

Cultivation and Characterization of Canine Skin-Derived Mast Cells

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ABSTRACT. It is essential to develop a technique to culture purified skin-derived mast cells (SMCs) to facilitate immunological research on allergic diseases in dogs. This study was performed to develop an efficient culture system for canine SMCs and to characterize the cells in comparison to canine bone marrow-derived mast cells (BMMCs). Enzymatically digested skin biopsy samples were cultivated in serum-free AIM-V medium supplemented with recombinant canine stem cell factor. Three to five weeks after the initiation of culture, mast cells were collected by a magnetic activated cell separation system using anti-c-Kit antibody. The collected cells were composed of a uniform population showing morphological characteristics of mast cells with a round or oval nucleus and abundant toluidine blue-positive metachromatic granules in the cytoplasm. The results of flow cytometric analysis for the presence of cell membrane c-Kit and Fc epsilon receptor 1 (FcεRI) indicated that approximately 90% of the cells were mast cells. The cytoplasmic granules were positive for both tryptase and chymase. Apparent dose-dependent degranulation was induced by antibody-mediated cross-linking of immunoglobulin E (IgE) bound to the cells. These cytological and immunological characteristics observed in SMCs were mostly similar to those observed in BMMCs; however, IgE-mediated degranulation was significantly lower in SMCs than BMMCs. The culture system for canine SMCs developed in this study would be useful in understanding the pathophysiology and developing anti-allergic therapeutics in canine allergic dermatitis.

KEY WORDS: bone marrow, canine, culture, mast cells, skin.

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Allergic dermatitis in dogs is a complex of inflammatory skin diseases primarily mediated by type I hypersensitivity [8]. Cross-linking of the surface Fc epsilon receptor 1 (FcεRI)-bound immunoglobulin E (IgE) with allergens results in the release of various mediators from mast cell granules, inducing type I hypersensitivity [5, 6]. Studies using an experimental allergic model have revealed that cytokines and chemokines released from mast cells induce infiltration of leukocytes, leading to a late-phase reaction [42].

In dogs, histopathological examination of the skin lesions of atopic dermatitis (AD) has revealed infiltration of mast cells, Langerhans cells, and lymphocytes in addition to a small number of eosinophils and neutrophils into the lesional skin [14]. The presence of allergen-specific IgE-bearing mast cells in the dermis of allergic dogs has been shown [13], and both the number of mast cells and the histamine concentration in the dermis have been shown to correlate with clinical symptoms of AD [28]. As shown in the spontaneously developed AD in dogs, in a canine experimental model of AD, epicutaneous application of an aller-

gen slurry has been shown to generate a penetration of allergen into the dermis, an elevated allergen-specific serum IgE, and localized pruritic dermatitis with typical histological changes by IgE-induced inflammation [32]. Moreover, since survival and growth of mast cells are known to be promoted by binding of IgE to FcεRI [2], it can be considered that interaction between mast cells and IgE is associated with the pathogenesis of allergic dermatitis in dogs. To elucidate the role of mast cells in allergic dermatitis in dogs, it has been required to develop a technique for the use of purified skin-derived mast cells (SMCs) from allergic and non-allergic dogs.

Precursors of mast cells originate from bone marrow and differentiate into mature cells after migration to various tissues [10, 20, 23, 37]. These mature cells express cell membrane FcεRI and c-Kit and contain cytoplasmic granules harboring neutral proteases and heparin [10]. Generally, c-Kit is expressed in mast cells, and interaction between c-Kit and its ligand, stem cell factor (SCF), is essential for the development, growth, survival, and maturation of mast cells [10, 29, 40, 41]. These mature mast cells exhibit heterogeneous phenotypes specific to their tissue environments [10, 15]. In rodents, histochemical stainings have been used to discriminate between connective tissue-type mast cells (CTMC) and mucosal mast cells (MMC); the former are predominantly found in the skin and peritoneal cavity, and

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the latter are mainly observed in the intestinal lamina propria [10]. In humans, the composition of neutral proteases has been utilized to define the subtypes of mast cells. Mast cells containing tryptase, chymase, mast cell carboxypeptidase, and cathepsin G are designated as MC_{TC}, whereas mast cells containing only tryptase are represented as MC_T. MC_{TC} and MC_T are known to be distributed in the skin and lung, respectively [10, 15]. Similar to humans, neutral proteases in dogs are also considered to define the tissue-specific subtypes of mast cells [21].

Culture techniques have been developed to obtain a pure population of human mast cells derived from bone marrow [37], cord blood [36], peripheral blood [35], the fetal liver [19], skin [18], and the intestine [3]. Kambe *et al.* [18] reported a successful culture system for proliferation of mature SMCs derived from dispersed human skin samples in a serum-free culture medium supplemented with recombinant human SCF. In dogs, cell lines derived from mast cell tumors have been used as a pure population of mast cells, but malignant transformation substantially alters the structure and function of mast cells [12, 24]. A procedure to culture canine bone marrow-derived mast cells (BMMCs) in order to obtain non-neoplastic progenitor cells has recently been reported [23]. Furthermore, de Mora *et al.* [7] have reported an assay system for histamine release from canine mast cells included in dispersed cells obtained from the skin tissues after treatment with collagenase, hyaluronidase, and protease. However, they reported that mast cells constituted only 1% of the total dispersed cells obtained from the enzymatically digested canine skin tissues. Therefore, a culture technique to obtain a pure population of canine SMCs has not been reported so far.

