

Characterization of 'adult-type' mast cells derived from human bone marrow CD34⁺ cells cultured in the presence of stem cell factor and interleukin-6. Interleukin-4 is not required for constitutive expression of CD54, FcεRIα and chymase, and CD13 expression is reduced during differentiation

Y. Shimizu, K. Sakai*, T. Miura†, T. Narita‡, H. Tsukagoshi, Y. Satoh§, S. Ishikawa§, Y. Morishita§, S. Takai¶, M. Miyazaki¶, M. Mori, H. Saito**, H. Xia†† and L. B. Schwartz††

Department of Internal Medicine, Gunma University School of Medicine, Maebashi, *Department of Nutrition, School of Medicine, University of Tokushima, †Kirin Brewery Incorp., Takasaki, ‡Laboratory of Molecular Genetics, Institute for Molecular and Cellular Regulation, Maebashi, §Department of Surgery, Gunma University School of Medicine, Maebashi, ¶Department of Pharmacology, Osaka Medical College, Osaka, **Department of Allergy, National Children's Medical Research Center, Tokyo, Japan, and ††Department of Internal Medicine, Virginia Commonwealth University, Richmond, Virginia, USA

Summary

Background *In vitro*-derived human mast cells exhibit different properties, depending in part on the source of progenitor cells. Most investigations have used fetal liver, cord blood or peripheral blood. Few have used adult bone marrow.

Objective Human mast cells derived *in vitro* from the CD34⁺ progenitors in bone marrow and cord blood that had been cultured with recombinant human stem cell factor (rhSCF) and recombinant human interleukin-6 (rhIL-6) were compared.

Methods and results After 12 weeks of culture, nearly all of the cells were mast cells, and nearly all of these had cytoplasmic granules containing both tryptase and chymase (MCTC type), stained metachromatically with acidic toluidine blue, and expressed CD117 on the cell surface. Both tryptase protein and mRNA were detected by two weeks of culture. Chymase mRNA and protein were detected at 4 weeks but not at 2 weeks of culture. By 12 weeks, chymase content per cell, measured by ELISA, was significantly higher ($P < 0.05$) in human bone marrow-derived mast cells (HBMMC) (5.6 ± 0.9 pg) than in cord blood-derived mast cells (CBMC) (2.4 ± 0.9 pg), whereas histamine and tryptase levels were not significantly different. Of the cluster designations tested, CD29, CD49d, CD51 and CD61 were strongly expressed on HBMMC. CD54 and FcεRIα also were expressed constitutively. Approximately half of CD34-sorted cells at day 0 were CD13⁺ and this diminished as mast cell maturation occurred. Electron microscopy revealed that 12-week-old HBMMC had many secretory granules that contained spherical electron dense cores surrounded by electron lucent space, consistent with previous reports of immature MCTC cells developing *in vivo*.

Conclusions CD34⁺ progenitors of human bone marrow are a rich source of mast cell progenitors capable of expressing granule and surface markers of mature mast cells in the presence of rhSCF and rhIL-6.

Keywords β-tryptase, CD13, chymase, differentiation, human bone marrow, mast cells

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Introduction

Human mast cells develop from haematopoietic progenitors found in cord blood [1, 2], fetal liver [3], peripheral blood [4] and bone marrow [5, 6]. Surface FcεRIα is expressed spontaneously when mast cells are developed from these progenitors *in vitro* with recombinant human stem cell factor (rhSCF) on all but those from fetal liver cells [1, 7]. IL-4 induces expression of FcεRI in fetal liver-derived mast cells, and upregulates

expression on cord blood-derived mast cells (CBMC). In both cases, the portion of chymase⁺ cells increases [8, 9]. Human mast cells (MC) are classified into two subtypes based on their content of two protease, tryptase and chymase [10]. MCT contains tryptase only and MCTC contains both tryptase and chymase together with cathepsin G and mast cell carboxypeptidase. Recently, however, there has been controversy about the origin of these mast cells and whether MCT and MCTC were reversible or not [11]. It was observed by Toru *et al.* [12] that IL-4 promoted cell maturation with the expression of both tryptase and chymase from tryptase-single positive mast cells.

Heterogeneity of mast cells with respect to phenotype also has been reported. CD54 is expressed on lung mast cells or HMC-1 [13], but expression is minimal or absent on cultured human

Correspondence: Yuji Shimizu, First Department of Internal Medicine, Gunma University School of Medicine, 3-39-15 Showa-machi, Maebashi, Gunma 371-8511, Japan. E-mail: yshimizu@med.gunma-u.ac.jp

fetal liver-derived mast cells [14, 15]. IL-4 increases the expression of CD18 and CD54 on CBMC, resulting in haemotypic aggregation [16]. Moreover, it has been shown that human mast cells arise from precursor cells that express CD34, CD117 and CD13 [17]. CD13 is detected on cultured mast cells derived from fetal liver [14] or cord blood [18], but is minimally or not expressed on mast cells dispersed from human tissues [19, 20]. CD13 is expressed on stem cells during early stages of myeloid and lymphoid cell development [21]. It is therefore of interest to characterize cultured mast cells derived from 'adult' bone marrow progenitors, particularly in comparison with those derived from 'young' cord blood or fetal liver-derived mast cells.

In the current study, we show several characteristics of human bone marrow-derived mast cells (HBMMC). Surface FcεRIα and CD54 are constitutively expressed, while CD13 surface expression is diminished during differentiation. All HBMMC derived with rhSCF and rhIL-6 are shown to be tryptase⁺ and chymase⁺ and the level of chymase to be significantly higher than in CBMC. The ultrastructural appearance of HBMMC showed well-granulated cells with electron-dense cores inside these granules, a characteristic of MCTC cells developing *in vivo*.

