Characterization of Cultured Mast Cells Derived From Ws/WS Mast Cell-Deficient Rats With A Small Deletion at Tyrosine Kinase Domain of c-kit

By Hideki Tei, Tsutomu Kasugai, Tohru Tsujimura, Shiro Adachi, Takuma Furitsu, Kazuo Tohya, Michio Kimura, Krisztina M. Zebo, George F. J. Newlands, Hugh R.P. Miller, Yuzuru Kanakura, and Yukihiko Kitamura

The Ws mutant allele of rats represents a 12-base deletion at the tyrosine kinase domain of the c-kit gene. Although homozygous Ws/WS rats were deficient in both connective tissue-type mast cells (CTMC) and mucosal-type mast cells (MMC), mast cells did develop when bone marrow cells of Ws/WS rats were cultured in the presence of concanavalin A-stimulated spleen cell conditioned medium (ConA-SCM). Although the proliferative response of rat cultured mast cells (RCMC) derived from Ws/WS rats to ConA-SCM was comparable to that of RCMC derived from control normal (+/+ +) rats, the proliferative response of Ws/WS RCMC to rat recombinant stem cell factor (rrSCF: a ligand for the c-kit receptor tyrosine kinase) was much lower than that of +/+ + RCMC. However, a slight c-kit kinase activity was detectable in Ws/WS RCMC, and the proliferation of Ws/WS RCMC was accelerated when rrSCF was added to ConA-SCM. Because CTMC contain mast cell protease-I (RMCP-I) and MMC contain RMCP-II, the phenotype of +/+ + and Ws/WS RCMC in various culture conditions was evaluated by immunohistochemistry of RMCPs. Both +/+ + and Ws/WS RCMC showed the MMC-like phenotype (RMCP-I+/II+) when they were cultured with ConA-SCM alone. Most +/+ + RCMC and about half of Ws/WS RCMC acquired a novel protease (RMCP-I+/II+) phenotype when they were cultured with rrSCF alone. However, because the number of Ws/WS RCMC dropped to one tenth in the medium containing rrSCF alone, the absolute number of Ws/WS RCMC with the RMCP-I+/II+ phenotype did not increase significantly. The effect of rrSCF in inducing the novel phenotype was suppressed when ConA-SCM was added to rrSCF. In contrast, +/+ + and Ws/WS RCMC cocultured with +/+ + fibroblasts showed the RMCP-I+/II+ phenotype even in the presence of ConA-SCM. Moreover, a fibroblast cell line derived from SI/SI mouse embryos that did not produce SCF did not support the survival of both +/+ + and Ws/WS RCMC but did induce the RMCP-I+/II+ phenotype in about half of +/+ + and Ws/WS RCMC when their survival was supported by the addition of ConA-SCM. The normal signal transduction through the c-kit receptor did not appear to be prerequisite for the acquisition of the RMCP-I+/II+ phenotype.

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There are two induction mechanisms for differentiation/proliferation of murine mast cells. One is dependent on T cells and the other is dependent on fibroblasts. The T-cell-derived cytokines, interleukin-3 (IL-3), IL-4, IL-9, and IL-10 have been shown to be involved in development of mast cells. The fibroblast-dependent mast cell development is mediated by a growth factor encoded by the Sl locus of the mouse. Hereafter, the growth factor encoded by the Sl locus is called stem cell factor (SCF). The receptor for SCF has been mapped to the W locus of the mouse; the W locus is identical with the c-kit proto-oncogene and encodes a receptor tyrosine kinase (hereafter called the c-kit receptor). A double gene dose of mutant alleles at either the W or Sl locus results in the depletion of both connective tissue-type mast cells (CTMC) and mucosal-type mast cells (MMC).

We found a rat mutant with a 12-base deletion in the tyrosine kinase domain of the c-kit gene. The mutant allele was designated as Ws (white spotting); the homozygous Ws/Ws rats are deficient in both CTMC and MMC. We recently investigated the role of the c-kit receptor in the development of CTMC and MMC by infecting Ws/Ws and control +/+ + rats with Nippostrongylus brasiliensis (NB), which induces T-cell-dependent mast cell development. Although mast cells did not develop in the skin of Ws/Ws rats, a significant number of mast cells did develop in the jejunum after NB infection. Mast cells that appeared in the jejunum of NB-infected rats were of MMC phenotype. MMC contain chondroitin sulfate proteoglycan and relatively small quantities of histamine, whereas CTMC contain heparin proteoglycan and relatively large amount of histamine. The granules of CTMC and MMC store mast cell protease-I (RMCP-I) and RMCP-II, respectively. Mast cells that developed in the jejunum of NB-infected Ws/Ws rats had the MMC protease phenotype (RMCP-I+/II+), although the number of MMC and the concentration of RMCP-II in the serum of NB-infected Ws/Ws rats were only 13% and 7% of the values in NB-infected +/+ + rats, respectively. The requirement for stimulation via the c-kit receptor appeared to be greater for development of CTMC in the skin than for development of MMC in the jejunum.