The present study was performed to develop a system to culture canine SMCs in order to facilitate immunological and pharmacological research on canine allergic skin diseases. Moreover, cytological and immunological characteristics of canine SMCs were compared to those of canine BMMCs.

MATERIALS AND METHODS

Dogs: Fourteen 1- to 2-year-old healthy Beagle dogs were used. They included 7 neutered males and 7 neutered females. All the experimental procedures were carried out according to the "Guidelines for the Care and Use of Laboratory Animals" issued by The University of Tokyo. Two to five grams of skin tissues at the lateral side of the abdomen or neck were obtained by surgical skin resection. Bone marrow specimens were obtained from the humerus or femur by using a bone marrow aspiration needle (15G, 2 inches; MDTech, Gainesville, FL, U.S.A.). Both skin resection and bone marrow aspiration were performed in the dogs under inhalation anesthesia with isoflurane after preanesthetic induction with acepromazine, butorphanol, and propofol.

Isolation and cell culture conditions for canine SMCs: Surgically resected skin tissues were placed in 70% ethanol for 5 min and then washed with phosphate buffer saline

(PBS). Subcutaneous fat was removed by blunt dissection, and the residual cutaneous tissues were minced with surgical scalpels. The minced tissues were treated with a solution containing a collagenase mixture [2.0 mg/ml collagenase from *Clostridium histolyticum* (Sigma-Aldrich, St. Louis, MO, U.S.A.), 700 U/ml hyaluronidase from bovine testes (Sigma-Aldrich), and 300 U/ml DNase I from the bovine pancreas (Sigma-Aldrich)], 20% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, U.S.A.), and 1% antibiotic antimycotic solution (ABAM; 100 units/ml penicillin G, 100 ng/ml streptomycin sulfate, and 250 ng/ml amphotericin B; Sigma-Aldrich) in Minimum Essential Medium Eagle alpha modification (α -MEM; Sigma-Aldrich) at 37°C for 90 min with constant shaking. After sequential filtration through 100- and 40- μ m cell strainers (BD Falcon, Bedford, MA, U.S.A.), the dispersed cells were suspended in Hanks' balanced salts solution with 10 mM HEPES, 20% FBS, and 1% ABAM and centrifuged at $300 \times g$ for 5 min. The cells obtained from 1 g of skin tissues were cultivated in serum-free AIM-V medium (Invitrogen) supplemented with 50 ng/ml recombinant canine SCF (rcSCF; R&D Systems, Minneapolis, MN, U.S.A.) and antibiotics (1% ABAM and 100 ng/ml Kanamycin) in a 75 mm² flask at 37°C in a humidified atmosphere with 5% CO₂. Half of the medium was renewed every 5 days. When the cells adherent to the flask reached confluence, they were subcultured after dispersing the cells with 0.02 mM EDTA-2Na.

After cultivation for 3–5 weeks, the cells were dispersed and filtered through 40- μ m cell strainers and then purified with a magnetic-activated cell separation system (MACS) (Miltenyi Biotec, Bergisch Gladbach, Germany). The cells were washed twice in PBS containing 5% bovine serum albumin (BSA), 0.02 mM EDTA-2Na, and 10 mM HEPES and then treated with anti-mouse c-Kit antibody-coated magnetic microbeads (Miltenyi Biotec) at 4°C for 10 min. In our preliminary experiment, the cells separated by MACS using the anti-mouse c-Kit antibody-coated magnetic microbeads were shown to be stained with an anti-mouse c-Kit antibody (clone ACK45; BD Pharmingen, San Diego, CA, U.S.A.) that has been reported to be cross-reactive to canine c-Kit [22–24]. The c-Kit-positive cells were isolated by an LS separation column (Miltenyi Biotec) and cultured at 1×10^5 cells/ml in conditioned AIM-V medium (50% fresh medium plus 50% used medium) supplemented with 100 ng/ml rcSCF and the antibiotics. Half of the culture medium was renewed every 3 days. After cultivation for 1–2 weeks, the purity of mast cells was assessed through analysis of c-Kit- and Fc ϵ RI-double positive cells by flow cytometry.

Isolation and cell culture conditions for canine BMMCs: The procedures to culture canine BMMCs were principally based on those reported previously [20, 23]. Using cells obtained by bone marrow aspiration, mononuclear cells were collected by Ficoll-Hypaque density gradient centrifugation and then diluted with PBS containing 0.6% anticoagulant citrate dextrose formula A and 0.5% BSA to cell concentration of 1.25×10^8 cells/ml. After blocking with heat-inactivated pooled canine sera at 4°C for 30 min, the

cells were treated with anti-canine CD34 antibody (clone 1H6; BD Pharmingen) and then reacted with anti-mouse IgG₁ antibody-coated microbeads (Miltenyi Biotec) at 4°C for 10 min. The CD34-positive cells were collected using an LS separation column, and the purity was assessed by flow cytometry using the combination of biotin-labeled anti-canine CD34 antibody (clone 2E9; BD Pharmingen) and streptavidin-PerCP-Cy5.5 (BD Pharmingen). Cells with more than 90% purity were cultured at 2.5×10^5 cells/ml in serum-free StemSpan H3000 medium (StemCell Technologies, Vancouver, BC, Canada) supplemented with rSCF (50 ng/ml) and human recombinant Flt-3 ligand (R&D Systems; 100 ng/ml) for 1 week. Subsequently, the serum-free StemSpan H3000 medium was changed to AIM-V medium supplemented with rSCF (50 ng/ml) and the antibiotics. After cultivation for 3–5 weeks, c-Kit-positive cells were isolated and cultivated in the same manner as canine SMCs.

