

Generation of a Considerable Number of Functional Mast Cells with a High Basal Level of FcεRI Expression from Cord Blood CD34⁺ Cells by Co-culturing Them with Bone Marrow Stromal Cell Line Under Serum-free Conditions

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Abstract

The number of mast cells (MC) that can be obtained from tissue is limited, making it difficult to study the role of MC. Cultured MC derived from cord blood (CB)-CD34⁺ cells proliferate well compared with those derived from adult CD34⁺ cells; however, they have been reported to be phenotypically or functionally immature regardless of culture system. For example, very few cells express FcεRI. To resolve this problem, we addressed the effect of human bone marrow stromal cell line on the development of cultured MC. CB-CD34⁺ (1×10^4) cells were cultured for 8 weeks in a serum-free medium containing rhIL-6 and rhSCF with or without a human bone marrow stromal cell line, namely, co-culture and liquid culture, and were compared in various regards. MC were basically determined by metachromatic staining of granules. The number of MC obtained ($60.3 \pm 15.8 \times 10^5$ versus $2.0 \pm 1.0 \times 10^5$), percentage of FcεRI⁺ cells ($29.3 \pm 9.4\%$ versus $1.9 \pm 0.8\%$), histamine content (9.7 ± 2.8 pg/cell versus 5.8 ± 2.3 pg/cell), and IgE-mediated histamine release ($46 \pm 10\%$ versus $17 \pm 7\%$) were higher ($P < 0.01$ and $P < 0.05$) in the co-culture than in the liquid culture. When CB-CD34⁺ cells were developed in liquid culture with the co-culture supernatant (CM), a significant increase ($P < 0.01$) in the percentage of FcεRI⁺ cells and in cell number was observed but these values were lower than those of co-cultured MC. We concluded that this co-culture system was useful for obtaining a considerable number of mature MC with a high basal level of functional FcεRI expression from CB-CD34⁺ cells. Yet unknown humoral factors in CM may partly mediate this effect.

Introduction

Human mast cells (MC) express high-affinity Fc receptors for IgE (FcεRI) on their surface and release various biologically active molecules including histamine and serotonin when FcεRI-bound IgE is cross-linked [1]. It is believed that MC play a central role in allergic reactions such as anaphylaxis, allergic rhinitis and asthma through these molecules.

Mast cells are classified into two types according to their protease expression pattern: those that express tryptase but not chymase and those that express both, desig-

nated MC_T and MC_{TC} respectively. To better understand the mechanisms of allergic reactions, it is important to obtain substantial numbers of fully mature MC. However, only limited numbers of MC can be obtained from animal or human tissue. Thus, there have been many attempts to establish an *in vitro* culture system for MC using different sources of progenitor cells and different culture conditions [2–7].

Cord blood-derived (CB)-CD34⁺ cells are one source of MC progenitors and it was reported that greater numbers of MC were obtained when CB-CD34⁺ cells were cultured in a serum-free medium containing recombinant

human stem cell factor (rhSCF) with or without other cytokines such as rhIL-6 than when cultured in serum-containing medium [6, 8–14]. However, compared with the latter MC, the former failed to express a high basal level of FcεRI, contained a very small amount of histamine, or showed little IgE-mediated histamine release and thus were functionally immature. In addition, although CB progenitors can produce far more MC than adult-type progenitors, MC generated from CB progenitors were reported to express less surface FcεRI and release less histamine in response to IgE-mediated stimulation than those generated from adult-type progenitors [12]. Therefore, it is desirable to establish a culture system that can provide a large number of phenotypically and functionally mature MC from CB-CD34⁺ cells.

Mast cells coexist with vascular endothelial cells and tissue fibroblasts *in vivo*. Thus, interaction with these cells may be necessary for the full development of MC. In fact, co-culturing MC with mouse skin-derived 3T3 fibroblasts or human umbilical vein endothelial cells has been shown to affect their proliferation, phenotype and responsiveness to stimuli [15, 16]. As MC are recruited from progenitor cells present in bone marrow, it is possible that bone marrow stromal cells having the potential to support haematopoiesis regulate the proliferation, differentiation and maturation of MC. We have already established a human bone marrow stromal cell line (hTERT-ST) by transfecting a human telomerase catalytic subunit gene and confirmed that it can support hematopoiesis in serum-free culture conditions [17]. Thus, we co-cultured CB-CD34⁺ cells with hTERT-ST in a serum-free medium containing rhSCF and rhIL-6 and obtained large numbers of fully mature MC.

