Mechanism of Mast Cell Deficiency in Mutant Mice of mi/mi Genotype: An Analysis by Co-Culture of Mast Cells and Fibroblasts

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Mutant mice of mi/mi genotype are osteopetrotic and are deficient in mast cells. The osteopetrosis of mi/mi mice can be cured by bone marrow transplantation from congenic normal (+/++) mice, and therefore, the cause of the osteopetrosis is attributed to a defect of osteoclasts. Since both osteoclasts and mast cells are the progeny of multipotential hematopoietic stem cells, we examined whether mast cells were defective in mi/mi mice. In spite of the deficiency of mast cells in tissues of mi/mi mice, mast cells did develop when spleen cells of mi/mi mice were cultured with pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM). The proliferative response of cultured mast cells (CMC) derived from mi/mi mice to PWM-SCM was comparable with that of CMC from +/+ mice. In contrast, when CMC were co-cultured with the NIH/3T3 fibroblast cell line in culture medium lacking PWM-SCM, only +/+ CMC entered into the S phase of the cell cycle and were maintained; mi/mi CMC gradually disappeared. Moreover, fibroblasts derived from the skin of mi/mi mice normally supported the proliferation of +/+ CMC. Thus, the mast cell deficiency of mi/mi mice appears to be due to the inability of mi/mi mast cells to respond to the proliferative stimuli presented by fibroblasts.

A DOUBLE GENE DOSE of mutant alleles at the mi locus (chromosome 6) produces the pleiotropic effects of microphthalmia, depletion of pigment in both hairs and eyes, and osteopetrosis.1,2 In addition to these abnormalities, Stevens and Louit3 and Stechschulte et al4 reported depletion of mast cells in mi/mi mice. Osteopetrosis of mi/mi mice can be cured by bone marrow transplantation from histocompatible normal (+/+) donors,5 and its cause is attributed to a defect of the osteoclasts. Although both osteoclasts6 and mast cells7,8 are the progeny of multipotential hematopoietic stem cells, the mechanism of mast cell deficiency in mi/mi mice has not been studied to our knowledge. The purpose of our study was to investigate the mechanism by using the co-culture system of mast cells and fibroblasts that was recently established by us.9,10

T cell-dependent and fibroblast-dependent growth of mast cells are known.11 T cell-dependent growth is mediated by diffusible factors, ie, interleukin-3 (IL-3) and interleukin-4 (IL-4). On the other hand, there are two modes of fibroblast-dependent growth of mast cells. We described the mast cell growth supported by direct contact with fibroblasts,11 whereas Jarboe et al12 reported that fibroblasts produced diffusible factor(s) that induced differentiation of mast cell-committed progenitors.

In spite of mast cell depletion in mutant mice of either W/W or SI/SI' genotypes,13,14 mast cells do develop when bone marrow cells of W/W and SI/SI' mice are cultured in the presence of IL-3 and IL-4.9,11,15,16 We analyzed the mechanism of mast cell depletion of W/W' and SI/SI'mice by using these artificially obtained cultured mast cells (CMC). When CMC derived from W/W' mice were co-cultured with the NIH/3T3 cells (an embryo-derived fibroblast line), the growth of W/W' CMC stopped, and then W/W' CMC disappeared within 2 weeks.10 In contrast, the proliferation of CMC derived from the bone marrow of SI/SI' mice is normally induced by the contact with mouse embryo-derived fibroblasts.11 Then fibroblast cell lines were established from SI/SI' and control +/+ embryos. All fibroblast cell lines derived from +/+ embryos supported the proliferation of +/+ CMC, whereas all fibroblast cell lines derived from SI/SI' embryos failed to support the +/+ CMC.15,16 Jarboe and HufT17 also investigated the mechanism of mast cell depletion of W/W and SI/SI' mice. They showed that W/W' mice did not produce mast cell progenitors that responded to fibroblast-conditioned medium and that fibroblasts derived from SI/SI' mice did not produce the activity that induced the differentiation of the committed mast cell progenitors of +/+ mice.

In the present study, we cultured the spleen cells of mi/mi mice in the presence of IL-3 and IL-4. In spite of the depletion of mast cells in tissues of mi/mi mice, CMC did develop as described in the cases of W/W' and SI/SI' mice. The response of mi/mi CMC to T cell-derived growth factors was normal, but the proliferation of mi/mi CMC was not induced by the co-culture with fibroblasts derived from +/+ mice.