Wright-Giemsa staining: Cells (1.0×10^4 cells) were cytocentrifuged onto glass slides using Shandon Cytospin[®] 4 (Thermo Fisher Scientific, Waltham, MA, U.S.A.). The preparations were air-dried, fixed in methanol, and stained with Wright-Giemsa solution [10% Wright's eosin methylene blue solution (Merck, Darmstadt, Germany) and 10% Giemsa's azur eosin methylene blue solution (Merck) in PBS].

Toluidine blue staining: Cytocentrifuged preparations of the cells were air-dried, fixed in Carnoy's solution (60% ethanol, 30% chloroform, and 10% acetic acid) and stained with 0.05% toluidine blue (pH 7.0; Muto Pure Chemicals, Tokyo, Japan).

Flow cytometry: The procedures to detect cell membrane FcεRI and c-Kit in canine mast cells were based on a previous report [30] with slight modifications. To examine the FcεRI expression on cultured cells, the cells were incubated with pooled serum derived from dogs that had been experimentally hypersensitized with Japanese cedar pollen as reported previously [43]. The titer of IgE specific to Japanese cedar pollen antigen in the pooled serum was shown to be more than 2,500 laboratory units by FcεRIα-based ELISA (Allercept[®] Definitive Allergen Panels, Heska, Ft Collins, CO, U.S.A.) [9]. Cells were incubated with 10% of the IgE-rich dog serum and 0.25 μg/ml ethidium monoazide (EMA; Molecular Probes, Eugene, OR, U.S.A.) in a wash buffer (PBS containing 2% FBS) for 10 min. The cells were then exposed to a fluorescent white-light source for 15 min. Thereafter, the cells were fixed with formaldehyde-containing permeabilizing solution (Cytofix/Cytoperm, BD Pharmingen). The fixed cells were washed twice with a saponin-containing wash buffer (Perm/Wash, BD Pharmingen) and then labeled with RPE-conjugated rat anti-mouse c-Kit antibody (clone ACK45; BD Pharmingen) and FITC-conjugated goat anti-dog IgE antibody (Bethyl Laboratories, Montgomery, TX, U.S.A.) for 30 min. The RPE-conjugated rat IgG_{2bκ} (clone A95-1; BD Pharmingen) and FITC-conjugated goat IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, U.S.A.) were used as the isotype controls.

The procedures were performed on ice in the dark except during the light exposure process. The percentage and mean fluorescent intensities (MFI) of the viable cells (1.0×10^4 cells) stained with FITC-conjugated anti-dog IgE antibody and RPE-conjugated anti-mouse c-Kit antibody were analyzed with a flow cytometer (FACSCalibur, Becton Dickinson, Franklin Lakes, NJ, U.S.A.) using the CellQuest software (Becton Dickinson). Dead cells were excluded by staining with EMA [34]. Cells with more than 80% purity were used for the following study.

Transmission electron microscopy: The cultured cells were fixed in 2% glutaraldehyde, washed in PBS and then post-fixed in 1% osmium tetroxide. After stepwise dehydration with ethanol (70–100%), the cells were embedded in Spurr's resin, and ultra thin sectioning was performed (800–1000 Å) using an ultratome (LKB U-5 Ultratome, LKB, Sweden). The cells were stained with 4% uranyl acetate and then with Reynolds lead citrate. The sections were observed with a transmission electron microscope (JEOL 1200EX, JEOL, Tokyo, Japan).

Immunocytochemistry for tryptase: For detection of tryptase, immunocytochemistry was performed using an R.T.U. Vectastain Universal Quick Kit (Vector Laboratories, Burlingame, CA, U.S.A.). Cytocentrifuged preparations were fixed in Carnoy's solution at 4°C for 10 min and washed twice in PBS. Endogenous peroxidase was blocked with 3% H₂O₂ in methanol at room temperature for 5 min. After blocking with 2.5% heat-inactivated horse serum, the preparations were incubated overnight with mouse anti-human tryptase monoclonal antibody (clone AAI; Serotec, Oxford, U.K.) or mouse IgG₁ isotype control (Rockland, Gilbertsville, PA, U.S.A.) diluted with 1.5% heat-inactivated horse serum in PBS at 4°C. The anti-human tryptase monoclonal antibody has previously been shown to be cross-reactive with canine tryptase by immunohistochemistry [31]. The preparations were incubated with biotinylated panspecific universal secondary antibody and then a biotin-streptavidin peroxidase complex method was carried out according to the manufacturer's protocol. The immunoreactivity was visualized by using a Diaminobenzidine (DAB) Substrate Kit (Vector Laboratories) and counterstaining with hematoxylin. To assess the percentage of tryptase-positive cells in each preparation, 200 cells on 2 glass slides were counted by light microscopic examination.

