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Contribution of the SgIGSF adhesion molecule to survival of cultured mast cells in vivo

Akihiko Ito,[a,*,1] Yu-ichiro Koma,[a,1] Kenji Watabe,[a] Tomoko Jippo,[b] Tomohiko Wakayama,[c] Shoichi Iseki,[c] and Yukihiko Kitamura[a]

[a] *Department of Pathology, Osaka University Medical School/Graduate School of Frontier Bioscience, Osaka 565-0871, Japan*
[b] *Department of Physiology, Senri Kinran University Faculty of Human Life Sciences, Osaka 565-0873, Japan*
[c] *Department of Histology and Embryology, Graduate School of Medical Science, Kanazawa University, Ishikawa 920-8640, Japan*

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Abstract

Spermatogenic immunoglobulin superfamily (SgIGSF) is a recently identified adhesion molecule, and the microphthalmia transcription factor (MITF) was essential for its expression in mast cells. Since the *tg* mutant allele is practically a null mutation of the MITF gene, cultured mast cells (CMCs) derived from (WB × C57BL/6)F_1 (F_1)-*tg*/*tg* mice did not express SgIGSF whereas CMCs from F_1-wild-type (+/+) mice expressed it abundantly. When cocultured with NIH/3T3 fibroblasts, F_1-*tg*/*tg* CMCs showed poor adhesion to NIH/3T3 fibroblasts. When injected intraperitoneally, F_1-*tg*/*tg* CMCs showed poor survival in the peritoneal cavity of mast cell-deficient F_1-W/W^v mice. SgIGSF was expressed in *tg*/*tg* CMCs ectopically through retroviral transfection and through expression of a transgene. The resulting *tg*/*tg* CMCs showed not only a better adhesion to NIH/3T3 fibroblasts but also a better survival in the peritoneal cavity than control F_1-*tg*/*tg* CMCs. SgIGSF-mediated adhesion seemed to play a role in the survival of CMCs in the peritoneal cavity.

Keywords: Intraperitoneal injection; Mesentery; MITF; Peritoneal cavity; Transgenic mice

We identified spermatogenic immunoglobulin superfamily (SgIGSF) as a new mast-cell adhesion molecule [1]. This molecule has an extracellular domain with significant homology to neural cell adhesion molecule-1 (NCAM-1) and NCAM-2 [2], and has a motif sequence in its intracellular domain that putatively connects to the actin cytoskeleton [3]. We further found that the microphthalmia transcription factor (MITF) was essential for expression of SgIGSF in mast cells [1].

MITF is a member of the basic-helix–loop–helix-leucine zipper family and encoded by the mouse *mi* locus [4,5]. More than 10 mutant alleles have been described in the *mi* locus [6]. The *tg* mutant allele is a transgene insertion mutation in the 5′ flanking region of the MITF gene [4,7]. C57BL/6 (B6)-*tg*/*tg* and (WB × B6)F_1 (F_1)-*tg*/*tg* mice have a white coat and lack mast cells in tissues other than the dermis, such as the peritoneal cavity, mesentery, and stomach [8]. Although the coding region of the MITF gene is normal in B6-*tg*/*tg* mice, significant amounts of MITF were not detectable in cultured mast cells (CMCs) derived from the spleen of B6-*tg*/*tg* mice [9]. As a result, B6-*tg*/*tg* CMCs did not practically express SgIGSF, whereas CMCs derived from B6 wild-type (+/+) mice expressed it abundantly [1]. In the coculture with NIH/3T3 fibroblasts, an appreciable number of B6-+/+ CMCs adhered to the fibroblasts, whereas the fewer B6-*tg*/*tg* CMCs adhered to the fibroblasts [1]. However, when B6-*tg*/*tg* CMCs were transfected with SgIGSF cDNA, the number of CMCs that adhered to NIH/3T3 fibroblasts increased to the normal level [1]. The number of CMCs adhering to fibroblasts paralleled with the expression level of SgIGSF.

* Corresponding author. Fax: +81-78-382-5479.
E-mail address: akito@med.kobe-u.ac.jp (A. Ito).
[1] Present address: Division of Surgical Pathology, Kobe University Graduate School of Medicine, Hyogo 650-0017, Japan.

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Decreased expression of SgIGSF appeared to cause the poor adhesion of B6-*tg*/*tg* CMCs to NIH/3T3 fibroblasts.

