

Establishment of a novel high-affinity IgE receptor-positive canine mast cell line with wild-type *c-kit* receptors

Yosuke Amagai^a, Akane Tanaka^a, Keitaro Ohmori^a and Hiroshi Matsuda^a  

^aLaboratory of Veterinary Molecular Pathology and Therapeutics, Division of Animal Life Science, Graduate School, Institute of Symbiotic Science and Technology, Tokyo University of Agriculture and Technology, 3-8-5 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan

Received 6 December 2007. Available online 18 December 2007.

Abstract

Much is known regarding participations of mast cells with innate and acquired immunity by secreting various cytokines and chemical mediators. However, details of mast cell biology still remain unclear. In this study, we successfully established a novel growth factor-independent mast cell line (MPT-1) derived from canine mast cell tumor. MPT-1 cells manifested factor-independent proliferation as floating cells containing a large amount of histamine, as well as chymase-like dog mast cell protease 3, in cytosolic granules. Particularly, MPT-1 cells expressed high-affinity IgE receptors (FcεRI) and wild-type *c-kit* receptors. Degranulation of MPT-1 cells was induced not only by stimulation with calcium ionophore but also by cross-linkage of the surface IgE. Given that MPT-1 is the first mast cell line with FcεRI which has no *c-kit* mutations, MPT-1 cells may provide great contribution for investigation of IgE-mediated activation mechanisms of mast cells, leading to development of effective treatment for allergic disorders.

Keywords: Mast cell; FcεRI; *c-kit*; Degranulation

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Mast cells have diverse effector functions in allergic reactions secreting various cytokines, chemokines, eicosanoids, and growth factors [1]. Mast cells release granules containing not only chemical mediators but also inflammatory cytokines after aggregation of the surface binding IgE [1]; therefore the control of mast cell degranulation is one of the most important strategies for treatment of allergic disorders. In order to provide reliable information, the useful *in vitro* systems of experiments including cell lines are necessary. Although HMC-1 cells are the widely distributed human growth factor-independent mast cell line, they rarely express FcεRI [2]. In addition, HMC-1 has mutated *c-kit* receptors, which causes constitutive activation without ligand binding [3]. Although LAD2 cells express FcεRI, stem cell factor is essential to maintain in culture, and it takes 2–3 weeks for LAD2 cells to double in number [4]. Mast cells derived from the cord blood, bone marrow, or lung have been provided as sources of human mast cells; however, researchers have to spend much time and cost to obtain sufficient cell numbers [5]. Although these mast cells express FcεRI, their proliferation is quite dependent on growth factors. RBL-2H3 cells, which were derived from rat basophilic leukemia (mucosal mast cells), express FcεRI and have been used in various experiments concerning IgE-mediated signaling pathways in mast cells [6]. However, RBL-2H3 cells have the gain-of-function mutation in the *c-kit* tyrosine kinase gene [7], therefore, FcεRI signaling may be modified by auto-activation of *c-kit* receptors. In addition, biological characters of mast cells are quite different between the human and the rodent [8]. Thus, there has been a great demand for connective tissue-type mast cell lines, which are derived from a higher animal, with reactivity to FcεRI-mediated stimulation.

Here, we first report a successful establishment of a novel canine mast cell line (MPT-1) with FcεRI and wild-type *c-kit* receptors. MPT-1 cells proliferated in growth factor-independent manner as floating cells. Furthermore, they contained chymase-like dog mast cell protease (dMCP)-3 [9] and [10], as well as abundant histamine in cytosolic granules than conventional cell lines including HMC-1 cells and BR cells [11]. We believe that MPT-1 cells may be potent tools and may bring new insights into the research of mast cell biology.

Materials and methods

Cell culture. MPT-1, derived from dog mast cell tumor, was maintained in α -minimum essential medium (α -MEM) (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Filtron, Brooklyn, Australia) and antibiotics. MPT-1 cells were subcultured at 1:10 dilution over 60 times, before they were used in this study. Cytospin preparations were stained with acid toluidine blue (pH 4.1). Histamine contents were quantified using a histamine cell culture research EIA kit (Labor Diagnostika Nord GmbH & Co. KG, Nordhorn, Germany).