Materials and methods

Reagents

Streptavidin-peroxidase, naphthol AS-MX phosphate, levamisole, collagenase, Fast Blue RR and AEC chromogen kit (Sigma Chemical Company, St Louis, MO, USA), Ficoll-Paque research grade (Pharmacia Biotech AB, Uppsala, Sweden), human serum AB (Cosmo Bio Company Ltd, Tokyo, Japan), L-glutamine, Tween-20, Phosphate buffer powder (WAKO Ltd, Osaka, Japan), ENVISION polymer and DAB tablets (DAKO, Glostrup, Denmark) and Media III, which contains insulin (10 µg/mL), transferrin (10 µg/mL), HEPES (4470 µg/mL), NaHCO₃ (2.3 mg/mL), penicillin (100 U/mL) and streptomycin (100 µg/mL) (Immuno-Biological Laboratories, Fujioka, Japan), were purchased from the indicated manufacturers. RhSCF was a kind gift from AMGEN Inc. (Thousand Oaks, CA, USA); rhIL-6 from Kirin Brewery Inc. (Takasaki, Japan).

Antibodies

For flow cytometry, cells were treated with several monoclonal antibodies (mAbs) recognizing various CD antigens. Most of them were provided from the 6th International Workshop and Conference on Human Leukocyte Differentiation Antigens [22]. CD29 (β1 chain of VLA family, 4B4) (Coulter Corporation, Hialeah, FL, USA), CD88 (C5aR, W17/1), PE-conjugated mouse anti-human CD117 (YB5.B8) (Pharmingen, San Diego, CA, USA), CD123 (IL-3R, 6H6) (Serotec, Kidlington, Oxford, UK), FITC-conjugated mouse anti-human CD13 (WM-47) (DAKO) and CRA-1, which is useful for the detection of FcεRIα by either immunohistochemistry or flow cytometric analysis [23, 24] (Kyokuto Pharmaceutical Industrial Company Ltd, Ibaraki, Japan), were purchased as indicated. Anti-tryptase mAb conjugated to alkaline phosphatase (G3/AP) and biotinylated antichymase mAb (B7/B) were prepared as reported previously [10]. FITC-conjugated F(ab')₂ fragment of rabbit anti-mouse immunoglobulins, dual colour reagent mouse

IgG1/FITC, mouse IgG1/RPE negative control (DAKO), MOPC 31C (irrelevant mouse IgG1 control), MOPC 141 (irrelevant mouse IgG2b control), ABPC22 (irrelevant mouse IgM control) (Sigma), and mouse anti-human IL-4 and biotinylated mouse anti-human IL-4 mAbs (Pharmingen) also were obtained as indicated.

Cell culture

Human bone marrow was obtained from patients without any haematological disorders who provided informed consent, and was collected in heparinized tubes. The protocol was approved by the Institutional Review Board of Gunma University. Bone marrow was incubated with 0.02% collagenase in DMEM, then filtered. Cell suspensions were carefully layered above Ficoll-paque and centrifuged at 400 g for 35 min at room temperature. The cell layer at the interface was transferred into a new tube and washed three times with PBS/EDTA. The CD34⁺ cells in this layer were magnetically sorted with CD34 progenitor cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) as the manufacturer's protocol described. Sorted cells were washed, resuspended in Media III supplemented with 10% human serum, 2 mM L-glutamine and 50 µM 2-mercaptoethanol, and seeded into plastic 48- or 96-well plates (Iwaki Glass, Tokyo, Japan) at a concentration of 5×10^4 cells/mL. Sorted cells were cultured with rhSCF and rhIL-6 at a final concentration of 100 ng/mL (rhSCF) and 50 ng/mL (rhIL-6), respectively, for up to 12 weeks in 5% CO₂ at 37 °C in a humidified atmosphere. Half of the media was exchanged once a week with the same concentration of rhSCF and rhIL-6. A portion of suspended cells was stained with toluidine blue as described [25] to determine the percentage of metachromatic cells at each time-point. Viability and cell number were determined by trypan blue stain. More than 95% of cells were viable at each time-point. CBMC were obtained from CD34⁺ cord blood progenitors, cultured with rhSCF + rhIL-6, as reported [1].

Immunocytochemical stain

Cytospins were prepared, fixed in methanol with 0.6% H₂O₂ for 30 min and subjected to immunocytochemistry within 1 week after cytospin preparations were obtained. Cytospins were first incubated with B7/B, then incubated with Streptavidin-peroxidase. A brown colour developed after incubation of labelled cells with AEC. For the detection of tryptase, cytospins were incubated with G3/AP; labelled cells became blue after incubation with Fast Blue RR. Detailed procedures for B7/B and G3/AP immunocytochemistry have been described [10].

Measurement of mediators

Different forms of tryptase (both total and β-tryptase) in HBMMC were measured by sandwich ELISA, as described previously [26]. Tryptase in CBMC was determined using the UniCAP tryptase fluoroenzyme-immunoassay (Pharmacia & Upjohn, Uppsala, Sweden).

IL-4 was quantified by a chemiluminescent enzyme immunoassay (CLEIA). Briefly, mouse anti-human IL-4 mAb (8D4-8) (Pharmingen) was incubated in a 96-well plate as a capture antibody. After blocking with PBS/BSA, standard rhIL-4 (R & D Systems, Minneapolis, MN, USA) or samples were added into each well. Subsequently, biotinylated rat anti-human IL-4 (Pharmingen) was added as a detection antibody.

