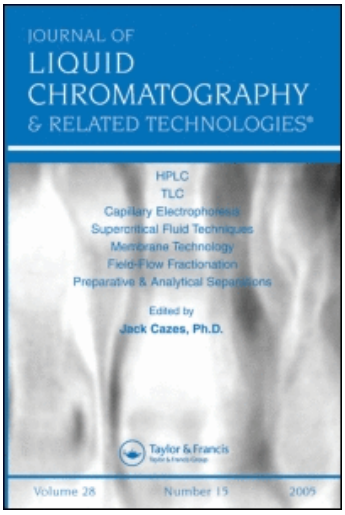

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Abstract: A novel, continuous cell separation method, based on density differences, is developed for application to transfusion medicine. Performance of a miniature separation column with ca. 8 mL capacity was examined on separation of human buffy coat (containing 10^7 nucleated cells and 10^{10} erythrocytes/mL). Differential leukocyte counts revealed that lymphocytes were concentrated in two fractions of density 1.065 to 1.070. Neutrophils were located in another fraction (density 1.080), while some cells present in early eluted low density fractions were difficult to identify by microscope examination. Flow cytometry analysis revealed that CD34-positive cells, which are considered to be stem cells, were relatively concentrated in an early eluted fraction.

Comparing the data obtained from hemocytometer and flow cytometry suggested the presence of immature CD45-negative nucleated cells in the same fraction. These results indicate that the present density method might be capable of collecting CD34-negative stem cells from peripheral blood. The present method was applied to the separation of cultured human mast cells. When the co-cultured cell suspension containing 3.5×10^6 cells (approximately 10% of mast cells and 90% of fibroblasts) was separated with a set of media (density: 1.065–1.085); fibroblasts were concentrated in a fraction of density 1.065 with a few young mast cells. Densely granulated matured mast cells were collected in a fraction of density 1.085. The method could also be used to detect minute changes in cell density under various physiological and pathological conditions.

Keywords: Continuous flow cell, Human mast cells

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INTRODUCTION

Separation of cells based on their densities is conventionally performed by test tube density gradient centrifugation, which is tedious and inefficient.[1] The method, however, has been improved by continuous flow centrifugation using a seal-less continuous-flow centrifuge equipped with a separation column with a circular channel.[2] The channel is interrupted at one portion so that the liquid introduced at one end is collected from the other end. A set of gradient media and a sample suspension is continuously eluted through 6 pairs of inlet and outlet tubes to produce a stable density gradient throughout the channel. Consequently, the cells are continuously separated according to their densities. This original separation column has a large capacity of 160 mL which is suitable for separation of a large number of cells. In order to apply this technique for the separation in a small scale, we recently developed a miniature separation column with a total capacity of 8 mL. The capability of the system was successfully demonstrated by separation of human buffy coat, which gave an excellent separation of nucleated cells with high reproducibility.[3]

In this paper, we report the separation of leukocytes from human buffy coat and peripheral blood, using our miniature continuous-flow cell centrifuge, and the application of the density method to purification of cord-blood derived mast cells.

EXPERIMENTAL

Apparatus and Separation Procedure

The density apparatus was designed in our laboratory and custom-made by Pharma-Tech Research Corporation, Baltimore, MD, USA. The design of the apparatus has been reported earlier. Briefly, the apparatus holds a separation disk that is equipped with a circular channel of about 8 mL capacity, which is interrupted at one portion so that the liquid introduced at one end is collected from the other end. The separation column is equipped with a set of 6 pairs of inlet or outlet tubes at each terminal. A set of isotonic Percoll media with different densities is continuously introduced into the inlet terminal and collected through the other terminal at a flow rate of 0.1 mL/min (for each density medium). The sample cell suspension is then eluted through the most proximal inlet at a flow rate of 0.3 mL/min. Under the centrifugal force field, cells gradually migrate into the density layers and finally suspend in the density layer corresponding to their own density before reaching the outlet of the channel. The present study was performed at 1,500 rpm of the separation disk.