Reagents. Signal inhibitors AG490, LY294002, rapamycin, and PD98059 were purchased from Calbiochem-Boehringer (La Jolla, CA). IMD-0354, a novel NF- κ B inhibitor [12], was kindly provided by Institute of Medicinal Molecular Design Inc. (Tokyo, Japan). Unless otherwise indicated, all chemicals used in this study were obtained from Sigma–Aldrich (Taufkirchen, Germany).

Electron microscopy. MPT-1 cells were fixed in 2% glutaraldehyde solution and examined with an electron microscope at Chobikeitai Laboratory (Yokohama, Japan).

Flow cytometry. To detect expression of *c-kit* receptors and CD23, MPT-1 cells were labeled with anti-mouse CD117 antibody or anti-mouse CD23 antibody (Pharmingen, San Diego, CA), then followed by labeling with fluorescein (FITC)-conjugated goat anti-mouse IgG (Bethyl Labs, Montgomery, TX). For detection of IgE receptors, cells were labeled with dog IgE (Bethyl Labs), then followed by labeling with goat anti-dog IgE (Bethyl Labs) and FITC-conjugated donkey anti-sheep IgG (Jackson ImmunoResearch, West Grove, PA). Positive reactions were analyzed by an EPICS XL flow cytometer (Coulter, Hialeah, FL).

Reverse transcription and polymerase chain reaction (RT-PCR). Total RNA was extracted with ISOGEN (Nippongene, Tokyo, Japan) and reverse-transcribed into cDNA by using oligo(dT) primers and SuperScript II (Invitrogen, Tokyo, Japan) according to the instructions. PCR was performed with Platinum *Taq* DNA polymerase (Invitrogen) and specific primers as follows; forward (5'-TCCAAGTCGGGCAGCTGAGAC-3') and reverse (5'-TCCAATTCCAAGCAGGCATC-3') (for dMCP-3), forward (5'-TGGCTGATCCTTCAAGCCTCT-3') and reverse (5'-CGCTGTTTTTGGTTGTGACGT-3') (for Fc ϵ RI γ -subunit), forward (5'-TTCTGGGGGTAACACAAAT-3') and reverse (5'-TGCAAACCCAGAAATAGT-3') (for Fc ϵ RI γ subunit), forward (5'-ATTTCTGCCGTGGTCTTGCTCCTG-3') and reverse (5'-GTCTCCTGGTTCCGGGTGCTCA-3') (for Fc ϵ RI γ -subunit), and forward (5'-CCCAGAGCTTGAACGAGAGAA-3') and reverse (5'-TCCTCGCCGAAGTAGTAGCAC-3') (for CD23).

Sequencing reaction and analysis. A *c-kit* gene was amplified with specific primers as follows; forward (5'-TCTCGATATCGCCACCGCGATGAGAGGCGCTCGCGGCGCCT-3') and reverse (5'-CTCTGCGGCCGCTCACACATCTTCGTGTACCAGCA-3'). PCR was carried out using KOD-Plus-Ver.2 (Toyobo, Osaka, Japan) according to the instructions.

Sequencing reaction was performed by using BigDye[®] Terminator ver.3.1 (Applied Biosystems, Tokyo, Japan) using primers for forward 243–263, 648–667, 1050–1069, 1484–1504, 1843–1862, 2205–2225, 2639–2657, and reverse 386–406 (parallel with base numbers of *c-kit* gene: GenBank Accession No. [AF044249](#)). The product was analyzed by ABI PRISM[®] 3100-Avant Genetic Analyzer (Applied Biosystems).

Activation of MPT-1 cells. β -Hexosaminidase (β -HEX) assay was performed according to the method described by Ortega et al. [13]. For IgE cross-linkage, cells were first sensitized with 5 μ g/ml human IgE (Chemicon international, Temecula, CA) for 12 h and stimulated by 10 μ g/ml anti-human IgE antibody for 1 h. The absorbance was measured with ImmunoMini NJ-2300 (Nalge Nunc International K.K., Tokyo, Japan).

Growth activity of MPT-1 cells. After serum deprivation for 12 h, cells were incubated with or without each reagent for 72 h with FBS. 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) assay was performed as described previously [12]. For 5-bromo-2'-deoxy-uridine (BrdU) incorporation assay, cell suspension (2×10^5 cells/ml) was incubated with or without various concentrations of each reagent for 48 h. BrdU incorporation assay was performed according to the manufacturer's instructions of a cell proliferation ELISA, BrdU kit (Roche Molecular Biochemicals, Mannheim, Germany).

