

ORIGINAL PAPER

A protocol for generating high numbers of mature and functional human mast cells from peripheral blood

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Clinical and Experimental Allergy

Summary

Background Mast cells (MCs) are multi-functional effector cells with an essential role in innate immunity and host defence, and under several pathological conditions, such as allergy. Here, we aimed at defining the culture conditions that would allow efficient generation of mature and functional human MCs from their progenitor cells.

Methods Human peripheral blood-derived CD34⁺ progenitor cells were cultured *in vitro* under serum-free conditions with human stem cell factor for 9 weeks. Growth and differentiation of the cells into MCs were optimized by selected cytokines and a combination of hypoxic and normoxic conditions. MCs were phenotypically characterized by immunocytochemistry, their preformed mediators were quantified, and their functional ability to degranulate and release histamine was tested.

Results On average, 20×10^6 mature MCs were generated from 0.5×10^6 progenitor cells during 9 weeks of culture, i.e. at least a 40-fold increase in cell number was achieved. The mature MCs had oval-shaped non-lobular nuclei, contained histamine, heparin, tryptase, chymase, and cathepsin G in their secretory granules, and strongly expressed c-kit (CD117) and Fc epsilon receptor I on their surface. Histamine release from the cells could be brought about by IgE–anti-IgE cross-linkage, compound 48/80, substance P, and anaphylatoxin C3a. The MCs remained functional for several weeks after their maturation.

Conclusion This study describes an efficient protocol for generating mature MCs from human peripheral blood with a functional phenotype of connective tissue-type MCs. Use of these cultured human MCs will increase our knowledge and understanding about human MC development and biology in human disease.

Keywords chymase, differentiation, histamine, *in vitro*, mast cells, tryptase

Submitted 1 October 2006; revised 2 April 2007; accepted 18 June 2007

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Introduction

Mast cells (MCs) are multi-functional tissue-dwelling cells involved in various pathological conditions such as allergy and asthma, and their contribution to cardiovascular diseases and rheumatoid arthritis has also been suggested [1–4]. MCs are filled with cytoplasmic secretory granules that contain preformed mediators including cytokines, heparin, histamine, and two neutral serine proteases: tryptase and chymase [5–8]. Mature MCs can be distinguished from other cells by their expression of high-affinity IgE receptor [Fc epsilon receptor I (FcεRI)] and c-kit (CD117) on their surface, and tryptase and heparin in their granules [1, 9, 10].

The development of MCs *in vivo* begins when the pluripotent progenitor cells leave the bone marrow as

CD34⁺ cells, and enter the circulation, and a subset of them later immigrate peripheral tissues where they differentiate into mature MCs [11–13]. Stem cell factor (SCF) expressed by stromal cells, including endothelial cells and fibroblasts, is an indispensable growth factor for human MC development and survival, and deprivation of SCF results in rapid apoptotic death of MCs [12, 14, 15]. The importance of SCF and its receptor, c-kit, in MC development is stressed by the facts that SCF-deficient mice lack MCs [16], and that injection of SCF into human subjects results in MC hyperplasia [17]. Besides SCF, the cytokine IL-3 has also been shown to induce the development of human MCs from the bone marrow [18], and to promote the growth of mature MCs [19]. Finally, the contribution of T-helper type 2 (Th2) cytokines to human MC development has been

investigated [14, 20–22], but their exact role in this setting remains to be elucidated.

To obtain viable human MCs for *in vitro* experimentation, two approaches are available: (i) isolation of mature MCs from tissues and (ii) isolation of immature MC progenitors from the bone marrow, cord blood, foetal liver or peripheral blood, and induction of their differentiation into mature MCs under defined conditions *in vitro*. Both established methods are laborious and they suffer from inherent limitations. Regarding isolation of human MCs from tissues, only small numbers of cells can be obtained. Moreover, because the tissue-derived mature MCs do not generally divide, and only survive for a short period of time in culture after their isolation [23], the time window available for studies using primary cultures of MCs is narrow. Similarly, it is cumbersome to generate high numbers of mature MCs from progenitor cells, because human MCs require an exceptionally long period for their development and maturation *in vitro*. Because of these limitations, human MC cell lines have also been used extensively for studies of MC biology. Available are the immature HMC-1 leukaemia cells [24], and the more mature LAD2 cells, which, in contrast to HMC-1 cells, respond to SCF and express functional FcεRI [25, 26].

To date, several methods have been described to obtain primary cultures of human MCs. These include the bone marrow [18], cord blood [27–29], peripheral blood [9, 30], and foetal liver [10, 31] as a source of MC progenitors. Depending on the source of the progenitor cells and the methodology used, the number of MCs generated, the degree of their maturity, and their phenotype and responsiveness towards various stimuli vary greatly [31–33]. Clearly, the challenge has been to develop a method that rapidly produces high numbers of mature and functional MCs.

Here, we present an efficient and reliable method for generating high numbers of mature and phenotypically uniform human MCs from peripheral blood-derived CD34⁺ progenitor cells under serum-free conditions in the presence of SCF. The protocol was standardized by selecting and optimizing the use of various cytokines and by combining hypoxic and normoxic culture conditions. We demonstrate that the MCs generated by this protocol express heparin and histamine, the neutral proteases chymase, tryptase and cathepsin G in their secretory granules, and the receptors c-kit and FcεRI on their surface. The cells are also functional as demonstrated by their ability to acutely release histamine in response to stimulation with IgE–anti-IgE, compound 48/80, substance P, and the anaphylatoxin C3a.

