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Novel Mast Cell Lines with Enhanced Proliferative and Degranulative Abilities Established from Temperature-Sensitive SV40 Large T Antigen Transgenic Mice

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Mast cells (MCs) play crucial roles in innate immunity to parasitic and bacterial infections as well as in hypersensitivity, such as the induction and exacerbation of allergy and autoimmune diseases. The regulatory mechanisms for MC development and effector functions are of great interest for developing novel therapeutic strategies against such disorders. Here we report the establishment of novel, immortalized MC lines from bone marrow (BM) cells of a temperature-sensitive mutant of SV40 large T antigen-transgenic mice (termed SVMCs). BM cells from tsSV40LT mice were cultured in the presence of interleukin (IL)-3 for 3 weeks, and then subjected to limiting dilution and single-cell cloning, yielding 27 independent MC clones, three of which were subjected to further analysis. On culture with nerve growth factor, stem cell factor and IL-3, these SVMC clones showed morphologic and biochemical changes from mucosal MC-like to connective-tissue MC-like phenotypes. These SVMC lines exhibited a significantly enhanced proliferation rate, and a higher responsiveness to the high affinity Fc receptor for IgE-mediated intracellular calcium mobilization and degranulation than those of BM-derived cultured MCs. These cell lines should facilitate studies on the mechanisms for the development, differentiation and effector functions of MCs in health and diseases.

Key words: degranulation, differentiation, murine mast cell line, SV40 large T, protease.

Abbreviations: B6, C57BL/6; BM, bone marrow; BMMC, bone marrow–derived cultured mast cell; CTMC, connective tissue mast cell; MC, mast cell; MMC, mucosal mast cell; NGF, nerve growth factor; SCF, stem cell factor; SVMC, temperature-sensitive SV40 large T antigen–transduced mast cell line; tsSV40LT, temperature-sensitive mutant of SV40 large T transgenic mice.

Mast cells (MCs) are distributed widely in the body, being particularly associated with connective and mucosal tissues, and have been recognized as critical tissue–based effector cells that mediate defense against parasitic and bacterial infections, and IgE-dependent and -independent allergic responses, and regulate the development of autoimmune diseases (*1*). After immunological activation via the high-affinity Fc receptor for IgE (FcεRI), for instance, MCs release a variety of cytokines, lipid-derived mediators, amines, proteases and proteoglycans—all of which can modulate adjacent tissues, such as changes in metabolism of the extracellular matrix of connective tissue (*1–5*). In addition, MCs play important roles in the initiation and regulation of inflammatory responses against parasites and microbes in innate immunity (*1*, *3–10*).

Immune responses usually involve the coordinated, sequential and redundant activities of different cell types in addition to MCs, such as dendritic cells, T helper cells, basophils and eosinophils (*1–6*). Under these circumstances, determining the MC-specific contributions to specific events, such as inflammation, is difficult. Therefore, it is important to establish a procedure for isolating MCs and for characterizing the functions of the cells *in vitro*. To date, bone marrow (BM)–derived cultured MCs (BMMCs) have been used as a model for studies on MC development and function (*11*). Although murine BMMCs are induced by culturing BM progenitors with interleukin (IL)-3 (*1–6*), cell viability and the functional ability to respond to degranulating stimuli decrease rapidly upon long-term culture, with the senescence and eventual death of all cells after a few months (*12*). Additionally, since MCs differentiated in the presence of IL-3 exhibit a broad range of phenotypes depending on the maturation and senescence phases, it is difficult to examine the characteristics of the MCs at a defined stage.

Accumulating evidence indicates that transgenic mice harboring a temperature-sensitive mutant of the SV40 large T antigen (tsSV40LT) gene have several advantages for establishing immortalized cell lines with differentiated functions. The transgenic mice show the stable and identifiable expression of the tsSV40LT gene in all tissues, and the cultured cells can be readily immortalized by activating the tsSV40LT gene at 33°C, a permissive temperature (*13*). The immortalized cells proliferate at 33°C because large T-antigen binds to the tumor suppressor gene product, p53,

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and/or the retinoblastoma gene product, Rb, which regulate cell proliferation (*13*). At temperatures over 37°C, which are non-permissive ones, large T antigen becomes unstable and is degraded, which stops the growth of the cells. Several cell lines, such as hepatocytes (*14*), kidney tubule cells (*15*), gastric surface mucous cells (*16*), brain capillary endothelial cells (*17*), bone marrow stromal cells (*18*), retinal pigment epithelium–derived cells (*19*), colonic epithelial cells (*20*), and smooth muscle cells (*21*), have been established from tsSV40LT mice, and these cell lines exhibit some of the specific differentiated phenotypes. Recently, we successfully established immortalized dendritic cells with sufficient antigen presentation ability by culturing BM cells from tsSV40LT mice (*22*) in the presence of granulocyte–macrophage colony–stimulating factor (GM–CSF). This was the first study on immortalization of myeloid-lineage cells with a specialized function.

Based on our previous experience of immortalization of dendritic cells, we attempted to isolate lines of MCs, other myeloid-lineage cells, which could be induced from BM cells in response to IL-3, instead of GM-CSF, for dendritic cell culture. In the present study, we report the establishment and characterization of MC lines from tsSV40LT mice (termed SVMC clones). Selected SVMC clones a, c, and E were analyzed in detail regarding their abilities as to differentiation and function *in vitro*. The availability of these murine MC lines will facilitate studies designed to obtain a better understanding of the mechanisms of the function and differentiation of MCs under normal and pathological conditions.

MATERIALS AND METHODS

Animals—The founder mice for the tsSV40LT mice (*13*) were donated by Dr. K. Ishibashi (Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan). Since tsSV40LT mice developed a colloid plexus tumor and died within 5 months, they were maintained by *in vitro* fertilization in our laboratory. Eight-week-old C57BL/6 (B6) mice were purchased from Charles River Japan, Inc. (Kanagawa, Japan). Mice were housed and bred in the Animal Facility of The Institute of Development, Aging and Cancer (Tohoku University, Sendai, Japan), an environmentally controlled and specific pathogen–free faculty, according to the guidelines for experimental animals defined by the faculty. All the experiments were performed using protocols approved by the institutional animal care committee.

Generation of BMMCs from B6 or tsSV40LT Mice—BM cells were obtained from both hind limbs (femur and tibia) of 8-week-old female C57BL/6 or tsSV40LT mice. After lysing the erythrocytes with lysis buffer (0.144 M ammonium chloride solution), BM cells were suspended at a density of 5×10^5 cells/ml in RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO), supplemented with 10% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids solution, and 5 ng/ml mouse rIL-3 (R&D Systems, Inc., Minneapolis, MN), and incubated at 37°C (for cells from B6 mice) or at 33°C (for cells from tsSV40LT mice) under a humidified atmosphere of 5% CO_2, and 95% air. The BM-derived non-adherent cells were transferred every 6–7 days to new culture flasks at a density of 3×10^5 cells/ml over a 4- to 10-week culture period. Over 90% of cells were identified as immature MCs at 4 weeks after initiation of the culture and were designated as the "SVMC bulk" culture.