MATERIALS AND METHODS

Mice and cells. The original stock of C57BL/6-mi/+ (hereafter called mi/+ ) mice was purchased from the Jackson Laboratory, Bar Harbor, ME, and the mice were maintained in our laboratory by repeated backcrosses to our own inbred C57BL/6 strain. Since the tail tip and paws of mi/+ mice are white, they are recognizable in most cases. Female mi/+ mice were crossed with male mi/+ mice, and the resulting mi/mi mice were selected by their white coat color. Mice of mi/mi genotype and their normal (+/+ ) littermates were used at 2 to 3 weeks of age. The origin of NIH/3T3 cells has been described.13 The cell line was adapted to grow in α-minimal essential medium (α-MEM; Flow Laboratories, Irvine, UK) containing 10% fetal calf serum (FCS; HyClone, Logan, UT), 100 U/mL penicillin, and 100 μg/mL streptomycin. Swiss-albino/3T3 fibroblast cells were obtained from the Japanese Cancer Research Resources Bank.
Tokyo, Japan. The WCB6F1, +/+ /3T3 and WCB6F1, −/−/3T3 fibroblast cell lines were established in our laboratory.

**Number of mast cells.** Mice were anesthetized with ether inhalation and killed by decapitation. Pieces of dorsal skin were removed, smoothed onto a piece of filter paper to keep them flat, fixed in Carnoy’s solution, and embedded in paraffin. Serial sections (5 μm thick) were stained with Alcian blue, acidified toluidine blue (pH 3.0), or berberine sulfate. Berberine sulfate is a fluorescent dye that binds to and practically all mast cells in the skin and peritoneal cavity of +/+ mice were stained with berberine sulfate. Mast cells between epithelium and panniculus carnosus were counted under the microscope, and the number was expressed as mast cells per centimeter. The specimens stained with berberine sulfate were examined with an Olympus epifluorescence microscope.

**Establishment of CMC.** Pokeweed mitogen-stimulated spleen cell conditioned medium (PWM-SCM) was prepared as described by Nakahata et al.13 Spleen cells (2 × 10^6/mL) were incubated for 5 days in α-MEM containing 1:300 dilution of PWM (GIBCO, Grand Island, NY), 10% FCS, and 10−4 mol/L 2-mercaptoethanol (Sigma Chemical Co, St Louis, MO). The conditioned medium was centrifuged, filtered through a 0.22 μm filter (Milipore Corp, Bedford, MA), and stored at −80°C. Culture flasks (Nunc, Roskilde, Denmark) containing 2 × 10^7 spleen cells and 5 mL α-MEM supplemented with 10−4 mol/L 2-mercaptoethanol, 10% FCS, and 10% PWM-SCM were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. 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.

**Fibroblasts derived from the skin.** Skin pieces from +/+ mice and mi/mi mice were diced into fragments (about 1 μL). Fragments were washed in Hanks’ balanced salt solution (HBSS) buffered with 10 mmol/L HEPES (pH 7.4) and were incubated in HBSS buffered with HEPES containing 1 mg/mL Type I collagenase (Sigma), and 1 mg/mL Type I hyaluronidase (Sigma), and 20% FCS at 37°C for 2.5 hours. After the incubation, the mixtures were passed through a nylon mesh, the cells were washed three times with α-MEM, and cell counts were made with a standard hemocytometer. Five milliliters of a culture mixture containing 2 × 10^3 dispersed skin cells, Dulbecco’s modified Eagle’s medium (Flow), and 10% calf serum (Hyclone) was plated in a 60-mm dish. The culture medium was aspirated and replaced with 5 mL fresh medium every 3 days. When cells were confluent, they were trypsinized, and then 10^3 cells were transferred to each 35-mm dish.

**Co-culture with fibroblasts.** Co-culture of CMC with fibroblasts was done as described previously.13,15 CMC suspended in 2 mL α-MEM containing 5% FCS and antibiotics (but not PWM-SCM) were added to a confluent culture of fibroblasts in a 35-mm dish. They were cultured at 37°C in a humidified atmosphere containing 5% CO2 in air. The culture medium was aspirated and replaced with 2 mL fresh culture medium without PWM-SCM every 2 days. Mast cells were identified by staining cytocentrifuge preparations of trypsinized cultures with Alcian blue. Results were expressed as the mean ± SE of four dishes.