Materials and methods

Antibodies

For immunocytochemistry, the following primary antibodies against human antigens were used: a mouse

monoclonal antibody (mAb) against chymase (clone CCI, IgG1 isotype) from Serotec (Oxford, UK); a mouse mAb against tryptase (clone AA1, IgG1) from Dako (Alstrup, Denmark); a mouse mAb against FcεRI (clone CRA-1, IgG2b) from eBioscience (San Diego, CA, USA); a rabbit polyclonal antiserum against cathepsin G from Abcam (Cambridge, UK); and a goat polyclonal antiserum against human IgE from Chemicon (Temecula, CA, USA). Isotype-matched mouse mAbs served as negative controls, and the secondary antibodies used will be described in the text. For flow cytometry, the antibodies were as follows: a phycoerythrin (PE)-conjugated mouse mAb against c-kit (clone YB5.B8, IgG1) and a mouse mAb against Bsp-1 (clone 155–228, IgM), both from BD Pharmingen (San Diego, CA, USA), and mouse mAbs against FcεRI (clone CRA-1, IgG2b) and IL-3Rα (clone 6H6, IgG1, biotin-conjugated), both from eBioscience. Negative controls were isotype-matched mouse IgG from eBioscience, Serotec, or ImmunoTools (Friesosythe, Germany). The appropriate secondary antibodies will be described in the text.

Cell culture

The CD34⁺ cells were obtained from fresh buffy coats prepared from peripheral blood of healthy volunteers and kindly supplied by the Finnish Red Cross Blood Transfusion Service (Helsinki, Finland). The buffy coats were first diluted with phosphate-buffered saline (PBS), layered over Ficoll-Paque (1.77 g/L) (Amersham Pharmacia, Uppsala, Sweden), and the interface containing mononuclear cells was harvested after centrifugation. The cells were then washed with PBS, and CD34⁺ progenitor cells were enriched by positive immunomagnetic selection according to the manufacturer's instructions (Miltenyi Biotec, Auburn, CA, USA). The isolated cells were then transferred into 12-well plates at a density of 0.5×10^6 cells/mL and placed in a humidified incubator at 37 °C.

For the development of the culture protocol described in this paper, the following basal conditions were used. All cytokines and growth factors were recombinant human proteins from PromoCell (Heidelberg, Germany). The CD34⁺ progenitor cells were grown under serum-free conditions using StemSpanTM medium (Stem Cell Technologies, Vancouver, Canada) supplemented with penicillin (100 U/mL) and streptomycin (100 µg/mL) (GIBCO BRL, Grand Island, NY, USA), human low-density lipoproteins (LDL) (10 µg/protein/mL) prepared from healthy volunteers in our laboratory as described [34], and SCF (100 ng/mL). We established a protocol in which four different cytokines (IL-3, IL-9, IL-6, and IL-4) were added to the culture medium sequentially for various periods of time and to give the final concentrations indicated in the text (see Fig. 1). The cytokine-supplemented medium was replaced biweekly during the first 2 weeks and weekly thereafter, and the non-adherent cells were passaged into fresh

medium containing the indicated cytokine(s). Each week, cell proliferation was estimated by counting the cells with a haemocytometer, and cell viability was estimated by the trypan blue exclusion test. Cell morphology was evaluated after staining with May–Grünwald–Giemsa and toluidine blue.

Immunocytochemistry

Identification of MC proteases, notably of tryptase, chymase, and cathepsin G, was performed at various stages of MC differentiation. Briefly, $1\text{--}2 \times 10^4$ cells/sample were centrifuged onto glass slides by Cytospin 2 (Shandon, Pittsburgh, PA, USA), allowed to air-dry, then fixed with methanol, and finally washed repeatedly with PBS. Next, the non-specific binding sites were first blocked with 3% rabbit or goat serum in 1% bovine serum albumin (BSA) in PBS (pH 7.4), washed, and incubated overnight at 4 °C with primary antibodies (3 µg/mL for chymase, 0.5 µg/mL for tryptase, and 1 : 350 dilution for cathepsin G) in a humid chamber. The slides were then washed four times

and incubated for 60 min with the following secondary antibodies: a goat anti-mouse Alexa-488 (IgG1) for detection of chymase and tryptase, a goat anti-rabbit Alexa-546 for cathepsin G, and a goat anti-mouse Alexa-568 (IgG2b) for FcεRI. The secondary antibodies were from Molecular Probes (Eugene, OR, USA) and were used at a 1 : 500 dilution.

Flow cytometry

For cytofluorographic analysis of cell surface epitopes, a fluorescence-activated cell sorter (FACScan, Becton Dickinson, San Jose, CA, USA) was used. Briefly, after removal of the cell culture medium, the cells ($2\text{--}3 \times 10^5$ per sample) were suspended in PBS containing 0.5% BSA and 0.05% NaN₃ (BSA–PBS). Non-specific binding sites were blocked by incubating the cells with 50 µg/mL of human IgG (Sigma, St Louis, MO, USA) along with primary antibodies for FcεRI or Bsp-1 or IL-3Rα, or with their isotype-matched controls for 30 min at 4 °C. Cells were then washed and incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse F(ab')₂ fragments (Serotec), streptavidin-FITC (Dako), or PE-conjugated goat anti-mouse IgM F(ab')₂ fragments (Abcam), washed again, and incubated with a PE-conjugated c-kit or isotype-matched control. After the final washes, the cells were suspended in BSA–PBS. For exclusion of necrotic cells, propidium iodide (10 µg/mL; Sigma) was added to samples of unstained cells before analysis.