Enzyme cytochemistry for chymase: Chymase was detected by enzyme cytochemistry using a Naphthol AS-D Chloroacetate Esterase Kit (Sigma-Aldrich) according to the manufacturer's protocol. The cytocentrifuged preparations were fixed with a citrate/acetone/formaldehyde mixture and then incubated in Naphthol AS-D chloroacetate substrate solution at 37°C for 15 min. The preparations were counterstained with hematoxylin. The percentage of chymase-positive cells was counted with the same method used for the analysis of tryptase-positive cells.

β-hexosaminidase release assay: Cross-linking of cell surface FcεRI on the mast cells was performed according to the method reported by Ohmori *et al.* [30]. Cells (1.5×10^5

cells) were incubated with 15% IgE-rich dog serum in Tyrode's buffer (Sigma Aldrich) containing 10 mM HEPES and 0.1% BSA at 37°C for 60 min and then stimulated with goat anti-canine IgE antibody (Bethyl Laboratories) at 37°C for 60 min with constant shaking. After centrifugation of the stimulated cells at $500 \times g$ for 3 min, the supernatant and cell pellet were separately collected to quantify β -hexosaminidase activity. The cell pellet was lysed with 0.1% Triton X-100 in Tyrode's buffer. β -hexosaminidase activity in the supernatant and lysate was measured by hydrolysis of *p*-nitrophenyl-*N*-acetyl- β -D-glucopyranoside (Sigma-Aldrich) at 37°C for 90 min. Absorbance of the hydrolyzed substrate was measured using a microplate reader (MPR-A4, TOSOH, Tokyo, Japan) at 405 nm. The percentage of β -hexosaminidase release was calculated as follows: (β -hexosaminidase release after stimulation – spontaneous β -hexosaminidase release)/(total β -hexosaminidase activity – spontaneous β -hexosaminidase release).

Statistical analysis: The Wilcoxon rank-sum test was performed to compare differences between canine SMCs and BMMCs in the levels of MFI analyzed by flow cytometry and in the percentage of β -hexosaminidase release through the cross-linking of Fc ϵ RI. *P* values less than 0.05 were considered significant. Statistical analysis was performed using the JMP statistical software version 4 (SAS Institute, Cary, NC, U.S.A.).

RESULTS

Culture of canine SMCs: During the early phase of culture, round cells with a round to oval nucleus were observed on adherent fibroblast-like cells. After isolation by MACS using anti-c-Kit antibody-coated magnetic microbeads, canine SMCs did not adhere to the culture flask, formed floating aggregates (Fig. 1a), and contained cytoplasmic toluidine blue-positive granules in the cytoplasm (Fig. 1c). The total number of canine SMCs was $9.8 \times 10^5 \pm 5.5 \times 10^5$ cells (mean $\pm$ standard deviation, SD) (number=10 dogs) from 1 g of wet skin tissues. The percentages of Fc ϵ RI- and c-Kit-double positive cells were 80.9–98.9% ($90.1 \pm 6.6\%$, *n*=10; Fig. 2a and 2b).

Culture of canine BMMCs: Canine CD34-positive cells ($2.8 \times 10^6 \pm 1.9 \times 10^6$ cells, *n*=6) were obtained after separation from bone marrow mononuclear cells by MACS using anti-CD34 antibody. After cultivation for 3–5 weeks, the cells were further isolated by MACS using anti-c-Kit antibody. These cells showed a dispersed growth pattern that was different from the aggregate pattern observed in canine SMCs, and they also had an oval nucleus and toluidine blue-positive cytoplasmic granules (Fig. 1d and 1f). The total number of canine BMMCs was determined to be $1.5 \times 10^6 \pm 1.1 \times 10^6$ cells from 1×10^6 cells of CD34-positive bone marrow cells (*n*=6), and the percentages of the double-positive cells were 94.0–99.5% ($97.3 \pm 1.9\%$, *n*=6; Fig. 2c and