CSPD (disodium 3-(4-methoxyspiro (1,2-dioxetane-3, 2'-(5'-chloro)-tricyclo(3.3.1.1^{3,7})decan)-4-yl) phenyl phosphate) (TROPIX, Bedford, MA, USA) was then used for the detection of alkaline phosphatase, and Emerald II (TROPIX) was used as a luminescence amplifying material. Chemiluminescence was analysed by microplate luminometer (Dynatec Inc., Yokohama, Japan). Histamine was measured by using an automatic histamine analyser having double HPLC columns (Tosoh, Tokyo, Japan), as reported [1].

Chymase was measured by sandwich ELISA, as described [27], using antichymase mAb (developed in the Department of Pharmacology, Osaka Medical College, Osaka, Japan) as a capture antibody, and biotinylated antichymase mAb (Chemicon International, London, UK) as a detection antibody.

Flow cytometry

For the analysis of surface antigen expression of HBMMC, CD34-sorted cells cultured with rhSCF + rhIL-6 for 12 weeks (94 to 99% of cells exhibited metachromasia and were labelled with G3/AP against tryptase) were transferred into plastic tubes (1×10^5 cells in each tube) and washed with PBS/1% BSA. Cells were then incubated with each mAb for 30 min at 4 °C, washed three times with PBS/BSA and incubated with FITC-conjugated F(ab')₂ fragment of rabbit anti-mouse immunoglobulins for 30 min at 4 °C. Excess antibody was washed three times with PBS/BSA. Washed cells were resuspended in sheath fluid and subjected to flow cytometry. Propidium iodide (PI) was added (final concentration 10 µg/mL) prior to the analysis to identify and exclude dead cells. Flow cytometry was performed in an EPICS ELITE Flow Cytometer (Coulter, Hialeah, FL, USA). At least 5000 cells were analysed for surface antigen expression. Data were further analysed by WinMDI software (Coulter). Dotplots (FSC and SSC) were first drawn, and PI-stained cells were gated out for the analysis. HBMMC were gated and overlay histograms with subtype-matched control immunoglobulins or mAb against each surface antigen were obtained with gated cells.

For double immunostaining with CD34-sorted cells (D0) and HBMMC populations (D28, D56 and D84), cells were incubated with FITC-conjugated anti-CD13 and RPE-conjugated CD117 simultaneously for 30 min at 4 °C. Dual colour reagent mouse IgG1/FITC, mouse IgG1/RPE negative control was used. Cells were washed three times with PBS/BSA, then analysed as shown above. Dotplots (FSC and SSC as parameters) were first drawn on the same scale as shown above and adequate gates were determined. As mean fluorescence intensity (MFI) with control antibody tended to increase as cells differentiated, the ratio of MFI (MFI with anti-CD13 mAb to MFI with control antibody) was calculated.

Competitive RT-PCR for tryptase and chymase mRNA

mRNA was isolated by guanidinium-phenol extraction, and competitive RT-PCR for tryptase and chymase was performed as previously described [28]. Primers used and the respective target and competitor fragment sizes were 5' (562)-GGAA AACCACATTTGTGACG-3' (sense) and 5' (712)-ATTCACC TTGCACACCAGGG-3' (anti-sense) for tryptase (151 bp and 114 bp), 5' (411)-ATCAGAATTCAACTTTGTCCACCT GG-3' (sense) and 5' (687)-TGCAGTCGACCGTCCATA

GGATACGATGC-3' (anti-sense) for chymase (277 bp and 175 bp). PCR was performed for 35 cycles, each cycle including denaturation (94 °C, 1 min), annealing (55 °C, 1 min) and extension (72 °C, 1 min). After the measurement of mRNA for tryptase, chymase and G3PDH, levels of tryptase and chymase mRNA were normalized to the corresponding level of G3PDH mRNA.

Electron microscopy

Pelleted samples of HBMMC at 12 weeks of culture in the presence of rhSCF + rhIL-6 were fixed in 0.1% cacodylate buffer, pH 7.4, containing 2.5% glutaraldehyde for 1 h at 4 °C. The specimens were then washed with the same buffer and post-fixed with 1% osmium tetroxide for 1 h. The samples were dehydrated in a graded ethanol series and embedded in Epon. Thin sections were cut with an ultramicrotome, and stained with a saturated aqueous solution of uranyl acetate and lead citrate, and observed with a JEM-1200EXII electron microscope (JEOL Ltd, Tokyo, Japan).

Results

Differentiation of human mast cells derived from bone marrow progenitors

CD34-sorted cells obtained from human bone marrow were cultured in the presence of rhSCF + rhIL-6 for up to 12 weeks, and the percentages of metachromatic cells were determined using toluidine blue. Cytospin preparations also were labelled with G3/AP. As shown in Fig. 1, the percentages of metachromatic cells at 2, 4, 8 and 12 weeks were 4.3 ± 2.9 , 25 ± 15 , 86 ± 9 and $99 \pm 1\%$, respectively. These percentages were similar to those obtained after G3/AP labelling (data not shown), as reported [10]. Total cell numbers ($\times 10^5$ cells) from 1×10^5 CD34⁺ progenitors at 2, 4, 8 and 12 weeks were 6.0 ± 2.8 , 14.6 ± 3.0 , 11.1 ± 4.5 and 6.8 ± 1.8 , respectively.