Quantitation of mast cell mediators

The contents for tryptase, heparin, and histamine were determined as follows: first, $4\text{--}10 \times 10^4$ cells were sedimented by centrifugation, the supernatants were removed, and the sedimented cells were resuspended in 0.1% Triton-X 100 for lysis. Tryptase was measured with a fluoroenzyme-immunoassay (UniCAP™, Pharmacia, Uppsala, Sweden) [35], and the results were expressed as picograms of tryptase per cell. Proteoglycan content was determined by a commercially available glycosaminoglycan assay (Blyscan™, Biocolor, Newtownabbey, Ireland). In this assay, 1,9-dimethylmethylene blue was used under conditions that produced a specific label for the sulphated polysaccharide component of proteoglycans and protein-free sulphated glycosaminoglycan chains, which could then be determined colorimetrically [36]. For quantitation, a standard curve was created with commercial heparin (Sigma), and the results were expressed as picogram of heparin per cell. As the assay is not heparin glycosaminoglycan specific, the proportion (%) of heparin glycosaminoglycans on the total glycosaminoglycan content of the cells was calculated from samples treated with heparinase I (Sigma) or with chondroitinase ABC (Seikagaku Corp, Tokyo, Japan) to degrade heparinase- and

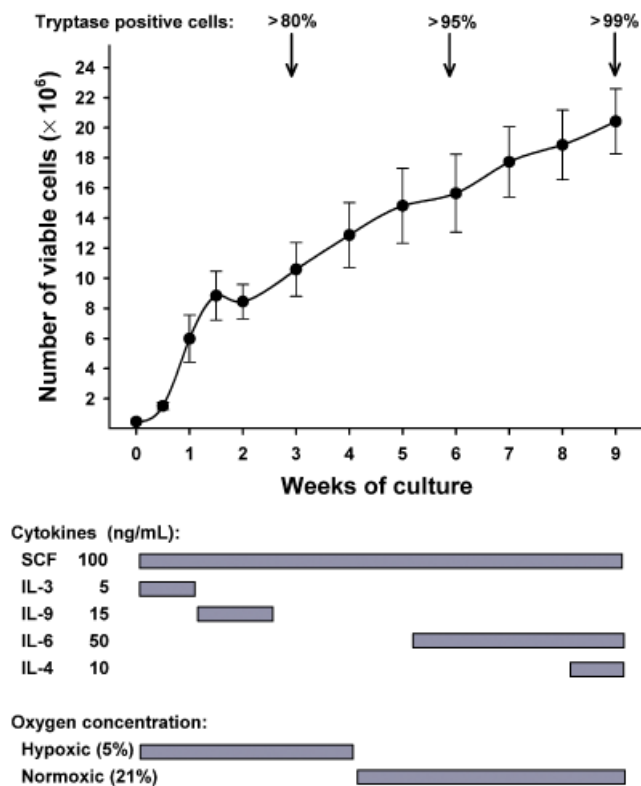


Fig. 1. Growth and differentiation of peripheral blood-derived CD34⁺ progenitor cells into mature mast cells (MCs). The cells were cultured in the presence of the indicated cytokines at the concentrations given for a total period of 9 weeks. Two different oxygen concentrations were used sequentially. The proportion of tryptase-positive cells was determined at weeks 3, 6, and 9 to identify the developing MCs. The time intervals allocated for each cytokine and oxygen concentration are shown. Data are means ± SEM for cells derived from six donors. SCF, stem cell factor; SEM, standard error of the mean.

chondroitinase-susceptible proteoglycans before analysis, respectively. For histamine measurement, the high-molecular-weight proteins were first precipitated by incubating the samples on ice for 20 min in the presence of trichloroacetic acid (TCA). Samples were then centrifuged at 10 000 g for 10 min at 4 °C, the supernatants were removed, and stored at -20 °C until histamine determination. Histamine was measured by registering the fluorescence generated by the *o*-phthalaldehyde complex according to Shore *et al.* [37]. A standard curve was created with commercial histamine (Sigma) and the results were expressed as picograms of histamine per cell.

Histamine release as a measure of mast cell activation

For immunological activation, the MCs were suspended in fresh culture medium supplemented with 100 ng/mL of SCF, and sensitized with purified human myeloma IgE (1 µg/mL, Chemicon) overnight at 37 °C. The supernatants were then removed by centrifugation and cells ($2-3 \times 10^4$ per sample) were washed with Ca²⁺-free PBS and suspended in warm Tyrode's buffer consisting of 10 mM HEPES [N-2-hydroxyethylenepiperazine-*N'*-2-ethanesulphonic acid], 137 mM NaCl, 2.7 mM KCl, 0.38 mM Na₂HPO₄ · 7 H₂O, 5.6 mM glucose, 1.8 mM CaCl₂ · 2 H₂O, 1.3 mM MgSO₄ · 7 H₂O, and 0.03% BSA (pH 7.4). Next, the IgE-sensitized cells were challenged with goat antiserum against human IgE (2 µg/mL, Chemicon) for 30 min at 37 °C. Negative controls were treated in the same way, except for omission of anti-IgE. In some experiments, aliquots of the sensitized MCs were stimulated with compound 48/80 (6.5 µM, Sigma), with substance P (10 µM, Sigma), or with affinity-purified C3a (1 µg/mL, Calbiochem, San Diego, CA, USA) for 30 min at 37 °C. After stimulation, the incubation mixtures were cooled down to at 4 °C and centrifuged to sediment the MCs. The supernatants were collected and the MCs were lysed in 0.1% Triton-X 100, freeze-thaw repeatedly, and high molecular weight proteins were precipitated with TCA. Histamine was measured as described above. Histamine release (%) was calculated as follows: histamine in medium/(histamine in cells+histamine in medium) × 100.

Data handling and analysis

For statistical analysis, the data were processed using SPSS software (SPSS Inc., Chigago, IL, USA). Differences between each experimental and control group were assessed using Student's *t*-test, and the level of statistical significance was set at *P* < 0.05. Data are expressed as mean ± standard error of the mean (SEM) for *n* independent experiments unless otherwise stated.