Our samples, such as buffy coat or peripheral blood, contain a large number of erythrocytes with a high density. Therefore, they must be diluted

with an isotonic phosphate-buffered saline to a hematocrit value at 20% so that the density of the sample suspension is significantly lower than that of the lightest density gradient layer. The sample suspension was fed through a separate pump at a flow rate of 0.3 mL/min and the supernatant was collected through another pump at a reduced flow rate of 0.24 mL/min (for hematocrit 20%). In order to maintain steady-state hydrodynamic equilibrium of the density gradient layers, the above adjustment of the flow rate is required (Fig. 1). In our typical experiment, 30 mL of diluted buffy coat with 20% hematocrit was fed into the separation column for about 100 min, followed by elution with isotonic saline for an additional 15~20 min to collect the cells still retained in the channel.

Preparation of Gradient Media

Isotonic Percoll stock media (d = 1.124, pH 7.4) containing EDTA at 0.01% (0.34 mM) was prepared by mixing Percoll (d = 1.130 g/mL, osmolality <30 mOsmol/kg H_2O) (Amersham Biosciences), 10 × concentrated phosphate-buffered saline (PBS) (d = 1.075 g/mL, pH 7.4) (Sigma-Aldrich, St. Louis, MO, USA) and EDTA (d = 1.104 g/mL) (Sigma-Aldrich). This isotonic Percoll stock medium was diluted with an isotonic PBS solution (d = 1.008 g/mL, pH 7.4) containing EDTA at 0.01% (0.34 mM) to prepare each density medium.

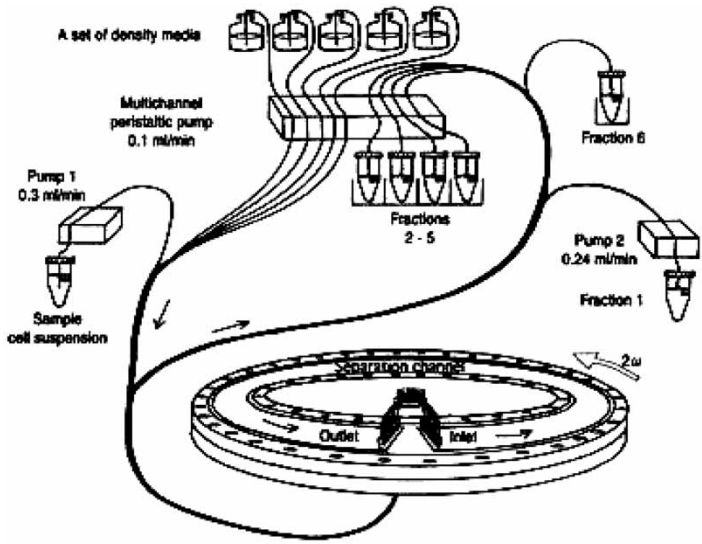

Figure 1. The present system for separating blood samples using a seal-less flow-through centrifuge.

Preparation of Sample Cell Suspension

Human buffy coat from a healthy donor was supplied from the Department of Transfusion Medicine, National Institutes of Health, Bethesda, MD, USA. Peripheral blood and human umbilical cord blood were donated from a healthy donor.

Cord blood-derived mononuclear cells differentiate into mast cells and are matured under a special condition when co-cultured with fibroblasts. A mast cell suspension was prepared as follows: after 20 weeks in co-culture, adherent cells left in a culture dish were freed by treating with trypsin (Sigma-Aldrich) and EDTA, followed by centrifugation. Harvested cells were re-suspended in culture medium.

Analysis of Cells

For buffy coat separation, nucleated cells were stained with Tuerk solution (Sigma-Aldrich) and manually counted using a hemocytometer. Differential leukocyte count for each separated fraction was performed by identifying 200 cells per smear which was stained with May-Giemsa's solution (Sigma-Aldrich). Flow cytometry was performed by Fast Systems, Gaithersburg, MD, USA.

Cells in each fraction separated from peripheral blood were placed onto a glass slide by cytospin centrifugation and stained with May-Giemsa's solution. The cytospin uses a proven, low-speed centrifugation technology to concentrate and deposit a thin layer of cells onto a clearly-defined area of a microscope slide and allows for the absorption of the residual fluid into a filter card to ensure capturing the target cells with excellent preservation of cell morphology.