**Clonal cell culture.** Methylocellulose culture was carried out according to the method described by Nakahata et al.15 At various times after initiation of the co-culture of CMC with NIH/3T3 cells, cells were harvested by trypsinization. One milliliter of a culture mixture in α-MEM containing 500 CMC, dispersed NIH/3T3 cells, 1% methylocellulose (Sigma), 30% FCS, 1% deionized bovine serum albumin (BSA; Sigma), 10−4 mol/L 2-mercaptoethanol, and 10% PWM-SCM was plated in 35-mm non-tissue culture dishes (Flow). The dishes were incubated at 37°C in a humidified atmosphere of 5% CO2 in air. NIH/3T3 cells did not form colonies, and mast cell colonies containing ≥50 cells were counted on day 16.

**Detection of mast cells in S phase.** The proportion of cells that incorporated bromodeoxyuridine (BrdUrd) was used as an index of cell proliferation, as described previously.21 Cells were incubated with 3 μg/mL BrdUrd (Wako Pure Chemical Ind, Osaka, Japan) at 37°C for 30 minutes. Cytocentrifuge preparations of trypsinized cultures were fixed with Carnoy’s solution. The specimens were first stained with Alcian blue. The cells that incorporated BrdUrd were then stained by using mouse anti-BrdUrd monoclonal antibody (Becton Dickinson, Mountain View, CA), biotin-conjugated horse anti-mouse-IgG antibody (Vector Laboratories, Inc, Burlingame, CA), and avidin-biotin-peroxidase complex (Vector). Mast cells in the S phase were identified by the presence of Alcian blue-positive granules in the cytoplasm and dark brown granules on the nucleus.

**RESULTS**

Paraffin sections of the dorsal skin of mi/mi mice and the littersmate +/+ mice were stained with Alcian blue, toluidine blue, or berberine sulfate. The staining methods did not significantly influence the number of mast cells in the skin of +/+ mice, but remarkably influenced the number of mast cells in the skin of mi/mi mice. When sections were stained with Alcian blue, the number of mast cells in the skin of mi/mi mice was about 40% that of +/+ mice. On the other hand, when sections were stained with berberine sulfate, the number of mast cells in the skin of mi/mi mice was about 2% that of +/+ mice. An intermediate result was obtained when sections were stained with toluidine blue (Table 1).

CMC were prepared from spleens of either three mi/mi mice or three control +/+ mice. The same number (3 × 10^6 cells/mL) of either mi/mi CMC or +/+ CMC were co-cultured with various fibroblast cell lines in the absence of PWM-SCM. The numbers of both mi/mi CMC and +/+ CMC increased in parallel with the concentration of PWM-SCM (Fig 1). No significant difference was detectable between mi/mi CMC and +/+ CMC. In the experiment shown in Fig 1, both mi/mi CMC and +/+ CMC were stimulated by PWM-SCM prepared with +/+ spleen cells; however, another experiment using PWM-SCM prepared with mi/mi spleen cells gave a comparable result (data not shown).