Although W/W- mutant mice are deficient in both CTMC and MMC, mast cells of W/W- genotype may be obtained by culturing bone marrow cells of W/W- mice in the presence of T-cell-derived cytokines. When mouse cultured mast cells (MCMC) of +/+ or W/W- genotype
were cocultured with fibroblasts in the absence of T-cell-derived cytokines. \(+/+\) MCMC continued proliferation, but \(W^+/W^+\) MCMC stopped proliferation and died out.\(^{29,30}\) Moreover, \(+/+\) MCMC surviving in the coculture acquired CTMC-like phenotype.\(^{31,32}\) In the present study, we obtained rat cultured mast cells (RCMC) from the bone marrow of \(Ws/Ws\) and control \(+/+\) rats and characterized \(Ws/Ws\) RCMC from the viewpoints of fibroblast-dependent proliferation and phenotypic change.

**MATERIALS AND METHODS**

**Rats, mice, and cell lines.** The origin and breeding procedure of \(Ws/Ws\) rats with white spots and a coat color dilution was first found in the inbred colony of the BN/Mai (hereafter BN) strain that was maintained in Yagi Memorial Park (Kani-gun, Japan). Because homozygous \(Ws/Ws\) rats were not obtained in the genetic background of the BN strain, spotted BN-\(Ws/Ws\) rats were crossed with normal \((+/+)\) rats of the Donryu strain. The resulting \(F_1\) rats with white spots were mated together, and \(F_2(+/+)\) rats were obtained (hereafter called \(Ws/Ws\) and \(+/+\) rats). The genotypes were identified by their coat color. The origin and breeding procedure of WB-\((+/+)\) mice have been described,\(^{14}\) and hereafter they are called \(W/\) and \(+/+\) mice, respectively. The WCB6\(+/+)\)/3T3-1 fibroblast cell lines were established in our laboratory\(^{39}\) (hereafter called \(+/+\) and \(S/S\) fibroblasts). The cell lines were adapted to grow in a-minimal essential medium (a-MEM; How Laboratories, Irvine, UK) containing 10% fetal calf serum and growth factors. RCMC were suspended in a-MEM containing 2-mercaptoethanol, 30% air.

**Bone marrow cell culture.** Bone marrow cells were obtained by flushing humeri, femurs, and tibias of \(Ws/Ws\) or \(+/+\) rats of 2 months of age with \(\alpha\)-MEM containing 5% HS (\(\alpha\)-MEM-HS). Mononuclear cells were isolated from spleen cells of \(+/+\) rats with Lymphocyte Separating Medium (Organon Teknika-Cappel, Durham, NC) and cultured for 3 days in 75-cm\(^2\) tissue culture flasks containing 3 \(\mu\)g/mL ConA (Sigma Chemical, St. Louis, MO), 20% heat-inactivated horse serum (HS; Irvine Scientific, Santa Ana, CA), and 10\(^{-4}\) mmol/L 2-mercaptoethanol (Sigma). The conditioned medium was centrifuged, filtered through a 0.22-\(\mu\)m filter (Millipore, Bedford, MA), and stored at -80°C until use.

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**Cell proliferation assay.** To quantify the proliferation of RCMC, we used MTT \(3(4,5\text{-dimethylthiazol-2-yl})\)-2,5-diphenyltetrazolium bromide; Sigma) rapid colorimetric assay with minor modifications.\(^{34}\) In brief, triplicate aliquots of \(4.0 \times 10^6\) RCMC were suspended in 100 \(\mu\)L of ASF-102 medium containing various amounts of rSCF and were cultured in 96-well flat-bottom microtiter plates for 48 hours at 37°C in a humidified atmosphere of 5% CO\(_2\) in air. For the MTT assay, 10 \(\mu\)L of 5 mg/mL solution of MTT in PBS was added, and plates were incubated for 4 hours. Acid isopropanol (100 \(\mu\)L of 0.04 N HCl in isopropanol) was added, and plates were mixed thoroughly to dissolve the dark blue crystals. The optical density was then measured on a Microplate reader (Corona Electric, Ibaragi, Japan) with a test wave length of 550 nm and a reference wave length of 630 nm.