Single-Cell Cloning—Cloning of single cells from the SVMC bulk culture was carried out by the limiting-dilution method. Cells were cultured in 96-well flat-bottom tissue culture plates at a density of 0.2 cell/well, 0.5 cell/well, and 1.0 cell/well. After 4–5 weeks of incubation at 37°C under a humidified atmosphere of 5% CO_2 and 95% air, 27 single-cell clones were identified, three of which were characterized further.

Polymerase Chain Reaction (PCR) Analysis—Genomic DNA was isolated from SVMC clones and BMMCs. 1×10^7 cells were collected by centrifugation at $300 \times g$ and resuspended in 200 μl of 150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.1% SDS, and 0.25 mg/ml proteinase K (Sigma-Aldrich). After 16 h incubation at 50°C, DNA was extracted with phenol-chloroform, precipitated with ethanol, and then resuspended in TE (10 mM Tris-HCl containing 1 mM EDTA). PCR analysis of the SV40 large T gene was performed using the following primer pair: 5′-GGAGGAGTAGAATGTTGAG-3′ and 5′-GTGTTGATGCAATGTACTGC-3′. The PCR reaction was performed in the presence of 0.1 mM dNTPs, 1 mM $MgCl_2$, 1 × PCR buffer, 0.1 U Taq polymerase (all from Sigma-Aldrich), 0.1 μM primers and 1 μl of DNA (0.1 μg equivalent) prepared as described above in a total volume of 20 μl. The reaction was performed under the following conditions: 30 s at 94°C, 30 s at 54°C, and 30 s at 72°C, for 30 cycles.

Western Blot Analysis—SVMC clones and BMMCs (1×10^6/ml) were solubilized in 100 μl of RIPA buffer, and lysates were prepared by direct boiling in reducing sample buffer supplemented with 1.0% of 2-mercaptoethanol, separated on a 12% acrylamide gel, transferred to a nitrocellulose membrane, and then blotted with anti-SV40 large T antigen mAb (Ab-2; Oncogene, Germany), followed by HRP-conjugated anti–mouse IgG2a antibodies [a Mo IgG2a-ADS (AFF)-PEROX] (The Binding Site, Birmingham, UK). Immunoblots were developed using ECL Western blotting detection reagents (GE Healthcare Bio-Sciences Corp., NJ).

Cytochemical Staining and Electron Microscopy—For assessment of morphological and cytochemical characteristics, cytocentrifuged preparations of SVMC clones and BMMCs were stained with Wright–Giemsa, toluidine blue and alcian blue/safranin according to the manufacturer's instructions, and then microscopically examined. For electron microscopy, cells were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), and postfixed in 1% osmium tetroxide. They were dehydrated in graded concentrations of ethanol, and then embedded in epoxy resin. Thick sections were firstly stained with toluidine blue for screening cells. Next, ultrathin sections (70 nm) were stained with uranyl acetate and a subsequent solution consisting of lead nitrate, lead citrate and lead acetate. These sections were examined under a transmission electron microscope (H-7600; Hitachi, Japan). Captured images of MCs were analyzed for granule numbers and contents using the image-processing software in the NIH image program available from the U.S. National Institutes of Health.

Induction of Phenotypic Changes of MCs by Nerve Growth Factor (NGF) and Stem Cell Factor (SCF)—SVMC clones and BMMCs were suspended at a density of 2×10^5 cells/ml in RPMI 1640 medium supplemented with 10% FCS, 50 IU/ml penicillin, 50 μg/ml streptomycin, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate, 0.1 mM MEM non-essential amino acids solution, 5 ng/ml mouse rIL-3 (R&D Systems), 50 ng/ml rβ-NGF (R&D Systems), and 100 ng/ml mouse rSCF (PeproTech EC Ltd., UK). The medium was changed every 4–5 days and cells were cultured for up to 20 days. The phenotypic changes of SVMC clones and BMMCs were examined histochemically with alcian blue/safranin staining that had been used to distinguish safranin connective tissue MC (CTMC)–like cells from alcian blue mucosal MCs (MMCs) at 4, 8, 12, 16, and 20 days after initiation of the culture. The viability of cells was maintained at nearly 100% under these culture conditions. For enzymatic assaying to evaluate the phenotypic change to CTMC-like cells, BMMCs, SVMC bulk, and clones (2×10^6 cells each) cultured in the presence of SCF and NGF were solubilized in 200 μl of lysis buffer (2 M NaCl and 0.5% Triton X-100 in PBS). The cell lysates were centrifuged ($10{,}000 \times g$) and supernatants were recovered, and aliquots were stored at –80°C for next experiments. One hundred microliter aliquots of cell extracts were mixed with 20 μl of a 1.8 mM solution (in H_2O) of a substrate (MeOSuc-Ala-Ala-Pro-Met-pNA; Bachem AG, Switzerland), followed by incubation for 24 h at 37°C. The chymotrypsin-like activity was determined as the amidolytic rate at which *p*-nitroaniline (pNA) was released by monitoring the absorbance at 405 nm in duplicate with an E_{max} precision microplate reader (Molecular Devices, USA) using SOFTmax PRO3.1 software.

Reverse Transcription–PCR (RT-PCR) Analysis—Total RNA was extracted from cultured SVMC clones and PMCs (5×10^6 cells) using an RNeasy kit (QIAGEN, Germany), according to the manufacturer's instructions. Five micrograms of RNA was converted to first-strand cDNA in 20 μl of 4 mM Tris-HCl, pH 7.5, containing 20 mM NaCl, 0.02 mM ETDA, 2 μM 2-mercaptoethanol, 0.002% NP-40, 10% glycerol, 0.1 mM dNTPs, 0.1 U RNase inhibitor, 0.1 U Moloney Murine Leukemia Virus reverse-transcriptase, and 1 μM oligo $(dT)_{12–18}$ primer (all from Toyobo, Osaka, Japan) under the following conditions: 10 min at 25°C, 60 min at 42°C, 5 min at 99°C, and 5 min at 4°C. PCR analyses of various mouse MC-specific proteases were performed using the following primer pairs: mMCP-1, 5′-ACCACACTCCCGTCCTTACAT-3′ and 5′-GGGCCACACCAGCACAC-3′; mMCP-5, 5′-CTTCATCTGCTGCTCCTCTCCTG-3′ and 5′-GGCTGGCTCATTCACGTTTGTTCC-3′; mMCP-6, 5′-TGCACCCCCACTATTACACG-3′ and 5′-CCATCCAAGAGAGGGAAAGG-3′; and mMC-CPA, 5′-CCTGTCCACTTTGACAGGGAG-3′ and 5′-CAGTGCCAATGCAGGTGGAGT-3′. All PCR reactions were performed in the presence of 0.1 mM dNTPs, 1 mM $MgCl_2$, 1 × PCR buffer, 0.1 U Taq polymerase (all from Sigma-Aldrich), 0.1 μM each primer and 1 μl of cDNA (250 ng RNA equivalent) prepared as described above in a total volume of 20 μl. All reactions were performed under the following conditions: 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C, for 30 cycles (mMCP-1, mMCP-6), 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C, for 30 cycles (mMCP-5), and 30 s at 94°C, 30 s at 58°C, and 30 s at 72°C, for 30 cycles (mMC-CPA).