In addition to the adhesion to fibroblasts, B6-*tg*/*tg* CMCs showed a poor survival in the peritoneal cavity of B6-*tg*/*tg* mice. B6-+/+ CMCs survived in the peritoneal cavity of B6-*tg*/*tg* mice, whereas B6-*tg*/*tg* CMCs did not [8]. We previously examined the survival of F_1-+/+ CMCs in the coculture with NIH/3T3 fibroblasts. F_1-+/+ CMCs survived on a monolayer of NIH/3T3 fibroblasts for more than a month without any T cell-derived growth factors, such as IL-3 and IL-4 [10,11]. However, F_1-+/+ CMCs did not survive in this coculture when they were separated from NIH/3T3 fibroblasts with a mesh filter [11]. CMCs appeared to require direct interaction with NIH/3T3 fibroblasts to survive in the coculture. We supposed that the poor adhesion of B6-*tg*/*tg* CMCs might be a cause of their poor survival in the peritoneal cavity.

In the present study, we examined whether ectopic expression of SgIGSF might improve the poor survival of *tg*/*tg* CMCs in the peritoneal cavity. As recipients, we also used F_1-W/W^v mice, a standard of mast cell-deficient mice [12]. We successfully obtained *tg*/*tg* CMCs that expressed SgIGSF ectopically by two methods. First, F_1-*tg*/*tg* CMCs were transfected with a retrovirus vector expressing SgIGSF. Second, two lines of mice carrying a SgIGSF transgene were generated and were backcrossed with B6-*tg*/*tg* mice. The resulting *tg*/*tg* CMCs that expressed the SgIGSF transgene did survive in the peritoneal cavity more efficiently than original F_1-*tg*/*tg* CMCs.

Materials and methods

Mice. The original stock of VGA-9-*tg*/*tg* mice, in which the mouse vasopressin-*Escherichia coli* β-galactosidase transgene was integrated at the 5′ flanking region of the MITF gene [4], was kindly given by Dr. H. Arnheiter (National Institute of Health, Bethesda, MD). The mutant mice were maintained by repeated backcrosses to our own inbred B6 and WB colonies more than 12 generations. B6-*tg*/+ mice were crossed to WB-*tg*/+ mice, and the resulting F_1-*tg*/*tg* mice were selected by their white coat color. F_1-W/W^v and F_1-+/+ mice were purchased from the Japan SLC (Hamamatsu, Japan).

Cells. Pokeweed mitogen-stimulated spleen cell-conditioned medium (PWM-SCM) was prepared according to the method described by Nakahata et al. [13]. Spleens were removed from 2- to 3-week-old mice, passed through a 23-gauge needle, 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-supply Center, Tokyo, Japan). Half of the medium was replaced every 7 days. Four weeks later, more than 95% of the cells were CMCs. Ψ2 helper virus-free packaging cells, NIH/3T3 mouse fibroblastic cells were maintained as described previously [1].

Transfection of CMCs with retroviral vector. We previously obtained blasticidin-resistant Ψ2 cell clones that produce high titers of retrovirus containing either the SgIGSF cDNA or no insert [1]. The procedure for transfection of CMCs with retrovirus vector was described previously [1]. Briefly, a freshly prepared spleen cell suspension was added onto the confluent monolayer of the Ψ2 cell clones that had been γ-irradiated at a single dose of 30 Gy on a day before. After spleen cells were incubated for 5 days in α-MEM containing 10% FCS and 10% PWM-SCM, blasticidin-resistant CMCs were selected by continuing the culture in the presence of blasticidin (1.5 μg/ml) for 4 weeks.

Antibodies. A rabbit polyclonal antibody against SgIGSF was made in Kanazawa University (by T.W. and S.I.). The method of preparation and the sensitivity of the antibody were described in detail previously [1,14]. Briefly, rabbits were immunized against the synthetic polypeptide containing 15 amino acids of the C-terminus of SgIGSF. Four months later, the rabbit sera were purified with an affinity column containing the synthetic polypeptide. The antibody specific for actin (AC-40) was purchased from Sigma (St. Louis, MO). Secondary antibodies used are peroxidase-labeled anti-rabbit or anti-mouse IgG antibodies (MBL, Nagoya, Japan) and fluorescein isothiocyanate (FITC)-labeled anti-rabbit IgG antibody (MBL).

Western blot analysis. CMCs and mouse tissues were lysed in a buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride. The resulting lysates were separated on 10% SDS–polyacrylamide gels, transferred to Immobilon (Millipore, Bedford, MA), and reacted with the anti-SgIGSF antibody. After washing, the blots were incubated with the peroxidase-labeled secondary antibody and then reacted with Western Lighting reagents (Perkin–Elmer Life Sciences, Boston, MA) before exposure. After stripping, the blots were probed with the anti-actin antibody.