**Immune complex kinase assay.** The assay was performed according to Majumder et al\(^{17}\) with minor modifications.\(^{38}\) Exponentially growing RCMC were washed three times with \(\alpha\)-MEM and incubated in ASF-102 medium for 18 hours at 37°C to eliminate serum and growth factors. RCMC \((5 \times 10^6)\) were suspended in 1 mL of ASF-102 medium and then exposed to rSCF \((100 \text{ ng/mL})\) at 37°C for 15 minutes. After stimulation, RCMC were washed with cold PBS, immediately lysed in 100 \(\mu\)L of lysis buffer (20 mmol/L Tris-HCl, pH 8.0, 137 mmol/L NaCl, 10% glycerol, 1% Nonidet P-40 [Nacalai Tesque Inc, Kyoto, Japan]) containing 21 mmol/L phenylmethylsulfonyl fluoride (Sigma), 0.15 U/mL apro- tinin (Sigma), 10 mmol/L EDTA, 10 \(\mu\)g/mL leupeptin (Sigma), 100 mmol/L sodium fluoride, and 2 mmol/L sodium orthovanadate (Sigma) at 4°C for 15 minutes. The lysates were centrifuged at 15,000 rpm for 10 minutes at 4°C to remove cell debris. For immuno precipitation, the extract was first incubated with normal rabbit serum and protein-G Sepharose beads for 45 minutes at 4°C and then cleared in an Eppendorf centrifuge. The supernatant was incubated with rabbit polyclonal antibody to the whole murine c-kit protein, a generous gift of Dr P. Besmer (Cornell University Graduate School of Medical Sciences, New York, NY), and protein-G Sepharose beads for 45 minutes at 4°C. The protein-G Sepharose
bead-bound immune complexes were washed once with lysis buffer, twice with washing buffer (20 mmol/L Tris-HCl, pH 7.4, 500 mmol/L NaCl, 5 mmol/L EDTA, 1 mmol/L sodium orthovanadate), and then once with 20 mmol/L Tris-HCl, pH 7.4 solution. The pellet was suspended in 40 μL of kinase buffer (20 mmol/L Tris-HCl, pH 7.4, 10 mmol/L MnCl2) and 1 μL of [γ-32P]-adenosine triphosphate (ATP; DuPont/NEN Research Products; 10 mCi/ mL). The reaction mixture was incubated for 15 minutes at 30°C and the reaction was terminated by adding 20 μL of 3X sodium dodecyl sulfate (SDS) sample buffer. The mixture was boiled for 5 minutes, and then analyzed by 5% to 20% gradient SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiography. 

Coculture with fibroblasts. +/+ and Sl/SI fibroblasts (1 x 10^6 cells) were plated into each well of 24-well tissue culture plates; confluent monolayer developed 2 days later. The medium of the confluent culture was aspirated, and 2 x 10^5 RCMC suspended in 0.4 mL α-MEM containing 5% FCS was added. ConA-SCM (30%) was not added unless mentioned specifically. In one experiment, antimurine NGF MoAb (250 ng/mL) was added to the culture medium. The concentration of MoAb was determined according to the result of Hirata et al who used the same MoAb. The culture was kept at 37°C in a humidified atmosphere of 5% CO2 in air. The culture medium was aspirated and replaced with 0.4 mL fresh culture medium every 2 days. At various times after the initiation of the coculture, the number of RCMC was determined.

The proportion of cells that incorporated bromodeoxyuridine (BrdUrd) was used as an index of cells in the S phase of the cell cycle, as described previously. Cells were incubated with 3 μg/mL BrdUrd (Wako Pure Chemical Inc, Osaka, Japan) at 37°C for 15 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 using mouse anti-BrdUrd MoAb (Becton Dickinson, Mountain View, CA), biotin-conjugated horse antimouse IgG antibody (Vector Laboratories Inc, Burlingame, CA), and avidin-biotin-peroxidase complex (Vector). RCMC in the S phase were identified by the presence of alcian blue-positive granules in the cytoplasm and dark brown granules on the nucleus.