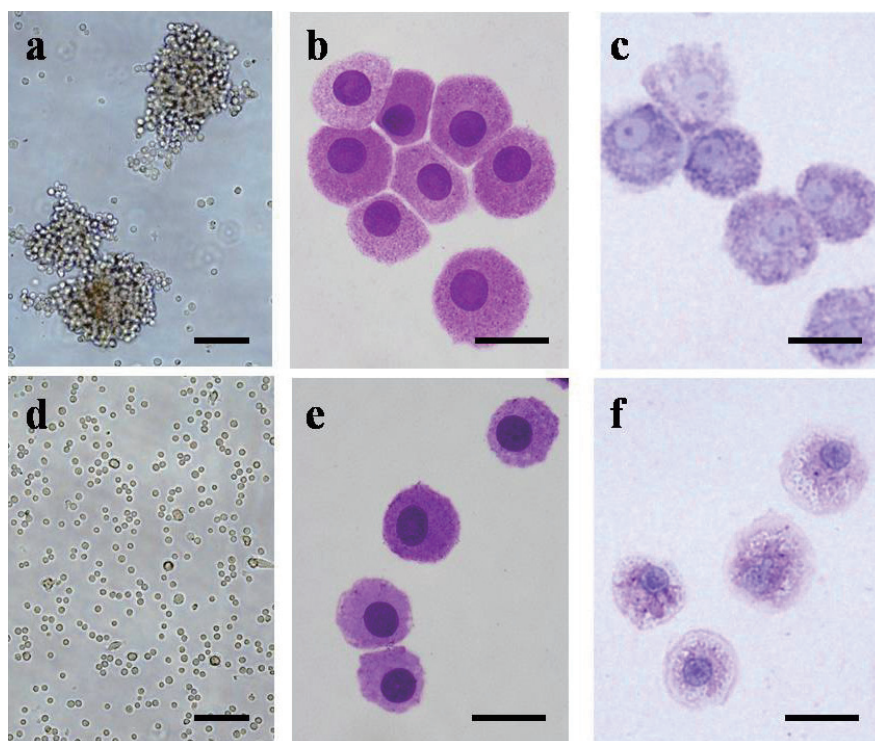


Fig. 1. Microscopic appearances of canine SMCs and BMMCs 5 weeks after initiation of culture. Phase-contrast microscopic appearances (a, d), Wright Giemsa-stained preparations (b, e) and toluidine blue-stained preparations (c, f) of the canine SMCs (a, b, c) and BMMCs (d, e, f). Bars=100 μ m (a, d), 20 μ m (b, c, e, f).

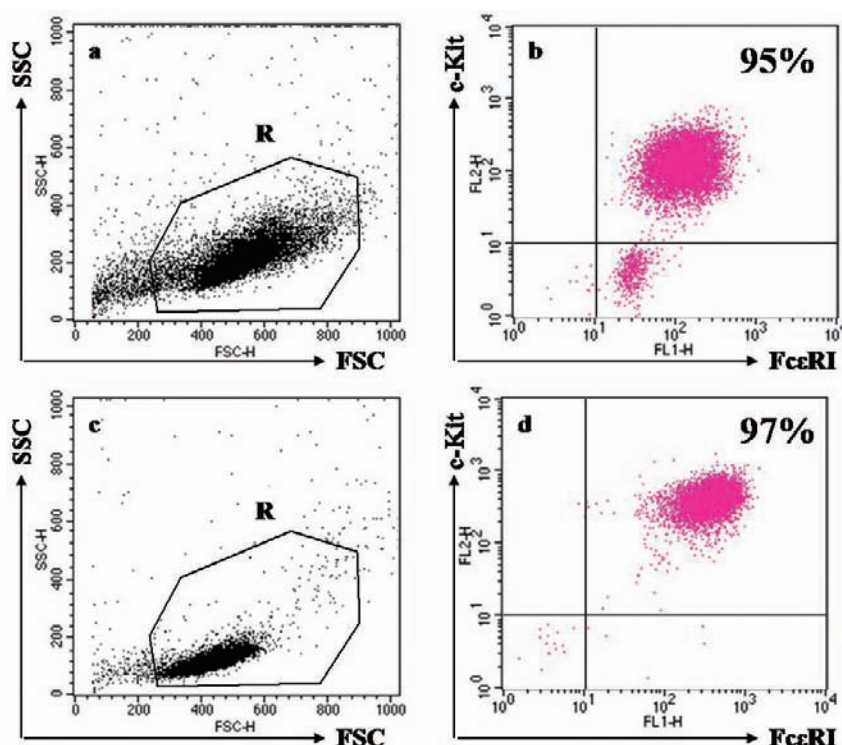


Fig. 2. Flow cytometric analysis for the expression of Fc ϵ RI and c-Kit in canine SMCs and BMMCs 6 weeks after initiation of culture. Canine SMCs and BMMCs were incubated with IgE-rich dog serum and then stained with FITC-conjugated goat anti-dog IgE antibody as well as RPE-conjugated anti-mouse c-Kit antibody. Dot plots of forward scatter (FSC) versus side scatter (SSC) for canine SMCs (a) and BMMCs (c) are shown. Dead cells stained with EMA in region R were excluded, and the other viable cells (1.0×10^4 cells) were analyzed in the quadrant plots of Fc ϵ RI versus c-Kit (b, d). The percentages of events indicating Fc ϵ RI and c-Kit double-positive cells in canine SMCs (b) and BMMCs (d) are shown in the figure. The threshold lines of the Fc ϵ RI- and c-Kit-positive cells were set from the events stained with each isotype control antibody. The data are representative of 3 independent experiments from individual dogs.

2d).

Light microscopic morphology: Both the canine SMCs and BMMCs were shown to be mostly uniform populations of single round cells having a round to oval nucleus of a relatively uniform size and abundant cytoplasm that contained a number of metachromatic granules (Fig. 1b and 1e). The diameters of the canine SMCs and BMMCs were $21.0\text{--}28.5\text{ }\mu\text{m}$ and $18.5\text{--}23.5\text{ }\mu\text{m}$, respectively.

Flow cytometry: The levels of MFI of Fc ϵ RI and c-Kit in the canine SMCs ($n=3$) were 115.8 ± 11.9 (mean $\pm$ SD) and 105.2 ± 72.3 , respectively, and those in the canine BMMCs ($n=5$) were 251.8 ± 132.2 and 152.7 ± 152.8 , respectively. No significant difference was observed between the canine SMCs and BMMCs 5–8 weeks after initiation of culture in the levels of MFI of both Fc ϵ RI and c-Kit; however, the level of MFI of Fc ϵ RI in the canine SMCs was relatively lower than that in the canine BMMCs ($P=0.17$; Fig. 2).