Results

Mast cell growth and differentiation

We established a protocol in which SCF at a concentration of 100 ng/mL was present throughout the culture period, and in which four different cytokines (IL-3, IL-9, IL-6, and IL-4) were added to the culture medium sequentially for various periods of time at the final concentrations indicated in Fig. 1. Briefly, IL-3 (5 ng/mL) was present during the first week of culture and IL-9 (15 ng/mL) for the next 10 days (days 8–17). IL-6 (50 ng/mL) was added at week 5 and IL-4 (10 ng/mL) at week 8 of culture. Using this protocol, an average of $20 \pm 2.2 \times 10^6$ mature MCs were developed from 0.5×10^6 peripheral blood-derived CD34⁺ progenitor cells after 9 weeks of culture (*n* = 6 donors) (Fig. 1). The cells proliferated rapidly during the first 10 days until their number was about 18-fold of that at the beginning of culture. Although a slight decrease in the number of viable cells was observed after 2 weeks of initiation of the culture, the proliferation rate remained stable until at least 9 weeks of culture. At this time-point, more than 99% of the cells were tryptase positive (Fig. 1).

To evaluate the effect of IL-9 on the growth and differentiation of CD34⁺ progenitor cells, we performed an experiment in which this cytokine was either present or absent during days 8–17 of culture. Addition of IL-9 at this early stage of culture was found to increase the total number of progeny, on average, by 1.4-fold as compared with cells grown with SCF alone (*n* = 3 donors) (Fig. 2). However, addition of IL-9 to the cells was without any influence on their differentiation, when examined by staining against tryptase (data not shown). In separate experiments, we also observed that inclusion of IL-9 at this early stage of culture for longer periods of time than for 10 days did not further improve cell proliferation when compared with cells cultured in the absence of IL-9 (data not shown). Based on these results, a 10-day period of stimulation with IL-9 was adopted to the final protocol (Fig. 1).

A small number of adherent cells survived in the culture despite the MC growth-supporting conditions. However, these cells were depleted by transferring the non-adherent cells to new culture plates each week. Furthermore, instead of weekly hemi-depletions, the entire volume of culture medium was replaced to exclude the possibility that growth factors secreted by the contaminating cells were interfering with MC growth. For instance, granulocyte-macrophage colony-stimulating factor (GM-CSF) secreted by macrophages has been shown to suppress human MC growth [27] and to support the development of other lineages [38].

After 6 weeks of culture, the cell population consisted of over 95% of MCs, as revealed by their tryptase

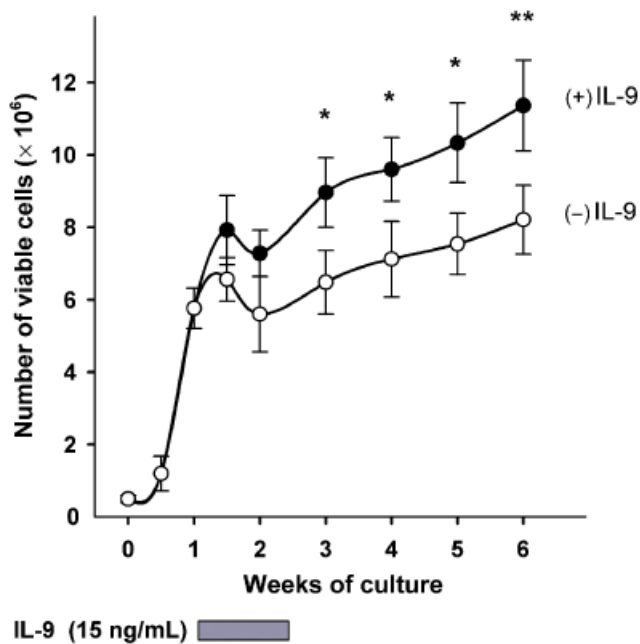


Fig. 2. Effect of IL-9 on human mast cell (MC) progenitor growth. Cells were cultured for 6 weeks as described in Fig. 1, except that on days 8–17 the cells were grown either in the absence (○) or presence (●) of IL-9. Data are means $\pm$ SEM from cells derived from three donors. Comparisons were made at the indicated time-points: * $P < 0.05$; ** $P < 0.01$. SEM, standard error of the mean.

positivity (see Fig. 1). Moreover, this fraction of cells had a granular appearance in light microscopy when stained with toluidine blue (not shown). By week 9, the cultures consisted of a fully homogenous population of cells (Fig. 3a). These cells even had a more distinct granular appearance than the cells at week 6, and the cytoplasmic granules stained metachromatically with toluidine blue, thus revealing the presence of highly sulphated proteoglycans, such as heparin (Fig. 3b). In contrast to the multi-lobed nuclei often observed in MCs grown in serum-containing media [31, 32], the cells had round, oval-shaped, and non-segmented nuclei (Fig. 3b).

Immunophenotyping

Immature MCs progenitor cells were identified at week 3 of culture, as evidenced by their positive staining for tryptase (Fig. 1).

To establish that the present system is suitable for generation of MCs, and not basophils, already at the early stage of culture (weeks 2–5), we identified the developing MCs by their high surface expression of c-kit. The basophils were detected by their expression of Bsp-1 and IL-3R α [39, 40]. At weeks 2 and 3, a small percentage of the total cells were identified as basophils (between 0.7 and 3.9%, mean 2.6%, $n = 4$ donors), but they disappeared after 4 weeks of culture (Figs 4e–h). The basophils were negative for c-kit (data not shown). Among the MC

progenitors, about 6–33% (mean 15.2%, $n = 4$ donors) were shown to express IL-3R α at week 2, and as the cells matured, the antigen expression gradually decreased (Figs 4a–d).