Attachment of RCMC. Attachment of RCMC to fibroblasts was investigated according to Adachi et al with a minor modification. To analyze the attachment to fibroblasts, 1.0 x 10^5 RCMC (or MCMC) were cocultured with fibroblasts as described above. At various times after the coculture, the culture plates were washed four times with warmed (37°C) α-MEM to remove nonattaching RCMC (or MCMC). Fibroblasts and attaching RCMC (or MCMC) were harvested after trypsin (0.1%) treatment (at 37°C for 15 minutes). Cytocentrifuged specimens were fixed with Carnoy's solution and stained with alcian blue and nuclear fast red. The proportion of remaining RCMC (or MCMC) to inoculated RCMC (or MCMC) was calculated.

RESULTS

Development of RCMC from the bone marrow of Ws/Ws rats. Cell suspensions were prepared from the bone marrow of Ws/Ws and control +/+ rats. Approximately 1% of +/+ marrow cells were alcin blue positive, and most of the alcin blue-positive cells were considered to be basophils because of the paucity of alcin blue-positive granules and the lobulated nucleus. Although heavily granulated mast cells were detectable in the bone marrow of +/+ rats, the number was much smaller than the number of basophils. Proportion of basophils to total nucleated cells in the Ws/Ws marrow was comparable to that of the +/+ marrow, but no mast cells were found in the bone marrow of Ws/Ws rats. When bone marrow cells of Ws/Ws and +/+ rats were cultured in the presence of ConA-SCM, alcin blue-positive cells increased and reached 40% of the total nucleated cells on day 10 after initiation of the culture (Fig 1). These alcin blue-positive cells were considered to be immature mast cells for the following two reasons. (1) The ultrastructural features were consistent with those of immature mast cells rather than those of basophils; the nucleus was round or oval; the surface processes were numerous, relatively uniformly distributed, elongated, and thin; the number of electron dense granules was much fewer than that observed in mature CTMC obtained from the peritoneal cavity of rats; and some vacuoles were observed in the cytoplasm (data not shown). (2) When cytocentrifugation preparations were stained with either anti-RMCP-I or anti-RMCP-II, the proportion of RMCP-I* cells was comparable to that of alcin blue-positive cells. In contrast, only few RMCP-I* cells were detectable (Table 1). These data indicated that alcin blue-positive cells were not basophils because basophils are RMCP-I*/II-. As shown in Fig 1, the number of total nucleated cells and the number of alcin blue-positive cells were comparable between +/+ and Ws/Ws rats. In both cases, the cultured bone marrow contained about 40% of RCMC on day 10 after initiation of the culture. Because the number of RCMC to total nucleated cells did not increase thereafter, we purified RCMC using the Sephadex G-10 beads column; the proportion of RCMC to total nucleated cells increased up to 60% because of the removal of macro-
**DIFFERENTIATION OF RAT MAST CELLS**

**Table 1. Development of RCMC in Suspension Culture of Bone Marrow Cells**

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Alcian Blue</th>
<th>RMCP-I</th>
<th>RMCP-II</th>
</tr>
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<tbody>
<tr>
<td>+/+</td>
<td>30 ± 3</td>
<td>&lt;1</td>
<td>29 ± 3</td>
</tr>
<tr>
<td>Ws/Ws</td>
<td>28 ± 3</td>
<td>&lt;1</td>
<td>27 ± 2</td>
</tr>
</tbody>
</table>

Bone marrow cells (2.0 × 10^6 cells/well) from +/+ or Ws/Ws rats were suspension cultured in the presence of ConA-SCM (30%). On day 10 of the culture, total cells were harvested and counted. Proportions of alcian blue-positive, RMCP-I+, and RMCP-II+ cells were determined in cytocentrifuge preparations. Values are the mean ± SE of four wells.

The impaired response of Ws/Ws RCMC to rrSCF suggested a defect in the activity of c-kit receptor tyrosine kinase. When the activity of c-kit kinase was estimated by in vitro immune complex kinase assay, the activity was apparently lower in Ws/Ws RCMC than in +/+ RCMC. However, c-kit kinase activity of a low level appeared to be present in Ws/Ws RCMC after stimulation by rrSCF (Fig 4).

**Fig 3.** Numbers of +/+ (solid symbols) and Ws/Ws (open symbols) RCMC at various days after the stimulation of rrSCF alone (50 ng/mL) (○, ■) or ConA-SCM alone (30%) (▲, □). Partially purified RCMC (approximately 60% of total nucleated cells) were used. Each point represents the mean of four wells; bars show SE.

