American Journal of Pathology, Vol. 151, No. 4, October 1997

Involvement of Transcription Factor Encoded by the Mouse *mi* Locus (MITF) in Apoptosis of Cultured Mast Cells Induced by Removal of Interleukin-3

Tohru Tsujimura,* Koji Hashimoto,* Eiichi Morii,* Ghulam Muhammad Tunio,* Kumiko Tsujino,* Taizo Kondo,* Yuzuru Kanakura,† and Yukihiko Kitamura*

From the Departments of Pathology and Hematology/Oncology,† Osaka University Medical School, Suita, Japan*

Mast cells develop when spleen cells of mice are cultured in the medium containing interleukin (IL)-3. Cultured mast cells (CMCs) show apoptosis when they are incubated in the medium without IL-3. We obtained CMCs from *tg/tg* mice that did not express the transcription factor encoded by the *mi* gene (MITF) due to the integration of a transgene at its 5′ flanking region. MITF is a member of the basic-helix-loop-helix-leucine zipper (bHLH-Zip) protein family of transcription factors. We investigated the effect of MITF on the apoptosis of CMCs after removal of IL-3. When cDNA encoding normal MITF (+-MITF) was introduced into *tg/tg* CMCs with the retroviral vector, the apoptosis of *tg/tg* CMCs was significantly accelerated. The mutant *mi* allele represents a deletion of an arginine at the basic domain of MITF. The apoptosis of *tg/tg* CMCs was not accelerated by the introduction of cDNA encoding *mi*-MITF. The overexpression of +-MITF was not prerequisite to the acceleration of the apoptosis, as the apoptotic process proceeded faster in +/+ CMCs than in *mi/mi* CMCs. The Ba/F3 lymphoid cell line is also dependent on IL-3, and Ba/F3 cells show apoptosis after removal of IL-3. The c-*myc* gene encodes another transcription factor of the bHLH-Zip family, and the overexpression of the c-*myc* gene accelerated the apoptosis of Ba/F3 cells. However, the overexpression of +-MITF did not accelerate the apoptosis of Ba/F3 cells. The +-MITF appeared to play some roles for the acceleration of the apoptosis specifically in the mast cell lineage. *(Am J Pathol 1997, 151:1043–1051)*

The *mi* locus of mice on chromosome 6 encodes a member of the basic-helix-loop-helix leucine zipper (bHLH-Zip) protein family of transcription factors that recognize a consensus sequence of CANNTG motif (hereafter called MITF).[1,2] The mutant *mi* allele encodes the transcriptional factor carrying a deletion of one of four consecutive arginine residues in the basic domain (hereafter *mi*-MITF).[1] Mutant mice of *mi/mi* genotype exhibit microphthalmus, depletion of pigment in both hair and eyes, osteopetrosis, and a decrease in the number of mast cells.[3–9] The VGA-9-*tg/tg* transgenic mouse possessing the transgene-insertional mutation at the 5′ flanking region of the *mi* gene was produced by Arnheiter and co-workers.[1,10] The expression of MITF transcripts was undetectable in various tissues of VGA-9-*tg/tg* mice except the embryonal retina. VGA-9-*tg/tg* mice exhibit microphthalmus, depletion of pigment in both hair and eyes, and the decrease of mast cells as noted in *mi/mi* mice, but they do not show osteopetrosis.[1,10] Recently, we demonstrated that the MITF directly regulated the expression of mouse mast cell protease 6 (MMCP-6) gene and c-*kit* gene, which encodes a receptor tyrosine kinase, by binding the CANNTG motifs in the promoter region of the MMCP-6 and c-*kit* genes.[11–16] The ligand of c-*kit* receptor, stem cell factor (SCF), is the most important growth factor of mast cells.[17] MITF also transactivates the tyrosinase gene, which encodes the key enzyme for production of melanin.[18,19]

The c-*myc* gene encodes another transcription factor of the bHLH-Zip family.[20] The product of the c-*myc* gene transactivates various genes, such as the ornithine decarboxylase, α-prothymosin, p53, and ECA39 genes.[21–24] Moreover, the overexpression of c-*myc* accelerated the apoptosis in some types of cells under specified conditions. In the presence of 10% serum, the Rat-1 fibroblasts overexpressing c-*myc* continued to proliferate. However, when compared with the untreated Rat-1 fibroblasts, the Rat-1 fibroblasts overexpressing c-*myc* showed the accelerated apoptosis in the culture medium containing 0.05% serum.[25] The Ba/F3 lymphoid cell line is dependent on interleukin (IL)-3, and Ba/F3 cells succumbed to apoptosis in the absence of IL-3.[26]

Supported by grants from the Japanese Ministry of Education, Science, Sports, and Culture, the Senri Life Science Foundation, the Mochida Memorial Foundation for Medical and Pharmaceutical Research, and the Ryoichi Naito Foundation for Medical Research.

Accepted for publication July 2, 1997

T. Tsujimura's present address: First Department of Pathology, Hyogo College of Medicine, 1–1, Mukogawa-cho, Nishinomiya, Hyogo 663, Japan.

Address reprint requests to Dr. Yukihiko Kitamura, Department of Pathology, Osaka University Medical School, Yamada-oka 2–2, Suita 565, Japan.

The overexpression of c-*myc* significantly accelerated the apoptosis of Ba/F3 cells. Cultured mast cells (CMCs) derived from either +/+ or *mi/mi* mice are dependent on IL-3 as are Ba/F3 cells.[9] In the present study, we examined whether the overexpression of normal MITF (+-MITF) also accelerated the apoptosis of CMCs in the absence of IL-3.

Materials and Methods

Mice

The original stock of C57BL/6-*mi*/+ (*mi*/+) mice was purchased from the Jackson Laboratory (Bar Harbor, ME), and *mi*/+ mice were maintained in our laboratory by repeated backcrosses to our own inbred C57BL/6 colony (more than 15 generations at the time of the present experiment). Female and male *mi*/+ mice were crossed together, and the resulting *mi/mi* mice were selected by their white coat color.[5,6] The original stock of VGA-9-*tg/tg* mice, which had the mouse vasopressin-*Escherichia coli* β-galactosidase transgene integrated at the 5′ flanking region of the *mi* gene, was kindly given by Dr. H. Arnheiter (National Institutes of Health, Bethesda, MD). The integrated transgene was maintained by repeated backcrosses to our own inbred C57BL/6 colony (more than five generations at the time of the present experiment). Female and male *tg*/+ mice were crossed together, and the resulting *tg/tg* mice were selected by their white coat color.[1,10]

Cells

Pokeweed-mitogen-stimulated spleen-cell-conditioned medium (PWM-SCM) was prepared according to the method described by Nakahata et al.[27] Two-week-old *mi/mi*, *tg/tg*, and normal C57BL/6-+/+ (+/+) mice were used to obtain CMCs. Mice were killed by decapitation after ether anesthesia and spleens were removed. Spleen cells were suspended as described previously and cultured in α-minimal essential medium (α-MEM; ICN Biomedicals, Costa Mesa, CA) supplemented with 10% PWM-SCM and 10% fetal calf serum (FCS; Nippon Bio-Supp Center, Tokyo, Japan). One-half of the medium was replaced every 7 days, and more than 95% of cells were CMCs 4 weeks after the initiation of the culture.[28] In one experiment, bone marrow cells of +/+ mice were used instead of the spleen cells to obtain CMCs. Four weeks after the initiation of the culture, appearance of the bone-marrow-derived CMCs was similar to that of the spleen-derived CMCs. The helper virus-free packaging cell line (ψ2)[29] was maintained in Dulbecco's modified Eagle's medium (DMEM; ICN Biomedicals) supplemented with 10% FCS. The Ba/F3 murine IL-3-dependent lymphoid cell line was maintained in α-MEM supplemented with 10% FCS and recombinant murine (rm)IL-3 at the concentration of 10 ng/ml.[30]