Materials and methods

Preparation of human cord blood (CB) cells. Cord blood was obtained from normal full-term deliveries. After sedimentation of red blood cells by incubating CB samples with the same volume of 6% (w/v) hydroxyethyl starch dissolved in Ringer's solution (Veen-D Inj., Nikken Chemical, Tokyo, Japan) at room temperature for 30 min, low-density (<1.077 g/ml) mononuclear cells were collected with Ficoll-Paque (Amersham-Pharmacia Biotech, Uppsala, Sweden). The cells were further enriched for CD34⁺ cells with a magnetic activated cell sorting (MACS) CD34 Progenitor Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions and the purity was verified by flow cytometry. In all experiments, the purity of CD34⁺ cells was more than 90%. Experiments using human umbilical cord blood were approved by the Committee of Hokkaido Cord Blood Bank. Only the cord blood units that did not fulfil criteria for banking were used for this study.

Co-culture of CB-CD34⁺ cells with a human bone marrow stromal cell line. A human bone marrow stromal cell line (hTERT-ST) was established [17]. One week before the co-culture, 1×10^5 hTERT-ST cells that had been prepared using a long-term culture medium containing minimum essential medium- α , 12.5% horse serum (Gibco BRL, Rockville, MD, USA), 12.5% fetal calf serum (Gibco BRL) and 1×10^{-6} M hydrocortisone (Sigma, St Louis, MO, USA) were plated in 12.5 cm² flasks in 2 ml of a long-term culture medium. When stromal cell monolayers became confluent, they were irradiated (1500 cGy) and washed with PBS three times to remove serum components. Then, 1×10^4 CB-CD34⁺ cells in 2 ml of serum-free medium, QSBF60 (Quality Biological, Inc. Gaithersburg, MD, USA) containing rhIL-6 (50 ng/ml) and rhSCF (100 ng/ml) (kindly provided by Kirin Brewery Co Ltd, Tokyo, Japan) were added into the flask to start the co-culture at 37 °C in a humidified atmosphere of 5% CO₂/95% air for 8 weeks. In a previous experiment, we confirmed that this cell line could survive in QSBF60 at least 8–10 weeks. In the case of the liquid culture, cells were cultured in the same medium in the absence of stromal cells. In both cases, after 1 week of culture, 2 ml of fresh medium containing these cytokines was added. After that, half of the medium was replaced with fresh medium every week. At the end of the culture, the cells that had attached weakly or not at all to stromal cells were collected by gentle pipetting. The supernatant in the co-culture system was collected at 8 weeks, and filtered to remove cellular debris. This was designated as conditioned medium (CM). In some experiments, liquid culture was carried out in the presence of 25% (v/v) CM.

Histological staining. Cultured cells (1×10^3 – 1×10^4 /100 μ l) were centrifuged onto slides with Cytospin (Shandon, Pittsburgh, PA, USA), and stained with May-Grünwald-Giemsa (Merck, Darmstadt, Germany). Metachromatic staining of centrifuged cells was performed using 0.05% Toluidine Blue Solution (Muto pure chemical Co. Ltd, Tokyo, Japan) after fixation with methanol. In the case of immunostaining for tryptase and chymase, mouse anti-tryptase or chymase antibody (Medical and Biological Laboratories Co. Ltd, Nagoya, Japan) and Histfine Simple Stain MAX-PO(M) with Simple Stain DAB Solution and Mayer's Hematoxylin Solution (Nihon Biorei Co., Tokyo, Japan) were used according to the manufacturer's instructions. As a control antibody, Universal negative control-mouse (Dako Japan, Kyoto, Japan) was used. MC were identified by the metachromatic staining of granules and morphology. The percentage of MC, tryptase-positive cells, or chymase-positive cells was determined by analysing at least 200 cells under a Nikon BX50 light microscope. The number of MC was estimated from the total cell count and percentage of MC. Microscopic images were captured by MP5Mc/OL digital

camera (Olympus) and processed using Win Roof software version 5.5.