**Fig 4.** In vitro kinase assay using [γ-^32P]-ATP. +/+ and Ws/Ws RCMC were incubated with or without rrSCF. The cell lysates of +/+ or Ws/Ws RCMC were immunoprecipitated with rabbit polyclonal antibody to the whole murine c-kit protein, incubated with [γ-^32P]-ATP, and then analyzed by SDS-PAGE and autoradiography. Appreciable autophosphorylation activity of the c-kit protein was observed in +/+ RCMC. Autophosphorylation activity of the c-kit protein was slightly detectable in Ws/Ws RCMC. The arrowhead shows the 145-kD c-kit protein.
WB-\(W/W\) mice (\(W/W\) MCMC) were cocultured with \(+/+\) fibroblasts. All cultures were performed without ConA-SCM. \(+/+\) and \(W_s/W_s\) RCMC attached to \(+/+\) fibroblasts as well as \(+/+\) MCMC, but attachment of \(W/W\) MCMC was apparently impaired (Fig 5A). There was no significant attachment of either \(+/+\) RCMC or \(+/+\) MCMC to \(S/S\) fibroblasts (Fig 5B).

In the next experiment, the duration of the coculture was extended to 48 hours and the proportion of RCMC in the S phase of the cell cycle was determined. The proportion was significantly higher for \(+/+\) RCMC than for \(W_s/W_s\) RCMC, but a few \(W_s/W_s\) RCMC in the S phase were detectable (Table 2). On the other hand, neither \(+/+\) RCMC nor \(W_s/W_s\) RCMC entered into the S phase when they were cocultured with \(S/S\) fibroblasts. Furthermore, the numbers of \(+/+\) and \(W_s/W_s\) RCMC were counted 8 days after the coculture with \(+/+\) or \(S/S\) fibroblasts. The number of \(+/+\) RCMC was maintained in the coculture with \(+/+\) fibroblasts, but the number of \(W_s/W_s\) RCMC decreased to one-tenth of the original population (Table 2). Neither \(+/+\) nor \(W_s/W_s\) RCMC were maintained in the coculture with \(S/S\) fibroblasts.

Phenotypes of RCMC in various culture conditions. The protease phenotypes of RCMC were evaluated by staining with anti–RMCP-I or anti–RMCP-II antibodies. Both \(+/+\) and \(W_s/W_s\) RCMC were RMCP-I/II* when cultured with ConA-SCM alone (Table 3 and Fig 6). However, when \(+/+\) RCMC were cultured in the medium containing rrSCF alone, most of \(+/+\) RCMC and half of \(W_s/W_s\) RCMC became RMCP-I/*II* (Fig 6). Because the number of \(W_s/W_s\) RCMC decreased to one-tenth in the medium containing rrSCF alone, the absolute number of \(W_s/W_s\) RCMC with the RMCP-I/*II* phenotype did not increase significantly. The phenotypic change of \(+/+\) and \(W_s/W_s\) RCMC induced by rrSCF was suppressed by the addition of ConA-SCM to rrSCF. The number of \(+/+\) RCMC increased 15-fold, but their phenotype remained RMCP-I/*II* in the presence of both ConA-SCM and rrSCF (Table 3). The number of \(W_s/W_s\) RCMC increased threefold when cultured with both ConA-SCM and rrSCF, and the phenotype remained RMCP-I/*II*. The phenotype of \(+/+\) RCMC changed from RMCP-I/*II* to RMCP-I/*II* when cocultured with \(+/+\) fibroblasts, with or without ConA-SCM. Most of \(W_s/W_s\) RCMC acquired the phenotype of RMCP-I/*II* when cocultured with \(+/+\) fibroblasts in the presence of ConA-SCM (Table 3). The number of \(W_s/W_s\) RCMC decreased to one-tenth when ConA-SCM was not added to the coculture with \(+/+\) fibroblasts, but one-fourth of the remaining \(W_s/W_s\) RCMC showed the RMCP-I/*II* phenotype (Table 3). Because neither \(+/+\) nor \(W_s/W_s\) RCMC were supported by \(S/S\) fibroblasts, they were cocultured with \(S/S\) fibroblasts in the presence of ConA-SCM. Both \(+/+\) and \(W_s/W_s\) RCMC were maintained, and approximately half of them acquired the phenotype of RMCP-I/*II* (Table 3).