The phenotypic change from MMCs to CTMC-like cells was also examined by RT-PCR of mMCP-4 mRNA, a mast cell–specific protease predominantly expressed in rodent CTMCs. After induction of a phenotypic change with NGF and SCF, total RNA was extracted from BMMCs or SVMC clones with an RNeasy kit (QIAGEN) according to the manufacturer's specifications. One microgram of total RNA was converted to first-strand cDNA by using a SuperScript first-Strand Synthesis System for RT-PCR (Invirtogen Corporation, Carlsbad, CA) according to the manufacturer's instructions, and the cDNA was then amplified for 20 s at 94°C, 2 s at 54°C, and 30 s at 74°C, for 30 thermocycles, in a 20 μl volume reaction mixture comprising 1 × PCR buffer, 0.1 mM dNTPs, and 0.5 U of KOD Dash DNA polymerase (Toyobo). The primer pairs used were: (i) mMCP-4 (700 bp) 5′-TGAGAGAGGGTTCACAGCTAC-3′ and 5′-CCCAAGGGTTATTAGAAGAGCT-3′; and (ii) β-actin (550 bp) 5′-GTGGGGCGCCCCAGGCACCA-3′ and 5′-CTCCTTAATGTCACGCACGATTTC-3′. The PCR products were analyzed on 2% agarose gels and the amounts of the products were determined.

Flow Cytometry—Expression of the high-affinity IgE receptor, FcεRI, and the SCF receptor, c-kit, was analyzed by flow cytometry. SVMC clones and BMMCs (1×10^6 cells/ml) were incubated first with 5 μg/ml mouse anti-trinitrophenyl (TNP) IgE (BD Biosciences, Franklin Lakes, NJ) for 2 h, and then with FITC-conjugated rat anti-mouse IgE (BD Biosciences) and PE-conjugated rat anti–c-kit monoclonal antibodies (mAbs) (BD Biosciences) for another 10 min. For measurement of cell-surface receptors, SVMC clones and BMMCs (1×10^6 cells/ml) were incubated with one of the following mAbs: PE-conjugated 0.5 μg/ml anti-FcγRIIB/III mAb (2.4G2), anti–PIR-A/B mAb (6C1), or anti–TLR-4 mAb (BD Biosciences). Isotype-matched PE-conjugated rat IgG1 or IgG2b was used as a negative control. Amounts equaling to 10,000 stained cells in each sample were analyzed by the use of a BDLSR (BD Biosciences) equipped with Cell Quest software.

Growth Curve and Cell Cycle Progression Analysis—SVMC clones and BMMCs were cultured in 24-well plates at 2.4×10^5 cells/ml/well, and collected at 1, 2, 3, 4, 5, and 6 days post-inoculation. The cell number and viability were determined by trypan blue dye exclusion analysis. For cell cycle analysis, asynchronously growing SVMC clones and BMMCs were fixed with 70% ethanol for 30 min, treated with RNase A (100 μg/ml) (Sigma-Aldrich), and then stained with propidium iodide (50 ng/ml) (Sigma-Aldrich). Cell cycle analysis was carried out with a BDLSR (BD Biosciences) using CellQuest software.

[^{3}H]-Thymidine Incorporation Assay—To measure the proliferative response of MCs to IL-3, the [methyl ^{3}H]-thymidine (GE Healthcare Bio-Sciences) incorporation assay was performed as follows: SVMC clones and BMMCs (0.6×10^6 cells/ml) were cultured for 48 h in 200 μl of enriched medium containing various concentrations of IL-3 in the wells of a 96-well microculture plate. During the last 24 h of culture, 1.0 MBq [methyl ^{3}H]-thymidine was added to each culture. The cells were then harvested on glass fiber filters (Hewlett-Packard, Co., CA), using a cell harvester (Hewlett-Packard), and

the incorporation of [^{3}H]-thymidine was measured with a gas scintillation counter (Hewlett-Packard).

Serotonin-Release Assay—IgE-mediated serotonin release assay was performed as follows: SVMC clones and BMMCs (0.6×10^6 cells/ml) were incubated with 5-hydroxy [G-^{3}H] tryptamine creatinine sulfate (5.0 μCi/ml; 370 kBq; GE Healthcare Bio-Sciences) for 16 h at 37°C, washed, incubated with mouse IgE anti-TNP mAb (5 μg/ml; BD Biosciences) for 2 h at 37°C, and then washed again. One hundred microliter aliquots of [^{3}H]-serotonin–loaded SVMC clones and BMMCs (1.0×10^6 cells/ml) were incubated for 1 h at 37°C on 96-well microculture plates containing 10 ng/ml or 100 ng/ml of TNP-OVA, and then centrifuged. [^{3}H]-Serotonin in the supernatant and precipitates was measured with a liquid scintillation counter (Beckman Coulter Inc., CA). Serotonin release as a percentage was calculated according to the following formula: % serotonin release = [(cpm of supernatant)/(cpm of supernatant + cpm of precipitates)] × 100.

Calcium Mobilization Analysis—Intracellular Ca^{2+} concentrations were measured after FcεRI cross-linking on SVMC clones and BMMCs. SVMC clones and BMMCs (1.0×10^6 cells/ml) were first sensitized at 37°C with mouse IgE anti-TNP mAb (5 μg/ml; BD Biosciences) for 2 h, washed two times to remove unbound IgE, and then incubated for 30 min at 37°C in RPMI 1640 containing 1% fetal calf serum and 2 μM Indo-1/AM (Sigma-Aldrich). The 400/510 nm fluorescence ratio in cells preloaded with the Indo-1/AM dye was determined with a BDLSR flow cytometer (BD Biosciences) equipped with a HeCd ion laser operating in the UV mode. After establishing the baseline, 100 ng of TNP-OVA was added to the dye-loaded cells as a cross-linker, and the increase in the fluorescence ratio was monitored.