Flow cytometric analysis. Aliquots of cells were stained with monoclonal antibodies in PBS/0.1% BSA at 4 °C for 30 min. The analysis was performed by using a BD LSR flow cytometer (BD Biosciences Immunocytometry System, San Diego, CA, USA). Monoclonal antibodies used were as follows: FITC-conjugated CD34 [Nippon Becton Dickinson Co, (BD), Tokyo, Japan] antibody, PE-conjugated CD117(c-kit) and CD203c (Immunotech, Marseille, France) antibodies, and APC-conjugated CD45 (BD) and FcεRI (eBioscience, San Diego, CA, USA) antibodies. FITC- and PE-conjugated mouse IgG1 antibodies (BD) and APC-conjugated mouse IgG1 (BD) and IgG2b (eBioscience) antibodies were used as isotype-matched controls. In the flow cytometric analysis, dead cells were gated out first with propidium iodide staining and then with a forward versus side scatter window. CD117(c-kit) (or CD203c) was used as a cell surface marker of MC. To evaluate the FcεRI expression or mean fluorescence intensity (MFI) of FcεRI on MC, CD117-positive cells were gated first, and then the analysis was conducted. In each analysis, at least 10,000 events were collected.

Analysis of IL-4-induced enhancement of FcεRI expression and histamine release assay. Cells were cultured for 8 weeks in co-culture or liquid culture, then, further cultured for 1 week under the same culture conditions in the presence or absence of 50 ng/ml of rhIL-4 (PeproTech EC Ltd, London, UK) to enhance FcεRI expression. After this, FcεRI expression on CD117-positive cells was analysed. For the histamine release assay, cells cultured for 8 weeks in each set of conditions were further cultured in the presence of rhIL-4 for 6 days, and then sensitized with 3 µg/ml of purified human myeloma IgE (Chemicon International, Temecula, CA, USA) at 37 °C for 24 h. After they were washed, the cells were suspended at a density of 4×10^4 cells/ml in a modified Tyrode solution (pH 7.4) containing 124 mM NaCl, 4 mM KCl, 0.64 mM NaH_2PO_4 , 1 mM CaCl_2 , 0.6 mM MgCl_2 , 10 mM HEPES and 0.03% human serum albumin. The cells were pre-incubated for 10 min and then challenged with 1.5 µg/ml of anti-human IgE (Chemicon International), 3 µM Ionomycin, 3 µM substance P, 3 µM Compound 48/80 (Sigma-Aldrich Japan, Tokyo, Japan) or the control Tyrode solution at 37 °C for 45 min. Next, the cells were centrifuged at 450 g for 10 min, the supernatant was collected, and the amount of histamine in the supernatant was measured using an histamine ELISA kit (Immunotech). To release all the histamine in cells, the cell suspension was boiled for 15 min and centrifuged at 450 g for 10 min. Then, the amount of histamine in the supernatant was measured and designated as the total histamine content. Per cent histamine release was calculated as (released histamine

content)/(total histamine content) $\times$ 100. The amount of histamine in each cell was calculated as (total histamine content)/(number of cells).

