or \(W/W^{Q}\)-CMC, expressing the extracellular domain of the c-kit receptor, attached to fibroblasts at levels comparable to \(+/+-\)CMC. Moreover, the attachment of \(+/+-\)CMC to fibroblasts was inhibited by the addition of ACK2 MoAb against the extracellular domain of the c-kit receptor. These results indicated that the extracellular domain of the c-kit receptor appeared to be necessary for attachment of CMC to fibroblasts.\(^{17}\) In the present study, we showed that the expression of c-kit receptor was significantly lower in \(mi/mi\)-CMC at both protein and message levels than in \(+/+-\)CMC. This finding suggests that the impaired attachment of \(mi/mi\)-CMC to fibroblasts is attributable to the low expression of the c-kit receptor in the cells.

The present study also showed that c-kit ligand, SCF, did not stimulate the proliferation of \(mi/mi\)-CMC, whereas it induced the proliferation of \(+/+-\)CMC in a dose-dependent manner. Because the growth of \(mi/mi\)-CMC was not supported by SCF alone but by IL-3 or the combination of IL-3 and SCF, we cultured \(mi/mi\)-CMC with both rrSCF\(^{16}\) and IL-3 to examine whether or not SCF was active in inducing phenotypic change in \(mi/mi\)-CMC. According to their safranin-staining properties and histamine content, \(mi/mi\)-CMC did not acquire CTMC-like phenotypes after treatment with SCF and IL-3, although \(+/+-\)CMC did. These results obtained by SCF were comparable with the results by the coculture with fibroblasts.

SCF has been shown to induce tyrosine phosphorylation of several proteins, including its receptor, in murine\(^{42-44}\) and human cell lines.\(^{39,45}\) Immunoblotting with an MoAb against phosphotyrosine showed that the treatment of \(+/+-\)CMC with SCF resulted in a marked increase in tyrosine phosphorylation of 145- to 165-Kd protein, c-kit, and tyrosine phosphorylation of c-kit was found to be dose-dependent and correlated precisely with the rate of cell proliferation. However, SCF induced little tyrosine phosphorylation of the protein in \(mi/mi\)-CMC. The bare induction of tyrosine phosphorylation of c-kit by its ligand was found to be due to the low expression of c-kit protein in \(mi/mi\)-CMC. It is possible that tyrosine kinase activity of c-kit receptor may be weaker in \(mi/mi\)-CMC than in \(+/+-\)CMC because of the low expression of c-kit receptor, and that the low kinase activity of c-kit may contribute to the unresponsiveness of \(mi/mi\)-CMC to SCF stimulation. Although it is not fully known yet how c-kit expression is regulated, the present findings raise the possibility that the molecule defined by the \(mi\) locus may play some roles in regulating c-kit expression as well as MMCP and MC-CPA expression. Also, as discussed above, the low c-kit expression of \(mi/mi\)-CMC may be attributable to their different stage of differentiation. Additional information about the mechanisms responsible for the expression of c-kit, MMC, and MC-CPA would be particularly helpful in understanding mast cell growth and function in \(mi/mi\) mice.

Recently, Dubreuil et al introduced c-fms gene, which encodes a tyrosine kinase receptor for macrophage colony-stimulating factor, into CMC derived from \(W\) and \(mi\) mutant mice, and examined the survival of the CMC expressing c-fms receptor in an in vitro mast cell/fibroblast coculture system.\(^{46}\) They found that ectopic expression of c-fms complemented mutations at the \(W\) locus, but could not overcome the inability of \(mi/mi\)-CMC to survive in the culture system. These results raised the possibility that a common signal pathway might be used by c-kit and c-fms to promote cell proliferation, and also suggested either that the \(mi\) locus might define a common step in the c-kit and
c-fms signal transduction pathways, or that mi might encode a parallel pathway necessary for c-kit and c-fms function. These findings seem to be at variance with our conclusions in the present study, but it is not sufficiently known yet if the impaired signaling of c-kit in mi/mi-CMC is because of low c-kit expression, a defect in one of downstream targets of c-kit receptor, or both of them. It would be of interest to examine whether ectopic expression of c-kit receptor by a heterologous promoter could complement the mi defect, and this examination could provide clues as to which molecules are critical for the impaired signaling of c-kit receptor. However, the phenotype of mi/mi mice is not completely identical to that of W mutant mice. For example, osteopetrosis, microphthalmus, and deficient natural killer cell activity18,40 are observed only in mi/mi mice, whereas anemia and deficiency of germ cells occur only in W mutant mice. Further studies will be necessary to understand the pleiotropic effects of mi mutation, and the differential phenotype of mi/mi mice and W mutant mice.

In summary, the results presented here show that the attachment of mi/mi-CMC to fibroblasts is significantly impaired, and that mi/mi-CMC do not proliferate or differentiate in response to the stimuli presented either by fibroblasts or by c-kit ligand. It is likely that the impaired response and attachment of the cells may be due, at least in part, to low c-kit expression on their surface. Identification of the molecule encoded by the mi locus will be important in further understanding of the growth regulation and differentiation of mast cells.

REFERENCES

Low c-kit expression of cultured mast cells of mi/mi genotype may be involved in their defective responses to fibroblasts that express the ligand for c-kit

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