By week 9, virtually all cells stained positively for tryptase, chymase, and cathepsin G (Figs 3c–e), thus resembling the MC_{TC} phenotype [41]. In addition, the developed cells stained positively for Fc ϵ RI (Fig. 3f), revealing MC maturity [1]. Flow cytometry also revealed that by 9 weeks of culture, all cells were positive for c-kit and that more than 90% of the cells strongly expressed Fc ϵ RI (Fig. 5).

Quantification of mast cell mediators and histamine release

After 9 weeks of culture, each MC contained, on average, 7.6 ± 1.8 pg/cell of histamine, 7.8 ± 0.8 pg heparin, and 16.1 ± 1.2 pg tryptase ($n = 6$ donors). The heparin content of the cultured MCs at week 9 was about $65 \pm 4.3\%$ and that of chondroitin sulphate was about $22 \pm 3.1\%$ ($n = 3$ donors) of the total glycosaminoglycan content. Following an overnight sensitization with human IgE, the 9-week-old MCs showed substantial activation and degranulation, as indicated by their ability to release histamine upon addition of anti-IgE (Fig. 6). Thus, the cultured mature MCs expressed functional Fc ϵ RI and contained the cellular machinery required for an Fc ϵ RI-mediated acute anaphylactic reaction. The cells also responded to the polycationic agents, compound 48/80 and substance P, and also mildly to C3a (Fig. 6).

In summary, after 9 weeks of culture of CD34⁺ progenitor cells, practically all cells derived from the CD34⁺ progenitor cells were tryptase positive, their cytoplasm was filled with granules, and the cells responded to various stimuli by acutely releasing histamine. When the MCs were allowed to grow under the culture conditions present at week 9 (see Fig. 1), they remained viable and functional for several weeks, and were ready for experimentation.

Discussion

In this study, a protocol was established that enabled the generation of a high number of mature human MCs from peripheral blood-derived CD34⁺ progenitor cells after 9 weeks of culture. Compared with previous methodological reports with peripheral blood-derived progenitor cells, the present protocol resulted in a higher culture efficiency, i.e. more MCs were generated per number of cells at the initiation of culture (Table 1). More importantly, the cultures consisted exclusively of cells with characteristics typical of mature human connective tissue-type MCs [1, 9, 42].

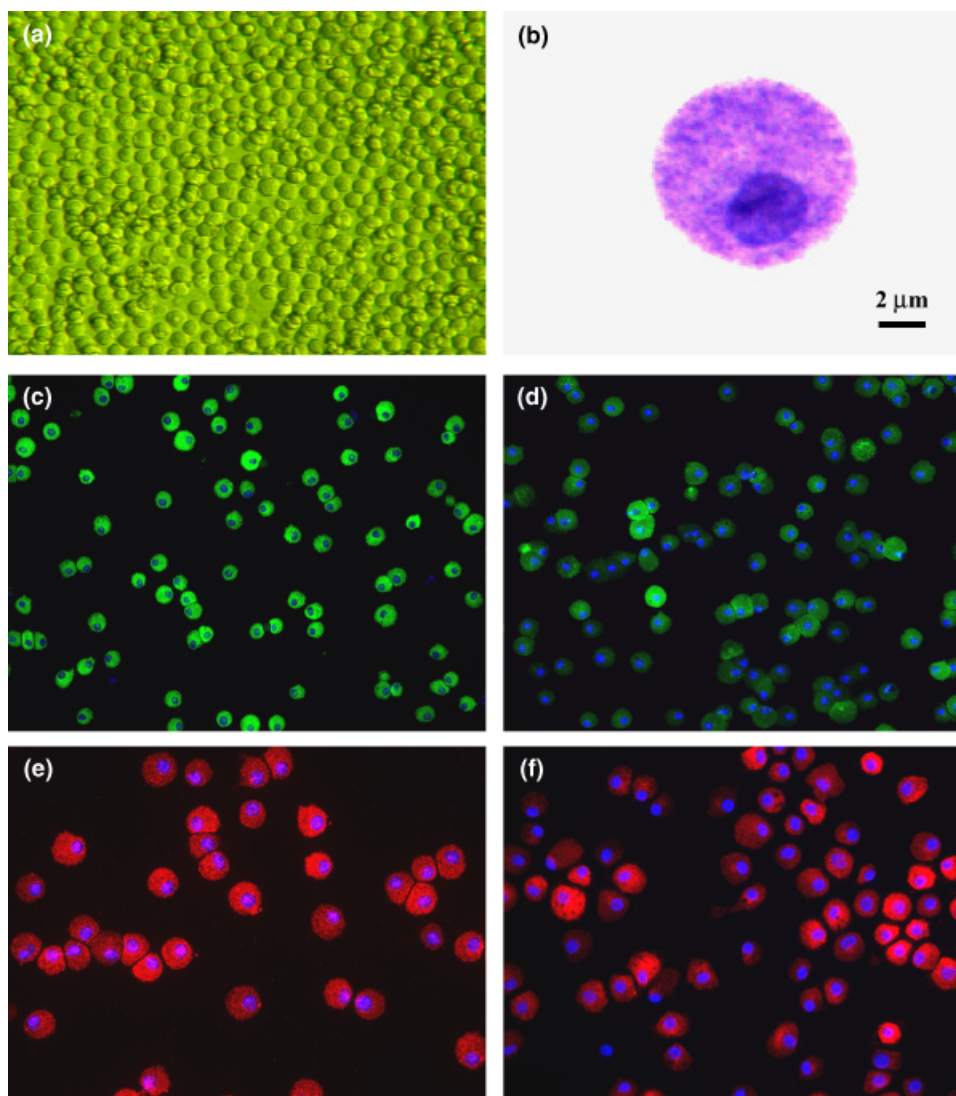


Fig. 3. Phenotypic appearance of human mast cells (MCs) after 9 weeks in culture. (a) MCs in culture. (b) Metachromatic staining of MCs with toluidine blue reveals the presence of heparin (one representative cell is shown at high magnification). Cells stained positively with monoclonal antibodies against tryptase (c), chymase (d), cathepsin G (e), and the alpha-chain of FcεRI (f). A fluorochrome-conjugated Alexa-488 IgG (c and d), Alexa-546 IgG (e), or Alexa-568 IgG (f) was used as the secondary antibody. Nuclei were stained with DAPI. Stainings with irrelevant Igs were negative in each case (data not shown). Original magnifications $\times 20$ (a, c), $\times 40$ (d–f), and $\times 60$ (b). FcεRI, Fc epsilon receptor I.