RESULTS

Generation of SVMC Clones—SVMCs were generated by culturing BM cells derived from female tsSV40LT mice in IL-3–supplemented medium at 33°C. After 30 days of culture, ~90% of the non-adherent cells appeared to be similar to the BMMCs from B6 mice according to morphological and cytochemical criteria, and were designated as the SVMC bulk culture. Single cell cloning from the SVMC bulk culture was carried out by the limiting-dilution method, which yielded 27 independent clones. Three of the SVMC clones containing an FcεRI and c-kit single population (Fig. 1A) and proliferating at a relatively

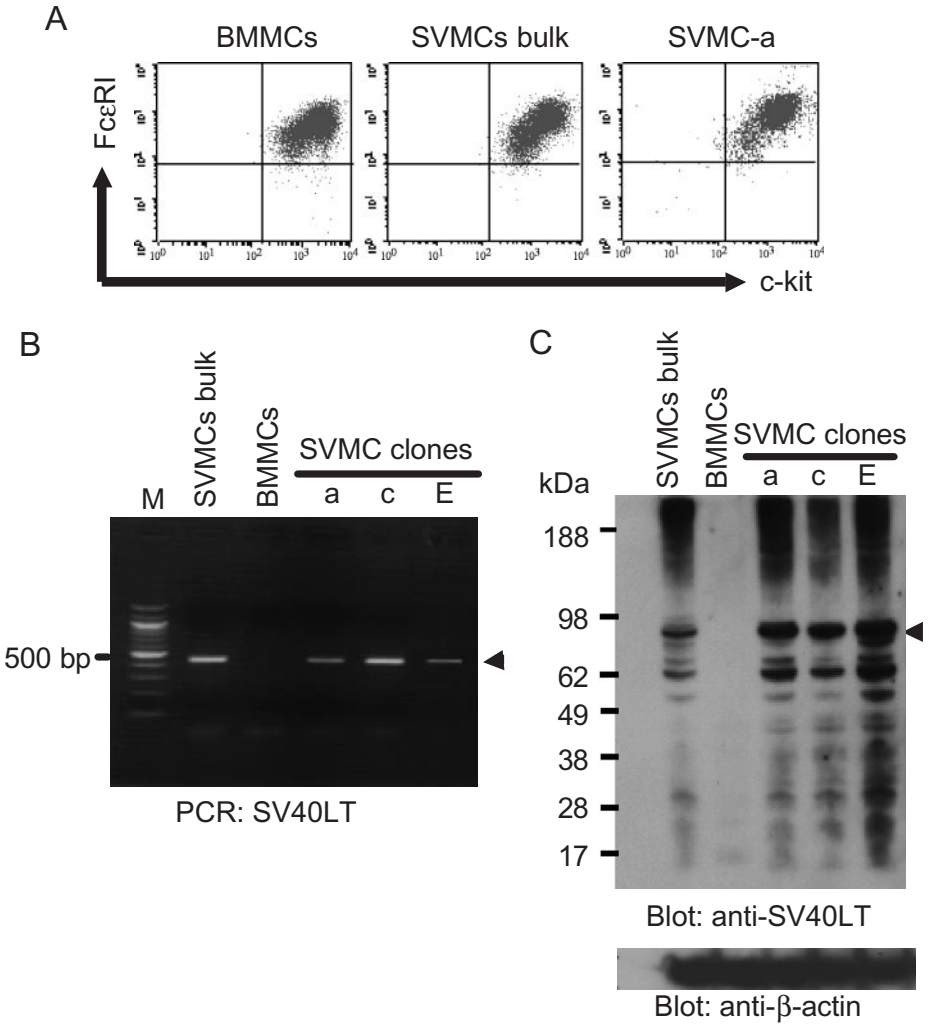

Fig. 1. **Generation of SVMC clones.** (A) Flow cytometric analysis of FcεRI and c-kit expression on SVMC clones and BMMCs. The SVMC clones and BMMCs were cultured for 2 h with mouse IgE anti-TNP mAb, washed, incubated with FITC-conjugated rat anti-mouse IgE and PE-conjugated rat anti–c-kit mAb, and then analyzed by FACS as described under "MATERIALS AND METHODS." Clones SVMC-c and -E gave flow cytometric profiles similar to SVMC-a. (B) PCR analysis for SV40 Large T antigen detection on SVMC clones. Genomic DNA from SVMC clones and BMMCs was subjected to PCR analysis as described under "MATERIALS AND METHODS." The position of an ~500 bp amplified product for the SV40 large T antigen gene is indicated by an arrowhead. (C) Western blot analysis. SVMC clones and BMMCs were subjected to Western blot analysis with monoclonal antibodies to mouse anti-SV40 large T antigen as described under "MATERIALS AND METHODS." The position of 94-kDa SV40 large T antigen is indicated by an arrowhead.

high rate (data not shown) were designated as SVMC-a, -c, and -E, and evaluated further. We confirmed, by PCR and Western blot analyses, that SVMC-a, -c, and -E possessed the SV40 large T antigen gene and expressed the protein product (Fig. 1, B and C).

Morphological and Cytochemical Characteristics of SVMC Clones—Cytocentrifuge preparations of SVMC clones and BMMCs stained with Wright–Giemsa, toluidine blue, and alcian blue/safranin comprised a roughly homogenous population of cells with cytoplasmic granules as well as round or oval nuclei (Fig. 2A). SVMC clones had, like BMMCs, a cytoplasm containing abundant dark violet granules upon Wright–Giemsa staining, exhibited discrete cytoplasmic metachromasia upon staining with toluidine blue, and cytoplasmic granules stained blue with alcian blue (Fig. 2A). These *in vitro*–differentiated cells were considered to resemble immature mucosal MCs (MMCs) because they failed to be counterstained with safranin, and also because they had a limited number of secretory granules.