Characteristics of CMCs

Characteristics of spleen-derived CMCs were compared with those of bone-marrow-derived CMCs. The concentration of histamine was determined according to the method described by Yamatodani et al.[31] CMCs were stained with berberine sulfate to evaluate the contents of heparin proteoglycan.[32] The expression of c-*kit* was measured by flow cytometry as described previously.[14] Expression of MMCP-2, -4, and -6 and mast cell carboxypeptidase A (MC-CPA) was evaluated by Northern blotting.[14,33]

CMC Number in Culture

CMCs were adapted to grow in α-MEM supplemented with rmIL-3 (10 ng/ml) and 10% FCS 2 weeks before starting the experiment. CMCs (5.0×10^5) were then cultured for 42 days in the medium containing rmIL-3 (10 ng/ml) alone or in the medium containing both rmIL-3 (10 ng/ml) and rmSCF (100 ng/ml). One-half of the culture medium was replaced twice a week. At various times after starting the experiment, the number of CMCs was determined with the hemocytometer.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNAs were prepared by the lithium chloride-urea method.[34] Total RNA (5 μg) obtained from spleen and thymus of +/+ mice and CMCs were reverse transcribed in 20 μl of the reaction mixture containing 20 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim Biochemica, Mannheim, Germany) and random hexamer. One microliter of each reaction product was amplified in 25 μl of PCR mixture containing 0.125 U of *Taq* DNA polymerase (Takara Shuzou, Kyoto, Japan) and each set of sense and antisense primers by 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. The following oligonucleotides were used for PCR: sense (5′-CTGATCTGGT GAATCGGATC-3′, 1051 to 1070) and antisense (5′-TCCTGAAGAAGAGAGGGA GC-3′, 1422 to 1441) primers for the *mi* gene,[1] sense (5′-ACACAG-GATCGAATGTG GAG-3′, 688 to 707) and antisense (5′-TAATGCAGGACTTCATGAGC-3′, 1358 to 1377) primers for the MC-CPA gene,[35] sense (5′-TAAAGACCTCTAT-GCCAACAC-3′, 950 to 970) and antisense (5′-CTCCT-GCTTGCTGATCCACAT-3′, 1143 to 1163) primers for the β-actin gene,[36] sense (5′-ATGACTGCCATGGAGGAGT-CACA G-3′, 123 to 146) and antisense (5′-AGGTGTG-GCGCTGACCCACAACTG-3′, 540 to 563) primers for the P53 gene,[37] sense (5′-CAAGGTAGTAATAGCATCTC-CGAG-3′, 113 to 136) and antisense (5′-GCACTTTCTTT-TCCGGTACTTTCG-3′, 608 to 631) primers for the Fas antigen (Fas) gene,[38] and sense (5′-ATGCAGCAGC-CCATGAAT TACCCA-3′, 125 to 148) and antisense (5′-CTCACGGAGTTCTGCCAGTTCCTT-3′, 443 to 466) primers for the Fas ligand (FasL) gene.[39] Ten microliters of the

PCR products was electrophoresed in 1% agarose gel containing 0.5 μg/ml ethidium bromide.

Semiquantitative RT-PCR

Total RNA (5, 0.5 and 0.05 μg) obtained from +/+ CMCs at various days after stimulation of rmIL-3 (10 ng/ml) alone, rmSCF (100 ng/ml) alone, or both rmIL-3 (10 ng/ml) and rmSCF (100 ng/ml) were reverse transcribed as mentioned above. One microliter of each reaction product was amplified in 25 μl of PCR mixture with sense and antisense primers for the *mi* gene[1] by 30 cycles.

Infection of Retrovirus Vector

The ψ2 cells that produced viruses containing either +-MITF or *mi*-MITF cDNA were prepared in our laboratory.[13,16] For gene transfer, spleen cells of *tg/tg* mice or BaF/3 cells were incubated on an irradiated (30 Gy) subconfluent monolayer of virus-producing ψ2 cells for 72 hours in each culture medium. Neomycin-resistant cells were obtained by continuing the culture in medium containing G418 (0.8 mg/ml; GIBCO BRL, Grand Island, NY) for 4 weeks. For the morphological examination, cells were collected and cytocentrifuged. The specimens were fixed with Carnoy's solution for 30 minutes and then stained with Alcian blue and nuclear fast red.

Northern Blotting Analysis

The fragments of the MMCP-2,[14,33] MMCP-4,[33] MCCPA,[14,33] *mi*,[1] c-*myc*,[40] β-actin,[36] *bcl-2*,[41] and *bax*[42] genes were used as probes after being labeled with α-[^{32}P]deoxycytidine triphosphate (dCTP; DuPont/NEN Research Products, Boston, MA; 10 mCi/ml) by random oligonucleotide priming. After hybridization at 42°C, blots were washed to a final stringency of 0.2X SSC (1X SSC is 150 mmol/L NaCl and 15 mmol/L trisodium citrate, pH 7.4) at 50°C and subjected to autoradiography.

Immunocytochemistry by Anti-MITF Antibody

A rabbit anti-MITF antibody was prepared in our laboratory.[43] Immunocytochemistry was carried out as described by Takebayashi et al[43] with minor modification. Briefly, cells were cytocentrifuged and fixed with 4% paraformaldehyde for 30 minutes at 4°C. After microwave treatment (H2500 microwave processor, Bio-Rad Laboratories, Hercules, CA) in 0.01 mol/L citrate buffer (pH 6.0) for 3 minutes, specimens were incubated with the rabbit anti-MITF antibody. The specimens were washed with phosphate-buffered saline (PBS) and then incubated with the biotin-conjugated goat anti-rabbit IgG antibody (DAKO, Glostrup, Denmark). Immunoreacted cells were visualized with streptavidin-biotin-peroxidase and 0.05% diaminobenzidine/0.02% H_2O_2 solution (DAKO) according to the manufacturer's instructions.

Assay of Viable Cells

CMCs were adapted to grow in α-MEM supplemented with rmIL-3 at the concentration of 10 ng/ml and 10% FCS 2 weeks before starting the experiment. After being washed with α-MEM, CMCs or Ba/F3 cells were seeded in 96-well culture plates (Iwaki Glass, Hunahashi, Japan) at a density of 1.0×10^5 cells in 100 μl of Cosmedium-001 (Cosmo Bio Co., Tokyo, Japan) and then maintained at 37°C in a humidified atmosphere of 95% air/5% CO_2 for an additional 3 days. Viability of CMCs and Ba/F3 cells was estimated by the exclusion of trypan blue.