Immunocytochemistry. CMCs were washed with phosphate-buffered saline (PBS, pH 7.4), attached to microscope slides by cytospin 2 centrifugation, and fixed with methanol. Fixed samples were blocked with 2% bovine serum albumin in PBS, incubated with the anti-SgIGSF antibody, and stained with FITC-labeled anti-rabbit IgG antibody. Cells were visualized using a confocal laser scanning microscope (LSM510; Carl Zeiss, OberKochen, Germany).

Generation of transgenic mice. The *Eco*RI–*Eco*RI cDNA fragment containing the full-length coding region of SgIGSF was described previously [1]. This fragment was inserted directionally into the pCAGGS vector via the *Eco*RI site. The resulting plasmid construct was digested with *Sal*I and *Hin*dIII to obtain the DNA fragment containing the cytomegalovirus enhancer, chicken β-actin promoter and intron, SgIGSF coding sequence, and rabbit β-globin poly-adenylation signal in this order. The excised DNA fragment was injected into fertilized eggs obtained from the mating of (B6 × DAB/2)F_1 male and female mice. In total, 311 DNA-injected eggs were transplanted to pseudo-pregnant mice, resulting in 77 newborns. Genomic DNA was extracted from tail tips of the mice and used as a template in polymerase chain reaction with primers specific for the SgIGSF cDNA (5′-CACTGAAGAAGCATC CTTGC-3′ and 5′-TCTGCTGGTAGTGGTCGGCG-3′). Eight out of 77 mice were found to carry the SgIGSF transgene. Expression levels of SgIGSF proteins were examined in protein lysates of tail tips of the 8 founder mice by Western blot analyses. Four mice that expressed higher levels of SgIGSF were mated with B6 tg/+ mice. Only two mice gave offspring and were named either A- or B-line transgenic mice.

Coculture of CMCs with fibroblasts and evaluation of attachment. Coculture of CMCs with NIH/3T3 cells was performed as described previously [10,11]. Briefly, CMCs (1×10^5 cells/dish) were suspended in 2 ml α-MEM containing 10% FCS and added to a confluent culture of NIH/3T3 cells in 35-mm culture dishes. After 3 h of coculture, the dishes were washed with warmed (37 °C) α-MEM to remove non-adherent CMCs. NIH/3T3 cells and adherent CMCs were harvested by trypsin treatment, attached to microscope slides using cytospin 2 centrifugation, fixed with Carnoy's solution, and stained with alcian blue and nuclear fast red. The proportion of alcian blue-positive mast cells to alcian blue-negative NIH/3T3 cells was determined. Each experiment was done in triplicate and repeated three times with similar results.

Intraperitoneal injection of CMCs and estimation of mast cell numbers in the peritoneal cavity and mesentery. CMCs (1.0×10^6) were suspended in 0.5 ml α-MEM and injected into the peritoneal cavity of the recipient mice. After 5 weeks, 2 ml of Tyrode's buffer [15] containing 0.1% gelatin (Sigma) was injected into the peritoneal cavity of mice, and the abdomen was massaged gently for 30 s to harvest

peritoneal cells. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells was aspirated with a Pasteur pipette. After centrifugation, the pellet was resuspended with Tyrode's buffer (1.0 ml), and 0.2 ml of the suspension was centrifuged at 600 rpm for 5 min with a cytospin 2 centrifuge (Shandon, Pittsburgh, PA) to attach cells to a microscope slide. The cytospin preparations were fixed in Carnoy's solution and stained with alcian blue and nuclear fast red. Proportion of alcian blue-positive cells in 1000 nucleated peritoneal cells was determined.

Immediately after collecting peritoneal cells, the mesentery with the intestine was dissected from the mice and spread on a microscope slide. Air-dry for 2 h allowed the mesentery to attach to the slide. After the intestine was removed, the slide with the mesentery was fixed in Carnoy's solution and stained with alcian blue and nuclear fast red. The number of alcian blue-positive cells in 1.0-cm^2 area of the stretched mesentery was counted as described previously [16]. All experiments included six mice per group and were reproduced twice.

The mast cell numbers in the peritoneal cavity and mesentery of 10-week-old transgenic *tg/tg* mice were determined with the same procedure as described above.

Results and discussion

We previously succeeded in expressing SgIGSF ectopically in B6-*tg/tg* CMCs by the transfection using a retrovirus vector [1]. In the present study, we transfected F_1-*tg/tg* CMCs with the same procedure, because we planned to use F_1-*tg/tg* and F_1-W/W^v mice as recipients that would receive intraperitoneal injection of the transfected CMCs. We examined SgIGSF expression in transfected and non-transfected CMCs by Western blot analyses using the anti-SgIGSF antibody. As we reported previously [1], the heavily glycosylated mature form of SgIGSF was detected in the lysate of F_1-+/+ CMCs at mobility size of 110 kDa, while F_1-*tg/tg* CMCs did not express it (Fig. 1). When F_1-*tg/tg* CMCs were transfected with SgIGSF cDNA, they overexpressed the mature form of SgIGSF (Fig. 1). In contrast, F_1-*tg/tg* CMCs transfected with MITF cDNA expressed a normal level of the form (Fig. 1). Transfection with an empty vector did not influence SgIGSF expression in F_1-*tg/tg* CMCs (Fig. 1).