Immunocytochemical stain with G3/AP and B7/B

By 12 weeks, all of the HBMMC cultured with rhSCF + rhIL-6 were stained with both G3/AP (Fig. 2a) and B7/B (Fig. 2b). The

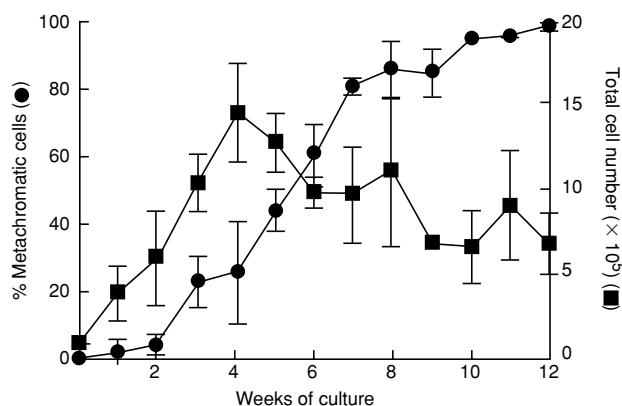


Fig. 1. Differentiation of human mast cells derived from bone marrow progenitors. CD34⁺ human bone marrow progenitors were cultured in the presence of rhSCF + rhIL-6 for up to 12 weeks, and metachromatic cells and total cell number were analysed each week. Data points show mean $\pm$ SEM values from four independent experiments.

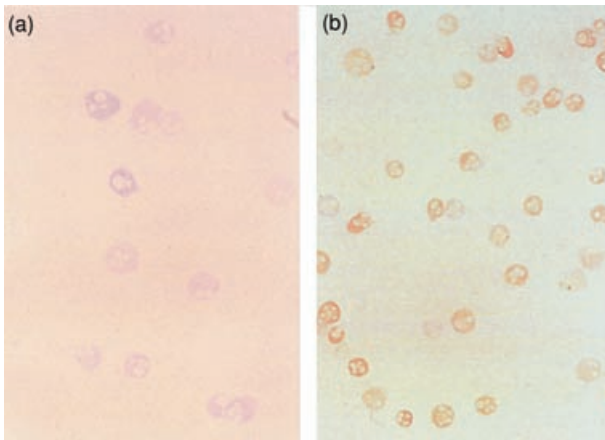


Fig. 2. Immunocytochemical stain with either G3/AP or B7/B. Cytospin preparations of 12-week-old HBMMC were either stained with G3/AP (a), or B7/B (b). All of HBMMC were stained well with B7/B. Macrophages, which were together with HBMMC, were not stained with either mAb and HBMMC were not stained with irrelevant mouse IgG (data not shown).

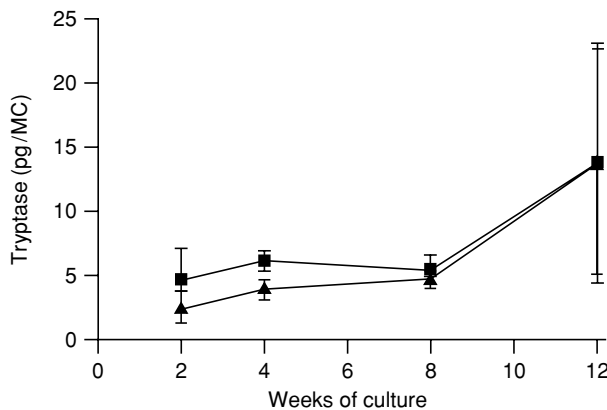


Fig. 3. Tryptase levels in human bone marrow-derived mast cells during culture. Tryptase (both total and b-tryptase) in HBMMC cultured with rhSCF + rhIL-6 for 2, 4, 8 and 12 weeks of culture were measured by sandwich ELISA, and tryptase levels per mast cells (not total cells) are shown. Data are mean ± SEM of three independent experiments.

expression of protease was selective for HBMMC among the cells developed, as macrophages, which were more evident at earlier time-points, were not labelled by either G3/AP or B7/B. HBMMC were not stained with irrelevant mouse IgG (data not shown). Thus, mast cells that develop from human bone marrow progenitors under these conditions are of the MCTC type of mast cell.

Quantification of mediators in HBMMC and CBMC

Total tryptase levels (mean ± SEM, pg/mast cell) in HBMMC at 2, 4, 8 and 12 weeks of culture were 4.5 ± 2.6 , 5.9 ± 0.8 , 5.1 ± 1.3 and 14 ± 9 , respectively. In most cases it appeared that the β -tryptase assay gave similar values to the total tryptase assay, as shown in Fig. 3, suggesting this form of tryptase may predominate inside these cells. To compare the granule contents in HBMMC and CBMC, tryptase, chymase and histamine were measured at comparable time-points during differentiation. As shown in Table 1, there was no significant differences in the

Table 1. Quantification of tryptase, chymase and histamine during differentiation of HBMMC as compared with that of CBMC. Cultured cells were lysed in 1M NaCl, and tryptase, chymase and histamine were measured as described in materials and methods. Data are mean ± SEM of three (HBMMC) and six (CBMC) independent experiments, respectively

	HBMMC	CBMC
Tryptase (pg/mast cell)		
4W	5.89 ± 0.76	5.28 ± 4.50
8W	5.14 ± 1.30	9.50 ± 13.45
12W	13.65 ± 8.75	14.42 ± 9.97
Chymase (pg/mast cell)		
4W	1.58 ± 1.59	1.01 ± 0.96
8W	$3.84 \pm 1.40^*$	1.56 ± 0.69
12W	$5.58 \pm 0.91^*$	2.44 ± 0.88
Histamine (pg/mast cell)		
4W	2.20 ± 0.53	3.62 ± 3.41
8W	3.10 ± 1.92	4.53 ± 3.95
12W	2.44 ± 1.22	5.48 ± 2.63