Detection of DNA Fragmentation

Genomic DNAs were prepared by the conventional phenol extraction method. Purified DNAs were electrophoresed on 1% agarose gel containing 0.5 μg/ml ethidium bromide and viewed under ultraviolet rays. For *in situ* detection of DNA fragmentation, CMCs were subjected to cytocentrifugation on glass slides, dried, and fixed in Carnoy's fixative overnight. Specimens were incubated at 37°C in 2.5 μg/ml proteinase K for 15 minutes, washed with PBS, and dehydrated by passage through a series consisting of 50, 75, 95, and 100% ethanol. Then specimens were quenched in 2% hydrogen peroxidase to block nonspecific staining and then reacted with a mixture of terminal deoxynucleotidyl transferase (TdT), digoxigenin-dUTP, and dATP to label the 3′-OH ends of DNA. Digoxigenin incorporated into the tails of DNA molecules was visualized by an immunoperoxidase staining kit (ApopTag, Oncor, Gaithersburg, MD). The nuclei containing multiple DNA fragments were stained reddish brown.[44] Nuclei of intact cells were counterstained with methyl green.

Transfection to BaF/3 Cells

pEF-BOS expression vectors containing the whole coding region of +-MITF or *mi*-MITF were constructed in our laboratory. pHMT-mycS containing the rat c-*myc* gene[40] was kindly provided by Dr. M. Obinata (Tohoku University, Sendai, Japan). The *Bam*HI-*Eco*RI fragment of pHMT-mycS was introduced into the blunted *Xba*I site of pEF-BOS. These linearized plasmids (100 μg) and pSTneoB (1 μg) were added to a cell suspension (1.0×10^7) in 0.7 ml of PBS, mixed gently, and incubated on ice for 10 minutes. The gene transfer technique by electroporation was described previously.[16] Two days after the electroporation, 800 μg/ml G418 was added to the complete medium to select neomycin-resistant cells. After confirming the proliferation of neomycin-resistant cells, the cells were used for further study.

Statistical Analysis

The proportion of viable of cells was compared by Student's *t*-test between the experimental and control groups.

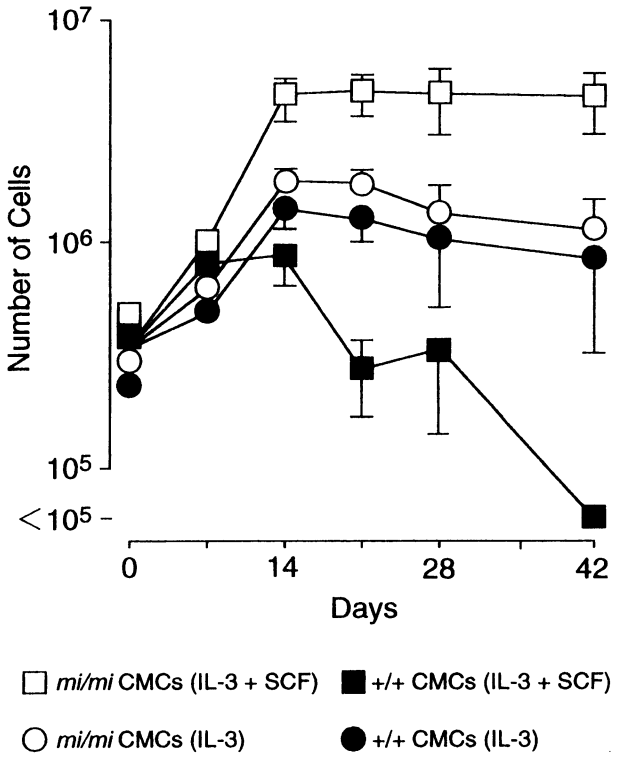

Figure 1. Numbers of +/+ and *mi/mi* CMCs in the medium containing rmIL-3 (10 ng/ml) or in the medium containing both rmIL-3 (10 ng/ml) and rmSCF (100 ng/ml). A total of 5.0×10^5 cells were cultured on day 0. Each point represents the mean of triplicate samples. Bars are the SE. In some points, the SE was too small to be shown by bars.

Results

Although the number of mast cells decreased in the skin of *mi/mi* mice, mast cells do develop when spleen cells of *mi/mi* mice are cultured in the medium containing IL-3.[9] We used spleen cells instead of bone marrow cells to obtain CMCs because bone marrow cells of *mi/mi* mice were difficult to harvest due to the presence of osteopetrosis. However, characteristics of CMCs derived from the spleen of +/+ mice were comparable to those of CMCs derived from the bone marrow of +/+ mice. Both of them contained a comparable amount of histamine and were not stained with berberine sulfate, suggesting no heparin content. Both of them expressed a comparable level of c-*kit* on the surface and contained comparable amounts of mRNAs of MMCP-2, -4, and -6 and MC-CPA (data not shown).

CMCs of *mi/mi* genotype survived neither on the fibroblasts that produced SCF nor in the medium containing SCF alone.[9,14,16] We obtained *mi/mi* and control +/+ CMCs from spleens of each genotype and examined the effect of SCF on *mi/mi* CMCs in the presence of IL-3. CMCs of *mi/mi* or +/+ genotype were cultured in the medium containing IL-3 alone or in the medium containing both IL-3 and SCF. In the medium containing IL-3 alone, the number of *mi/mi* CMCs and that of +/+ CMCs were comparable by 42 days after starting the experiment (Figure 1). On the other hand, a significant difference was observed between the number of *mi/mi* CMCs and that of +/+ CMCs on day 14 and thereafter in the medium containing both IL-3 and SCF. CMCs of +/+ genotype gradually decreased by day 42 (Figure 1). There is a possibility that this unexpected decrease may be due to an accelerated apoptosis of +/+ CMCs.

In the next experiment, the effect of SCF on the expression of MITF mRNA was compared with semiquantitative RT-PCR assay. RNAs were extracted from +/+ CMCs at 3, 7, and 14 days after stimulation of IL-3 alone, SCF alone, or both IL-3 and SCF. The method is not sensitive, and a 10-fold augmentation is necessary to substantiate the significant increase. Although the expression of MITF mRNA was confirmed in all +/+ CMCs stimulated with IL-3 alone, those stimulated with SCF alone, and those stimulated with both IL-3 and SCF, the magnitude of MITF expression was comparable among the three groups (data not shown).

Apoptosis of +/+ CMCs was reported when IL-3 was removed from the culture medium.[45–47] We compared survival of *mi/mi* and +/+ CMCs in this simple experimental condition. The +/+ and *mi/mi* CMCs were cultured in the medium containing rmIL-3 alone for 2 weeks and then thoroughly washed. The CMCs were incubated in the absence of IL-3, and the proportion of viable cells was measured. The *mi/mi* CMCs survived significantly longer than +/+ CMCs in the absence of IL-3 (Figure 2A). DNA fragmentation was not observed either in +/+ or *mi/mi* CMCs 24 hours after removal of IL-3 (data not shown) and was observed only in +/+ CMCs 48 hours after the removal (Figure 2B). When apoptosis was detected by an *in situ* DNA fragmentation kit, no difference was detected between +/+ and *mi/mi* CMCs 24 hours after the removal of IL-3; no nuclei were stained reddish brown by the DNA fragmentation kit in both +/+ and *mi/mi* CMCs (data not shown). However, the nuclei of +/+ CMCs but not the nuclei of *mi/mi* CMCs were stained reddish brown 48 hours after the removal (Figure 2C).