CMC of either mi/mi or +/+ mouse origin were co-cultured with various fibroblast cell lines in the absence of PWM-SCM; proportion of mast cells in S phase was determined 48 hours after the initiation of the co-culture. The value was significantly smaller in mi/mi CMC than in +/+ CMC when co-cultured with the NIH/3T3, Swiss-albino/3T3, or WCB6F1, −/+ /3T3-2 cell line (Table 2). In the co-culture with the WCB6F1, −/− /3T3-1 cell line, neither +/+ CMC nor mi/mi CMC entered into the S phase (Table 2). Since the proportion of mast cells in the S phase was the largest when +/+ CMC were co-cultured with the NIH/3T3 cell line, either +/+ CMC or mi/mi CMC were co-cultured with the NIH/3T3 cell line, either +/+ CMC or mi/mi CMC were co-cultured with the NIH/3T3 cell line, either +/+ CMC or mi/mi CMC were co-cultured with the NIH/3T3 cell line, either +/+ CMC or mi/mi CMC were co-cultured with the NIH/3T3 cell line, either +/+ CMC or mi/mi CMC were co-cultured with the NIH/3T3 cell line, either +/+ CMC or mi/mi CMC were co-cultured with the NIH/3T3 cell line, either +/+ CMC or mi/mi CMC were co-cultured with the NIH/3T3 cell line, either +/+ CMC or mi/mi CMC were co-cultured with the NIH/3T3 cell line, either +/+ CMC or mi/mi CMC were co-cultured with the NIH/3T3 cell line, either +/+ CMC or mi/mi CMC were co-cultured with the NIH/3T3 cell line, either +/+ CMC or mi/mi CMC were co-cultured with the NIH/3T3 cell line, either +/+ CMC or mi/mi CMC were co-cultured with the NIH/3T3 cell line, either +/+ CMC or mi/mi CMC were co-cultured with the NIH/3T3 cell line, either +/+ CMC or mi/mi CMC were co-cultured with the NIH/3T3 cell line, either +/+ CMC or mi/mi CMC were co-cultured with the NIH/3T3 cell line, either +/+ CMC or mi/mi CMC were co-cultured with the NIH/3T3 cell line, either +/+ CMC or mi/mi CMC were co-cultured with the NIH/3T3 cell line, either +/+ CMC or mi/mi

| Table 1. Number of Mast Cells in the Skin of mi/mi and Control +/+ Mice Stained with Various Dyes |
|----------|------------------|------------------|
| Dye      | +/+              | mi/mi            |
| Alcian blue | 248 ± 42        | 96 ± 35*         |
| Toluidine blue | 257 ± 40        | 46 ± 12*         |
| Berberine sulfate | 233 ± 69        | 4 ± 3*          |

Values are mean ± SE of six mice.

*P < .01, when compared with the value of +/+ mice by t test.

†P < .02, when compared with the number of mast cells in the skin of syngenic mice stained with Alcian blue.
Differntiation of mast cells

The number of +/+ CMC was maintained in this condition over a 4-week period, whereas the number of mi/mi CMC dropped to about 1% that of +/+ CMC at the end of the fourth week (Fig 2). We also determined the numbers of cells that form mast cell colonies in methylenoxide (CFU-Mast). The change in the number of CFU-Mast paralleled the change in the number of morphologically identifiable mast cells, regardless of the genotype (Fig 2).

The proportion of mast cells in the S phase of cell cycle was determined at various times after the initiation of the co-culture. The proportion of mi/mi CMC in the S phase progressively decreased after the co-culture, and the value became less than 0.1% that of +/+ CMC 2 weeks after initiation of the co-culture (Fig 3). When the result shown in Fig 2 was compared with the result shown in Fig 3, the decrease in the proportion of CMC in the S phase was faster than the decrease in the number of CFU-Mast.

In the previous report,₁₀ we showed that contact with NIH/3T3 fibroblasts drove +/+ CMC to transit the G₁ phase of the cell cycle and initiate DNA synthesis but did not drive W/W CMC to enter the S phase. Exponentially growing +/+ CMC and mi/mi CMC were transferred from medium containing PWM-SCM to the medium lacking PWM-SCM; both +/+ CMC and mi/mi CMC were synchronized at the G₁ phase after 24 hours. The synchronized CMC were divided into two parts: one part was transferred to the medium containing PWM-SCM and the other part was co-cultured with NIH/3T3 fibroblasts. Both +/+ CMC and mi/mi CMC started to incorporate BrdUrd in the presence of PWM-SCM, but only +/+ CMC entered into the S phase in the co-culture with NIH/3T3 cells (Fig 4).

In the next experiment, CMC were individually prepared from five mi/mi mice and from five +/+ mice to examine whether poor growth of mi/mi CMC was reproducibly observed. For each CMC, the proportion of mast cells in the S phase was determined 48 hours after initiation of the co-culture with the NIH/3T3 cell line, and the number of mast cells was determined on day 14. CMC derived from mi/mi mice showed a comparable degree of poor growth in all five cases (data not shown).