Detection of cytokines in culture supernatant. Culture supernatants of both the co-culture and liquid culture were collected at 8 weeks and the detection of cytokines was carried out using Human Cytokine Antibody Arrays (RayBiotech, Inc, Norcross, GA, USA) according to the manufacturer's direction. The cytokines addressed were as follows: ENA-78, G-CSF, GM-CSF, GRO, GRO- α , I-309, IL-1 α , IL-1 β , IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, IL-12, IL-13, IL-15, IFN- γ , MCP-1, MCP-2, MCP-3, M-CSF, MDC, MIG, MIP-1 β , MIP-1 δ , β -NGF, RANTES, SCF, TARC, TGF- β 1, TNF- α , TNF- β , EGF, IGF-I, Angiogenin, OncostatinM, Thrombopoietin, VEGF, PDGF-BB, Leptin, BDNF, BLC, Ckb8-1, Eotaxin, Eotaxin-2, Eotaxin-3, FGF-4, FGF-6, FGF-7, FGF-9, Flt3-Ligand, Fractalkine, GCP-2, GDGF, HGF, IGFBP-1, IGFBP-2, IGFBP-3, IGFBP-4, IL-16, IP-10, LIF, LIGHT, MCP-4, MIF, MIP-3 α , NAP-2, NT-3, NT-4, Osteoprotegrin, PARC, PIGF, TGF- β 2, TGF- β 3, TIMP-1 and TIMP-2.

Statistics. Results were expressed as the mean $\pm$ SD. The significance of differences was assessed with either the Student's *t*-test or a repeated measures ANOVA.

Results

Expansion capacity and phenotype of cultured cells

At 8 weeks, the cell viability in each culture was no less than 95% and Toluidine blue and May-Grünwald-Giemsa staining revealed that more than 95% of cultured cells had a large oval or round nucleus and a cytoplasm filled with metachromatic granules, features consistent with MC (Fig. 1A). Metachromatic granules were more abundant in co-cultured cells than in liquid-cultured cells. No less than 95% of the cultured MC were positive for tryptase (data not shown). Approximately 6×10^6 MC could be obtained from 1×10^4 CD34⁺ cells by co-culturing them with hTERT-ST for 8 weeks. This was 30 times more than that obtained by liquid culture ($60.3 \pm 15.8 \times 10^5$ versus $2.0 \pm 1.0 \times 10^5$) (Table 1). Cell surface analyses performed at the end of 8 weeks revealed that the percentage of CD117 (c-kit) and CD203c-positive cells was 93.5% and 22.9% in liquid culture, and 95.9% and 73.3% in co-culture (Fig. 1B). In addition, no more than 0.1% of cells were CD34-positive (data not shown), suggesting that CD117-positive cells were not haematopoietic progenitors any more. These results further confirmed that most of the cells generated by our culture system were MC. The percentage of chymase-positive cells was only $9.0 \pm 5.4\%$ in liquid culture and $1.7 \pm 0.5\%$ in co-culture (data not shown).

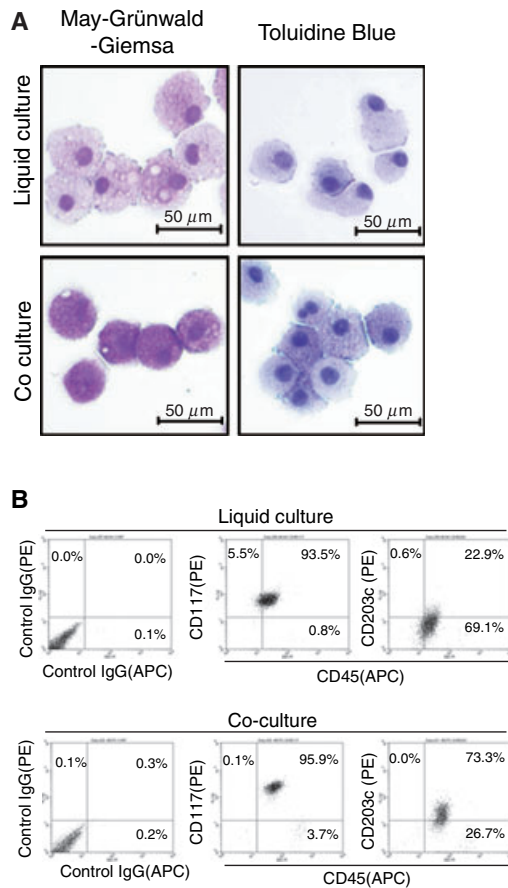


Figure 1 Morphology and cell surface antigens of cultured cells. (A) Cells obtained at 8 weeks were stained with Toluidine blue and May–Grünwald–Giemsa. More than 90% of the cells had an oval round nucleus and cytoplasm filled with metachromatic granules, consistent with the features of mast cells. Original magnifications at the time of photomicroscopy was $\times 600$. (B) Flow cytometric analysis of cell surface antigen showed that almost 100% of cells were positive for CD117 (c-kit) and a substantial percentage were positive for CD203c. Staining is expressed as $\log_{10}$ fluorescence intensity. Representative examples out of five separate cultures were shown.