Electron micrographic analysis of SVMC-a revealed that a typical cell of clone SVMC-a had many protrusions from the cell surface and stored a large number of granules with different electron densities like a BMMC (Fig. 2B). Computerized image analysis showed that there was no difference in the area as a percentage for granules per cell body area between SVMC-a and BMMCs, whereas the average number of granules in SVMC-a was

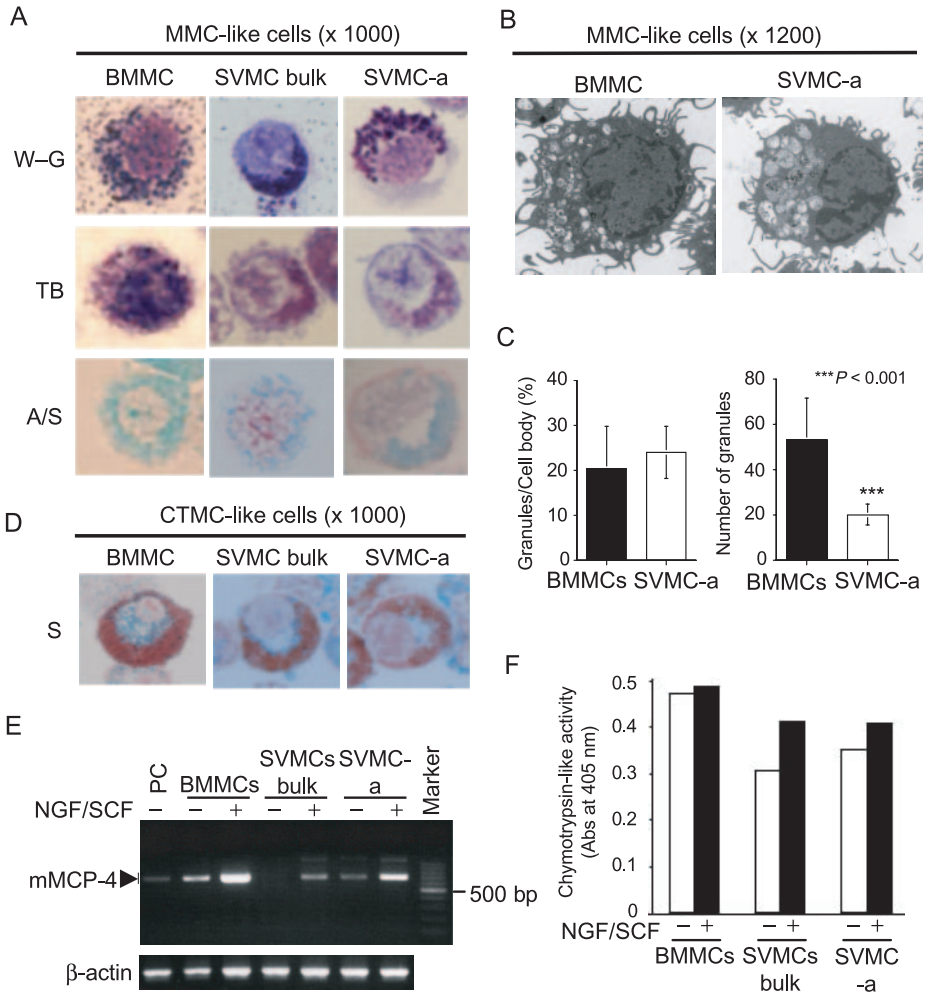

Fig. 2. **Morphological and cytochemical characterization of SVMC clones and BMMCs.** (A) Cytochemical staining of BMMCs, SVMC bulk culture, and SVMC-a with Wright–Giemsa stain (W–G), toluidine blue (TB), or alcian blue, followed by safranin (A/S). Original magnification, ×1,000. SVMC-a, -c, and E exhibited similar morphological and cytochemical characteristics to those of BMMCs. (B) Electron microscopic analysis of BMMCs and SVMC-a. SVMC-a had a similar morphology but their granules were relatively larger than those of BMMCs. Original magnification, ×1,200. (C) Areas and numbers of cytosolic granules in BMMCs and SVMC-a. Electron microscopic digital images of MCs (BMMCs, $n = 10$; SVMC-a, $n = 11$) were captured and analyzed with the image-processing software of the NIH image program. There was no difference in the gross area of granules per cell body between SVMC-a and BMMCs, whereas the average number of granules in SVMC-a was significantly lower than that in BMMCs. Statistical analysis was carried out by means of Student's t test. $^{***}P < 0.001$. (D–F) Induction of phenotypic changes from mucosal MC (MMC)–like to connective tissue-type MC (CTMC)–like cells. When SVMC clones were cultured for 14 days in the presence of IL-3, NGF, and SCF, their cytochemical profiles changed to resemble CTMC-like cells by changing from alcian blue to safranin (D), accompanied by up-regulation of the mMCP-4 mRNA levels (E) and increases in chymotrypsin-like activities (F). Original magnification, ×1,000 (D). PC, peritoneal cells (E).

significantly lower than that in BMMCs, indicating that the size of individual granules in SVMC-a was larger than that in BMMCs (Fig. 2C).

BMMCs and SVMC clones developed in the presence of IL-3 resemble MMCs because they are counterstained with alcian blue but not safranin and have a limited number of secretory granules (Fig. 2A). Culturing of BMMCs in the presence of IL-3, NGF and SCF for 7 days induced a phenotypic change from MMC-like to CTMC-like cells (Fig. 2D). Likewise, after 14 days of culture with IL-3, NGF, and SCF, about 40% of SVMC clones changed cytochemically to become safranin, as observed for BMMCs (Fig. 2D). It is known that the specific expression pattern for MC-specific proteases varies between different MC subtypes. In particular, CTMCs express MC-specific protease mMCP-4, a functional homolog of human chymotrypsin (*23*). Chymotrypsin is a serine protease that hydrolyzes peptide bonds with an aromatic or large hydrophobic side chain (*e.g.*, Tyr, Trp, Phe, and Met) at the carboxyl end of the bond. mMCP-4 mRNA levels were up-regulated in SVMCs bulk and SVMC-a (Fig. 2E), and chymotrypsin-like activity was increased in these SVMCs, consistent with mMCP-4 up-regulation (Fig. 2F). Thus, SVMC clones had the potential to differentiate further into CTMC-like cells in the presence of NGF and SCF.