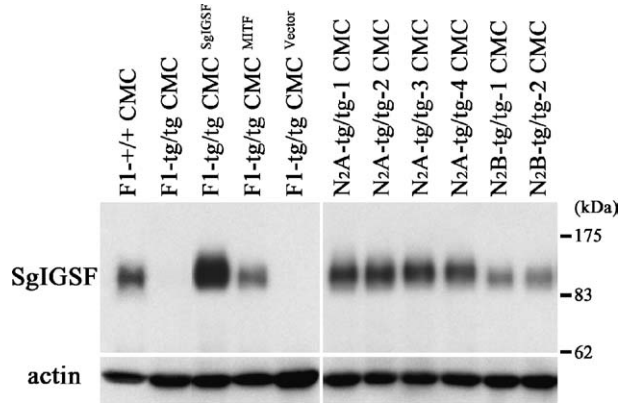

Fig. 1. Expression of SgIGSF proteins in various types of CMCs. Protein lysates were prepared from non-transfected CMCs (F_1-+/+ and F_1-*tg/tg* CMCs), transfected CMCs [F_1-*tg/tg* CMCs transfected with SgIGSF cDNA (F_1-*tg/tg* CMC^{SgIGSF}), MITF cDNA (F_1-*tg/tg* CMC^{MITF}), or an empty vector (F_1-*tg/tg* CMC^{Vector})], and transgenic *tg/tg* CMCs (N_2A-*tg/tg*-1 to 4, and N_2B-*tg/tg*-1 and 2 CMCs). The lysates were electrophoresed and blotted with the SgIGSF antibody and then probed again with the anti-actin antibody to indicate the total amount of proteins loaded per lane. The molecular weight scale is shown to the right of the blot.

We cocultured the transfected CMCs with NIH/3T3 fibroblasts and counted the number of CMCs that adhered to NIH/3T3 fibroblasts. F_1-*tg/tg* CMCs transfected with SgIGSF cDNA and those transfected with MITF cDNA showed a normal adhesion (Table 1), consistent with our previous results obtained by using B6-*tg/tg* CMCs [1]. Transfection with an empty vector did not influence the adhesion of F_1-*tg/tg* CMCs (Table 1).

We intraperitoneally injected these five types of CMCs into F_1-*tg/tg* and F_1-W/W^v mice, and counted the number of mast cells that survived in the peritoneal cavity 5 weeks after the injection. The number of mast cells was expressed as the proportion of alcian blue-positive cells per 1000 nucleated cells contained in the peritoneal lavage fluid (Table 2). In the peritoneal cavity of F_1-*tg/tg* and F_1-W/W^v mice which received the intraperitoneal injection of F_1-+/+ CMCs, approximately 40 and 60 mast cells were detected, respectively (Table 2). These numbers were comparable with the number of mast cells found in the peritoneal cavity of intact adult F_1-+/+ mice [8]. The number of F_1-*tg/tg* CMCs that survived in the peritoneal cavity of either F_1-*tg/tg* or F_1-W/W^v mice was 6% that of surviving F_1-+/+ CMCs (Table 2). In contrast, when F_1-*tg/tg* CMCs transfected with SgIGSF cDNA were injected, the number of surviving mast cells increased to 33% that of surviving F_1-+/+ CMCs (Table 2).

We also examined the survival of CMCs in the mesentery. Immediately after collecting peritoneal cells, we removed the mesentery from F_1-*tg/tg* and F_1-W/W^v

Table 1
Adhesion of *tg/tg* CMCs that express ectopic SgIGSF to NIH/3T3 fibroblasts

CMCs	No. of adhering CMCs per NIH/3T3 cell[a]
F_1-+/+	0.141 ± 0.004
F_1-*tg/tg*	$0.059 \pm 0.002^{*}$
F_1-*tg/tg* CMCs transfected with SgIGSF	$0.138 \pm 0.004^{**}$
F_1-*tg/tg* CMCs transfected with MITF	$0.135 \pm 0.003^{**}$
F_1-*tg/tg* CMCs transfected with vector	$0.049 \pm 0.002^{*}$
N_2A-*tg/tg*-1	$0.092 \pm 0.006^{*,**}$
N_2A-*tg/tg*-2	$0.103 \pm 0.004^{*,**}$
N_2B-*tg/tg*-1	$0.078 \pm 0.001^{*,**}$
N_2B-*tg/tg*-2	$0.071 \pm 0.002^{*}$

[a] Means ± SE of three dishes.