Matsuda et al detected NGF in the coculture of MCMC with 3T3-Swiss albino fibroblasts and found that the addition of anti-NGF MoAb to this coculture suppressed the phenotypic change of MCMC to CTMC-like mast cells that contained heparin proteoglycan and a relatively large amount of histamine. We examined the effect of anti-NGF MoAb on the protease phenotype of RCMC. Although the

### Table 2. Defective Proliferation of \(W_s/W_s\) RCMC in the Coculture With \(+/+\) Fibroblasts

<table>
<thead>
<tr>
<th>Genotypes of Cells</th>
<th>Proportion of RCMC in S Phase (%)</th>
<th>No. of RCMC (x10³/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fibroblasts RCMC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(+/+)</td>
<td>(+/+)</td>
<td>10.2 ± 0.4</td>
</tr>
<tr>
<td>(+/+)</td>
<td>(W_s/W_s)</td>
<td>1.3 ± 0.3*</td>
</tr>
<tr>
<td>(S/S)</td>
<td>(+/+)</td>
<td>0</td>
</tr>
<tr>
<td>(S/S)</td>
<td>(W_s/W_s)</td>
<td>0</td>
</tr>
</tbody>
</table>

Values are the mean ± SE of four wells.

* Percentage of RCMC incorporating BrdUrd 48 hours after the initiation of the coculture with each fibroblast cell line.
○ Number of RCMC 8 days after the inoculation of 2.0 x 10⁴ RCMC to the culture of each fibroblast cell line.
† P < .01 when compared with the value of \(+/+\) RCMC.
addition of anti-NGF MoAb slightly reduced the number of RCMC cocultured with +/+ or Sl/Sl fibroblasts, the protease phenotype of +/+ and Ws/Ws RCMC was not influenced by the presence of anti-NGF MoAb (Table 4).

**DISCUSSION**

When bone marrow cells of +/+ rats were cultured with ConA-SCM, RCMC developed. This is consistent with the results reported by Broide et al. and Haig et al. However, the proportion of RCMC to total nucleated cells did not exceed 50% in the present study, although the proportion of more than 90% was reported by Broide et al. who used ConA-SCM as in the present study. When the culture medium containing ConA-SCM alone was changed to the medium containing rrSCF alone or rrSCF and ConA-SCM, the proportion of RCMC increased up to 80%. There is a possibility that the serum used by Broide et al. contained more SCF than the serum used in the present study. Another possibility is the difference in the rat strains used in each experiment.

The proportion of RCMC to total nucleated cells was comparable between Ws/Ws and +/+ marrow cells stimulated by ConA-SCM alone. When the cell suspensions containing RCMC were stimulated by rrSCF alone, the proliferative response of Ws/Ws RCMC was much lower than that of +/+ RCMC. This is partially consistent with the result reported by Tsai et al., who examined the effect of rrSCF on the proliferation of MCMC derived from WBB6F1-W/W (hereafter called W/W) mice. The magnitude of mast cell deficiency was comparable between W/W mice and Ws/Ws rats. Although W/W MCMC did not respond to 500 ng/mL of rrSCF at all, Ws/Ws RCMC responded slightly to 200 ng/mL of rrSCF. The number of Ws/Ws RCMC was not maintained by rrSCF alone, but the number increased threefold (compared with the culture containing ConA-SCM alone) when rrSCF was added to ConA-SCM. Moreover, a weak but detectable level of c-kit kinase activity was observed in the lysate of Ws/Ws RCMC.

Taken together, the Ws mutation appeared to abolish most but not all of the c-kit kinase activity.

The Ws mutation of mice also abolishes most but not all of the c-kit kinase. However, abnormalities in development of melanocytes, mast cells, erythrocytes, and germ cells are not comparable between C57BL/6-W/W (hereafter called W/W) mice and Ws/Ws rats. (1) W/W mice frequently have pigmented areas in the auricle, whereas no Ws/Ws rats have ever shown comparable pigmentation. (2) Adult W/W mice have a considerable number of mast cells in the skin (ie, 17% that of control +/+ mice), whereas adult Ws/Ws rats have very few mast cells in the skin (ie, 0.3% that of control +/+ rats). (3) The anemia of adult W/W mice is severe (the number of erythrocytes is 60% that of control +/+ mice), whereas the anemia of Ws/Ws rats anemia ameliorates with age (the number of erythrocytes of adult Ws/Ws rats was 80% that of control +/+ rats). (4) Most male and female W/W mice are sterile, and only a few W/W female mice may be pregnant only once in the young age. On the other hand, most female and male Ws/Ws rats are fertile. The signal through the c-kit receptor may serve different functions in the two species in regard to the development of the above-mentioned four cell types.