Expression of Fc ϵ RI on cultured cells and its enhancement by IL-4

The percentage of Fc ϵ RI-positive MC in liquid culture at 4, 6 and 8 weeks was $2.2 \pm 1.3\%$, $2.0 \pm 0.7\%$ and $1.9 \pm 0.8\%$ respectively. In contrast, that in co-culture was $19.3 \pm 5.1\%$, $22.7 \pm 8.3\%$ and $29.3 \pm 9.4\%$ respectively (Fig. 2A). The latter increased gradually and was obviously higher ($P < 0.01$) than the former. When Fc ϵ RI expression was measured by MFI, the same tendency was observed (Fig. 2B). Furthermore, IL-4-induced enhancement of Fc ϵ RI expression was more vigorous in co-cultured than liquid-cultured MC (Fig. 2C).

Evaluation of histamine release and histamine content

As shown in Table 1, the amount of histamine in the cells increased gradually during the 8 weeks of culture. At 8 weeks, co-cultured cells contained more histamine than the cells in liquid culture ($P < 0.05$). The IgE-mediated release of histamine was greater in co-cultured cells than liquid-cultured cells ($46 \pm 10\%$ versus $17 \pm 7\%$, $P < 0.05$, Table 2). Furthermore, these cells released a substantial amount of histamine in response to non-immunological stimuli, such as substance P and compound 48/80, regardless of the culture conditions (Table 2).

Effect of co-culture supernatant on basal levels of Fc ϵ RI expression and proliferation

To see whether the co-culture supernatant (conditioned medium, CM) had the potential to upregulate the basal level of Fc ϵ RI expression on cultured cells, cell cultures were carried out for 8 weeks in three different conditions, namely, co-culture, liquid culture in the presence of 25%(v/v) CM (Liq + CM), and liquid

	Total cells ($\times 10^5$)		Mast cells ($\times 10^5$)		Histamine content (pg/cell)	
	Liquid culture	Co-culture	Liquid culture	Co-culture	Liquid culture	Co-culture
Pre	0.1	0.1	—	—	—	—
4 weeks	1.9 ± 1.3	$18.4 \pm 4.5^*$	1.4 ± 1.0	$6.6 \pm 1.6^*$	0.7 ± 0.5	1.7 ± 1.3
6 weeks	2.1 ± 1.4	$42.1 \pm 22.6^*$	1.7 ± 1.1	$29.2 \pm 14.7^*$	1.9 ± 0.6	3.2 ± 0.7
8 weeks	2.1 ± 1.1	$63.4 \pm 16.7^*$	2.0 ± 1.0	$60.3 \pm 15.8^*$	5.8 ± 2.3	$9.7 \pm 2.8^{**}$

Mast cells were identified based on the typical staining pattern shown in Fig. 1A. In the case of cell number, the data represent the mean $\pm$ SD of five experiments with five separate cord blood samples ($n = 5$) and in the case histamine content, the data represent those of nine experiments with nine cord blood samples ($n = 9$). $^*P < 0.01$, $^{**}P < 0.05$.

Table 1 Expansion of mast cells and their histamine content.