* $P < 0.05$.

cellular levels of tryptase and histamine at any time-point. However, the cellular level of chymase was significantly higher at 8 and 12 weeks of culture in HBMMC (3.8 and 5.6 pg/mast cell) than in CBMC (1.6 and 2.4 pg/mast cell).

mRNA for tryptase and chymase in HBMMC

Cellular levels of mRNA for tryptase and chymase were quantified by competitive RT-PCR. As shown in Fig. 4(a and b), respectively, tryptase and chymase mRNA were detected with 12-week-old HBMMC. As shown in Fig. 4(c), mRNA for tryptase was detected at 2 weeks and appeared to peak at 4 weeks and decline at 12 weeks. Tryptase mRNA values as percentage of G3PDH at 2, 4 and 12 weeks of culture were 487 ± 300 , $15\,700 \pm 14\,500$ and 3065 ± 703 , respectively. On the contrary, chymase mRNA was below the detectable level at 2 weeks of culture, but increased to 168 ± 157 at 4 weeks and 375 ± 142 at 12 weeks of culture, which is compatible with the expression of chymase protein noted above.

Surface antigen expression on HBMMC cultured in the presence of rhSCF + rhIL-6 for 12 weeks

Surface antigen expression on cultured HBMMC at 12 weeks was next investigated. As shown in Fig. 5, integrin subunits such as CD29, CD49d, CD51 and CD61, as well as CD117, were strongly expressed, consistent with human mast cells from other sources. Surface Fc ϵ RI α was detected without exogenous IL-4 or IgE. Sensitization with IgE and challenge with anti-IgE induced dose-dependent histamine release from HBMMC (data not shown), suggesting that the Fc ϵ RI α expressed reflected functional Fc ϵ RI. CD54 (ICAM1), reportedly expressed on lung and uterine mast cells and on CBMC after exposure to IL-4 [16], but not on fetal liver-derived mast cells, was expressed on 12-week-old HBMMC. IL-4 in recovered culture medium at 4, 8 and 12 weeks of culture was undetectable by CLEIA (< 2 pg/mL), suggesting there was little, if any, endogenous IL-4 present to affect expression of either CD54 or Fc ϵ RI. As anticipated, surface CD11b, CDw17, CD18, CD28, CD31, CD35, CD40, CD40L, CD88, CD116, CD123 and CD131 were not detected.

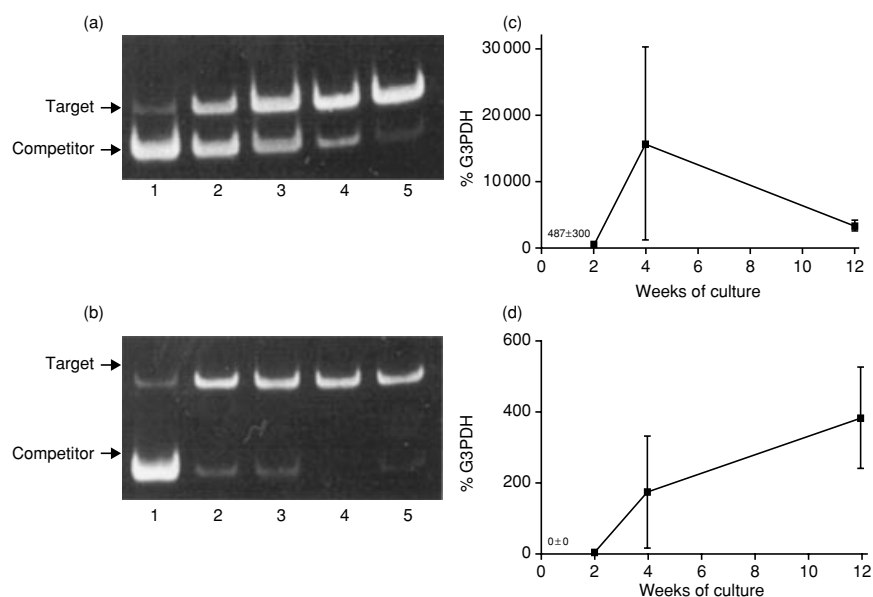


Fig. 4. Competitive RT-PCR for tryptase and chymase mRNA during culture. Representative electrophoresis and autoradiography for tryptase (a) and chymase (b) mRNA in 12-week-old HBMMC are shown, respectively. In both (a) and (b), 1 microgram of total RNA was as target. For tryptase, lanes 1 to 5 correspond with competitor RNA amounts of 220, 73, 24, 8.1, 2.7 attomoles, respectively. For chymase, lane 1 to 5 correspond with competitor RNA amounts of 132, 44, 15, 4.9, 1.6 attomoles, respectively. Changes in the amount of mRNA for tryptase (c) and chymase (d), respectively, are also shown. Data are mean $\pm$ SEM of three independent experiments.