Mice of *tg/tg* were used to examine more directly the effect of +- or *mi*-MITF on the apoptosis of CMCs. The expression of MITF was not detectable in *tg/tg* CMCs due to the transgene-insertional mutation at the 5′ flanking region of the *mi* gene (Figure 3). We then introduced cDNA encoding +-MITF or *mi*-MITF into *tg/tg* CMCs. For this purpose, spleen cells of *tg/tg* mice were co-cultured for 72 hours with $\psi 2$ cells that produced viruses containing either +-MITF or *mi*-MITF cDNA. The neomycin-resistant cells were selected by culturing for 4 weeks in α-MEM containing G418 and PWM-SCM. More than 95% of the surviving cells contained Alcian blue$^+$ granules, indicating that almost all cells are CMCs, as in the case of *tg/tg* CMCs that were established in the absence of $\psi 2$ cells. Northern blot analysis and immunocytochemistry using the anti-MITF antibody showed the apparent expression of introduced +-MITF or *mi*-MITF cDNA in CMCs (Figure 4, A and B). Moreover, as reported previously,[13,43] +-MITF localized in the nucleus and *mi*-MITF in the cytoplasm (Figure 4B).

Untreated *tg/tg* CMCs, *tg/tg* CMCs overexpressing +-MITF, and *tg/tg* CMCs overexpressing *mi*-MITF were cultured in the presence of rmIL-3 for 2 weeks. The CMCs were then thoroughly washed and incubated in the ab-

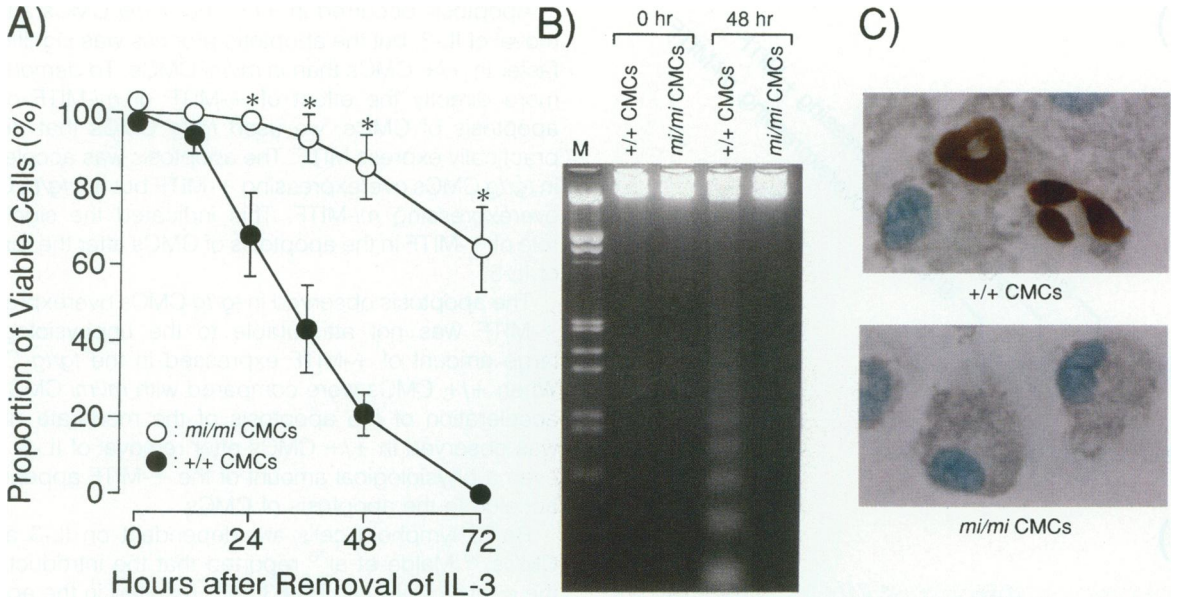

Figure 2. Retardation of apoptosis in *mi/mi* CMCs after removal of IL-3. **A:** Proportion of viable CMCs at various times after removal of IL-3. Each point represents the mean of three wells. Bars indicate the SE. In some cases, the SE was too small to be shown by bars. $^{*}P < 0.01$ by *t*-test when compared with the values of +/+ CMCs. **B:** DNA fragmentation in +/+ CMCs but not in *mi/mi* CMCs 48 hours after removal of IL-3. Genomic DNAs were extracted from +/+ CMCs and *mi/mi* CMCs before and 48 hours after removal of IL-3. The *Eco*RI- and *Hin*dIII-digested bacteriophage M DNA was used as a size marker (M). **C:** *In situ* demonstration of DNA fragmentation in +/+ CMCs 48 hours after removal of IL-3. Nuclei of intact cells were counterstained with methyl green. Original magnification, ×1350.

sence of IL-3. The proportion of viable cells in each cell population was measured by staining with trypan blue at various times after removal of IL-3. The untreated *tg/tg* CMCs started dying 36 hours after removal of IL-3, and approximately 50% of untreated *tg/tg* CMCs survived 72 hours after removal of IL-3 (Figure 5A). On the other hand, the *tg/tg* cells overexpressing +-MITF started dying 6 hours after removal of IL-3, and most cells died by 48 hours after removal of IL-3. The proportion of viable cells was comparable between untreated *tg/tg* CMCs and *tg/tg* CMCs overexpressing *mi*-MITF (Figure 5A). DNAs were extracted from various *tg/tg* CMCs before and 24 hours after removal of IL-3. The apparent DNA fragmentation was observed only in *tg/tg* CMCs overexpressing +-MITF 24 hours after removal of IL-3 (Figure 5B).

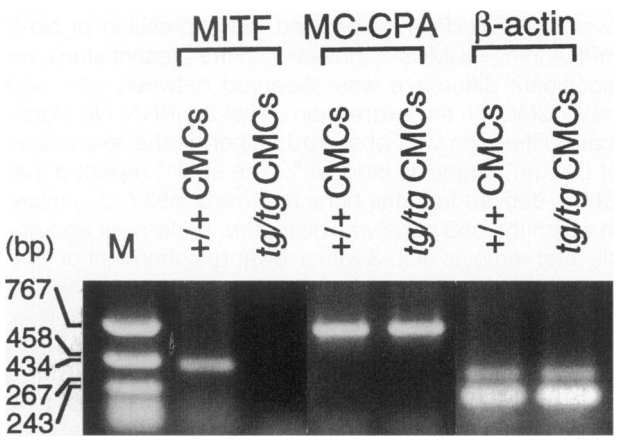

Figure 3. Expression of MITF mRNA in +/+ CMCs but not in *tg/tg* CMCs. Total RNA (5 μg) from +/+ and *tg/tg* CMCs was reverse transcribed and PCR amplified with MITF, MC-CPA, or β-actin primer for 30 cycles. DNA was stained with ethidium bromide on the gel. The sizes of the amplification bands of MITF, MC-CPA, and β-actin are 391, 690, and 214 bp, respectively.

We examined whether the acceleration of the apoptosis by +-MITF was specific to the mast cell lineage. The Ba/F3 cell line, as well as CMCs, is dependent on IL-3, and the overexpression of c-*myc* accelerates the apoptosis of Ba/F3 cells in the absence of IL-3.[26] The cDNA encoding +-MITF or *mi*-MITF was introduced into Ba/F3 cells. As a positive control, the c-*myc* gene was also introduced to Ba/F3 cells. The mRNA expression of +-MITF, *mi*-MITF, or c-*myc* was confirmed by Northern blotting (Figure 6A). Although overexpression of c-*myc* accelerated the apoptosis of Ba/F3 cells in the absence of IL-3, that of +-MITF or *mi*-MITF did not (Figure 6B).