Despite identical growth conditions, human cord blood-derived progenitor cells have been shown to generate several-fold more progeny than their bone marrow or peripheral blood counterparts [43, 44]. It is not clear, as to what causes the higher proliferation efficiency, but it may be related to a favourable cell cycle status or to the autocrine production of MC growth-inducing cytokines by the cord blood cells. Furthermore, Nomura *et al.* [45] also reported an inverse correlation between the age of the donors and proliferation potency of MC progenitors, which may partly account for the difference. However, our primary goal was to obtain mature and functional MCs for experimentation. Accordingly, we chose to use peripheral blood-derived progenitor cells because they have been shown to

mature faster and to express higher levels of functional FcεRI, as compared with cord blood cells [44, 46].

The rationale for using hypoxic conditions at the early stage (weeks 1–4) of our protocol was to support the clonogenic activity of the haematopoietic progenitor cells [47]. Although the effects of oxygen environment on the development of human haematopoietic cells are mainly limited to the early clonogenic activity of progenitor cells, hypoxic conditions have been reported to enhance the proliferation of cells also in long-term cultures [47, 48]. The generation of human MCs from CD34⁺ cord blood cells using a combination of hypoxic and serum-free conditions in the presence of SCF has resulted in a very high number of cells (1000-fold increase) within 20 weeks of culture [29], and the