Ultrastructural analysis: The ultrastructural morphology of canine SMCs was compared with that of canine BMMCs by transmission electron microscopy. Both canine SMCs

and BMMCs had a round to oval nucleus, irregular cell membranes with cytoplasmic projections, and abundant electron-dense cytoplasmic granules (Fig. 3a and 3c). The diameters of the canine SMCs and BMMCs were $8.0\text{--}13.0\text{ }\mu\text{m}$ and $7.5\text{--}11.0\text{ }\mu\text{m}$, respectively. In both the canine SMCs and BMMCs, the majority of the cytoplasmic granules were homogeneous and electron-dense particulates (Fig. 3b); however, a few granules were discrete scroll particulates (Fig. 3d).

Neutral protease phenotypes: The presence of both tryptase and chymase in the cytoplasmic granules was examined 4–7 weeks after initiation of culture. The percentages of tryptase-positive cells in the canine SMC and BMMC preparations were $96.4 \pm 2.3\%$ ($n=4$; mean $\pm$ SD) and $95.2 \pm 4.2\%$ ($n=6$), respectively. The percentages of chymase-positive cells in the canine SMC and BMMC preparations were $97.7 \pm 2.1\%$ ($n=5$) and $99.5 \pm 0.9\%$ ($n=6$), respectively. These results indicated that both the canine SMCs and BMMCs were MC_{TC} cells (Fig. 4).

Reactivity to Fc ϵ RI-mediated stimulation: A β -hex-

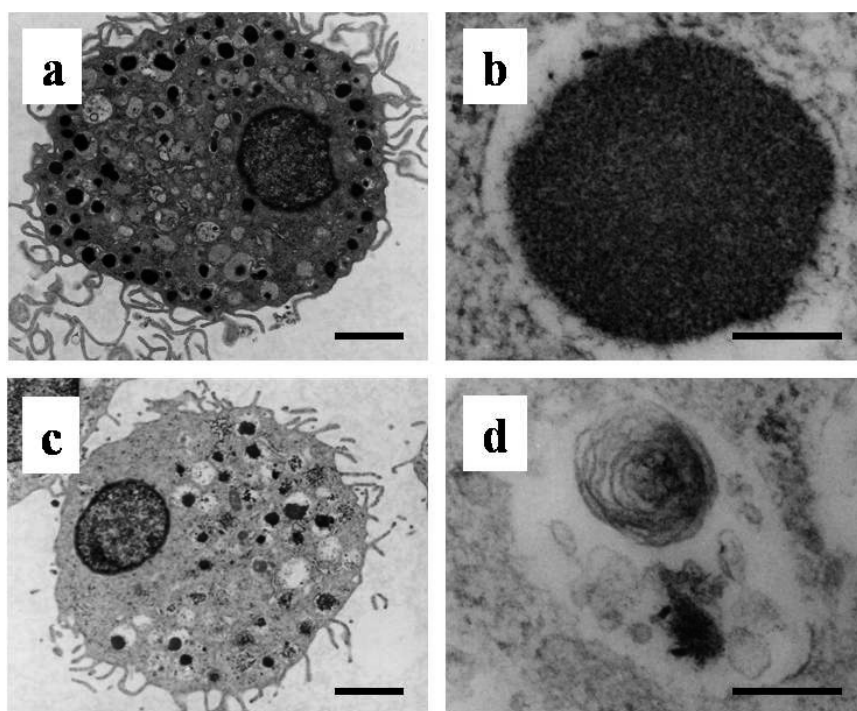


Fig. 3. Transmission electron micrographs of canine SMCs and BMMCs 6 weeks after initiation of culture. (a) A canine SMC, (b) homogeneous electron-dense granule in a canine SMC, (c) canine BMMC, (d) discrete scroll granule in a canine BMMC. Bars = 2 μ m (a, c), 200 nm (b, d).

osaminidase release assay was performed to examine the degranulation ability of canine SMCs and BMMCs through the cross-linking of Fc ϵ RI using goat anti-IgE antibody. β -hexosaminidase release through the cross-linking of Fc ϵ RI was observed in both the canine SMCs and BMMCs in a dose dependent manner (Fig. 5). The maximum release was observed after incubation with 3.0 μ g/ml of goat anti-dog IgE in both the canine SMCs and BMMCs. The percentage of β -hexosaminidase release was significantly less in the canine SMCs than in the canine BMMCs after incubation with 0.1, 0.3, 1.0, 3.0 and 10.0 μ g/ml of goat anti-dog IgE ($P < 0.05$).

DISCUSSION

In this study, successful cultivation of canine SMCs was achieved by employing a culture method similar to that used for culturing human SMCs [18]. The percentage of c-Kit- and Fc ϵ RI-double positive cells in the flow cytometric analysis was approximately 90%, indicating that most of the culture cells were mast cells. In addition, the cultured cells showed characteristics of mast cells such as metachromatic and tryptase- and chymase-positive granules in the cytoplasm. Furthermore, the cultured cells showed degranulation through cross-linking of Fc ϵ R, indicating a functional feature of mast cells.

In humans, cultivation of mast cells in serum-containing medium reduces the proliferation capacity of the mast cells

because the serum-containing medium promotes growth of other cell types that englobe mast cells [18, 19]. Lin *et al.* [23] successfully established a culture method for generation of canine BMMCs using a serum-free medium. A serum-free medium was also used in the present study, which allowed the proliferation of cells with typical characteristics of mast cells. It is conceivable that a serum-containing medium is not appropriate for culture of canine mast cells. Therefore, the culture technique employed in this study is conceivably appropriate for culture of mast cells from skin tissues in dogs.