Several proteins have been implicated to play a role in the process of apoptosis. Among them, *bcl-2* family proteins, p53, and FasL/Fas are known to play an important role.[48–51] We therefore examined the expression of *bcl-2*, *bax*, p53, Fas, and FasL mRNAs in +/+ and *mi/mi* CMCs. The expression of *bcl-2* and *bax* was comparable between +/+ and *mi/mi* CMCs (Figure 7A). The mRNA expression of p53 was undetectable in both +/+ and *mi/mi* CMCs even by using RT-PCR. The mRNA expression of Fas and FasL was barely detectable in both +/+ and *mi/mi* CMCs (Figure 7B).

Discussion

The number of +/+ CMCs decreased more rapidly than that of *mi/mi* CMCs in the medium containing both IL-3 and SCF. This was inconsistent with our previous experiment, in which +/+ or *mi/mi* CMCs were co-cultured with fibroblasts in the medium containing IL-3.[9,14] The number

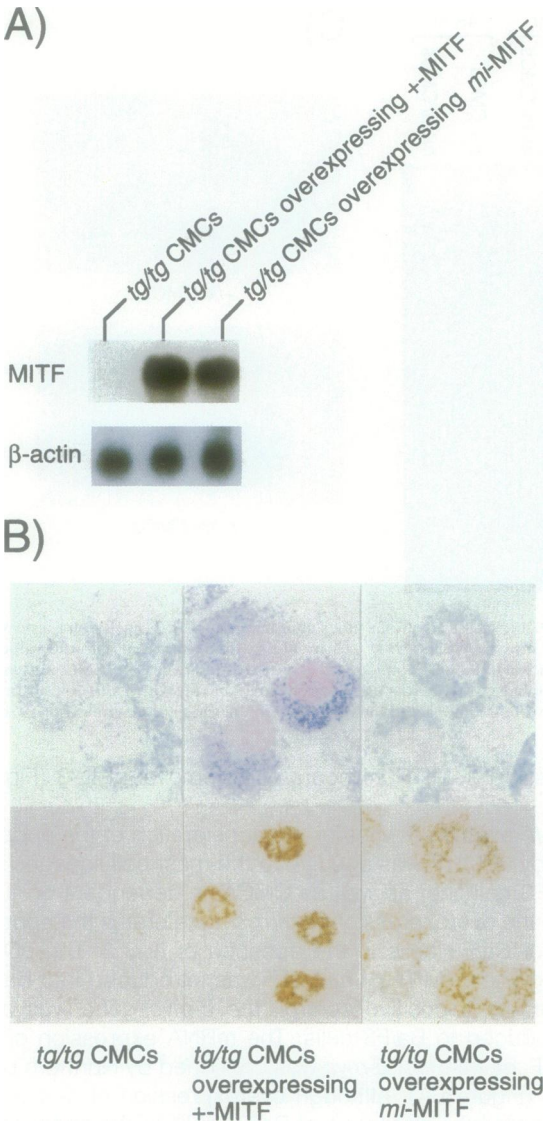

Figure 4. Expression of +-MITF or *mi*-MITF in *tg/tg* CMCs after introduction of respective cDNA. **A:** Northern blot. The blot was hybridized with ^{32}P-labeled cDNA probe of MITF or β-actin. The β-actin probe was used to verify that an equal amount of RNA was loaded in each lane. **B:** Localization of MITF protein in CMCs. The presence of alcian-blue-positive granules is shown in the **upper panel**, and the localization of MITF is demonstrated by immunohistochemistry using anti-MITF antibody in the **lower panels**. There was no detectable MITF protein in untreated *tg/tg* CMCs; localization of +-MITF protein was in the nuclei, and retention of *mi*-MITF was in the cytoplasm. Original magnification, ×1000.

of *mi/mi* CMCs decreased more rapidly than that of +/+ CMCs. A cause of this inconsistency may be deficient attachment of *mi/mi* CMCs with fibroblasts.[9,14] The *in vivo* result that the number of mast cells was greater in the skin of +/+ mice than in the skin of *mi/mi* mice is consistent with the result obtained by the co-culture experiment. Although the suspension culture containing IL-3 and SCF did not reflect the *in vivo* situation, the decrease of +/+ CMCs suggests their accelerated apoptosis in the suspension culture. The accelerated apoptosis of +/+ CMCs was also observed in the simple experimental system, ie, the apoptosis of CMCs induced by the removal of IL-3.[45–47]

Apoptosis occurred in +/+ and *mi/mi* CMCs after removal of IL-3, but the apoptotic process was significantly faster in +/+ CMCs than in *mi/mi* CMCs. To demonstrate more directly the effect of +-MITF or *mi*-MITF on the apoptosis of CMCs, we used *tg/tg* CMCs that did not practically express MITF. The apoptosis was accelerated in *tg/tg* CMCs overexpressing +-MITF but not *tg/tg* CMCs overexpressing *mi*-MITF. This indicated the significant role of +-MITF in the apoptosis of CMCs after the removal of IL-3.

The apoptosis observed in *tg/tg* CMCs overexpressing +-MITF was not attributable to the unphysiologically large amount of +-MITF expressed in the *tg/tg* CMCs. When +/+ CMCs were compared with *mi/mi* CMCs, the acceleration of the apoptosis of the moderate degree was observed in +/+ CMCs after removal of IL-3. Thus, even a physiological amount of the +-MITF appeared to accelerate the apoptosis of CMCs.

Ba/F3 lymphoid cells are dependent on IL-3 as are CMCs.[30] Malde et al[26] reported that the introduction of the c-*myc* cDNA into Ba/F3 cells resulted in the acceleration of apoptosis that occurred after removal of IL-3. In contrast, Ba/F3 cells overexpressing +-MITF did not show the accelerated apoptosis. The acceleration of the apoptosis induced by +-MITF appeared to be limited to the mast cell lineage.

The Ba/F3 cells overexpressing the c-*myc* show the accelerated apoptosis after removal of IL-3. As untreated Ba/F3 cells express the physiological amount of the c-*myc*,[26] the deregulated expression of the c-*myc* and/or the unphysiologically large amount of the c-*myc* protein appear to be important for the acceleration of the apoptosis. On the other hand, the physiological amount of +-MITF was enough to accelerate the apoptosis of CMCs after removal of IL-3. Therefore, there is a possibility that the mechanism of apoptosis induced by +-MITF may be distinct from that of the c-*myc*.

