

Research paper

Buffy coat preparation is a convenient source of progenitors for culturing mature human mast cells

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Received 16 May 2005; received in revised form 8 September 2005; accepted 15 November 2005

Available online 27 December 2005

Abstract

Mast cells are unique immune cells that release a spectrum of chemical mediators contributing to the inflammatory symptoms of allergic disorders. Mature mast cells have recently been cultured from CD34⁺ progenitors isolated from fresh umbilical cord blood and adult peripheral blood. In the current study, we investigated whether buffy coat preparations, which are readily available from blood banks, could be used as a convenient and more abundant source of progenitors for culturing human mast cells. We were able to culture a homogeneous population of human mast cells from progenitor cells isolated from human buffy coat. Morphologically, our cultured mast cells contained abundant cytoplasmic granules which stained positively using antibodies against human mast cell tryptase and, to a lesser extent, with those against human mast cell chymase. Functionally, these cultured mast cells responded to anti-human-IgE by releasing histamine in a dose-dependent manner after sensitization with human IgE. Taken together, buffy coat preparations can be a convenient source for culturing human mast cells which are predominantly tryptase positive only and express functional high-affinity IgE receptors.

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Keywords: Human mast cells; Buffy coat; Human-IgE; Tryptase; Chymase

1. Introduction

Based on the metachromatic staining properties of their prominent cytoplasmic granules, the mast cell was first identified in human connective tissues by Ehrlich in 1898. Mast cells are widely distributed throughout the body as terminally differentiated resident cells in

connective tissues and, unlike the basophils, they do not circulate in the blood stream (Kawakami and Galli, 2002). By virtue of their high-affinity receptors for immunoglobulin E (IgE) and their content of potent inflammatory mediators, mast cells are now recognized to be a major inducer of allergic and inflammatory reactions (Stassen et al., 2002). The immunological activation of mast cells through cross-linking of the high-affinity IgE receptors (Fc_εRI) initiates the release of preformed granular content, such as histamine, neutral proteases and proteoglycans within minutes of activation and is followed by the de novo synthesis of lipid mediators, such as cysteinyl-leukotrienes (cys-LTs) and prostaglandin (PG) D₂, and cytokines, such as TNF-α and IL-8 (Galli et al., 2005).

Abbreviations: Ig, immunoglobulin; Fc_εRI, high-affinity IgE receptor; PBS, Dulbecco's phosphate buffered saline; IMDM, Iscove's modified Dulbecco's medium; FBS, Fetal bovine serum; BSA, bovine serum albumin; SCF, human stem cell factor; IL, interleukin; APAAP, alkaline phosphatase anti-alkaline phosphatase.

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Mast cells are known to arise from a unique lineage of CD34⁺ pluripotent progenitor cells in the bone marrow which are distinct from the common haematopoietic progenitors for neutrophils, basophils and eosinophils (Valent et al., 1989a; Kirshenbaum et al., 1991). As a result of stimulation mechanisms yet to be identified, these mast cell progenitors migrate from the bone marrow into the circulation and finally enter the tissues where they differentiate and mature under the influence of local environmental factors. Early attempts to obtain human mast cells from human bone marrow cells by repeating the procedures of culturing murine mast cells from bone marrow cells using interleukin (IL)-3 alone resulted in the proliferation and maintenance of human basophils which expressed different cell surface markers from mast cells and contained no human tryptase (Valent et al., 1989b; Kirshenbaum et al., 1992). Significant advances in the development of human mast cell cultures took place after the observation that co-culturing of human bone marrow cells with murine 3T3 fibroblasts produced human mature mast cells which stained positive for human mast cell tryptase (Furitsu et al., 1989). The key fibroblast-derived mast cell growth factor has now been identified as stem cell factor (SCF) or *c-kit* ligand and is crucial for promoting colony growth of crude human hemopoietic cells from bone marrow, fetal liver and peripheral as well as cord blood (Irani et al., 1992; Valent et al., 1992; Mitsui et al., 1993). However, incubation of CD34⁺ progenitor cells with SCF alone induces only mast cells which are immature in many aspects and additional cytokines such as IL-3, IL-6 and GM-CSF have been shown by various investigators to enhance the growth, maturation and survival of human mast cell cultures (Kirshenbaum et al., 1992; Yanagida et al., 1995; Saito et al., 1996). Subsequently, culture methods for human mast cells using fresh human peripheral blood and cord blood as sources of progenitor cells were established (Yanagida et al., 1995; Kirshenbaum et al., 1999). However, such sources are not always available and their use often requires special permission. Human buffy coat preparations generated during the preparation of donated blood are readily available from blood banks and can potentially be used as a more convenient and abundant source of mast cell progenitors. Hence we investigated whether a method previously reported for culturing mature mast cells from human peripheral blood (Ahn et al., 2000; Nomura et al., 2001) could be adapted to produce functionally viable human mature mast cells from progenitor cells isolated from blood bank buffy coats. In this method, a combination of SCF, IL-6 and IL-3 was initially used in the first two weeks to induce differen-