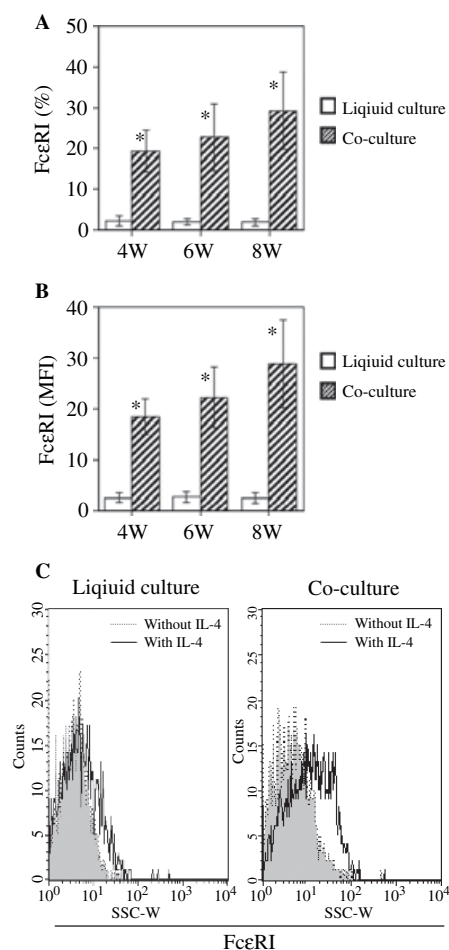


Figure 2 Flow cytometric analysis of FcεRI expression on CD117-positive cells and its enhancement by IL-4. (A,B) Cells cultured in each set of conditions were collected at 4, 6 and 8 weeks. CD117-positive cells were gated, then the percentage of FcεRI-positive cells and MFI of FcεRI of the gated cells were analysed by flow cytometry. During the culture, both the percentage of FcεRI-positive cells and MFI of FcεRI of the co-cultured cells kept increasing compared with that in the liquid culture. Experiments were carried out five times with five separate cultures. Values were expressed as the mean $\pm$ SD ($n = 5$) (* $P < 0.01$). (C) Cells were cultured in each set of conditions for 8 weeks and further cultured in the presence or absence of rhIL-4 for 1 week. Then, FcεRI expression on CD117-positive cells was analysed by flow cytometry. An enhancement of FcεRI expression was observed in both cells; however, it was more vigorous in the co-cultured cells. Staining is expressed as log₁₀ fluorescence intensity. Representative examples out of five experiments with five separate cultures were shown.

culture in the absence of CM (Liq) (Table 3). After 8 weeks in culture, the cell number, percentage of FcεRI-positive cells, and MFI of FcεRI were all significantly higher in Liq + CM condition than Liq condition but lower in Liq + CM condition than co-culture condition. Therefore, CM had the potential to increase the basal level of FcεRI expression as well as proliferation, although it did not completely replace the stromal cell line.

Table 2 The release of histamine from mast cells in response to immunological and non-immunological stimuli.

	Liquid culture (%)	Co-culture (%)	P-value
Anti-IgE	17 $\pm$ 7	46 $\pm$ 10	<0.05
Substance P	80 $\pm$ 8	65 $\pm$ 4	<0.05
Compound 48/80	77 $\pm$ 6	66 $\pm$ 13	0.18
ionomycin	73 $\pm$ 13	79 $\pm$ 15	0.3

Mast Cells obtained at 8 weeks were further incubated with IL-4, then sensitized with IgE. Subsequently they were stimulated by anti-IgE or non-immunological stimuli. The data represent the mean $\pm$ SD ($n = 3$) of three experiments with three separate cord blood.

Detection of cytokines in culture supernatant

To search for humoral factors in CM that were involved in the upregulation of FcεRI expression and proliferation, a semi-quantitative human cytokine assay was carried out (Fig. 3). We assumed that the candidates for the factor were present to a greater extent in the co-culture supernatant. Among 12 cytokines detected, four cytokines, IGFBP-2, Angiogenin, Osteoprotegrin, and PARC, were preferentially expressed in CM. GM-CSF, IL-3, IL-4, IL-5, IL-9, IL-10, IL-11 and β -NGF were reported to have some biological effects on MC [18–21]. However, none of them was detected.

Discussion

It was obvious that the stromal cell line hTERT-ST enhanced the generation of MC from CB-CD34⁺ cells (Table 1) under serum-free co-culture conditions. In fact, approximately 6×10^6 MC could be obtained with more than 95% purity from 1×10^4 CB-CD34⁺ cells in only 8 weeks of culture. As far as the rate of proliferation is concerned, this serum-free co-culture system appeared to be superior to previously reported serum-free culture systems [5–10].