We next examined whether SVMC clones express mRNA for mouse MC-specific proteases. Like BMMCs, SVMC clones exhibited similar profiles of expression of mRNA for MC-specific proteases, mMCP-1, mMCP-5, and mMCP-6, and MC carboxypeptidase A (Fig. 3A). In addition, flow cytometric analyses of the SVMC clones with monoclonal antibodies to FcγRIIB/III (2.4G2), PIR-A/B (6C1), or TLR-4 revealed expression levels of these cell-surface receptors roughly comparable to those in BMMCs (Fig. 3, B–D). Thus, SVMC clones were found to have grossly similar phenotypes to BMMCs in the criteria

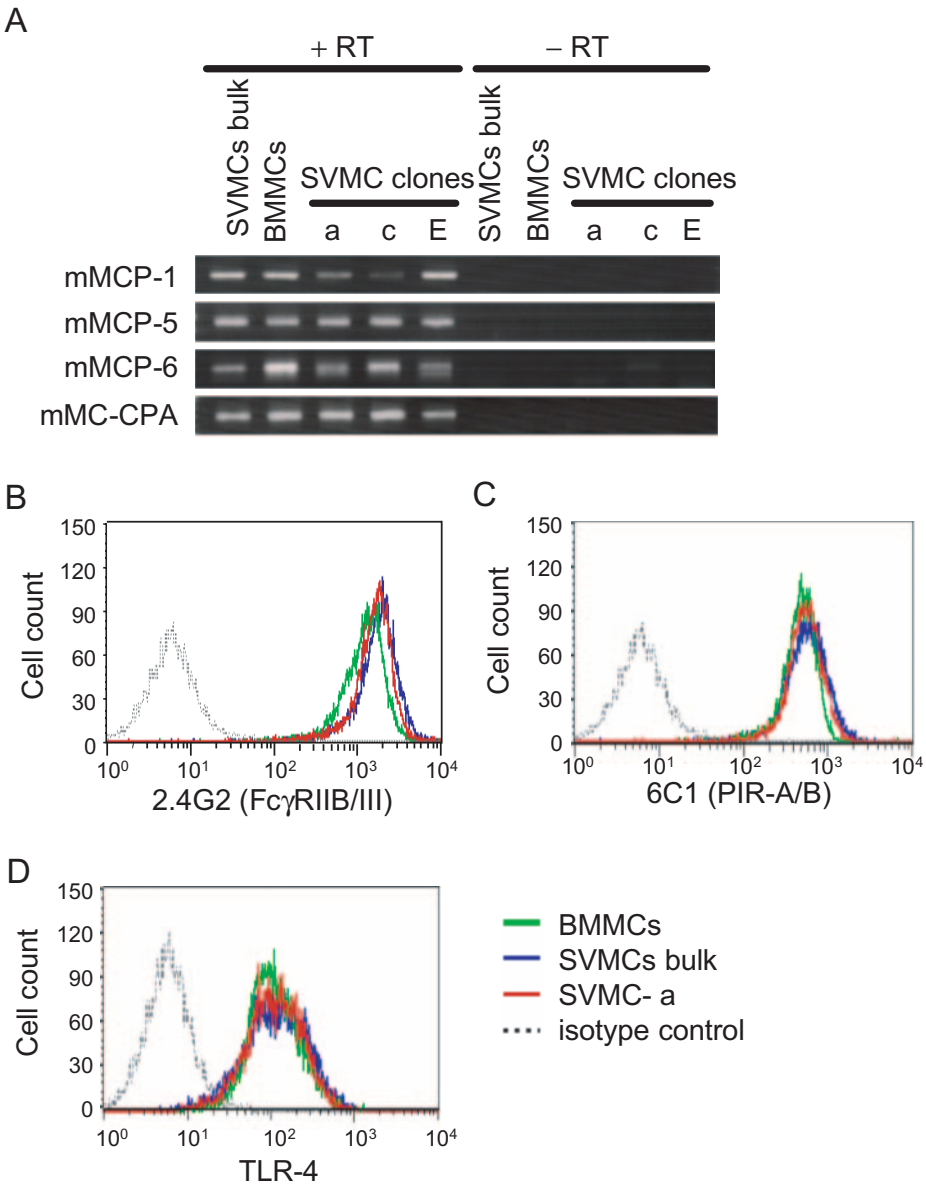

Fig. 3. **Expression of mouse MC–specific protease mRNAs and cell-surface receptors in SVMC clones and BMMCs.** (A) Verification of mRNAs for mouse MC–specific proteases (mMCP-1, -5, and -6) and MC carboxypeptidase A (mMC-CPA) by RT–PCR analysis. (B–D) Flow cytometric analysis (10^4 cells/conditions) of FcγRIIB/III, PIR-A/B, and TLR-4 expression on SVMC-a and BMMCs. Cells were labeled with the monoclonal antibody, 2.4G2, specific for FcγRIIB/III, 6C1 for PIR-A and -B, or the anti–TLR-4 monoclonal antibody, and then analyzed by FACS as described under "MATERIALS AND METHODS." Clones SVMC-c and -E gave flow cytometric profiles similar to SVMC-a.