Ws/Ws RCMC attached to +/+ fibroblasts as efficiently as +/+ RCMC and +/+ MCMC. Because normal expression of the extracellular domain of the c-kit receptor is necessary for the attachment, Ws/Ws RCMC appeared to express the extracellular domain of the c-kit receptor. This is consistent with molecular nature of the c-kit protein encoded by the Ws mutant allele, ie, a small deletion at the tyrosine kinase domain. The number of Ws/Ws RCMC dropped to one-tenth of the original population when cocultured with +/+ fibroblasts without ConA-SCM. However, because even +/+ RCMC disappeared completely when cocultured with Sl/Sl fibroblasts that do not produce SCF at all, Ws/Ws RCMC appear to have limited support from +/+ fibroblasts. This is also consistent with the low but de-
Fig 6. Paired immunofluorescence study of RMCP-I and RMCP-II. (A and B) +/+/ RCMC cultured in the presence of ConA-SCM alone, showing the RMCP-I⁻/II⁺ phenotype. (C and D) +/+/ RCMC cultured in the presence of rrSCF alone, showing the RMCP-I⁺/II⁺ phenotype. The Olympus confocal laser scanning microscope was used. (A and C) Ar⁺ laser of 488 nm wave length was used. (B and D) Ar⁺ laser of 514.5 nm wave length was used.

tectable level of c-kit kinase activity observed in Ws/Ws RCMC. Most +/+/ RCMC maintained by ConA-SCM alone were RMCP-I⁻/II⁺, as reported by Macmenamin et al.52 When +/+/ RCMC were stimulated by rrSCF alone, most of +/+/ RCMC showed the phenotype of RMCP-I⁺/II⁺. Because the number of +/+/ RCMC increased 11-fold, the absolute number of +/+/ RCMC with the RMCP-I⁺/II⁺ phenotype

<table>
<thead>
<tr>
<th>Genotypes of RCMC</th>
<th>Culture Conditions</th>
<th>No. of RCMC (&gt;10⁴/well)</th>
<th>Anti-RMCP-I</th>
<th>Anti-RMCP-II</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+/</td>
<td>With +/+ fibroblasts + ConA-SCM</td>
<td>5.5 ± 0.2</td>
<td>80 ± 3</td>
<td>93 ± 2</td>
</tr>
<tr>
<td></td>
<td>With +/+ fibroblasts + ConA-SCM + anti-NGF</td>
<td>4.8 ± 0.3</td>
<td>80 ± 4</td>
<td>90 ± 4</td>
</tr>
<tr>
<td></td>
<td>With S/S fibroblasts + ConA-SCM</td>
<td>2.0 ± 0.1</td>
<td>38 ± 3</td>
<td>85 ± 3</td>
</tr>
<tr>
<td></td>
<td>With S/S fibroblasts + ConA-SCM + anti-NGF</td>
<td>1.8 ± 0.1*</td>
<td>34 ± 4</td>
<td>72 ± 4</td>
</tr>
<tr>
<td>Ws/Ws</td>
<td>With +/+ fibroblasts + ConA-SCM</td>
<td>2.5 ± 0.1</td>
<td>83 ± 2</td>
<td>88 ± 3</td>
</tr>
<tr>
<td></td>
<td>With +/+ fibroblasts + ConA-SCM + anti-NGF</td>
<td>1.7 ± 0.1*</td>
<td>76 ± 3</td>
<td>90 ± 1</td>
</tr>
<tr>
<td></td>
<td>With S/S fibroblasts + ConA-SCM</td>
<td>1.9 ± 0.1</td>
<td>45 ± 3</td>
<td>87 ± 2</td>
</tr>
<tr>
<td></td>
<td>With S/S fibroblasts + ConA-SCM + anti-NGF</td>
<td>1.7 ± 0.1*</td>
<td>43 ± 4</td>
<td>70 ± 4</td>
</tr>
</tbody>
</table>

RCMC (2.0 × 10⁴) were cultured in various conditions. The number of RCMC and the protease phenotype were determined 8 days later. The concentration of ConA-SCM was 30% and that of anti-NGF MoAb was 250 ng/mL. Values are the mean ± SE of four wells.