Lin *et al.* [23] reported that apparent proliferation of canine BMMCs was observed after cultivation for 4–7 weeks; however, the cells did not survive beyond 8 weeks after initiation of culture. In the present study, we observed that canine SMCs began to decrease in number approximately 8 weeks after initiation of culture and could not survive for more than 12 weeks in most instances.

In the light microscopic examinations, both the canine SMCs and BMMCs were morphologically similar to mature mast cells having a round to oval nucleus of a relatively uniform size and abundant cytoplasm containing a large number of metachromatic and tryptase- and chymase-positive granules. The cytological characteristics of the canine SMCs and BMMCs were quite different from those of the cells of the canine mast cell tumor cell line (HRMC) [30] in which an increased nuclear-cytoplasmic ratio, anisokaryosis, and decreased number of cytoplasmic granules have

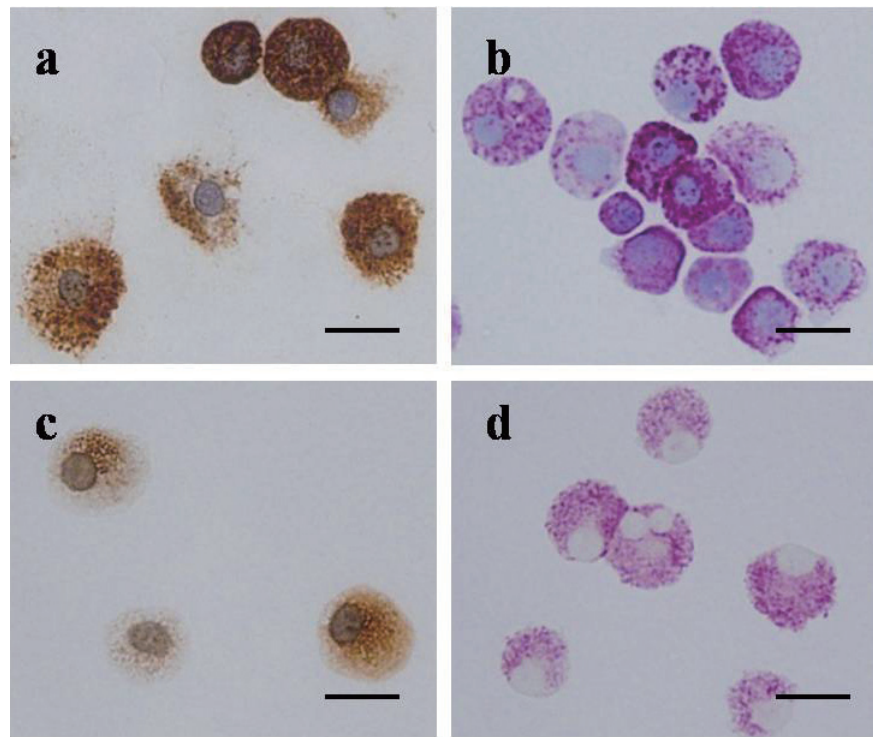


Fig. 4. Detection of cytoplasmic tryptase and chymase activity in canine SMCs and BMMCs 6 weeks after initiation of culture. Immunocytochemistry for detection of tryptase (a, canine SMCs; c, canine BMMCs). The tryptase-positive granules are stained brown. Enzyme cytochemistry for detection of chymase (b, canine SMCs; d, canine BMMCs). The chymase-positive granules are stained red. Bars =20 μ m.

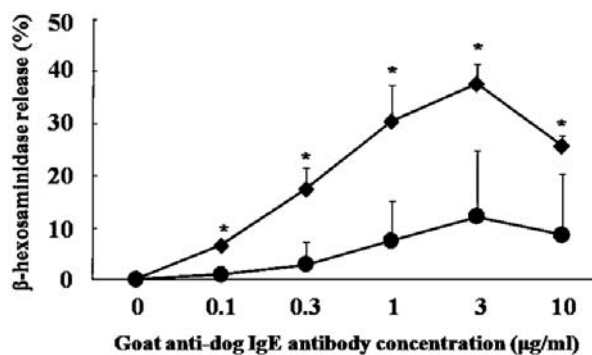


Fig. 5. β -hexosaminidase release assay in canine SMCs and BMMCs 6 weeks after the initiation of culture. Canine SMCs and BMMCs were sensitized with IgE-rich dog serum and then treated with various concentration of goat anti-dog IgE antibody. The percentage of β -hexosaminidase release induced by cross-linking of Fc ϵ RI was measured 1 hr after treatment with anti-dog IgE antibody. The symbols (● for canine SMCs, ◆ for canine BMMCs) and bars represent the means and SDs, respectively, determined from 3 experiments using individual dogs. * $P < 0.05$ was considered to indicate a significant difference between the canine SMCs and BMMCs.

been observed. These findings indicate that both canine SMCs and BMMCs retain the morphological characteristics observed in mature mast cells.

Garcia *et al.* [12] compared the ultrastructural morphology between the cells of the canine mast cell tumor cell line (BR) and mature cutaneous mast cells isolated from enzymatically digested skin tissue of healthy dogs. The numbers of electron-dense cytoplasmic granules and cytoplasmic projections were larger in the mature cutaneous mast cells than in the BR cells. These cytological characteristics of BR cells are essentially similar to those of the cells of other canine mast cell tumor cell lines previously reported [1, 30]. In contrast to the tumor cell lines, the ultrastructural morphology of the canine SMCs in the present study was similar to that of the mature cutaneous mast cells reported by Garcia *et al.* [12], having a number of cytoplasmic granules and cytoplasmic projections. These results indicate that the cultured canine SMCs obtained in this study had the structure characteristics of canine mature mast cells.