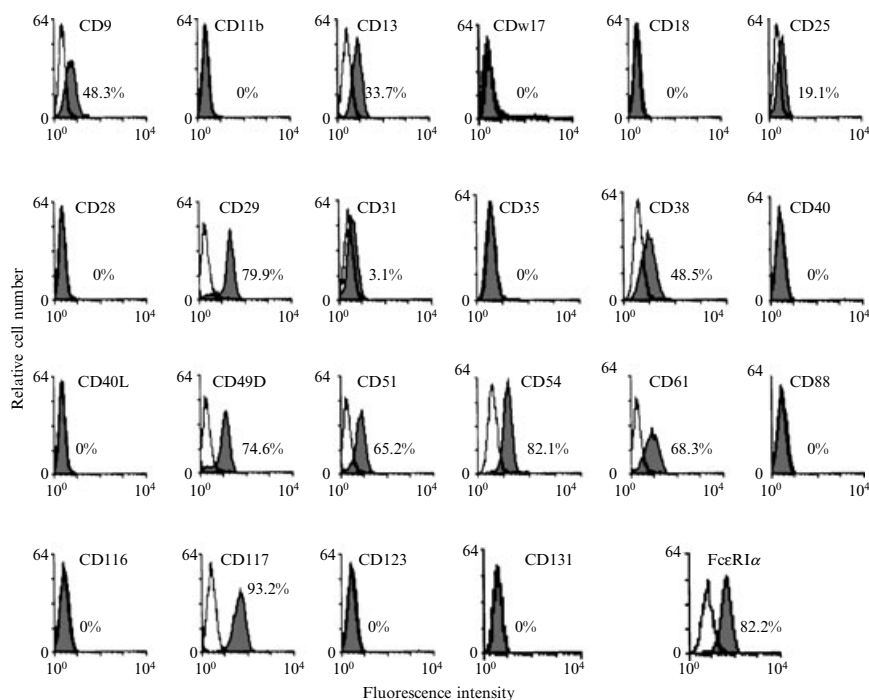


Fig. 5. Surface antigen expression of HBMMC cultured in the presence of rhSCF + rhIL-6 for 12 weeks. The histograms with mAb against each antigen were overlaid with those with isotype-matched mouse IgG or IgM non-immune mAb. Horizontal axes represent log of fluorescence and vertical axes indicate relative cell number. Data are representative of three or four independent experiments. Note that CD54 and FcεRIα are strongly expressed without addition of either IL-4 or IgE.

Surface CD9, CD13, CD25 and CD38 were detected at low fluorescence intensities. Incubation of HBMMC with IL-2 at a concentration of 100 ng/mL did not cause histamine and IL-13 release (data not shown), suggesting that CD25 expressed on HBMMC was not functional when stimulated alone.

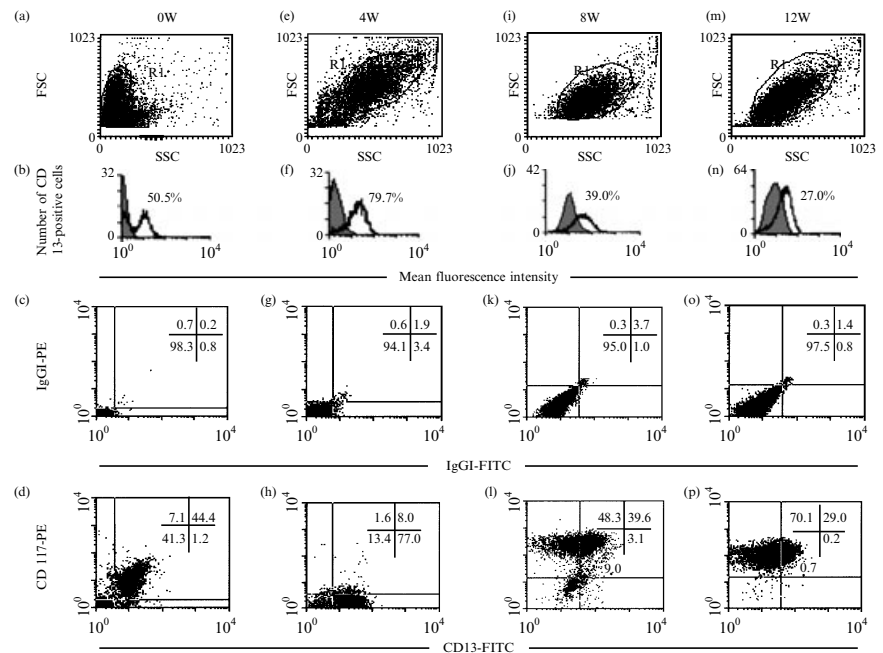
Reduced expression of CD13 with the differentiation of mast cells

Human mast cells arise from CD13⁺ progenitors [17] and the expression of CD13 continues during the maturation of mast cells from cord blood progenitors [18]. However, the expression

of CD13 on HBMMC was weak, as shown above. Figure 6 shows CD13 expression during the differentiation of HBMMC. Among CD34-sorted cells, 44% were CD13/CD117-double positive cells by flow cytometry (Fig. 6d).

When CD34-sorted cells were cultured with SCF + IL-6 for 4 weeks, the major portion of these cells shifted to higher FSC and SSC values (Fig. 6e). Such cells (85%) were positive for CD13 (Fig. 6f). CD117 expression appeared to be negligible (Fig. 6h) even though 15 to 37% of cells were tryptase⁺. Perhaps Kit expressed on the cell surface had been internalized with the excess of SCF in the culture media [29].