Several proteins have been implicated to be involved in the apoptosis of CMCs. Tsai et al[52] demonstrated that *bcl-2* protein, an apoptosis suppressor, is expressed at a significantly higher level in a growth-factor-independent C1.MC/C57.1 mouse mast cell line than in CMCs. Moreover, IL-3 significantly induced the expression of *bcl-2* mRNA in +/+ CMCs.[47] However, in the present study, no significant difference was observed between +/+ and *mi/mi* CMCs in the expression of *bcl-2* mRNA. No significant difference was observed, either, in the expression of *bax*, an apoptosis inducer.[42] Yee et al[47] reported that CMCs derived from the bone marrow of p53 (−/−) mice, in which the p53 gene was disrupted, underwent apoptosis after removal of IL-3 with a lower rate than that of +/+ CMCs. This indicates that the apoptosis of CMCs induced by deprivation of IL-3 is partly dependent on p53. However, in the present study, the expression of p53 was hardly detectable in both +/+ CMCs and *mi/mi* CMCs even by using RT-PCR. Therefore, the p53 might not play an important role for the acceleration of the apoptosis by +-MITF in +/+ CMCs. Fas is expressed on the surface of the p815 mouse mastocytoma cell line.[53] However, the expression of Fas and FasL was barely detectable in both +/+ and *mi/mi* CMCs. Therefore, +-MITF did not appear

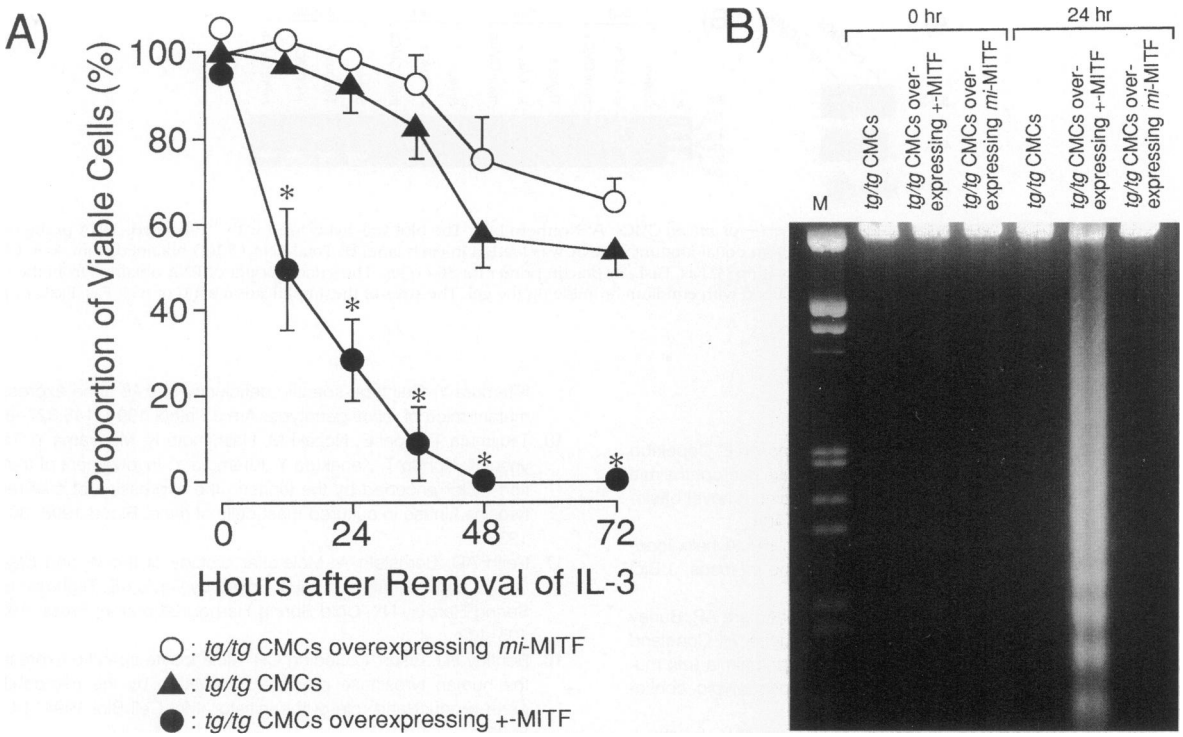

Figure 5. Accelerated apoptosis in *tg/tg* CMCs overexpressing +-MITF but not in *tg/tg* CMCs overexpressing *mi*-MITF. **A:** Proportion of viable cells at various times after removal of IL-3. Each point represents the mean of three wells. Bars indicate the SE. In some cases, the SE was too small to be shown by bars. $^{*}P < 0.01$ by *t*-test when compared with the values of untreated *tg/tg* CMCs. **B:** DNA fragmentation in *tg/tg* CMCs overexpressing +-MITF 24 hours after removal of IL-3. Genomic DNAs were extracted from various *tg/tg* CMCs before and 24 hours after removal of IL-3. The *Eco*RI- and *Hin*dIII-digested bacteriophage M DNA was used as a size marker (M).

to be involved in the expression of both Fas and FasL. Fas and FasL may not be related to the accelerated apoptosis observed in +/+ CMCs. Taken together, the present result showed the apparent lack of involvement of the major elements of other apoptotic pathways in acceleration of apoptosis observed in +/+ CMCs after the removal of IL-3. Target genes, through which +-MITF induces the acceleration of apoptosis, should be obtained to clarify the mechanism.

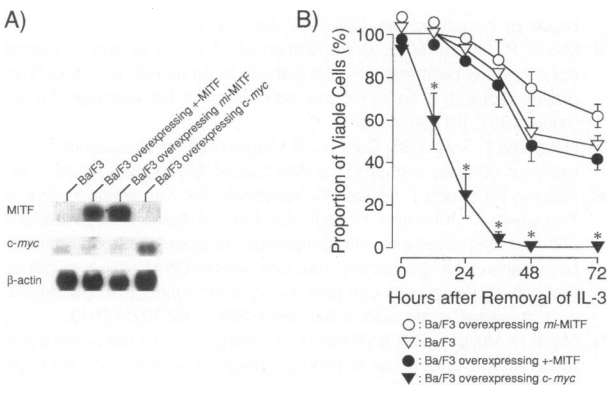

Figure 6. Accelerated apoptosis in Ba/F3 cells overexpressing the c-*myc* but not in Ba/F3 cells overexpressing +-MITF. **A:** Northern blot. The blot was hybridized with ^{32}P-labeled cDNA probe of MITF, c-*myc*, or β-actin. The β-actin probe was used to verify that an equal amount of RNA was loaded in each lane. **B:** Proportion of viable cells at various times after removal of IL-3. Each point represents the mean of three wells. Bars indicate the SE. In some cases, the SE was too small to be shown by bars. $^{*}P < 0.01$ by *t*-test when compared with the values of untreated Ba/F3 cells.

The acceleration of apoptosis by +-MITF may have a physiological significance. The cytoplasmic granules of mast cells contain biological mediators, such as vasoactive amines, proteases, proteoglycans, hydrolases, and tumor necrosis factor-α.[54] The extracellular release of these mediators from necrotic mast cells induces a substantial local inflammatory response. In contrast, cells undergoing apoptosis are rapidly recognized and removed by macrophages before the constituents of the apoptotic cells are released.[55] Therefore, mast cells might be expected to undergo apoptosis when the immunological or pathological responses cease, as suggested by Iemura et al.[46] The apoptosis represents a particularly suitable mechanism for reducing the number of mast cells. When the level of IL-3 decreases in association with the resolution of immunological or pathological reactions, +-MITF might contribute to the induction of the apoptosis in mast cells.

