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

By Yoshitaka Ebi, Tsutomu Kasugai, Yoshiki Seino, Hitoshi Onoue, Tomoko Kanemoto, and Yukihiko Kitamura

Mutant mice of $mi/mi$ genotype are osteoprototic 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 stimulus 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 Loutit\(^3\) and Stechschulte et al\(^4\) 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 osteoclasts\(^6\) and mast cells\(^7,8\) 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.\(^1\) 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 al\(^12\) 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,\(^13_1\) 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.\(^8,10\) 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.\(^13\) 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.\(^13,16\) Jarboe and Huff\(^17\) 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. Mouse of $mi/mi$ genotype and their normal (+/+) litters 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 WCB6F<sub>1</sub>, +/+ and WCB6F<sub>1</sub>, −/−/3T3 and WCB6F<sub>1</sub>, +/+/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 were made with HEPES containing 10 mmol/L HEPES (pH 7.4) and were incubated in HBSS buffered with 2.5 hours. After the incubation, the mixtures were passed through a nylon mesh, the cells were washed three times with a-MEM, and cell counts were made with a standard hemocytometer. Five milliliters of medium was replaced every 7 days, and more than 95% of cells were viable. Mast cell colonies containing ≥50 cells were counted on day 16.

### Detection of mast cells in S phase

The proportion of mast cells that incorporated bromodeoxyuridine (BrdUrd) was used as an index of cell proliferation, as described previously. Cells were incubated with 3 μg/mL BrdUrd (Wako Pure Chemical Ind, Osaka, Japan) at 37°C for 30 minutes. Cyto centrifuge 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 littermate +/+ 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).

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

CVC 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 coculture. The value was significantly smaller in mi/mi CVC than in +/+ CVC when cocultured with the NIH/3T3, Swiss-albino/3T3, or WCB6F<sub>1</sub>, +/+ /3T3-2 cell line (Table 2). In the co-culture with the WCB6F<sub>1</sub>, −/−/3T3-1 cell line, neither +/+ CVC nor mi/mi CVC entered into the S phase (Table 2). Since the proportion of mast cells in the S phase was the largest when +/+ CVC were cocultured with the NIH/3T3 cell line, either +/+ CVC or mi/mi CVC was used as the control.

### Table 1. Number of Mast Cells in the Skin of mi/mi and Control +/+ Mice Stained with Various Dyes

<table>
<thead>
<tr>
<th>Dye</th>
<th>mi/mi</th>
<th>+/+</th>
</tr>
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<tbody>
<tr>
<td>Alcian blue</td>
<td>248 ± 42</td>
<td>96 ± 35&lt;sup&gt;*&lt;/sup&gt;</td>
</tr>
<tr>
<td>Toluidine blue</td>
<td>257 ± 40</td>
<td>46 ± 12&lt;sup&gt;‡‡&lt;/sup&gt;</td>
</tr>
<tr>
<td>Berberine sulfate</td>
<td>233 ± 69</td>
<td>4 ± 3&lt;sup&gt;††&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

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.
CMC were co-cultured with this cell line for a longer period. 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 methylcellulose (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, 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

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**Table 2. Defective Proliferation Response of mi/mi Mouse-Derived CMC to Various Fibroblast Cell Lines**

<table>
<thead>
<tr>
<th>Cell Lines</th>
<th>Proportion of S Phase (%)</th>
<th>+/+</th>
<th>mi/mi</th>
</tr>
</thead>
<tbody>
<tr>
<td>NIH/3T3</td>
<td></td>
<td>8.2 ± 0.5</td>
<td>0.6 ± 0.3</td>
</tr>
<tr>
<td>Swiss-Albino/3T3</td>
<td></td>
<td>1.3 ± 0.5</td>
<td>0</td>
</tr>
<tr>
<td>WCB/C, +/+/3T3</td>
<td></td>
<td>5.3 ± 1.1</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>WCB/C, S1/S7/3T3-1</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Results are mean ± SE of four dishes.

*Percentage of CMC incorporating BrdUrd at 48 hours after the initiation of the co-culture with each fibroblast cell line.
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. 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 rats and mice, 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>
</tr>
</thead>
<tbody>
<tr>
<td>mi/mi 1</td>
<td>2.8 ± 0.4</td>
</tr>
<tr>
<td>mi/mi 2</td>
<td>3.2 ± 0.6</td>
</tr>
<tr>
<td>mi/mi 3</td>
<td>3.7 ± 0.6</td>
</tr>
<tr>
<td>mi/mi 4</td>
<td>3.4 ± 0.7</td>
</tr>
<tr>
<td>mi/mi 5</td>
<td>1.6 ± 0.1</td>
</tr>
<tr>
<td>mi/mi 6</td>
<td>3.9 ± 0.8</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>
</tr>
</tbody>
</table>

*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.
Differention of Mast Cells

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


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