tiation and proliferation of CD34⁺ progenitor cells whereas SCF and IL-6 were used to mediate the subsequent development of mast cell progenitors to mature mast cells.

2. Materials and methods

2.1. Antibodies and reagents

Microbeads conjugated with monoclonal mouse anti-human antibodies against CD4 (cat. no.130045101), CD8 (130045201), CD11b (130049601), CD14 (130050201) or CD19 (130050301) were purchased from Miltenyi Biotec (Bergisch Gladbach, Germany). Silica (23305) was from Immuno-Biological Labs., Gunma, Japan. Dulbecco's phosphate buffered saline (PBS, 21600010), Iscove's modified Dulbecco's medium (IMDM, 1244 0053), fetal bovine serum (FBS, 16000036), insulin–transferrin–selenium (41400045), 2-mercaptoethanol (21985023), penicillin–streptomycin (15140122), bovine serum albumin (BSA: A7403), human myeloma IgE (401152) were from Gibco-BRL, Gaithersburg, MD. Lymphocyte separation medium (50434) was from ICN Pharmaceutical, Costa Mesa, CA. Serum-free methylcellulose medium (METHOCULT™ SF^{BIT} H4236) was from StemCell Technologies, Vancouver, BC, Canada. Human SCF (30007), IL-6 (20006) and IL-3 (20003) were from PeproTech Asia, Rehovot, Israel. Mouse anti-human immunoglobulin E antibody (-chain specific, 10632) was from Sigma Co. Ltd., St. Louis, MO. Anti-human mast cell tryptase (MAB1222) and chymase (MAB1254) monoclonal antibodies were from Chemicon International, Temecula, CA. May-Gruenwald's (EM1013520025) and Giemsa's (EM1092040100) solution were from Merck, Darmstadt, Germany. The alkaline phosphatase anti-alkaline phosphatase (APAAP) kit used was from Dako Corp., Glostrup, Denmark (DAKK067011).

2.2. Isolation of pluripotent stem cells and culture of mast cells from buffy coat

Fresh buffy coats (not more than 24 h old) were obtained from the Hong Kong Red Cross. 60 ml of buffy coat lymphocytes were first incubated with 9 ml of silica for 1 h at 37 °C to facilitate the removal of phagocytes and were subsequently diluted with double volume of PBS. Each 30 ml of diluted lymphocyte preparation was then layered gently on top of 13 ml lymphocyte separation medium. After centrifugation (180 ×g, 30 min, room temperature), the interface containing mononuclear cells was collected and washed

with PBS. For concentrating CD34⁺ progenitor cells, CD4⁺ CD8⁺ CD11b⁺ CD14⁺ and CD19⁺ cells were removed from the mononuclear cells collected using a magnetic separation column (Super Macs II, Miltenyi Biotec, Bergisch Gladbach, Germany, 130042901) and a mixture of magnetic microbeads conjugated with antibodies against CD4, CD8, CD11b, CD14 and CD19 (Miltenyi Biotec) according to the manufacturer's instructions. While monocytes expressing the above CD markers were retained in the magnetic column, lineage negative cells containing CD34⁺ progenitors were eluted and collected from the column. After washing with PBS, the lineage negative cells were resuspended in 1.6 ml of IMDM supplemented with 1% insulin–transferrin–selenium, 5×10^{-5} M 2-mercaptoethanol, 1% penicillin–streptomycin and 0.1% BSA. The cells were then mixed thoroughly with 14.4 ml of serum free methylcellulose medium containing SCF, IL-6 and IL-3. The concentrations of methylcellulose, SCF, IL-6 and IL-3 in the final methylcellulose cell suspension were 1.25%, 200, 50 and 1 ng/ml, respectively. Aliquots (300 μ l) of the methylcellulose cell suspension were incubated in a 24-well plate at 37 °C in a 5% CO₂ incubator for six weeks during which 300 μ l of fresh methylcellulose medium containing SCF (100 ng/ml) and IL-6 (50 ng/ml) were layered over the methylcellulose cultures every 2 weeks. After six weeks of culturing in methylcellulose, cells in the wells were collected by dissolving methylcellulose in PBS. Cells from 8 wells were pooled together and resuspended in 6 ml of complete IMDM supplemented with SCF (100 ng/ml) and IL-6 (50 ng/ml) and 10% FBS. The cells were maintained in the liquid medium for another 6–8 weeks for maturation and the culture medium was renewed every 10 days during this period.