$^{*}P < 0.01$ by t test when compared with the values of F_1-+/+ CMCs.

$^{**}P < 0.01$ by t test when compared with the values of F_1-tg/tg CMCs.

Table 2
Number of mast cells in the peritoneal cavity and mesentery of F_1-*tg*/*tg* and F1-W/W^v mice which received intraperitoneal injection of various CMCs (10^6) 5 weeks before

Type of CMCs injected	No. of mast cells		
	Peritoneal cavity (no./10^3 peritoneal cells)[a]		Mesentery (no./cm^2 mesentery)[b]
	F_1-*tg*/*tg*	F_1- W/W^v	F_1- W/W^v
F_1-+/+ CMCs	42.1 ± 4.5	61.0 ± 9.6	875.3 ± 69.2
F_1-*tg*/*tg* CMCs	$2.6 \pm 0.6^*$	$3.5 \pm 0.5^*$	$9.8 \pm 3.3^*$
F_1-*tg*/*tg* CMCs transfected with SgIGSF	$14.2 \pm 1.2^{*,**}$	$19.9 \pm 3.7^{*,**}$	$86.6 \pm 28.4^{*,**}$
F_1-*tg*/*tg* CMCs transfected with MITF	$23.2 \pm 2.8^{*,**}$	$36.2 \pm 6.8^{*,**}$	$462.4 \pm 52.1^{*,**}$
F_1-*tg*/*tg* CMCs transfected with vector	$3.1 \pm 0.7^*$	$4.3 \pm 0.9^*$	$10.0 \pm 2.6^*$
N_2A-*tg*/*tg*-1 CMCs	NE	$15.4 \pm 1.9^{*,**}$	$122.4 \pm 40.2^{*,**}$
N_2A-*tg*/*tg*-2 CMCs	NE	$1.4 \pm 0.5^{*,**}$	$5.8 \pm 0.5^*$
N_2A-*tg*/*tg*-3 CMCs	NE	$17.4 \pm 3.1^{*,**}$	$210.5 \pm 68.2^{*,**}$
N_2A-*tg*/*tg*-4 CMCs	NE	$2.1 \pm 0.2^*$	$9.0 \pm 0.7^*$
N_2A-*tg*/*tg*-5 CMCs	NE	$14.7 \pm 1.6^{*,**}$	$82.0 \pm 23.7^{*,**}$
N_2A-*tg*/*tg*-6 CMCs	NE	$<0.1^*$	$<0.1^*$
N_2B-*tg*/*tg*-1 CMCs	NE	$12.1 \pm 1.3^{*,**}$	$110.2 \pm 16.8^{*,**}$
N_2B-*tg*/*tg*-2 CMCs	NE	$<0.1^*$	$<0.1^*$

NE, not examined.
[a] Cytospin samples of peritoneal lavage fluid were stained with alcian blue and nuclear fast red. Proportion of alcian blue-positive cells to 10^3 nucleated cells was calculated. Means and SE of six mice are shown.
[b] The number of mast cells in 1-cm^2 area of stretched mesentery. Means and SE of six mice are shown.
$^*P < 0.01$ by *t* test when compared with the values of F_1-+/+ CMCs.
$^{**}P < 0.01$ by *t* test when compared with the values of F_1-*tg*/*tg* CMCs.

mice that had been injected with CMCs, and counted the number of mast cells that survived there. As reported previously [8], no mast cells were detected in the mesentery of F_1-*tg*/*tg* mice even after the transplantation of F_1-+/+ CMCs (data not shown). In the mesentery of F_1-W/W^v mice injected with F_1-+/+ CMCs, approximately 900 mast cells were detected in 1-cm^2 area of the stretched mesentery (Table 2). This number was comparable with the number of mast cells found in the mesentery of intact adult F_1-+/+ mice [8]. The number of F_1-*tg*/*tg* CMCs that survived in the mesentery was only 1% that of surviving F_1-+/+ CMCs (Table 2). In contrast, when F_1-*tg*/*tg* CMCs transfected with SgIGSF cDNA were injected, the number of surviving mast cells increased to 10% that of surviving F_1-+/+ CMCs (Table 2). F_1-*tg*/*tg* CMCs transfected with MITF cDNA survived half as efficiently as F_1-+/+ CMCs in both the peritoneal cavity and the mesentery (Table 2). Transfection with an empty vector did not alter the survival efficiency of F_1-*tg*/*tg* CMCs in either site (Table 2).