Acknowledgments

We thank Dr. S. Nagata of Osaka University for pEF-BOS, pMF1 carrying mouse Fas cDNA, and pBL-MFLW4 carrying mouse FasL cDNA, Dr. M. Obinata of Tohoku University for pHMT-mycS, Dr. Y. Tsujimoto of Osaka University for pmbcl-2β3.3 and pSKmBaxβpn, Dr. H. Arnheiter of NIH for VGA9-*tg/tg* mice, and Kirin Brewery Co. Ltd. for rmIL-3 and rmSCF.

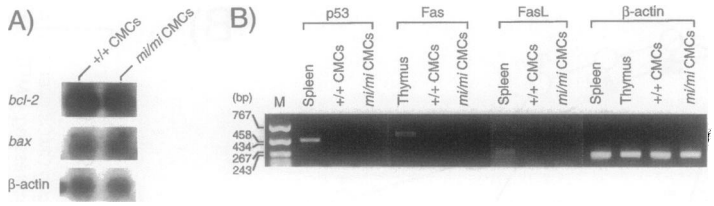

Figure 7. Expression of *bcl-2*, *bax*, p53, Fas, and FasL in +/+ or *mi/mi* CMCs. **A:** Northern blot. The blot was hybridized with ^{32}P-labeled cDNA probe of *bcl-2*, *bax*, or β-actin. The β-actin probe was used to verify that an equal amount of RNA was loaded in each lane. **B:** Total RNA (5 μg) obtained from +/+ CMCs or *mi/mi* CMCs was reverse transcribed and PCR amplified with p53, Fas, FasL, or β-actin primer for 30 cycles. The same amount of RNA obtained from the thymus or spleen of +/+ mice was used as controls. DNA was stained with ethidium bromide on the gel. The sizes of the amplification band of p53, Fas, FasL, or β-actin are 441, 519, 342, and 214 bp, respectively.

References

1. Hodgkinson CA, Moore KJ, Nakayama A, Steingrimsson E, Copeland NG, Jenkins NA, Arnheiter H: Mutations at the mouse microphthalmia locus are associated with defects in a gene encoding a novel basic-helix-loop-helix-zipper protein. Cell 1993, 74:395–404
2. Hughes JJ, Lingrel JB, Krakowsky JM, Anderson KP: A helix-loop-helix transcription factor-like gene is located at the *mi* locus. J Biol Chem 1993, 268:20687–20690
3. Steingrimsson E, Moore KJ, Lamoreux ML, Ferre-D'Amare AR, Burley SK, Zimring DCS, Skow LC, Hodgkinson CA, Arnheiter H, Copeland NG, Jenkins NA: Molecular basis of mouse microphthalmia (*mi*) mutations helps explain their developmental and phenotypic consequences. Nature Genet 1994, 8:256–263
4. Hemesath TJ, Steingrimsson E, McGill G, Hansen MJ, Vaught J, Hodgkinson CA, Arnheiter H, Copeland NG, Jenkins NA, Fisher DE: Microphthalmia, a critical factor in melanocyte development, defines a discrete transcription factor family. Genes Dev 1994, 8:2770–2780
5. Silvers WK: The Coat Colors of Mice: A Model for Mammalian Gene Action and Interaction. New York, Springer-Verlag, 1979
6. Green MC: Catalog of mutant genes and polymorphic loci. Genetic Variants and Strains of the Laboratory Mouse. Edited by Lyon MF, Searle AG. Stuttgart, Germany, Gustav Fischer Verlag, 1981, pp 158–161
7. Stevens J, Loutit JF: Mast cells in spotted mutant mice (*W*, *Ph*, *mi*). Proc R Soc Lond 1982, 215:405–409
8. Stechschulte DJR, Sharma KN, Dileepan KM, Simpson N, Aggarwal J, Clancy JR, Jilka RL: Effect of the *mi* allele on mast cells, basophils, natural killer cells, and osteoclasts in C57BL/6J mice. J Cell Physiol 1987, 132:565–570
9. Ebi Y, Kasugai T, Seino Y, Onoue H, Kanemoto T, Kitamura Y: Mechanism of mast cell deficiency in mutant mice of *mi/mi* genotype: an analysis by co-culture of mast cells and fibroblasts. Blood 1990, 75:1247–1251
10. Tachibana M, Hara Y, Vyas D, Hodgkinson C, Fex J, Grundfast K, Arnheiter H: Cochlear disorder associated with melanocyte anomaly in mice with a transgenic insertional mutation. Mol Cell Neurosci 1992, 3:433–445
11. Kasugai T, Oguri K, Jippo-Kanemoto T, Morimoto M, Yamatodani A, Yoshida K, Ebi Y, Isozaki K, Tei H, Tsujimura T, Nomura S, Okayama M, Kitamura Y: Deficient differentiation of mast cells in the skin of *mi/mi* mice: usefulness of *in situ* hybridization for evaluation of mast cell phenotype. Am J Pathol 1993, 143:1337–1347
12. Morii E, Takebayashi K, Motohashi H, Yamamoto M, Nomura S, Kitamura Y: Loss of DNA binding ability of the transcription factor encoded by the mutant *mi* locus. Biochem Biophys Res Commun 1994, 205:1299–1304
13. Morii E, Tsujimura T, Jippo T, Hashimoto K, Takebayashi K, Tsujino K, Nomura S, Yamamoto M, Kitamura Y: Regulation of mouse mast cell protease 6 gene expression by transcription factor encoded by the *mi* locus. Blood 1996, 88:2488–2494
14. Ebi Y, Kanakura Y, Jippo-Kanemoto T, Tsujimura T, Furitsu T, Ikeda H, Adachi S, Kasugai T, Nomura S, Kanayama Y, Yamatodani A, Nishikawa SI, Kitamura Y: 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*. Blood 1992, 80:1454–1462
15. Isozaki K, Tsujimura T, Nomura S, Morii E, Koshimizu U, Nishimune Y, Kitamura Y: Cell type-specific deficiency of c-*kit* gene expression in mutant mice of *mi/mi* genotype. Am J Pathol 1994, 145:827–836
16. Tsujimura T, Morii E, Nozaki M, Hashimoto K, Moriyama Y, Takebayashi K, Kondo T, Kanakura Y, Kitamura Y: Involvement of transcription factor encoded by the locus in the expression of c-*kit* receptor tyrosine kinase in cultured mast cells of mice. Blood 1996, 88:1225–1233
17. Reith AD, Bernstein A: Molecular biology of the *W* and *Steel* loci. Genes and Phenotypes, vol 3. Edited by Davis KE, Tilghman S. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1991, pp 105–133
18. Bentley NJ, Eisen T, Goding CR: Melanocyte-specific expression of the human tyrosinase promoter: activation by the microphthalmia gene product and role of the initiator. Mol Cell Biol 1994, 14:7996–8006
19. Yasumoto KI, Yokoyama K, Shibata K, Tomita Y, Shibahara S: Microphthalmia-associated transcription factor as a regulator for melanocyte-specific transcription of the human tyrosinase gene. Mol Cell Biol 1994, 14:8058–8070
20. Vätrik I, Mäkelä TP, Koskinen PJ, Klefstrom J, Alitalo K: Myc protein: partners and antagonists. Crit Rev Oncog 1994, 5:59–68
21. Bello-Fernandez C, Packham G, Cleveland JL: The ornithine decarboxylase gene is a transcriptional target of c-Myc. Proc Natl Acad Sci USA 1993, 90:7804–7808
22. Eilers M, Schirm S, Bishop JM: The Myc protein activates transcription of the α-prothymosin gene. EMBO J 1991, 10:133–141
23. Reisman D, Elkind NB, Roy B, Beamon J, Rotter V: c-Myc transactivates the p53 promoter through a required downstream CACGTG motif. Cell Growth Differ 1993, 4:57–65
24. Bevenisty N, Leder A, Kuo A, Leder P: An embryonically expressed gene is a target for c-Myc regulation via the c-Myc-binding site. Genes Dev 1992, 6:2513–2523
25. Evan GI, Wyllie AH, Gilbert CS, Littlewood TD, Land H, Brooks M, Waters CM, Penn LZ, Hancock DC: Induction of apoptosis in fibroblasts by c-myc protein. Cell 1992, 69:119–128
26. Malde P, Collins MKL: Disregulation of Myc expression in murine bone marrow cells results in an inability to proliferate in sub-optimal growth factor and an increased sensitivity to DNA damage. Int Immunol 1994, 6:1169–1176
27. Nakahata T, Spicer SS, Cantey JR, Ogawa M: Clonal assay of mouse mast cell colonies in methylcellulose culture. Blood 1982, 60:352–361
28. Nakano T, Sonoda T, Hayashi C, Yamatodani A, Kanayama Y, Asai H, Yonezawa T, Kitamura Y, Galli SJ: Fate of bone marrow-derived cultured mast cells after intracutaneous, intraperitoneal, and intravenous transfer into genetically mast cell deficient *W/W^v* mice: evidence that cultured mast cells can give rise to both "connective tissue-type" and "mucosal" mast cells. J Exp Med 1985, 162:1025–1043
29. Mann R, Mulligan RC, Baltimore D: Construction of a retrovirus packaging mutant and its use to produce helper-free defective retrovirus. Cell 1983, 33:153–159
30. Kitayama H, Kanakura Y, Furitsu T, Tsujimura T, Oritani K, Ikeda H, Sugahara H, Mitsui H, Kanayama Y, Kitamura Y, Matsuzawa Y: Constitutively activating mutations of c-*kit* receptor tyrosine kinase confer factor-independent growth and tumorigenicity of factor-dependent hematopoietic cell line. Blood 1995, 85:790–798
31. Yamatodani A, Maeyama K, Watanabe H, Wada H, Kitamura Y: Tissue distribution of histamine in a mutant mouse deficient in mast