2.3. May-Gruenwald and Giemsa staining

Cell smears were prepared by cytocentrifugation (250 $\times$ g) using a Shandon Cytospin 3 and were subsequently stained with 0.25% of May-Gruenwald solution for 5 min followed by a further 10 min incubation after diluting the solution with an equal volume of 0.1 M PBS (pH 6.4). The smears were then rinsed under running water and stained with 0.1% of Giemsa solution for 15 min.

2.4. Immunocytochemical staining

Staining for tryptase and chymase protein expression in mast cells was performed by the alkaline phosphatase

antialkaine phosphatase (APAAP) method described by Craig et al. (1986). Cytocentrifuged smears were fixed in Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid) for 90 s, air-dried and then incubated with a 1/1000 dilution of anti-human mast cell tryptase or chymase monoclonal antibodies overnight at 4 °C in a humidified chamber. After washing with Tris buffered saline, the smears were subjected to sequential 30 min incubation at room temperature with rabbit anti-mouse IgG and APAAP immune complex with washing in between. Finally, immunoreactivity was detected by incubating the slides with freshly prepared alkaline phosphatase substrate (containing naphthol AS-MX phosphate, Fast Red TR and levamisole in 0.1 M Tris–HCl of pH 8.2) for 20 min. The smears were counterstained in Mayer's haematoxylin and finally covered with a coverslip using aqueous mounting medium. The percentages of positively stained cells were estimated by counting 200 cells randomly chosen on the smears of 9 different mast cell cultures. Images were taken using a Zeiss Axioskop Microscope at 400 $\times$ magnification.

2.5. Response of mast cells to IgE cross-linking

After culture for 12–14 week, mature mast cells were harvested, washed and then incubated with human myeloma IgE (1 μ g/ml) for 2 h at 37 °C in IMDM. The sensitized mast cells were washed and resuspended in HEPES buffered Tyrode's solution supplemented with 0.03% human albumin. Activation of the sensitized mast cells was induced by addition of 40 μ l of different concentrations of anti-IgE to tubes containing 4×10^3 mast cells suspended in 400 μ l of HEPES buffered Tyrode's solution supplemented with 0.03% human albumin. After 30 min of incubation with anti-IgE at 37 °C, histamine release was stopped by the addition of 600 ml ice-cold buffer followed immediately by centrifugation (180 $\times$ g) for 5 min at 4 °C. The cell pellets and supernatants were then collected separately for histamine assay.

2.6. Histamine assay

Histamine contents in mast cell pellets and supernatants were measured spectrofluorometrically using a Bran+Luebbe AutoAnalyzer (Bran+Luebbe GmbH, Norderstedt, Germany) which chemically extracted histamine from the samples as described by Siraganian (1974). The sum of histamine contained in these two fractions from the same experiment tube gives the total histamine content in the 4×10^3 mast cells contained in

that tube. For the anti-IgE experiments, histamine released into the supernatant was expressed as a percentage of total cellular histamine content and all histamine release values were corrected for the spontaneous histamine release in buffer alone.

All results are expressed as mean $\pm$ standard error of mean values for n independent mast cell cultures studied and statistical significance was analyzed using Student's t -test for paired data and values were considered significantly different at $P < 0.05$.