It has been reported that the CB-derived MC developed under serum-free conditions proliferated well compared with those developed in the presence of serum. However, they contained little histamine and expressed a very low level of surface FcεRI. Therefore, the MC generated in serum-free conditions were considered to be immature. For example, Dahl *et al.* reported the histamine content of CB-derived MC cultured in the presence and absence of serum to be 3.8 ± 1.1 pg/ml, and 0.7 ± 0.1 pg/ml, respectively, and the percentage of FcεRI-positive cells to be 33.8% and 7.2% respectively [11]. By contrast, despite the serum-free culture conditions, MC obtained with our co-culture system would be quite mature compared with MC generated by a serum-free liquid culture system, based on the following observations; first, the percentage of FcεRI-positive cells (or MFI of FcεRI) was dramatically increased ($29.3 \pm 9.4\%$

	Before culture	After 8 weeks of culture		
	CD34 ⁺ cells	No. of mast cells	FcεRI (%)	FcεRI (MFI)
Liq	1.0×10^4	$1.1 \pm 0.2 \times 10^5$	1.7 ± 0.05	2.1 ± 0.61
Liq+CM	1.0×10^4	$9.1 \pm 1.5 \times 10^5$	11.1 ± 2.10	13.1 ± 3.34
Co-culture	1.0×10^4	$32.5 \pm 7.2 \times 10^5$	30.1 ± 3.34	21.7 ± 5.49

Cell cultures were carried out for 8 weeks in three different conditions: co-culture, liquid culture in the presence of 25% (v/v) CM (Liq + CM), and liquid culture in the absence of CM (Liq). In the case of the number of mast cells, the data represent the mean $\pm$ SD of three experiments with three separate cord blood samples ($n = 3$) and in the case of FcεRI (%) or FcεRI (MFI), the data represent those of five experiments with five separate cord blood samples ($n = 5$). * $P < 0.01$, ** $P < 0.05$

Table 3 Number of mast cells generated from 1×10^4 CB-CD34⁺ cells and their expression of FcεRI by liquid culture in the presence or absence of CM and by co-culture.

Li Co		Li Co		Li Co	
ENA-78		MIP-1δ		Fractalkine	
G-CSF		β-NGF		GCP-2	
GM-CSF		RANTES		GDGF	
GRO		SCF		HGF	
GRO-α		TRAC		IGFBP-1	
I-309		TGF-β1		IGFBP-2	
IL-1α		TNF-α		IGFBP-3	
IL-1β		TNF-β		IGFBP-4	
IL-2		EGF		IL-16	
IL-3		IGF-1		IP-10	
IL-4		Angiogenin		LIF	
IL-5		OncostatinM		LIGHT	
IL-6		Thrombopoietin		MCP-4	
IL-7		VEGF		MIF	
IL-8		PDGF-BB		MIP-3α	
IL-10		Leptin		NAP-2	
IL-12		BDNF		NT-3	
IL-13		BLC		NT-4	
IL-15		Ckb8-1		Osteoprotegrin	
IFN-γ		Eotaxin		PARC	
MCP-1		Eotaxin-2		PIGF	
MCP-2		Eotaxin-3		TGF-β2	
MCP-3		FCF-4		TGF-β3	
M-CSM		FCF-6		TIMP-1	
MDC		FCF-7		TIMP-2	
MIG		FCF-9		IL-9	
MIP-1β		Flt3-Ligand		IL-11	

Figure 3 Semi-quantitative assay for cytokines in culture supernatants. Liquid culture supernatant and co-culture supernatant were collected at 8 weeks and a cytokine assay was performed. The cytokines detected are shaded. Representative examples out of two experiments with two separate cultures were shown. Liq = liquid culture supernatant, Co = co-culture supernatant.

versus $1.9 \pm 0.8\%$) (Fig. 2A,B); second, IL-4-induced enhancement of FcεRI expression was more vigorous (Fig. 2C); and third, both histamine content and anti-IgE-mediated histamine release were significantly greater (Tables 1 and 2). The greater histamine release may reflect the enhanced FcεRI expression. Finally, these values appeared to be no less than those reported for CB-derived MC in a serum-containing medium [11, 12, 22].