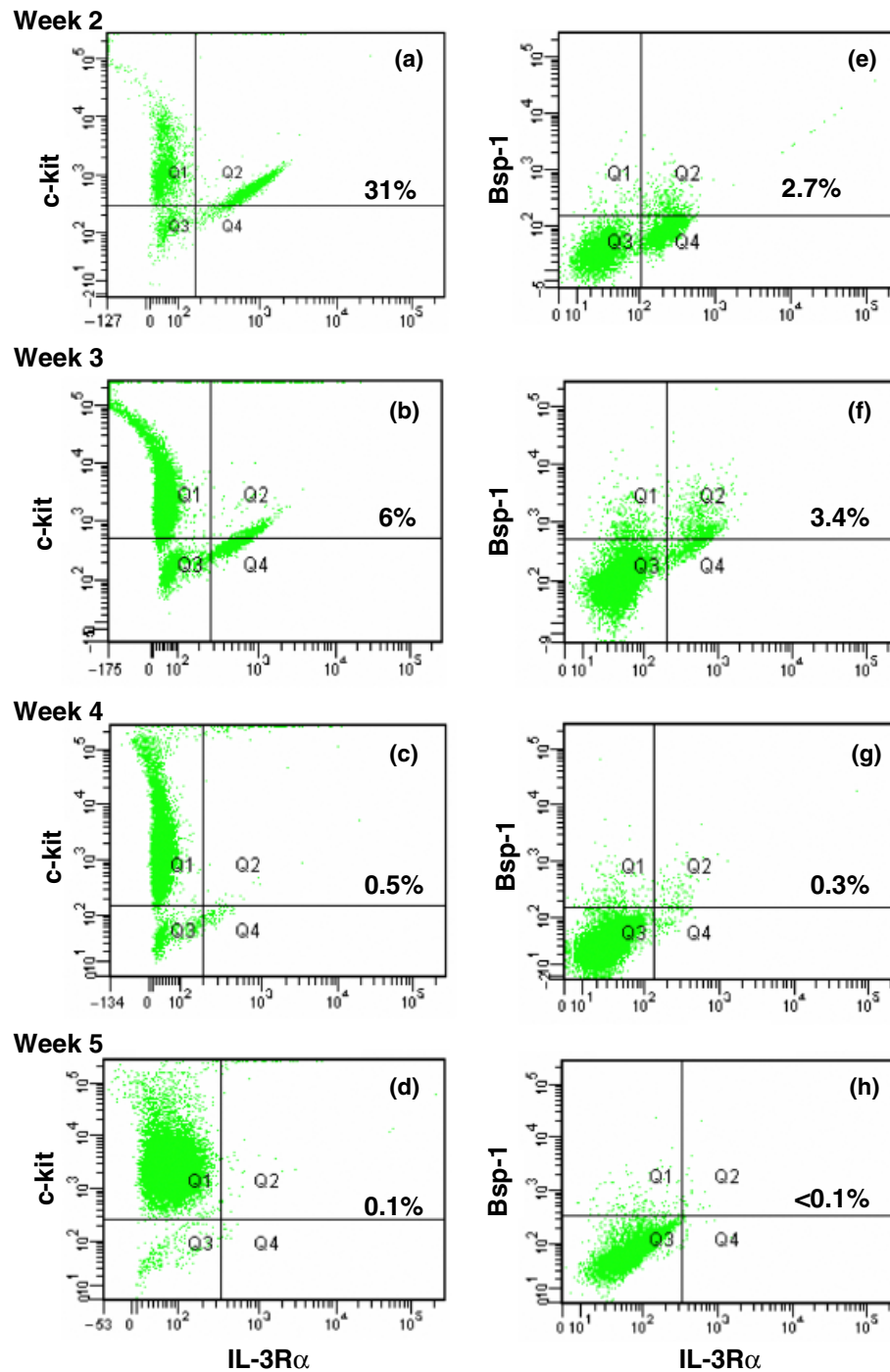


Fig. 4. A representative plot of flow cytometric analysis of the developing mast cells and basophils at early stages of the culture. The cells were cultured as shown in Fig. 1. At weeks 2 to 5 (from top to bottom), samples of cells were incubated with monoclonal antibodies and subsequently with FITC- and PE-conjugated immunoglobulins, or with a PE-conjugated antibody. Quadrant plots were created to evaluate the proportion and kinetics of cells double-positive for c-kit and IL-3R α (a–d), or Bsp-1 and IL-3R α (e–h). Fluorescence thresholds for positive staining were set by isotype-matched immunoglobulins.

addition of IL-6 further modulated the developing MCs towards a more mature phenotype. In addition, a low dose of IL-3 in serum-free medium has been shown to improve stem cell maintenance synergistically with hypoxia [47].

Both serum-free and serum-containing media have been used in systems for culturing human MCs. However, previous reports provide evidence that serum-free conditions allow for more efficient development of human MCs

than do serum-containing conditions [31, 32]. Thus, to minimize potentially suppressing or even inhibiting effects of serum on MC development [49], serum-free culture conditions were selected for the present protocol.

Regarding the various cytokines and their concentrations used in this protocol, the following points merit comment. Because SCF is of critical importance for the development and survival of cultured human MCs, this cytokine was present in the medium throughout the culture

period. In the majority of published reports, SCF has been used at a concentration of 100 ng/mL, and we also selected this widely used concentration for our protocol.

We used IL-3 at a low concentration (5 ng/mL) to support the growth of primitive MC progenitors, as this has shown to be optimal for comitogenesis [20, 50, 51]. Moreover, in accordance with a recent report by Scherthaner et al. [52], we observed that the MC progenitors were expressing detectable amounts of the alpha chain of the IL-3 receptor (CD123), which supports responsiveness to IL-3 stimulation at the early stage of their development. However, despite the enhancing effects of IL-3 on MC growth, this cytokine was added only at the early stage of culture (week 1) because, if administered for longer periods of time, it also supports substantial

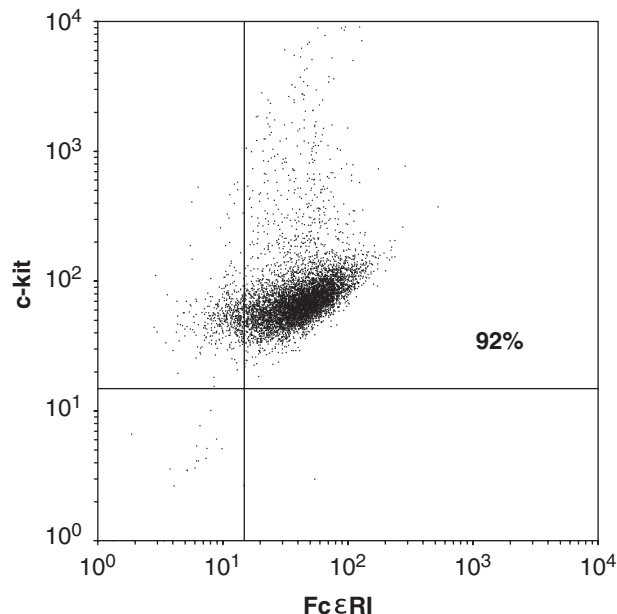


Fig. 5. Representative plot of flow cytometric analysis for c-kit and FcεRI expression in human mast cells after 9 weeks of culture. The cells were cultured as shown in Fig. 1. The cells were labelled with fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-conjugated antibodies, and a quadrant plot was created to calculate the proportion of cells strongly positive for both c-kit and FcεRI. Fluorescence thresholds for positive staining were set by isotype-matched Igs. FcεRI, Fc epsilon receptor I.

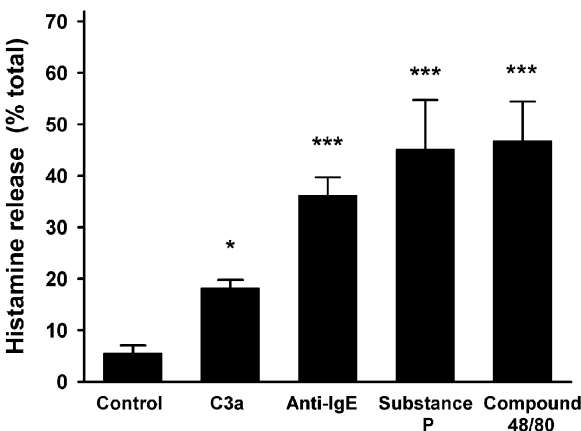


Fig. 6. Histamine release after immunological and non-immunological stimulation of mast cells (MCs) cultured for 9 weeks. The MCs were sensitized by overnight pre-incubation with human IgE. The sensitized cells were then incubated for 30 min with the indicated stimulatory ligands after which histamine in the incubation medium and in the cells was measured. Histamine release was expressed as % of total cellular histamine released into the medium (% total). Data are means $\pm$ SEM from cells derived from three donors. Compared with control: * P < 0.05; *** P < 0.001. SEM, standard error of the mean.