The number of F_1-+/+ CMCs surviving in the peritoneal cavity of F_1-W/W^v mice was significantly larger than the number of F_1-+/+ CMCs surviving in the peritoneal cavity of F_1-*tg*/*tg* mice. This result may have relation to the fact that no mast cells developed in the mesentery of F_1-*tg*/*tg* mice even after intraperitoneal injection of F_1-+/+ CMCs [8]. F_1-*tg*/*tg* mice apparently have a microenvironmental defect in the mesentery. Such a defect could not be attributable to the lack of SgIGSF expression in the mesentery, because mesenteric mesothelial cells of F_1-*tg*/*tg* mice expressed normal levels of SgIGSF (unpublished data, Ito and Watabe, 2004). In addition, F_1-*tg*/*tg* CMCs transfected with MITF cDNA showed an even better survival than those transfected with SgIGSF cDNA (Table 2). MITF appears to be involved in transactivation of other molecule(s) than SgIGSF, which express in CMCs themselves and in the mesentery and are necessary for the adhesion and/or survival of CMCs in the mesentery. Defective expression of such molecule(s) in the mesentery may explain the smaller number of F1-+/+ CMCs surviving in the peritoneal cavity of F_1-*tg*/*tg* mice.

SgIGSF appeared to contribute to the survival of CMCs in the peritoneal cavity and mesentery, but F_1-*tg*/*tg* CMCs transfected with SgIGSF showed still poor survival at both sites when compared with F_1-+/+ CMCs. Even the transfection with MITF cDNA did not normalize the survival of F_1-*tg*/*tg* CMCs. There was a possibility that expression of ectopic proteins could not be maintained in retrovirus-infected CMCs for 5 weeks. To establish *tg*/*tg* CMCs that expressed ectopic SgIGSF more stably, we obtained two founder transgenic mice that expressed SgIGSF under the control of the chicken β-actin promoter and cytomegalovirus enhancer, and backcrossed the mice to B6-*tg*/*tg* or B6-*tg*/+ mice. Offspring of one founder were named A-line, while those of the other B-line. Some offspring were white and judged as mice of *tg*/*tg* genotype, while others were black and judged as mice of *tg*/+ or +/+ genotype. SgIGSF expression was examined in the protein lysates from the tail tips of *tg*/*tg* offspring, and mice with high levels of SgIGSF expression were judged as the transgenic *tg*/*tg*

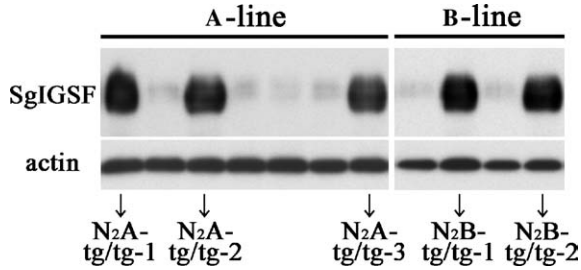

Fig. 2. Identification of A- or B-line transgenic *tg/tg* mice by Western blot analyses with the anti-SgIGSF antibody. Protein lysates were prepared from tail tips of A- and B-line offspring with white coat, and were blotted with the SgIGSF antibody. After striping the antibody, the blots were probed again with the anti-actin antibody to indicate the total amount of proteins loaded per lane. Mice with high levels of SgIGSF expression were judged as the transgenic *tg/tg* mice. The blots show that three A-line and two B-line offspring are transgenic mice. These mice were named serially N_2A-*tg/tg*-1, 2, and 3 and N_2B-*tg/tg*-1 and 2.

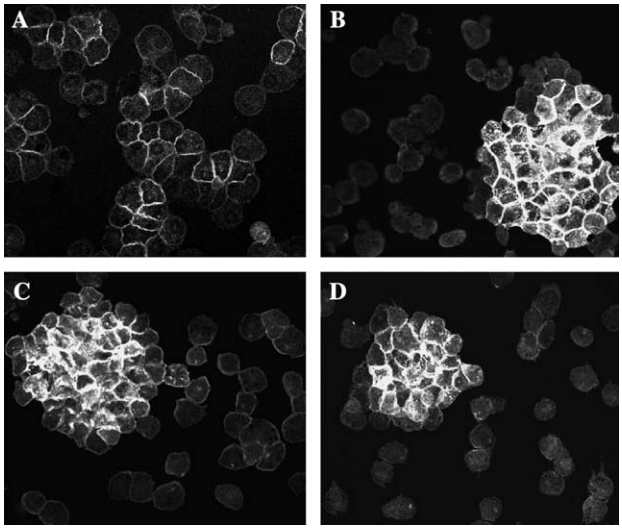

Fig. 3. Immunolocalization of SgIGSF in CMCs. Cytospin preparations of CMCs were fixed with methanol, reacted with the anti-SgIGSF antibody, and stained with FITC-labeled secondary antibody. (A) F_1-+/+ CMCs; (B) F_1-*tg/tg* CMCs transfected with SgIGSF cDNA; (C) N_2A-*tg/tg*-1 CMCs; and (D) N_2B-*tg/tg*-1 CMCs.