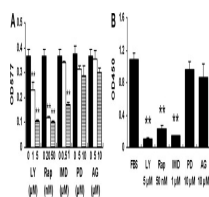
Statistical analysis. A two-tailed Student *t*-test was performed for statistical analysis of the data, and $p < 0.05$ was taken as the level of significance.

Results

Characters of MPT-1 cells

MPT-1 cells proliferated as a single, floating cell and doubled in number in 40.3 ± 3.1 h without any addition of cytokines (Fig. 1A). Their nucleus was oval and each cell had a metachromatic granules noticed by acid toluidine blue staining (Fig. 1B). Under the electron microscopy, numerous homogenous small electron-dense granules, partially condensed granules with small

vesicles, and immature vesicles were observed in their cytoplasm (Fig. 1C). Abundant microvilli were also noticed along the cell membrane (Fig. 1C). Since histamine is one of the most common mediators in mast cells involving in immediate hypersensitivity [14], we measured its contents in MPT-1 cells. The enzyme-immunoassay revealed that each MPT-1 cell contained a larger amount of histamine (0.82 ± 0.04 pg/cell) than HMC-1 cells or BR cells in their cytoplasm (Fig. 1D). The expression of *c-kit* receptors and IgE receptors were also detected by flow cytometry (Fig. 1E). To compare expression levels of two different types of IgE receptors; a high-affinity receptor (FcεRI) and a low-affinity receptor (CD23) [15], we performed RT-PCR analysis and revealed the expression of α-, β-, and γ-subunits of FcεRI, and CD23 using canine bone marrow as a control (Fig. 1F). Moreover, mRNA of chymase-like dMCP-3, which is a well-characterized enzyme of connective tissue-type mast cells [9] and [10], was also detected using BR cells [11] as a control (Fig. 1G). Although mRNA was detected, surface expression of CD23 was hardly detected by flow cytometry (data not shown).



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Fig. 1. Characters of MPT-1 cells. Growth curve of MPT-1 cells (A). Data represent means $\pm$ SE of two different experiments ($n = 5$ in each time).

Morphological characterization of MPT-1 cells stained with acid toluidine blue (pH 4.1) (magnification: 1000 $\times$) (B). Electron microscopic features of a MPT-1 cell with a transmission electron microscope (magnification 10,000 $\times$) (C).

Histamine content of a MPT-1 cell (D). The histamine contents were measured by using an EIA kit. Data represents means $\pm$ SE of two different experiments.

** $p < 0.01$. Flow cytometric analysis of surface expression of *c-kit* receptors (E, left) or FcεRI (E, right). MPT-1 cells were first labeled with (black line) or without (gray line) anti-mouse *c-kit* antibody or dog IgE. Data are the representative of two independent experiments. Expression of FcεRI α-, β-, and γ-subunit, CD23 (F), and dMCP-3 (G) mRNA in MPT-1 cells. Data are representative of two independent experiments. DNA sequence of exon 11–12 of *c-kit* gene (H). The arrow indicated the point mutation in codon 575 (T1752C) of BR cells. In other location, MPT-1 cells also had no mutations.

Evaluation of *c-kit* sequence of MPT-1 cells

Previous studies of canine mast cell tumors have revealed mutations consisting of internal tandem duplications in exons 11 and 12, encoding the juxtamembrane domain of *c-kit* receptors [16]. Several cell lines of human or rodent mast cells such as HMC-1 and RBL-2H3 also have mutation in exon 17 of *c-kit* receptors, encoding the tyrosine kinase domain [3] and [7]. Therefore, we carried out sequencing analyses of the whole *c-kit* gene to evaluate whether MPT-1 cells have any mutation. We identified no mutation not only in the juxtamembrane domain but also in the tyrosine kinase domain in MPT-1 cells (Fig. 1H). We did not find any other mutation in MPT-1 cells, either (data not shown).

Degranulation of MPT-1 cells

Next we evaluated degranulation of MPT-1 cells after chemical or immunologic stimulation by the β -HEX release. When stimulated with calcium ionophore A23187, MPT-1 cells markedly degranulated in a dose-dependent manner (Fig. 2A). On the other hand, although there were statistically significant differences in each concentration, effects of compound 48/80 and substance P were much weaker than A23187 (Fig. 2A). Since MPT-1 cells highly express Fc ϵ RI, we investigated whether IgE cross-linkage induced degranulation. Aggregation of surface of IgE with anti-IgE antibody significantly induced the β -HEX release as compared to control cells without anti-IgE stimulation (Fig. 2B).