cells: clear evidence for the presence of non-mast-cell histamine. Biochem Pharmacol 1982, 31:305–309
32. Enerbäck L: Berberine sulfate binding to mast cell polyanions: a cytofluorometric method for the quantitation of heparin. Histochemistry 1974, 42:301–313
33. Jippo T, Tsujino K, Kim HM, Kim DK, Lee YM, Nawa Y, Kitamura Y: Expression of mast-cell-specific proteases in tissues of mice studied by *in situ* hybridization. Am J Pathol 1997, 150:1373–1382
34. Auffray C, Rougenon F: Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. Eur J Biochem 1980, 107:303–314
35. Reynolds DS, Stevens RL, Gurley DS, Lane WS, Austen KF, Serafine WE: Isolation and molecular cloning of mast cell carboxypeptidase A: a novel member of the carboxypeptidase gene family. J Biol Chem 1989, 264:20094–20099
36. Tokunaga K, Taniguchi H, Yoda K, Shimizu M, Sakiyama S: Nucleotide sequence of a full-length cDNA for mouse cytoskeletal β-actin mRNA. Nucleic Acids Res 1986, 14:2829
37. Jenkins JR, Rudge K, Redmond S, Wade-Evans A: Cloning and expression analysis of full length mouse cDNA sequences encoding the transformation associated protein p53. Nucleic Acids Res 1984, 12:5609–5626
38. Watanabe-Fukunaga R, Brannan CI, Itoh N, Yonehara S, Copeland NG, Jenkins NA, Nagata S: The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. J Immunol 1992, 148:1274–1279
39. Takahashi T, Tanaka M, Brannan CI, Jenkins NA, Copeland NG, Suda T, Nagata S: Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. Cell 1994, 76:969–976
40. Kume TU, Takada S, Obinata M: Probability that the commitment of murine erythroleukemia cell differentiation is determined by the c-*myc* level. J Mol Biol 1988, 202:779–786
41. Negrini M, Silini E, Kozak C, Tsujimoto Y, Croce CM: Molecular analysis of *mbcl-2*: structure and expression of the murine gene homologous to the human gene involved in follicular lymphoma. Cell 1987, 49:455–463
42. Oltvai ZN, Milliman CL, Korsmeyer SJ: Bcl-2 heterodimerizes in vivo with a conserved homolog, Bax, that accelerates programmed cell death. Cell 1993, 74:609–619
43. Takebayashi K, Chida K, Tsukamoto I, Morii E, Munakata H, Arnheiter H, Kuroki T, Kitamura Y, Nomura S: The recessive phenotype displayed by a dominant negative microphthalmia-associated transcription factor mutant is a result of impaired nuclear localization potential. Mol Cell Biol 1996, 16:1203–1211
44. Gavrieli Y, Sherman Y, Ben-Sasson SA: Identification of programmed cell death *in situ* via specific labeling of nuclear DNA fragmentation. J Cell Biol 1992, 119:493–501
45. Mekori Y, Oh CK, Metcalfe DD: IL-3-dependent murine mast cells undergo apoptosis on removal of IL-3. J Immunol 1993, 151:3775–3784
46. Iemura A, Tsai M, Ando A, Wershil BK, Galli SJ: The c-*kit* ligand, stem cell factor, promotes mast cell survival by suppressing apoptosis. Am J Pathol 1994, 144:321–328
47. Yee NS, Paek I, Besmer P: Role of *kit*-ligand in proliferation and suppression of apoptosis in mast cells: basis for radiosensitivity of *white spotting* and *steel* mutant mice. J Exp Med 1994, 179:1777–1787
48. Stewart BW: Mechanisms of apoptosis: integration of genetic, biochemical and cellular indicators. J Natl Cancer Inst 1994, 86:1286–1296
49. Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML, Wyllie AH: Thymocyte apoptosis induced by p53-dependent and independent pathways. Nature 1993, 362:849–852
50. Lowe SW, Schmitt EM, Smith SW, Osborne BA, Jacks T: p53 is required for radiation-induced apoptosis in mouse thymocytes. Nature 1993, 362:847–849
51. Itoh N, Yonehara S, Ishii A, Yonehara M, Mizushima S-I, Sameshima M, Hase A, Seto Y, Nagata S: The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis. Cell 1991, 66:233–243
52. Tsai M, Tam S-Y, Veis DJ, Korsmeyer SJ, Galli SJ: Bcl-2 and mast cell survival. Bcl-2 is overexpressed in a growth factor-independent mouse mast cell line but mast cells develop in Bcl-2 null mice. FASEB J 1994, 8:A742
53. Hughes DPM, Crispe IN: A naturally occurring soluble isoform of murine Fas generated by alternative splicing. J Exp Med 1995, 182: 1395–1401
54. Galli SJ: New concepts about the mast cell. N Engl J Med 1993, 328:257–265
55. Savill J, Fadok V, Henson P, Haslett C: Phagocyte recognition of cells undergoing apoptosis. Immunol Today 1993, 14:131–136