mice (Fig. 2). A- and B-line transgenic *tg/tg* mice used in the present study were obtained in the second generation of backcrosses, and described as N_2A-*tg/tg* and N_2B-*tg/tg* mice, respectively. In general, A- and B-line transgenic *tg/tg* mice were indistinguishable from their non-transgenic *tg/tg* littermates with the exception of their small size.

CMCs were established from spleen cells of four N_2A-*tg/tg* (N_2A-*tg/tg*-1, 2, 3, and 4) and two N_2B-*tg/tg* (N_2B-*tg/tg*-1 and 2) sucking mice. As shown in Fig. 1, expression of SgIGSF was comparable among N_2A-*tg/tg*-1 to 4 CMCs and between N_2B-*tg/tg*-1 and 2 CMCs. The expression level in N_2A-*tg/tg*-1 to 4 CMCs was somehow higher than that in F_1-+/+ CMCs but significantly lower than that in F_1-*tg/tg* CMCs transfected with SgIGSF cDNA (Fig. 1). On the other hand, N_2B-*tg/tg*-1 and 2 CMCs expressed somehow lower levels of SgIGSF than F1-+/+ CMCs (Fig. 1).

We examined the adhesion of N_2A-*tg/tg*-1 and 2 CMCs and N_2B-*tg/tg*-1 and 2 CMCs to NIH/3T3 fibroblasts by coculturing these CMCs with NIH/3T3 fibroblasts. The adhesion of all these four types of CMCs was better than that of F_1-*tg/tg* CMCs but poorer than that of F_1-+/+ CMCs (Table 1). When the adhesion to NIH/3T3 fibroblasts was compared among *tg/tg* CMCs that expressed SgIGSF ectopically, the rank order was as follows: F_1-*tg/tg* CMCs transfected with SgIGSF cDNA > A-line transgenic *tg/tg* CMCs > B-line transgenic *tg/tg* CMCs (Table 1). This order was consistent with that of the expression levels of ectopic SgIGSF as revealed by Western blot analyses (Fig. 1). To function as efficiently as endogenous SgIGSF, the overexpression of ectopic SgIGSF appeared to be required.

In addition to functional defect, ectopic SgIGSF seemed to have an abnormality in cell surface localization. When cytospin preparations of F_1-+/+ CMCs were stained with the anti-SgIGSF antibody, SgIGSF-specific signals were evenly distributed on the cell membrane at every cell–cell contact site (Fig. 3A). Consistent with the result of Western blot analyses, no specific signals were detectable in F_1-*tg/tg* CMCs (data not shown). In contrast, F_1-*tg/tg* CMCs transfected with SgIGSF cDNA showed a unique staining pattern: strong signals were detected at cell–cell contact sites of aggregated CMCs, but CMCs outside the aggregates were negative for SgIGSF even when they were in touch with each other (Fig. 3B). This staining pattern was also shown by all A-line transgenic *tg/tg* and B-line transgenic *tg/tg* CMCs (two examples were shown in Figs. 3C and D). On the other hand, intracellular localization and expression levels of KIT in tg/tg CMCs were not influenced by ectopic expression of SgIGSF (data not shown). These results suggested that ectopic SgIGSF was expressed on the cell surface of some CMCs but not of all CMCs, and that its overexpression resulted in aggregation of CMCs. Abnormal cell surface localization may be a cause that ectopic SgIGSF was less functional than endogenous one.

CMCs derived from six N_2A-*tg/tg* (N_2A-*tg/tg*-1 to 6) and two N_2B-*tg/tg* (N_2B-*tg/tg*-1 and 2) mice were injected intraperitoneally into F_1-W/W^v mice, and were examined for their survival in the peritoneal cavity and mesentery. With respect to the survival in either site, these CMCs were divided into two groups. N_2A-*tg/tg*-1, 3, and 5 CMCs, and N_2B-*tg/tg*-1 CMCs survived more efficiently than F_1-*tg/tg* CMCs, whereas the survival of CMCs from the other transgenic mice was as poor as or poorer than that of F_1-*tg/tg* CMCs (Table 2). There was a difference in the survival efficiency among CMCs of the former group and between these CMCs and

SgIGSF-transfected F_1-*tg*/*tg* CMCs. This may be attributable to the variety of the genetic backgrounds of the transgenic mice, because the mice were obtained in the second generation of backcrosses to B6 mice. CMCs of the latter group, especially N_2A-*tg*/*tg*-6 and N_2B-*tg*/*tg*-2 CMCs, were probably rejected by F_1-W/W^v hosts.