Mast cells were stained with toluidine blue solution (Merck, Whitehouse Station, NJ, USA) metachromatically. Using a mouse anti-human mast cell tryptase monoclonal antibody (Chemicon, Temecula, CA, USA), tryptase antigens were detected by a fluorescein (FITC)-conjugated anti-mouse IgG antibody (Jackson ImmunoResearch, West Grove, PA, USA) for identification of human mast cells. The cell viability was determined by the dye exclusion test with trypan blue solution (Sigma-Aldrich).

RESULTS AND DISCUSSION

Separation of Human Buffy Coat

Cell distribution of each fraction from human buffy coat is shown in Table 1. Sample buffy coat diluted to a hematocrit value at 20% contained approximately 1×10^7 mononuclear cells and 2×10^9 erythrocytes/mL. These

Table 1. Nucleated cell distribution of each fraction separated from human buffy coat

Experimental number	Sample[a] (d < 1.050)	Fraction 1 (supernatant)	Fraction 2 (d = 1.060)	Fraction 3 (d = 1.065)	Fraction 4 (d = 1.070)	Fraction 5 (d = 1.075)	Fraction 6 (d = 1.080)
Cell counts ($\times 10^7$ cells/fraction)							
1	15.93	0	1.05	1.56	2.74	0.90	3.58
2	45.36	0	2.44	10.83	5.07	0.43	8.79
3	28.08	0	1.72	4.53	5.72	2.13	7.03
4	52.93	0	1.50	9.20	4.33	0.27	18.44
5	19.59	0	0.58	2.36	3.53	0.17	4.39
Mean	32.37	0	1.46	5.70	4.28	0.78	8.45
SD	16.15	0	0.70	4.13	1.19	0.80	5.96
Distribution	–	0%	7.1%	27.6%	20.7%	3.8%	40.9%

[a]Sample buffy coats were diluted at hematocrit value of 20%.

erythrocytes mostly flowed out to fraction 6; some of them were retained at the periphery of the separation channel, while no cells were observed in fraction 1. As to the cell distribution in each fraction, a relatively small number of cells were found in fractions 2 and 5, whereas fractions 3, 4, and 6 contained 25.1%, 23.6%, and 39% of the recovered cells, respectively. The average recovery of nucleated cells was 63.8 $\pm$ 4.8% in these five experiments, i.e., about 2/3 of the initial cells. Table 1 shows the high cell number variation from one experiment to another.

A differential leukocyte count in each fraction was performed microscopically on the May-Giemsa's stained smears for five samples. The results are shown in Fig. 2: A differential leukocyte count of sample buffy coat was similar to the normal value of peripheral blood, except for a slightly smaller number of heavy neutrophils. Almost all platelets (not counted) were eluted in fraction 2 (not shown). Monocytes were observed in fractions 2 and 3, lymphocytes were enriched in fractions 3 and 4, and neutrophils were concentrated in fraction 6 at 95.4 $\pm$ 6.4% (mean $\pm$ SD, n = 5). Rare basophils were observed in fraction 5 (density: 1.075) at 16.0 $\pm$ 13.3% (mean $\pm$ SD, n = 4), while eosinophils were found in fraction 6. Some nucleated cells present in fraction 2 (density: 1.060) were difficult to identify microscopically; we assume they are immature cells, i.e., erythroblasts, myeloblasts, or stem cells.

Each fraction separated from the buffy coat was analyzed by flow cytometry (Table 2). These data represents three analyses of fractions (the total nucleated cell count was obtained using a hemocytometer). In these analyses, many lymphocytes were found in fractions 3 and 4, while a number of neutrophils were in fraction 6. CD34-positive cells, which are believed to be stem cells, were relatively concentrated in fraction 2.