3. Results and discussion

3.1. Production of mast cells from buffy coat

In the current study, a negative selection method was used in order to increase the yield of CD34⁺ cells. Initially, $1.7 \pm 0.2 \times 10^9$ ($n=11$) monocytes were collected from 60 ml of buffy coat and subsequently $3.5 \pm 0.6 \times 10^8$ ($n=11$) lineage negative cells were obtained after removal of CD4⁺ CD8⁺ CD11b⁺ CD14⁺ and CD19⁺ cells. These cells were then cultured in serum-free methylcellulose medium supplemented with SCF, IL-6 and IL-3 at a cell density of $1.1 \pm 0.1 \times 10^7$ per well ($n=11$). After two weeks,

small cell colonies began to form as observed under phase-contrast microscopy. At the end of the six week methylcellulose culture, a large number of differentiated cells including mast cells were generated. Two weeks after transferring the cells into liquid medium, the cultured cells appeared to be homogeneous under phase-contrast microscopy. By the end of the 12th week, an average of $2.1 \pm 1.0 \times 10^7$ ($n=8$) mature mast cells were obtained per 60 ml of buffy coat.

3.2. Morphological features of cultured mast cells

Under the light microscope, mature mast cells collected after 12–14 weeks of culture generally showed centrally placed, round or indented nuclei which distinguishes them from basophils, which have 2–3 nuclear lobes (Tadokoro et al., 1983). The cultured mast cells contained numerous granules of different sizes in their cytoplasm which were stained purple with May-Gruenwald and Giemsa stain (Fig. 1A).

Two phenotypes of human mast cell can be identified by immunohistochemical detection of neutral protease content in the secretory granules (Irani et al., 1986; Schwartz et al., 1987). Mast cells that contain only tryptase are termed MC_T and are abundant in

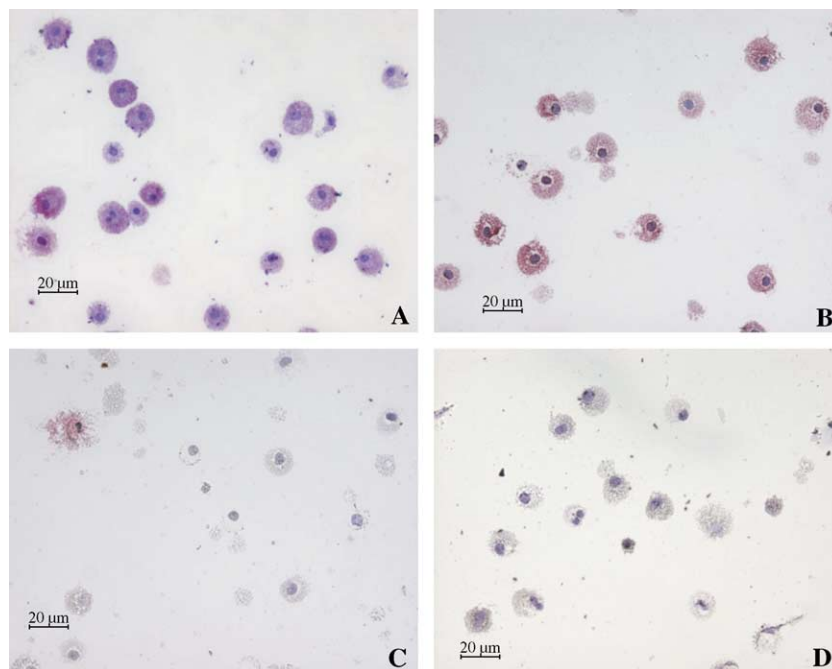


Fig. 1. Phenotypic characterisation of buffy coat-derived mast cells after 12–14 weeks of culture (400 $\times$). All cells contain numerous cytoplasmic granules which were stained with May-Gruenwald and Giemsa stain (A). More than 97% of cytoplasmic granules were positively stained against anti-tryptase antibody (B). Only 20–30% of cells were stained with anti-chymase antibody (C). Cells without addition of either antibody were taken as negative control (D). In B–D, nuclei were counterstained blue with hematoxylin. (Bar represents cell diameter).

mucosal tissues of the respiratory and gastrointestinal tract. Mast cells that contain both tryptase and chymase are termed MC_{TC} and predominate in the connective tissues of the bronchial and intestinal submucosa and skin. These two subtypes of human mast cells demonstrate differing responses to different activating stimuli and pharmacological modulators of mast cell activation (Forsythe and Ennis, 2000). In this study, 97–100% of cultured cells collected after 12 to 14 weeks of culture were stained positively for tryptase (Fig. 1B) but only 20–30% of mast cells showed positive staining for chymase (Fig. 1C). This result indicates that the majority of mast cells cultured from buffy coat phenotypically belong to the MC_T subtype. Our results are comparable to the 98% tryptase positive and 18% chymase positive cells reported by Saito et al. (1995) for mast cells cultured from umbilical cord blood.