Fig. 2. Degranulation of MPT-1 cells. Degranulation responses of MPT-1 cells. Cells (2×10^6 cells/ml) were incubated with or without A23187, compound 48/80, or substance P as indicated concentrations (A). Although degranulation of MPT-1 cells was induced with statistical significance ($p < 0.01$) by compound 48/80 or substance P when compared to medium alone, A23187 manifested most potent effect of all. Comp, compound 48/80; SP, substance P. Cells were also sensitized with human IgE and stimulated by goat anti-human IgE antibody (B). Data represent means $\pm$ SE of three individual experiments. ** $p < 0.01$, when compared with control.

PI3K pathway and transcription factor NF- κ B contribute cell proliferation

Mast cells have several major signal transduction pathways such as PI3K [12], Ras/mitogen-activated protein kinase (MAPK) [17], and Janus kinase/signal transducers and activators of transcription (JAK/STAT) [17] for their proliferation or functional activation. Therefore, we investigated which pathways mainly contributed to proliferation of MPT-1 cells. A MTT assay revealed that inhibitors for the PI3K signaling pathway, LY294002 and rapamycin, significantly abrogated proliferation of MPT-1 cells (Fig. 3A). Since constitutive activation of NF- κ B in human mast cell line HMC-1 seriously related to their proliferation [12], we investigated the effect of a novel inhibitor of I κ B kinase beta on proliferation of MPT-1 cells [12]. IMD-0354 inhibited cell proliferation as well as LY294002 and rapamycin. In contrast, inhibition of MAPK or JAK2 had little or no effect (Fig. 3A). Besides cellular activation, the effects of these inhibitors on the DNA synthesis were determined by a BrdU incorporation assay. These results highly correlated with those in a MTT assay (Fig. 3B).

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Fig. 3. Inhibitory effects of cellular proliferation in MPT-1 cells. Inhibitory effects of several signal inhibitors in cell proliferation of MPT-1 cells. Cells were incubated with or without each reagent for 72 h and cell activity was analyzed with a MTT assay (A). After cells were incubated with or without each reagent for 24 h, BrdU incorporation (DNA synthesis) was analyzed (B). Data represent means $\pm$ SE of three individual experiments. ** $p < 0.01$, when compared with medium alone. LY, LY294002; Rap, rapamycin; IMD, IMD-0354; PD, PD98059; AG, AG490.

Discussion

In this study, we successfully established a novel mast cell line derived from the connective tissue-type mast cell tumor in a dog. MPT-1 cells had basophilic granules in their cytoplasm when stained with toluidine blue. Their characteristics included both mature granules with dense material and immature granule chambers in the cytoplasm were identified under electron microscopy. They also contained larger amount of histamine than HMC-1 cells and carried chymase-like dMCP-3 as well as BR cells. Moreover, they expressed wild type *c-kit* receptors and Fc ϵ RI. These results indicated that MPT-1 cells had major characteristics of connective tissue-type mast cells.

Among mast cell lines that have been established, there were few cell lines that had wild-type *c-kit* receptors. Although RBL-2H3 cells express FcεRI, activation of signaling molecules induced by mutated *c-kit* receptors may influence FcεRI-mediated cellular responses [18]. Therefore, the use of such cell lines for investigation of IgE-mediated events might be limited. We revealed that MPT-1 cells had wild-type *c-kit* receptors and they degranulated by IgE cross-linkage, indicating that this cell line must be useful for the elucidation of IgE-mediated mast cell activation.

We also examined signaling pathways that contributed to cell proliferation. Our results indicated that as well as human mast cells, the contribution of PI3K signaling pathway and NF-κB should be important in proliferation of MPT-1 cells. These data may support the significance of MPT-1 cells as a model system for human mast cells.

In conclusion, MPT-1 cells are useful for analysis of general function of connective tissue-type mast cells. We believe that MPT-1 cells may provide the high priority into *in vitro* researches of mast cells.

Acknowledgment

This work was supported by the grant from Grant-in-Aid for Scientific Research on Priority Areas A provided by Japan Society for the Promotion of Science, Japan.

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 Corresponding author. Fax: +81 42 367 5916.