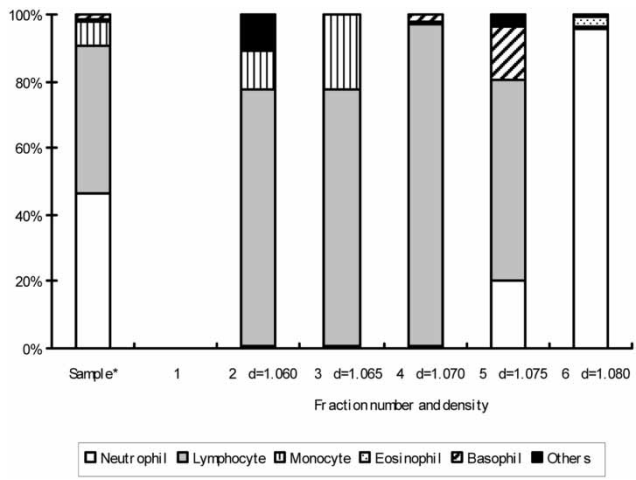

Figure 2. Differential leukocyte count in each fraction obtained from buffy coat. *Sample buffy coats were diluted at hematocrit value of 20%.

Table 2. Analytic results by hemocytometer and flow cytometry

	Hemocytometer [Total cells (nucleated cells) ($\times 10^6$ cells)]	Flow cytometry				
		Total cells CD45+ ($\times 10^6$ cells)	Monocytes CD45+, CD14+ ($\times 10^6$ cells)	Lymphocytes CD45+, CD14− ($\times 10^6$ cells)	Neutrophils/Poly CD45±, CD14− ($\times 10^6$ cells)	Stem cells CD34+ ($\times 10^6$ cells)
Sample Ht = 20%	165.2	172.3	20.4	149.2	2.7	2.7
Fraction 1 d < 1.060	0	0.6	0	0.3	0.3	0.2
Fraction 2 d = 1.060	10.5	2.2	0.8	1.2	0.2	1.6
Fraction 3 d = 1.065	15.6	14.0	0.2	12.8	1.1	0.5
Fraction 4 d = 1.070	27.4	25.1	0.0	23.1	2.0	0.4
Fraction 5 d = 1.075	9.0	14.5	0.0	5.2	9.3	—
Fraction 6 d = 1.080	35.8	70.8	0.8	26.4	43.7	—

Poly: Polymorphonuclear granulocytes.

Comparison of the total cells in fraction 2 obtained from these two methods suggests the presence of immature CD45-negative cells in this fraction. The number of CD34-positive cells in fraction 2 is approximately 15.5% among the total cells counted with a hemocytometer.

Separation of Peripheral Blood

As shown in Fig. 3, peripheral blood contains a large number of erythrocytes or approximately one thousand times that of leukocytes. Fraction 2 contained a large number of platelets with a few nucleated cells, some of which could not be identified as lymphocytes or monocytes. Fewer platelets were present in fraction 3, but no platelets were found in fraction 4. Lymphocytes were concentrated in fractions 3 and 4. Rare cells, such as basophils, were enriched in fraction 5. Neutrophils were mostly isolated in fraction 6 and eosinophils were found among neutrophils. Erythrocytes were observed in fraction 5 and they