Fig. 6. CD13/CD117 expression on CD34-sorted (D0) cells, and cultured mast cell/mast cell progenitors with rhSCF + rhIL-6 for 4, 8 and 12 weeks. CD34-sorted (at 0 weeks) (a–d), cultured with rhSCF + rhIL-6 for 4 (e–h), 8 (i–l) and 12 weeks (m–p), respectively, were labelled simultaneously with FITC-conjugated anti-CD13 and RPE-conjugated CD117. Dot plots were prepared with FSC and SSC as x-axis and y-axis parameters, respectively (a, e, i and m). Predominant (gated) cells at each time-point were then reanalysed using overlay histogram for the expression of CD13 (unshaded area) or control (shaded area) (b, f, j and n). Percentages of positive cells are shown as percentage of CD13⁺ cells subtracted by that of mouse IgG1/RPE negative control. Quadrant dot plots with dual colour reagent mouse IgG1/FITC, mouse IgG1/RPE negative control (c, g, k and o) and that with FITC-conjugated anti-CD13 and RPE-conjugated CD117 (d, h, l and p) and percentages of cells in each area are shown, respectively. Data are representative of three independent experiments.



After 8 weeks of culture, 84% of cells expressed CD117, but only 43% of gated cells expressed CD13.

Ninety-nine per cent of cells were positive for CD117 at 12 weeks of culture, but only 29% of cells expressed CD13. The MFI ratio of such cells labelled with anti-CD13 mAb compared with those labelled with a negative control mAb was 7.8 ± 0.9 , 7.5 ± 0.5 , 4.0 ± 0.9 and 3.0 ± 0.2 at 0, 4, 8 and 12 weeks, respectively, in three independent experiments. After an additional 2 weeks of culture there was no further reduction in the mean fluorescence intensity after labelling with anti-CD13 mAb (data not shown).

The changes in the MFI ratios of anti-CD13-labelled cells to those labelled with a negative control mAb and in the percentages of tryptase⁺ cells are shown in greater detail in Fig. 7.

Morphological appearance

Ultrastructural analysis by transmission electron microscopy of 12-week-old HBMMC developed in culture was consistent with these cells being mast cells (Fig. 8a). The surface folds were narrow and distributed over the entire surface of each mast cell. Each cell had a large and irregular nucleus with partially condensed chromatin and cytoplasm abundant granules. Almost every granule contained electron-dense cores, with most of the remaining portion of the granule being electron lucent. Discrete scrolls were rare in these HBMMC (Fig. 8b). Thus, the morphology of HBMMC corresponded with the ultrastructure of previously reported immature MCTC [30, 31], and that observed in IL-4-treated CBMC [12].

Discussion

The current study demonstrates several characteristics of human mast cells derived from CD34⁺ bone marrow progenitors in the presence of rhSCF and rhIL-6, including granule

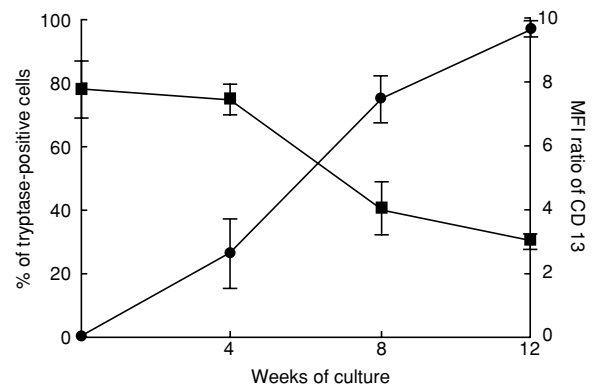


Fig. 7. Kinetics of mean fluorescence intensity (MFI) ratio of CD13 to control and percentage of tryptase-positive cells. MFI ratios of CD13 to control are shown together with the percentages of tryptase⁺ cells. Note that MFI ratio was not reduced to 1, which means that expression of CD13 on human bone marrow-derived mast cells was not completely inhibited. Mean $\pm$ SEM of three independent experiments are shown.

mediator contents, surface antigen expression and morphological appearance. Maturation proceeded in the absence of exogenously added or endogenously detected IL-4. Approximately 25% of cultured cells are mast cells by 4 weeks, while 98% are mast cells by 12 weeks. All mast cells are labelled with antitryptase and antichymase mAb, and all mast cells exhibited granules with electron-dense cores, features of the MCTC type of mast cell *in vivo* [30].

Essentially, all mast cells in the foreskin of newborns had such electron-dense cores by ultrastructural analysis, whereas most of the mast cells in adult skin had granules with electron-dense grating and lattice structure [31], features of mature mast cell granules that were not evident in the HBMMCs. What factor or factors might be needed for *in vitro*-derived mast cells to more

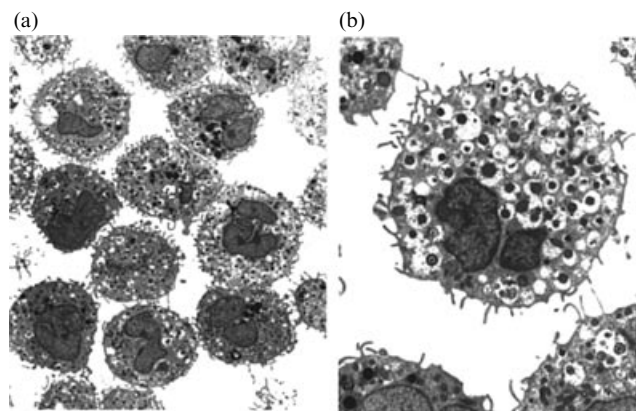


Fig. 8. Ultrastructural analysis of HBMMC cultured in the presence of SCF + IL-6 for 12 weeks. HBMMC derived from human bone marrow CD34⁺ progenitors were cultured in the presence of rhSCF + rhIL-6 for 12 weeks and subjected to transmission electron microscopy. Representative electron micrographs are shown. (Original magnifications: a, $\times 1500$; b, $\times 5000$.)

fully mature is not known. No mast cell with granules that were predominantly composed of complete scroll structures, a feature of MCT cells in lung and bowel mucosa [32], were observed in the current study.