Expression of ectopic SgIGSF, whether it was achieved by the transfection using a retrovirus vector or by the generation of transgenic mice, only partially improved the poor survival of *tg*/*tg* CMCs in the peritoneal cavity of F_1-W/W^v mice. According to the comparison between the number of surviving F_1-+/+ CMCs and the number of surviving *tg*/*tg* CMCs that expressed ectopic SgIGSF, the degree to which SgIGSF contributed to the survival of CMCs was estimated to be at most 30%. However, this might be underestimated because of the following reasons. (1) *tg*/*tg* CMCs expressed an appreciable amount but still significantly reduced level of KIT [17]. Normal expression of KIT might be a prerequisite for SgIGSF to support the survival of CMCs. (2) SgIGSF overexpression results not only in cell aggregation but also in poor cell proliferation [1]. There is a possibility that some *tg*/*tg* CMCs expressing ectopic SgIGSF aggregated in the peritoneal cavity, and consequently died. (3) Although expression levels of SgIGSF in A-line transgenic *tg*/*tg* and B-line transgenic *tg*/*tg* CMCs were high enough, these CMCs could not adhere to NIH/3T3 fibroblasts as efficiently as F_1-+/+ CMCs. Ectopic SgIGSF might be functionally defective. Considering these negative factors, SgIGSF might contribute to mast cell survival more significantly than estimated here.

When N_2A-*tg*/*tg* and N_2B-*tg*/*tg* transgenic mice grew up to be 10-week-old, the number of mast cells was examined in the peritoneal cavity and mesentery. Mast cells were practically absent from both sites, as is the case with B6-*tg*/*tg* and F_1-*tg*/*tg* mice [8]. Transgenic expression of SgIGSF did not appear to normalize the mast cell development in the peritoneal cavity or mesentery of *tg*/*tg* mice, although ectopic SgIGSF significantly contributed to the intraperitoneal survival of *tg*/*tg* CMCs. Restoration of SgIGSF expression in mast cells or their progenitor cells may be insufficient for development of mast cells in *tg*/*tg* mice. Alternatively, transgenic expression of SgIGSF in other types of cells may give rise to a defect in microenvironment that is necessary for mast cells to develop and proliferate in the peritoneal cavity and mesentery. To exclude such a microenvironmental factor, we are planning to perform bone marrow transplantation using A- or B-line *tg*/*tg* transgenic mice as donors and B6-*tg*/*tg* mice as recipients. If mast cell deficiency in *tg*/*tg* mice is attributable solely to decreased expression of SgIGSF in mast cells or their progenitor cells, this transplantation may result in development of mast cells in the recipients. For successful transplantation, we are now backcrossing A- and B-line transgenic mice to inbred B6 mice consecutively.

In conclusion, the present results clearly indicated that SgIGSF not only mediated the adhesion of CMCs to fibroblasts but also contributed to the survival of CMCs in the peritoneal cavity of F_1-W/W^v mice. Since SgIGSF has been characterized as an intercellular adhesion molecule [1–3], it is reasonable to speculate that SgIGSF might be involved primarily in the adhesion and secondarily in the survival. This speculation is consistent with our previous conclusion that CMCs required direct interaction with NIH/3T3 fibroblasts to survive in the coculture [11]. To survive in the peritoneal cavity, CMCs may require the adhesion to cells lining the peritoneal cavity, and SgIGSF may mediate this type of adhesion. In fact, when *tg*/*tg* CMCs that expressed ectopic SgIGSF were injected into the peritoneal cavity, they survived not only in the peritoneal cavity but also in the mesentery. This indicates that some intraperitoneally injected CMCs interacted with mesenteric mesothelial cells during the process of settlement in the mesentery. Interestingly, mesenteric mesothelial cells express a membrane-bound form of stem cell factor, a cognate ligand of KIT, as abundantly as NIH/3T3 fibroblasts (unpublished data, Ito et al., 2003). SgIGSF may help mast cells receive a growth-stimulating signal through KIT by mediating the adhesion of mast cells to mesenteric mesothelial cells. Although the precise mechanism for the survival of mast cells in the peritoneal cavity is still unknown, the present study suggests that adhesion of mast cells may play a role in their in vivo survival.

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