3.3. Functional study of cultured mast cells

Mast cells express high-affinity receptors (Fc_εRI) for IgE and cross-linking of these receptors triggers an intracellular biochemical chain reaction which results in the degranulation of mast cells. Immunologically, cross-linking of the Fc_εRI is mediated through the binding of antigen molecules to the Fab part of adjacent IgE molecules anchored to Fc_εRI on the mast cell surface. Upon activation, mast cells rapidly release their granular store of preformed mediators and initiate the de novo synthesis of eicosanoids and cytokines (Tkaczyk and Gilfillan, 2001). Histamine is the earliest identified preformed mediator stored in mast cell granules and when released can trigger a series of immunological responses such as bronchoconstriction, oedema and mucus secretion (Galli et al., 2005). The histamine content of mast cells cultured from buffy coat in the current study was 4.3 ± 0.7 pg/cell ($n=12$), which is similar to the histamine content in mast cells isolated from lung parenchyma or cultured from umbilical cord blood (Schwartz et al., 1987; Saito et al., 1995). In order to investigate whether the mast cells cultured from buffy coat were functionally active, the cells were first sensitized with human myeloma IgE and then stimulated with anti-IgE antibodies. As illustrated in Fig. 2, the buffy coat derived mast cells released histamine in a dose-dependent manner when challenged with increasing concentrations of anti-IgE. At a dilution of 1/100, anti-IgE induced around 50% of histamine release. Similar levels of anti-IgE induced histamine release have also been reported for human mast cells cultured from umbilical cord blood (Saito et al., 1995).

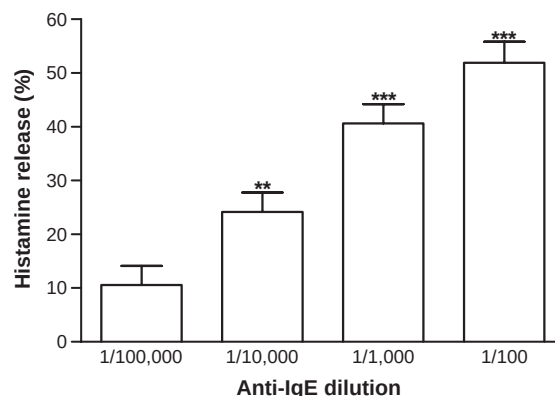


Fig. 2. Anti-IgE induced histamine release from buffy coat-derived mast cells. Mast cells were sensitized with human IgE, and then challenged with different dilutions of goat anti-human IgE for 30 min at 37 °C. Results were corrected for spontaneous histamine release and expressed as the mean $\pm$ SEM of 13 experiments. The spontaneous histamine release was $8.0 \pm 1.2\%$. Significant differences between the spontaneous and anti-IgE-induced histamine release are indicated by asterisks: ** $P < 0.01$, *** $P < 0.001$.

The current investigation has clearly demonstrated that an established method for the isolation and the culturing of mast cell progenitors from fresh peripheral blood (Ahn et al., 2000; Nomura et al., 2001) can be adapted for the same processes using buffy coat preparations from a blood bank. The blood bank buffy coat preparations are indeed a more abundant and less restrictive source of human mast cell progenitors than many of the earlier sources which would be restricted by ethics committees in most parts of the world. We have further demonstrated that the buffy coat derived mast cells resemble the MC_T mast cells in human lung and intestinal mucosa with reference to their expression of human tryptase as the only granule associated neutral protease and their content of histamine. Similar to all human tissue mast cells and mast cells cultured from umbilical cord blood, the buffy coat derived mast cells also express functional Fc_εRI receptors and their cross-linking through the attached IgE molecules can lead to the release of preformed mediators. These mast cells should be a useful model for future studies on human mast cell biology and for the screening of potential anti-allergic compounds.

Acknowledgement

The work described in this paper was fully supported by grants from the Research Grants Council of the Hong Kong Special Administration Region, China, Project no. CUHK4064/00M and CUHK 4337/03M.

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