There is a possibility that fibroblasts derived from mi/mi mice were defective as well as mi/mi CMC. Fibroblast cultures were prepared from the skin of individual +/+ and mi/mi mice. CMC of +/+ mouse origin were co-cultured with NIH/3T3 cells without PWM-SCM. At indicated times after initiation of the co-culture, the numbers of surviving mast cells were determined. Each point is the mean of four samples; bars are SE.
with each confluent fibroblast layer. Although some variations were observed among individual fibroblast layers, proportion of +/+ mast cells in the S phase was comparable between co-cultures with +/+ fibroblasts and co-cultures with mi/mi fibroblasts (Table 3).

DISCUSSION

When histologic sections were stained with Alcian blue, the number of mast cells in the skin of mi/mi mice was about 40% that of +/+ mice. On the other hand, when stained with berberine sulfate, the number in the skin of mi/mi mice was only 2% that of +/+ mice. Alcian blue stains both connective tissue-type mast cells (CTMC) and mucosal mast cells (MMC), whereas berberine sulfate stains only CTMC that contain heparin proteoglycan.11 Although practically all mast cells in the skin of +/+ mice showed the phenotype of CTMC, mast cells of MMC phenotype were predominant in the skin of mi/mi mice. Since differentiation of MMC-like mast cells to CTMC-like mast cells occurs in connective tissues of rats19 and mice,20 this step of differentiation appears to be impaired in mi/mi mice. The mechanism is now under investigation.

In spite of the deficiency of mast cells in tissues of mi/mi mice, CMC did develop when spleen cells of mi/mi mice were cultured in the presence of T cell-derived growth factors. The response of mi/mi CMC to PWM-SCM was comparable with that of +/+ CMC. Furthermore, when T cells of mi/mi mice were stimulated by PWM, active growth factors were produced. Therefore, the mast cell proliferation stimulated by T cell-derived growth factors does not appear to be involved in the mast cell deficiency in tissues of mi/mi mice.

In contrast to the normal T cell-dependent growth, the fibroblast-dependent growth of mi/mi CMC showed a marked

Table 3. Response of +/+ Mast Cells to Fibroblasts Established from the Skin of mi/mi and Control +/+ Mice

<table>
<thead>
<tr>
<th>Fibroblast Layer* (Origin/No.)</th>
<th>Proportion of S Phase (%)†</th>
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<tbody>
<tr>
<td>mi/mi</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>2</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>3</td>
<td>3.7 ± 0.8</td>
</tr>
<tr>
<td>4</td>
<td>3.4 ± 0.7</td>
</tr>
<tr>
<td>5</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>6</td>
<td>3.9 ± 0.8</td>
</tr>
<tr>
<td>+/+</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.5 ± 1.0</td>
</tr>
<tr>
<td>2</td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>3</td>
<td>3.5 ± 0.8</td>
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*Each fibroblast layer was prepared from the skin tissue of an individual mouse.
†Percentage of CMC incorporating BrdUrd at 48 hours after the initiation of the co-culture.
defect. Proliferation of mi/mi CMC was not induced by contact with +/+ mouse-derived fibroblast cell lines. Moreover, by synchronizing CMC before co-culture, the defect in mi/mi CMC was characterized as an inability to transit the G1 phase and enter the S phase upon contact with fibroblasts.

As reported in the case of W/W" mice,15.17 fibroblasts derived from mi/mi mice showed normal function to support proliferation of +/+ CMC. On the other hand, the defect of CMC derived from mi/mi mice was similar to that of CMC derived from W/W" mice.8.10 Recently, the W locus was shown to be identical with the c-kit proto-oncogene,22,26 which is a receptor tyrosine kinase. There is a possibility that the protein encoded by the mi locus may be involved in the further transduction of a signal received by the receptor encoded by the c-kit (W) gene. However, the phenotype of W/W" mice is not identical to that of mi/mi mice. Osteopetrosis, microphthalmus, and deficient natural killer cell activity27 are observed only in mi/mi mice, whereas the anemia and deficiency of germ cells occur only in W/W" mice.1,2 This discrepancy is not sufficiently explained by the above mentioned hypothesis, and further studies are necessary to understand functions of the mi gene at the molecular level.

REFERENCES

Mechanism of mast cell deficiency in mutant mice of mi/mi genotype: an analysis by co-culture of mast cells and fibroblasts

Y Ebi, T Kasugai, Y Seino, H Onoue, T Kanemoto and Y Kitamura