* P < .05 when compared with the culture without the addition of anti-NGF MoAb by t-test.
increased 882-fold. The RMCP phenotype of CTMC is RMCP-I+/II−.25-27,46 Therefore, rSCF did not induce the exact CTMC phenotype within 8 days of the culture, but did induce the CTMC-like RMCP-I+/II+ phenotype. This is consistent with the result of Tsai et al47 that +/+ MCMC acquired CTMC-like phenotype when cultured with rSCF. Gurish et al53 also reported a similar phenotypic change of MCMC using mouse mast cell protease (MMCP) as a marker. MCMC supported by IL-3 alone was MMCP-4+ but they became MMCP-4+ when stimulated with rSCF alone.53 However, the present results are at variance with those of Haig et al.46 who describe the development of mast cells with the RMCP-1+/II+ phenotype from CTMC but not from RCMC. The reasons for these discrepancies require further analysis that may include different sources of conditioned medium and, probably, rat strain differences.

Most Ws/WS RCMC maintained by ConA-SCM alone showed the RMCP-I+/II+ phenotype as in the case of +/+ RCMC. However, the number of Ws/WS RCMC decreased to one-tenth of the original population when they were cultured in the medium containing rSCF alone. About half of the remaining Ws/WS RCMC showed the RMCP-I−/II+ phenotype, but the absolute number of Ws/WS RCMC with the RMCP-I+/II+ phenotype did not increase significantly. Taken together, we cannot conclude whether rSCF may induce the CTMC-like phenotype in Ws/WS RCMC.

The presence of both ConA-SCM and rSCF increased +/+ RCMC 15-fold and Ws/WS RCMC threefold when compared with the culture stimulated by ConA-SCM alone. This is consistent with the result observed in Ws/WS rats infected with NB. Although MMC developed in the jejunum of NB-infected Ws/WS rats, the number was 13% that of NB-infected +/+ rats.17 The phenotype of RCMC stimulated by both ConA-SCM and rSCF remained RMCP-I+/II+. The inhibitory effect of T-cell factor(s) on the expression of CTMC-specific proteases has been described in MCMC. Gurish et al53 showed that the addition of IL-3 inhibited the expression of MMCP-4 induced by rSCF. There is a possibility that the MMC phenotype of mast cells at the NB infection site may be sustained by the relative abundance of T-cell factors.

The number of Ws/WS RCMC decreased to one-tenth of the original population when they were cultured on +/+ fibroblasts without ConA-SCM. However, when ConA-SCM was added to the coculture, Ws/WS RCMC survived and their phenotype changed from RMCP-I−/II+ to RMCP-I+/II+. There are at least two explanations. (1) The low c-kit kinase activity of Ws/WS RCMC may be sufficient for the phenotypic change in this experimental condition. (2) Fibroblasts produce activities other than SCF that may induce the expression of RMCP-I. Although Matsuda et al44 reported that fibroblasts produced NFG that induced the CTMC-like phenotype in MCMC, we could not suppress the expression of RMCP-I by the addition of anti-NFG MoAb. This suggested that the substance responsible for the phenotypic change was not NFG, at least in the present experiment. The reason of the discrepancy is not clear at the present time, and further investigations are necessary to clarify the discrepancy between MCMC used by Matsuda et al44 and RCMC used in the present experiment. The S/ mutant allele is a large deletion of the S/ locus,9,10 and therefore SCF was not produced by S/+ fibroblasts. However, about half of +/+ and Ws/WS RCMC may acquire the RMCP-I+/II+ phenotype on S/+ fibroblasts if their survival was supported by the addition of ConA-SCM. The addition of anti-NGF MoAb did not suppress the acquisition of the RMCP-I+/II+ phenotype. These results suggested that S/+ fibroblasts may produce some activities other than SCF and NFG that induce the CTMC-like phenotype. The present data on the phenotypes of Ws/WS RCMC is consistent with some results reported in tissues of W/W+ mice. Mast cells with CTMC-like phenotype developed in the skin of W/W+ mice in association with a chronic idiopathic dermatitis24 or in response to repeated epicutaneous application of phorbol 12-myristate 13-acetate.25 Moreover, perfusion with recombinant IL-3 resulted in development of CTMC-like mast cells in the skin of W/W+ mice.56 The normal signal transduction through the c-kit receptor does not appear to be prerequisite for the acquisition of the CTMC-like phenotype either in cultures or in tissues.

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Characterization of cultured mast cells derived from Ws/Ws mast cell-deficient rats with a small deletion at tyrosine kinase domain of c-kit

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