As far as the expression of CD203c is concerned, mature MC were reported to express very little of this surface marker compared with immature MC [23, 24]. Therefore, higher expression of this marker on co-cultured cells than on liquid cultured cells (Fig. 1B) may

contradict the discussion described above. At this point, no explanation for this can be put forward. However, the expression of CD203c on MC might not always associate with immaturity, because CD203c on primary MC have been reported to be upregulated under some pathophysiological conditions [25].

We also confirmed that cells obtained by both co-culture and liquid culture could release histamine in response to non-immunological stimuli, such as compound 48/80 and substance P (Table 2). This is consistent with a recent report by Moon *et al.* [26]. However, the responsiveness to substance P was significantly lower in co-cultured cells than in liquid cultured cells. The reason for this is not clear. It might be possible that this reflects the lower expression of substance P receptor, the so-called neurokinin 1 receptor (NK1R), in the former compared with the latter [27].

Therefore, we believe that the MC generated in our co-culture system were phenotypically and functionally fully developed. Referring to the staining for tryptase and chymase at 8 weeks, no less than 95% of MC obtained with each culture system were tryptase positive but chymase negative (data not shown). Thus, they were MC_T-type cells. Considering all these observations, our serum-free co-culture system is promising for obtaining considerable numbers of phenotypically and functionally mature MC_T-type MC from CB-CD34⁺ cells.

As has been previously described, MC derived from CB-CD34⁺ cells in serum-free conditions express only a very small amount of FcεRI. This may be explained in part by a report describing that the expression of FcεRI was restricted at the mRNA level in cord blood-derived MC [12]. However, it should be stressed again that substantial expression of a functional FcεRI and proliferation were observed when CB-CD34⁺ cells were developed in our co-culture system (Table 3).

We were specifically interested in the upregulation of the basal level of FcεRI expression. To address the factors that contributed to this effect, CB-CD34⁺ cells were cultured in liquid culture in the presence of CM (25%, v/v) for 8 weeks. We found that the MC in this culture expressed clearly a higher level of FcεRI (and prolifer-

ation) than MC cultured in the absence of CM, although the level failed to reach that of co-cultured MC (Table 3). Although we did not address the importance of direct cell-to-cell interactions, this suggested that humoral factors in CM attributed to the enhancement of FcεRI expression to a certain extent. So far, only two factors have been reported to possess biological activity to enhance FcεRI expression on MC. One of them is IL-4 [28, 29], and the other is IgE [30, 31]. However, neither IL-4 protein in the culture supernatant nor the IL-4 message in the cellular RNA preparation from cultured cells was detected. In addition, no IgE was detected by an enzyme immunoassay in the culture supernatant (data not shown).

As far as the four cytokines preferentially detected in CM are concerned, to the best of our knowledge, none of these cytokines has been reported to have FcεRI-enhancing ability. Nevertheless, their contribution to FcεRI expression remains to be investigated. In addition, the possibility that other cytokines, such as IL-18 [32] or some other non-cytokine stimulatory molecules released by hTERT-ST, such as lysophosphatidic acid [33], are involved cannot be ruled out in this study.

It may be argued that CM or hTERT-ST just accelerates the maturation process. However, when cells were cultured in liquid culture for up to 12 weeks, no increase in the basal level of FcεRI expression was observed at all.

In conclusion, co-culturing CB-CD34⁺ cells with hTERT-ST in rhSCF and rhIL-6-containing serum-free medium for 8 weeks can generate a considerable number of quite mature human MC with a phenotype similar to that of MC_T. Besides direct cell-to-cell interactions, yet unknown humoral factors may contribute to these effects. These MC would be useful for analysing the maturation of MC as well as functional aspects of MC in patients with diseases such as atopic dermatitis and anaphylaxis-like reactions.

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