Table 1. Comparison of the present protocol with previously published protocols to generate human mast cells from peripheral blood-derived progenitor cells

Generation efficiency*	Culture period (weeks)	Cytokine(s) used	Protease phenotype	Heparin content (pg/cell)	Histamine content (pg/cell)	Response to FcεRI-mediated stimulation	References
40 ×	9	SCF IL-3, -4, -6, -9	Tryptase ⁺ Chymase ⁺ (> 95%)	7.8 $\pm$ 0.8	7.6 $\pm$ 1.8	Histamine release	Current study
0.17 ×	6	SCF IL-3	Tryptase ⁺ Chymase ⁺ (< 3%)	ND	4.8 $\pm$ 1.3	ND	[30]
26 ×	8	SCF IL-3, -6	Tryptase ⁺ Chymase ⁺ (85%)	ND	2–3 [†]	Histamine release	[9]
0.13 ×	6	SCF IL-6	Tryptase ⁺ Chymase ND	ND	ND	Histamine release	[45]
11.2 ×	8	SCF	Tryptase ⁺ Chymase ND	ND	4.3 $\pm$ 0.6	β-hexosaminidase release	[21]
0.06 ×	12	SCF IL-3, -6	Tryptase ⁺ Chymase ⁺ (30%)	ND	4.3 $\pm$ 0.7	Histamine release	[33]

*Defined by the average number of MCs generated per number of cells at the initiation of culture.

[†]Data available at week 4 only.

ND, not determined; MCs, mast cells; SCF, stem cell factor.

development of basophils and eosinophils [51, 53, 54]. Hence, only a small number of basophils were detected at weeks 2 and 3, and they disappeared by week 5. As such, the assay is also suitable for analysing the biology of MC progenitors.

We were also able to confirm previous observations by Matsuzawa et al. [22], who showed that addition of IL-9 to SCF-supplemented serum-free cultures of human MC progenitors increased the number of MC progeny already at an early stage of culture. In our experiments, however, the increase in cell number was less prominent, which may be explained by the fact that we used peripheral blood-derived progenitor cells, whereas Matsuzawa et al. [22] used cord blood cells.

The rationale for including IL-6 in our protocol was based on the previous observation that the comitogenic effects of IL-6 in combination with SCF supports MC growth [27, 29, 55], and this cytokine has been reported to increase MC mediator content (e.g. histamine) [29]. Indeed, this MC maturity-promoting propensity was the rationale of its use during the later stages of MC differentiation (weeks 6–9).

We only added IL-4 at the final stage of MC development (week 9) to prime the cells for FcεRI expression [44, 56] and so to up-regulate their responsiveness to immunological stimuli [57, 58]. Of note is that IL-4 inhibited the development of immature MCs when added at the initiation [21] or an early stage [55] of culture. Toru et al. [59] have reported that together with SCF, IL-4 also promotes the conversion of MC_T cells into MC_{TC} cells, and Ahn et al. [51] observed that IL-4 further up-regulated the expression of chymase in MC_{TC} cells. In our system, the developing MCs were expressing chymase by week 3, and the protein stably persisted throughout the MC maturation, which accords with previous reports [20, 60]. These results contrast those of Wang et al. [33], and further emphasize earlier observations according to which all human MCs are able to express chymase under serum-free conditions [51]. Although the source of the progenitor cells may partly account for their ultimate chymase content [42], it seems that the serum-free culture environment may also have facilitated chymase expression in the mature cells [31]. In addition to chymase, the mature tryptase-containing cells were also positive for cathepsin G, which served as an additional marker of mature MCs.

The cytoplasmic secretory granules of mature human MCs contain substantial amounts of histamine, heparin, and tryptase [61]. We quantified these mediators, and found that, at 9 weeks, the tryptase and histamine contents of the mature MCs were comparable with or higher than those reported previously in cultured human MCs derived either from peripheral blood, cord blood, or bone marrow [21, 29, 30, 33, 42]. We also analysed the nature of the glycosaminoglycans in the mature MCs, and observed that the heparin content of the cells was higher than in

human lung-derived MCs [8, 62]. The proportion of heparin glycosaminoglycan to other glycosaminoglycans such as chondroitin sulphate was in agreement with lung-derived MCs [63], but in contrast to cord blood-derived MCs by Nilsson et al. [64]. Presumably, the MCs used in that study were still immature as they were also reported to be negative for the expression of the FcεRI [64]. To our knowledge, the present study is the first quantitative report of heparin in cultured human MCs.

The most striking hallmark of maturity of MCs is their ability to release granule contents upon appropriate stimulation. The classical type of activation of MCs occurs following antigen-induced IgE cross-linking [7], but MCs can also be stimulated by a variety of synthetic agents [65]. Based on the protease phenotype of MCs, certain differences have been observed in their responsiveness to degranulate and release histamine, and other preformed mediators following exposure to various stimuli [65, 66]. In our study, the IgE-sensitized MCs released substantial amounts of histamine when challenged with anti-IgE after sensitization with IgE, and the values of released histamine were comparable with those obtained previously with peripheral blood-derived MCs [33, 45]. In addition, we demonstrated that the MCs responded to other secretagogues, such as C3a, substance P, and compound 48/80, by releasing histamine similar to MCs derived from the skin, which, like the present cells, also express the MC_{TC} phenotype [66].

Characterization of the conditions for developing mature and functional human MCs *in vitro* is important to facilitate reliable and successful research on human MCs. The present study introduces an efficient protocol to generate high numbers of mature MCs of the MC_{TC} phenotype from human peripheral blood. As these cells fulfil several criteria required for mature human MCs, they will hopefully serve as a useful model in studies of MC biology in human health and disease.

Acknowledgements

The Wihuri Research Institute is maintained by the Jenny and Antti Wihuri Foundation. The authors are grateful to Jaana Tuomikangas, Leena Saikko, Mari Jokinen, and Suvi Mäkinen for skilful technical assistance.

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