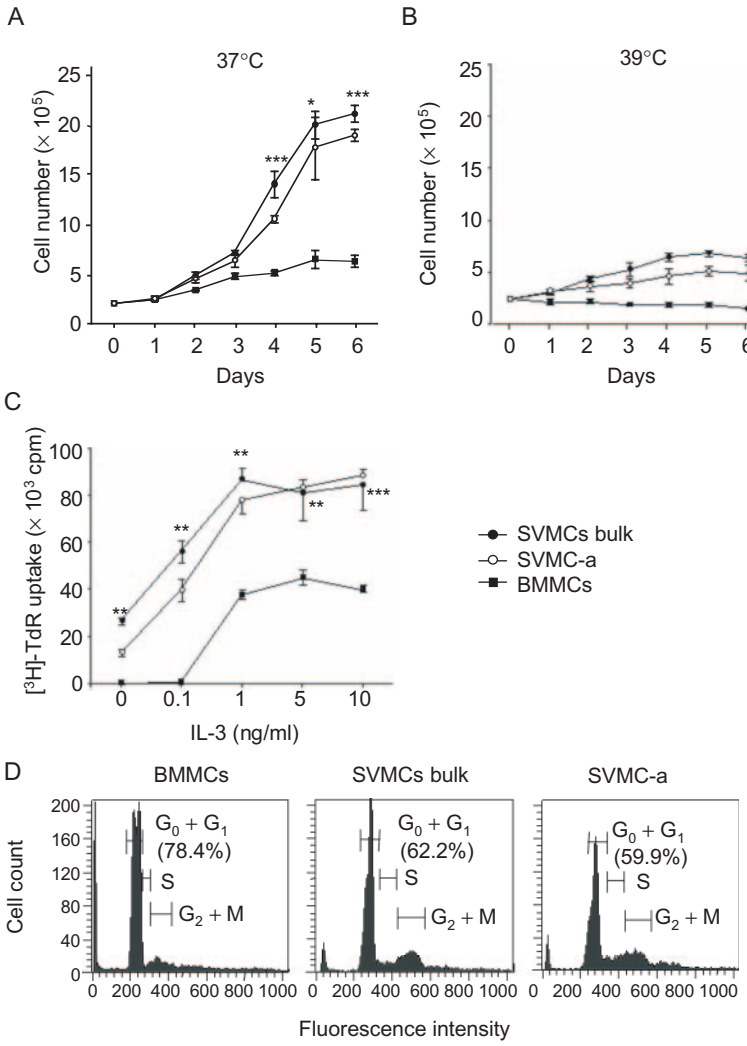

Fig. 4. **Growth characteristics of SVMC clones.** (A, B) Growth curves for SVMC-a and BMMCs. Growing cells were re-plated in medium supplemented with 5.0 ng/ml of IL-3 at 37°C (A) or 39°C (B). Viable cells were counted every day after plating. The cell number shown at each point is the mean ± SD of triplicate cultures. $*P < 0.05$, and $***P < 0.001$, when comparing BMMCs and SVMC-a under identical conditions by means of Student's *t* test. Clones SVMC-c and -E gave similar growth profiles to SVMC-a. (C) [^{3}H]-thymidine incorporation, as a measure of DNA synthesis, in SVMC-a and BMMCs incubated with various concentrations of IL-3. After washing growing cells twice with medium without IL-3, the cells were re-plated in 96-well microculture plates and cultured for 24 h in the presence of various concentrations of IL-3. Cells were incubated for an additional 24 h with [^{3}H]-thymidine. $**P < 0.01$, and $***P < 0.001$, when comparing BMMCs and SVMC-a under identical conditions by means of Student's *t* test. Clones SVMC-c and -E gave similar profiles to SVMC-a. (D) Cell cycle profiles of SVMC-a and BMMCs. Representative cell cycle histograms are shown. Cells were stained with propidium iodide, and then the percentage in either the G_1, S, or G2–M phase was determined by FACS analysis as described under "MATERIALS AND METHODS." Clones SVMC-c and -E gave similar cell cycle profiles to SVMC-a.

of cytochemical profiles, microscopic morphologies, MC-specific protease and receptor expression, and differentiation into CTMC-like cells.

Growth Potential of SVMC Clones at a Permissive Temperature—tsSV40LT mice harbor the SV40 large T antigen gene from SV40 temperature-sensitive mutant tsA58, whose proliferation is restricted at a permissive temperature (33–37°C). In fact, the SVMC clones showed a significantly enhanced growth compared with BMMCs at 37°C (Fig. 4A), but they lost their viability at a non-permissive temperature, 39°C (Fig. 4B). It is noteworthy that the expression of SV40 large T antigen in SVMC clones enhanced their growth potential at 37°C without causing any gross alterations in their differentiated, MMC-like phenotypes. We also analyzed the IL-3 dependency of the proliferation of SVMC clones and BMMCs by [^{3}H]-thymidine incorporation assaying in the presence of varying concentrations of IL-3. SVMC clones, compared to BMMCs, exhibited higher proliferation ability in the presence of identical levels of exogenous IL-3 (Fig. 4C). The phases of the cell cycle were assessed by flow cytometry with propidium iodide staining (Fig. 4D). Although the presence of SV40 large T antigen significantly reduced the population in the (G_0 + G_1) phase, which probably led to the enhanced proliferation, similar histogram profiles were obtained for SVMC clones and BMMCs (Fig. 4D).

FcεRI-Mediated Degranulation and Ca^{2+} Mobilization of SVMC Clones—For FcεRI-mediated degranulation studies, SVMC clones and BMMCs were sensitized with mouse anti-TNP IgE and then challenged with TNP-OVA to aggregate FcεRI. SVMC clones exhibited marked enhancement of serotonin release via FcεRI stimulation compared to BMMCs (Fig. 5A). SVMC-a, -c, and -E exhibited serotonin release varying from 66 to 88% (Fig. 5A and data not shown). Since the aggregation of FcεRI induces an immediate increase in the intracellular Ca^{2+} concentration, which is required for degranulation, we next compared the induction of Ca^{2+} flux in response to FcεRI aggregation in SVMC clones and BMMCs. As was observed in the degranulation assay, SVMC clones exhibited a marked increase in intracellular Ca^{2+} elevation compared to BMMCs (Fig. 5B).

DISCUSSION

In the present study, we attempted to develop MC lines that retain their growth factor responsiveness, proliferation, and functional ability to respond to degranulation