In the present study, canine BMMCs were obtained from CD34-positive cells by using recently reported culture techniques for canine BMMCs [23] and human BMMCs [20]. Lin *et al.* [23] reported light microscopic observations of canine BMMCs and described that they had an oval nucleus and an abundant cytoplasm containing a large number of

metachromatic and tryptase- and chymase-positive granules. In the present study, we also examined the ultrastructural morphology of canine BMMCs: they were shown to have a round to oval nucleus, apparent cytoplasmic projections, and abundant electron-dense cytoplasmic granules. These light microscopic and ultrastructural observations for the canine BMMCs in this study were generally similar to those of human BMMCs [20, 37]. These findings indicate that canine BMMCs should be considered useful as a source of canine mature mast cells.

Two types of mast cells have been recognized depending on their phenotypes of different combinations of tryptase and chymase in the cells of humans and rodents [10, 15]. Mast cells containing tryptase are called MC_T and are mainly found in mucosal tissues (also called MMC). Mast cells with both tryptase and chymase in the cytoplasmic granules are called MC_{TC} and are found in connective tissues (also called CTMC) [10]. In the present study, since it was revealed that the cultured canine SMCs contained both tryptase and chymase, the cells were considered to have a CTMC-like phenotype. In mice, CTMC can be generated from bone marrow-derived mast cells under culture conditions supplemented with SCF and nerve growth factor [26]. IL-3 and SCF are also known to maintain the CTMC phenotype in the rat [41]. Considering these previous studies, the culture conditions supplemented with SCF in this study might be prone to generate CTMC rather than MMC from both skin tissue and bone marrow. Propagation of MMC is known to depend on several T-cell derived cytokines [10]. Some culture conditions different from those used in this study may be appropriate for generation of MMC, in which appropriate cytokines or factors are often added.

Our results showed that canine SMCs had lower susceptibility to FcεRI-mediated degranulation than canine BMMCs. Degranulation of mast cells is known to be under the control of signaling cascades after activation by cross-linking of IgE bound to the cell surface FcεRI [17]. In the present study, flow cytometric analysis showed that the amount of expression of FcεRI in the canine SMCs was slightly lower than that in the canine BMMCs. Takano *et al.* [39] established a culture system for murine mast cells that exhibited characteristics of CTMC-like cells by co-cultivation of murine BMMCs with Swiss 3T3 fibroblasts. These CTMC-like cells showed lower susceptibility to FcεRI-mediated degranulation and lower FcεRI expression than BMMCs. They speculated that the differences between the CTMC-like cells and BMMCs might be associated with either the expression level of FcεRI or intracellular signaling following stimulation by FcεRI. The different levels of FcεRI-mediated degranulation in the canine SMCs and BMMCs may be explained by a difference of the amount of expression of cell surface FcεRI or intracytoplasmic positive and negative regulators for the signaling.

The culture system for canine mast cells developed in the present study allowed us to use a relatively pure population of mast cells. Apparent FcεRI-mediated degranulation from the cells suggested that the cells could be used for functional

assays of mast cells. Previous studies on canine cultured mast cells by de Mora *et al.* [8, 11, 33] reported the efficacy of anti-allergic drugs on chemically induced or IgE-dependent histamine release from mast cells included in the dispersed cells obtained from enzymatically digested canine skin tissues. In their reports, they described that mast cells constituted only 1–2% of the skin-derived dispersed cells. However, in the present study, approximately 90% of the cultured cells were shown to be mast cells, and the other 10% of the cells were considered to be Langerhans cells or macrophages because they expressed low levels of cell surface FcεRI and were negative for c-Kit (Fig. 2b). The characteristics of these cells were similar to those of epidermal Langerhans cells in dogs reported by Bonkobara *et al.* [4]. The purity of the SMCs obtained in the present study was much higher than those in the studies by de Mora *et al.* [7, 8, 11, 13], suggesting the merit of our SMC-culture system for future immunological and pharmacological research on canine mast cells.

Mediators released from activated mast cells after FcεRI-mediated stimulation are known to cause aggravation of allergic symptoms [8, 17]. In humans, various novel strategies have been developed to prevent the degranulation and activation of mast cells, and these include anti-allergic drugs that target a decrease in serum IgE [38], inhibition of the positive regulators in the signaling cascade induced by FcεRI-mediated stimulation [25, 27], and inhibition of c-Kit receptor signaling [16]. The cultured canine SMCs and BMMCs obtained in the present study would be useful in examining the pharmacological efficacy of novel anti-allergic drugs in relation to the physiological function of mature mast cells. Moreover, the procedure to obtain canine SMCs from normal skin tissues developed in this study would be applicable to obtaining the same population from lesional skin tissues of the dogs with allergic dermatitis. Differences between the SMCs from normal dogs and those from dogs with allergic dermatitis would be of interest.

The present study should provide valuable culture techniques of purified canine mature mast cells for future immunological and pharmacological research into understanding and controlling the problematic allergic skin diseases in dogs.

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