Human mast cells have been divided into two types based on the protease phenotype of their granules. MCTC cells contain tryptase, chymase, cathepsin G and mast cell carboxypeptidase, and are the predominant type of mast cells in the skin and bowel submucosa. MCT cells contain tryptase without the other protease [33] and are the predominant type of mast cells in the lung alveolar walls and small bowel mucosa. Because both MCT and MCTC types of mast cells originate from bone marrow progenitors, it is of interest that all mast cells arising from bone marrow progenitors in the current study become MCTC cells. One possibility is that all progenitors are capable of becoming either type of mast cell, but that the culture conditions used in the current study favour the development and/or survival of MCTC cells. Another possibility is that the MCT progenitor was lost during the isolation of CD34⁺ cells. Other possibilities relate to the unknown abilities of either mature or committed MCT or MCTC cells to change their phenotype.

The predominant type of tryptase in HBMMC appears to be β -tryptase, because comparable amounts of tryptase were measured by one immunoassay previously shown to measure β -tryptase far better than α -protryptase, and another immunoassay shown to measure both types of tryptase about equally well. This finding is consistent with what has been proposed for normal mast cells that express mRNA for both α and β -tryptase genes. The hypothesis is that β -protryptase is processed efficiently to mature β -tryptase and is stored in secretory granules, whereas α -protryptase is processed less efficiently, if at all, and is spontaneously secreted by mast cells. Thus, even though both types of tryptase might be expressed by mast cells, the mature β -tryptase would accumulate inside the secretory granules and be the predominant form in cell extracts, as it appears to be in the current study.

Notably, chymase was abundantly expressed in HBMMC granules without the addition of IL-4. Levels of chymase per mast cell were about twice as high as in CBMC by 8 and

12 weeks of culture, and were comparable with levels reported in skin-derived mast cells (4.5 pg/mast cell). Cellular levels of tryptase and histamine in HBMMC compared with CBMC were not significantly different, as shown in Table 1. Chymase mRNA expression occurred later than that of tryptase, but progressed from 4 to 12 weeks of culture [34, 35], consistent with the appearance of chymase protein. Also, cellular levels of tryptase mRNA far exceeded those of chymase, while protein levels of tryptase were only several-fold higher than those of chymase. This suggests that post-transcriptional events may dictate the ultimate level of each protein in mast cells. Ahn et al. reported that the level of chymase mRNA was expressed higher in immature mast cells than in mature tissue-derived mast cells that were chymase protein-rich [11]. The late appearance of chymase expression has been reported in other systems. For example, when human fetal liver progenitors were cultured in serum-free AIM-V media, 40% of cells showed strong chymase immunocytochemical staining by 10–12 weeks [36]. Our results raise the possibilities that the differentiation of tryptase- and chymase-double positive mast cells is regulated at the progenitor level independent of cytokine presentation as well as culture condition.

Mast cells are also heterogeneous in the expression of surface markers. CD54 is expressed on mast cells in lung and skin [13] but not on fetal liver-derived mast cells [15]. IL-4 stimulates the appearance of CD54 on CBMC. CD88, the receptor for C5a, is expressed on skin mast cells and a subset of cardiac mast cells but not on other types of mast cells, including the HBMMC of the current study [37]. For the most part, the pattern of surface antigen expression on HBMMC resembles that reported on tissue-derived [13] or cultured mast cells [14, 38], as shown in Fig. 5.

Of possible interest in the current study was the observation that mast cell progenitors were predominantly CD13⁺, and this surface marker diminished but did not disappear as the bone marrow-derived mast cells matured. CD13 is strongly expressed on fetal liver-derived mast cells [14] and CBMC [18] but not on mature tissue mast cells.

CD13 antigen is a 150-kDa cell surface glycoprotein that is identical to aminopeptidase N, a membrane-bound glycoprotein thought to be involved in the metabolism of regulatory peptides by diverse cell types [39, 40]. CD13 was shown to be expressed on human bone marrow progenitors from mastocytosis patients as well as CD34 and CD117 [17]. CD13 expression during a cell's development may vary among different cell lineages. The antigen is expressed by cells of the granulocyte and monocyte lineages at all stages of differentiation [41]. For monocytes/macrophages, IL-4 induces CD13 to increase on mature cells such as alveolar macrophages, while immature monocytes express little, if any, CD13 [42]. CD13 is expressed on eosinophils and dendritic cells in human bronchial epithelium but not on mast cells and T lymphocytes [14, 19, 43]. CD13 is expressed on stem cells and during most developmental stages of myeloid cells [21]. Also, cells committed to the earliest stages of B or T cell differentiation are CD13⁺ [44] but become CD13⁻ upon maturation. In mice, CD13 also is expressed on mast cells as well as other cell types [45], suggesting that the biological activity of this aminopeptidase might be evolutionarily conserved. The expression of CD13 in human monocytes, macrophages and endothelial cells is upregulated by exposure to IL-4, providing an opportunity for such cells to be involved

in Th2-mediated inflammation. It is therefore possible to postulate that surface antigen expression may reflect both the developmental and activation state of a particular cell type.

In conclusion, mast cells derived from human bone marrow CD34⁺ cells express surface CD54 and FcεRIα as well as chymase, tryptase and histamine in their granules. CD13 is expressed on the surface of mast cell progenitors and, though diminished during the development of mast cells, persists on the immature type of mast cell that emerges from bone marrow-derived progenitors *in vitro*.

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