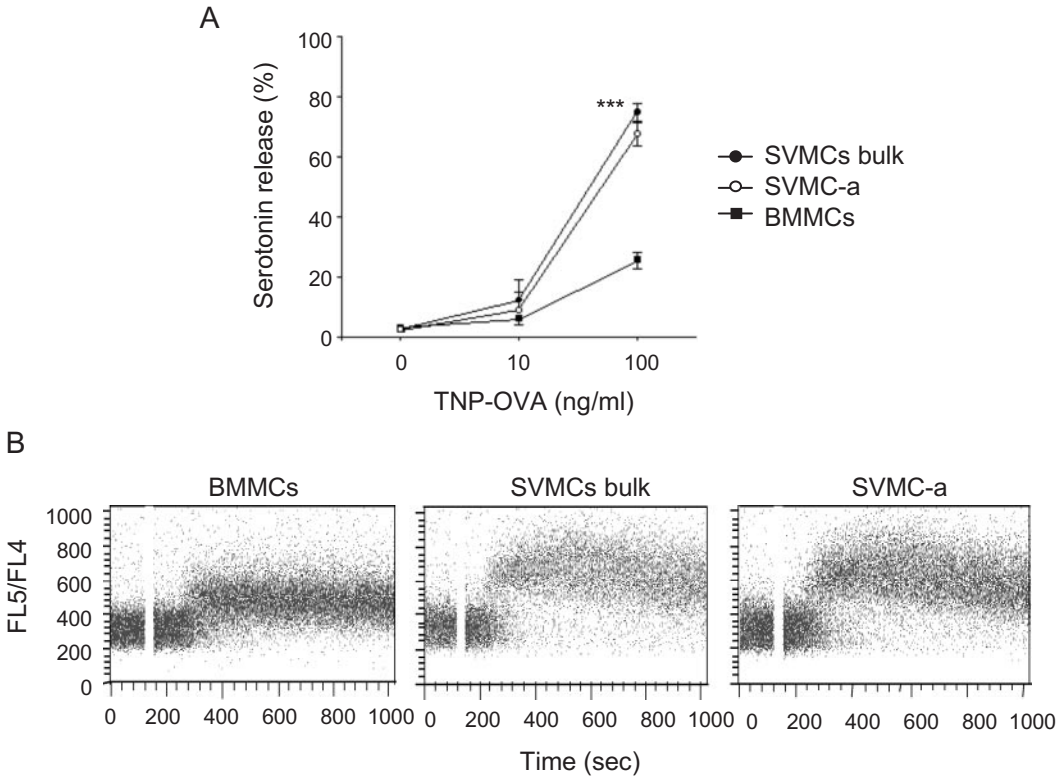

Fig. 5. **FcεRI cross-linking–induced degranulation in SVMC clones.** (A) Serotonin release. [^{3}H]-Serotonin–preloaded SVMC-a and BMMCs were incubated for 2 h with mouse IgE anti-TNP mAb. Antibody-primed cells were then stimulated with TNP-OVA as a cross-linker, and the [^{3}H]-serotonin release was measured as described under "MATERIALS AND METHODS." ***$P < 0.001$, when comparing BMMCs and SVMC-a under identical conditions by means of Student's t test. Clones SVMC-c and -E gave similar serotonin release profiles to SVMC-a. (B) Comparison of FcεRI cross-linking–induced Ca^{2+} mobilization between SVMC-a and BMMCs. Cells were cultured for 2 h with mouse IgE anti-TNP mAb, washed, loaded with Indo-1AM, and then stimulated with TNP-OVA at the time indicated by the blank space. Intracellular Ca^{2+} mobilization is shown as the FL4:FL5 fluorescence intensity ratio over time. Clones SVMC-c and -E gave similar Ca^{2+} mobilization profiles to SVMC-a.

stimuli in long-term cultures in order to investigate the biological functions of homogenous MCs *in vitro*. We established such MC lines, SVMC-a, -c, and -E, from BM cells of tsSV40LT mice. The SVMC clones appeared to be grossly similar to BMMCs in morphological and cytochemical characters, expression levels of surface markers, and in the cell cycle profiles.

Moreover, phenotypic changes from MMC- to CTMC-like cells could be induced in the presence of IL-3, NGF and SCF, as observed for BMMCs. These cell lines, however, showed some differences in morphology and biochemical responses compared to those of BMMCs, such as enlargement of its granules, an increased growth rate, and enhanced degranulation and intracellular Ca^{2+} influx.

Day *et al.* (*24*) reported that Rb-deficient murine prostate epithelial cell lines exhibited a higher rate of cell growth and a similar histogram profile on cell cycle analysis with an elevated S-phase DNA content, and that these cell lines developed into fully differentiated and morphologically normal prostate tissue. Our results agree with their report, and it is suggested that the phenotypic characteristics and normal differentiation of SVMC clones, which are comparable with those of BMMCs, are attributable to a similar profile of cell cycle progression, such as major G_0/G_1 and short G_2 + M distributions. However, SVMC clones exhibit an increase in IL-3–stimulated [^{3}H]-thymidine incorporation compared to BMMCs. It is possible that the enhanced DNA synthesis by SVMC clones is due to a slight decrease in the percentage of G_0/G_1 and accelerated S-phase progression. These data are consistent with previous studies, in which SV40 large T antigen was found to enhance G_1/S progression by inactivating the Rb tumor suppressor protein, which regulates the progression through the G_1/S transition of the cell cycle (*25*, *26*).

The MCs that reside in the gastrointestinal mucosa of mouse and rat, termed MMC, can be distinguished by several criteria from those that reside in the serosal cavity, termed CTMC. Functionally active MCs can differentiate and proliferate when progenitor cells from BM or other hematopoietic tissues are cultured in the presence of IL-3 (*1–6*). These *in vitro*–differentiated cells were considered to be MMC-like cells based on the criteria that they failed to be counterstained with safranin, because they preferentially synthesize chondroitin sulfate proteoglycans rather than heparin proteoglycans, and they had a limited number of secretory granules (*1–6*). Recent experiments have shown that NGF and SCF induce a phenotypic change of the cells from MMC- to CTMC-like cells *in vitro*, although their contribution to MC development *in vivo* is rather obscure (*11*, *27–32*). In accordance with previous findings for BMMCs, our SVMC clones, when NGF and

SCF were added to the culture medium containing IL-3, showed increased cytoplasmic granule contents and an augmented phenotypic change from alcian blue MMC-like to safranin CTMC-like cells with similar efficiency to BMMCs, which was accompanied by up-regulated mMCP-4 mRNA expression and increased chymotrypsin-like activities. This is the first description of the generation of immortalized murine MC lines able to change phenotypically from MMC-like to CTMC-like ones (*31–36*). These results suggest that the expression of SV40 large T antigen in MCs is sufficient for immortalization of these cells without any gross alterations in their differentiated phenotypes. Unexpectedly, however, the stimulation of SVMC clones mediated by FcεRI cross-linking yielded a significantly higher level of degranulation than for BMMCs. As was observed in the assay on serotonin release, SVMC clones exhibited a marked increase in the intracellular Ca^{2+} elevation. Currently, the mechanism underlying the enhanced FcεRI-mediated stimulation of SVMC clones is not known.

It has been reported that IL-3–independent mouse MC lines were established by co-culturing splenocytes with fibroblasts shedding Kirsten sarcoma virus (KiSV) (*37*) or infection of fetal liver cells with Abelson-MuLV (Ab-MuLV) (*38*). In addition, IL-3–dependent mouse MC lines have also been generated by infection of splenocytes with Harvey sarcoma virus (HaSV) (*39*), or by infection of BMMCs with recombinant retrovirus expressing the proto-oncogene c-myc (*40*) or adenovirus 12–SV 40 (Ad12-SV40) (*12*). These results show that the ability to confer growth factor independency appears to be a relatively specific effect of the viruses, and that SV40 large T antigen is not sufficient to eliminate the requirement of growth factors. Furthermore, the morphological and cytochemical features of these virus-infected immortalized MCs are similar to those of BMMCs, as observed for SVMC clones. Indeed, KiSV-transformed MC lines generated from splenocytes exhibited characteristics similar to those of CTMC-like cells, while Ad12-SV40–transformed ones generated from BMMCs exhibited characteristics similar to those of MMC-like cells. However, SVMC clones have the advantage over virus-infected MCs in that SVMC clones can be induced to show a phenotypic change from MMC- to CTMC-like cells, and that they do not shed infectious virus into the medium. We suppose that these differences are attributable to the differences in the culture conditions, such as co-culturing with virus-producer fibroblasts or exposure to virus-containing supernatants, and the cells used as the source of the culture, such as splenocytes or BMMCs.

To date, information is limited regarding the specific molecule and gene expression profile in primary mast cells with exogenous stimuli, such as antigens and growth factors. SVMCs exhibit high sensitivity to antigen stimulation and can differentiate into CTMC-like cells in response to appropriate growth factors like primary mast cells. Our data reveal that SVMCs, especially SVMC clones, can provide unique systems for studying the kinetics of specific molecule and gene expression in a homogeneous population. Also, these SVMC clones will serve as useful *in vitro* models for investigating the process of differentiation and maturation of murine MCs, which is important for delineating novel approaches toward the conquest of MC-based disorders such as allergy and autoimmune diseases.

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