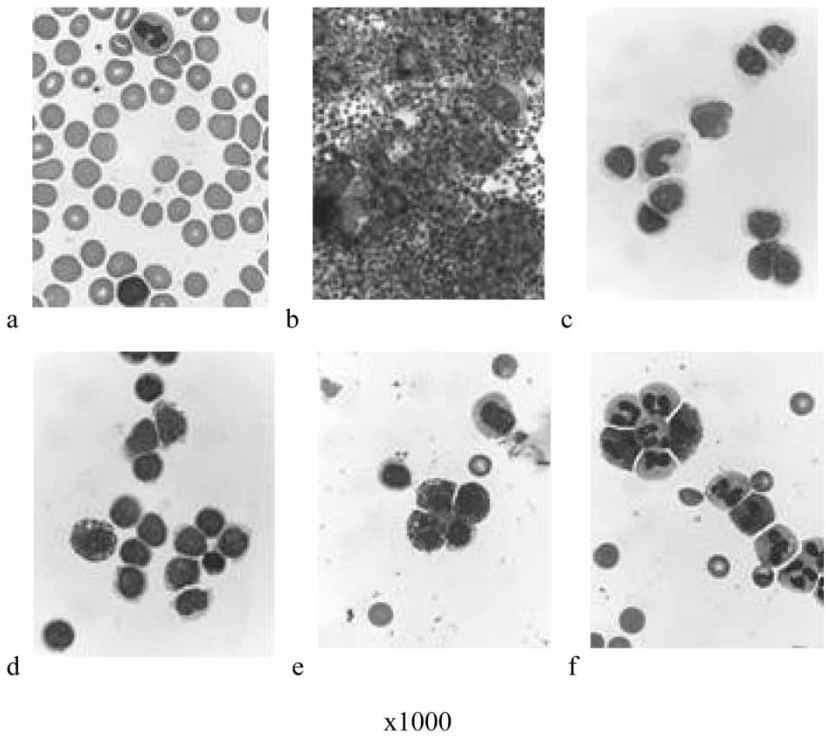

Figure 3. Cell morphological characteristics in each fraction separated from peripheral blood. a: Peripheral blood(hematocrit: 20%). b: Fraction 2(density: 1.060). c: Fraction 3 (density: 1.065). d: Fraction 4(density: 1.070). e: Fraction 5(density: 1.075). f: Fraction 6 (density: 1.080).

increased in fraction 6. Average cell recovery of 4 peripheral blood separations was 72.4%.

Nucleated Cells in Fraction 2 (density: 1.060)

Nucleated cells in fraction 2, separated from both buffy coat and peripheral blood, are shown in Fig. 4. Unusual nucleated cells were often observed among platelets in both samples. Some cells had nucleoli and their nuclei showed fine or loose chromatin structures like those seen in immature cells. Considering the results obtained by flow cytometry and morphological

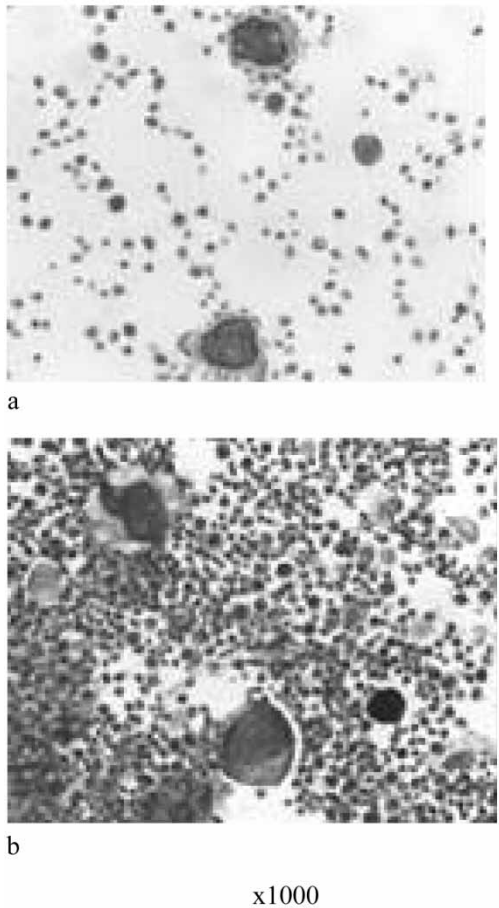

Figure 4. Cell morphological characteristics in fractions 2 obtained from buffy coat and peripheral blood. a: Bufft coat (density: 1.060). b: Peripheral blood (density: 1.060).

characteristics, CD34-positive cells were collected in this fraction together with other immature cells.

Separation of Cultured Human Mast Cells

A cultured cell suspension containing 3.5×10^6 cells (ca. 10% of mast cells and 90% of fibroblasts) was separated with a set of media with densities ranging from 1.065 to 1.085 (Fig. 5). Toluidine blue solution stains fibroblasts in light blue and mast cells containing metachromatic granules in violet. Mast cells were a few times larger than leukocytes as observed under microscopic magnification. A large majority of fibroblasts were present in fraction 2 (density: 1.065), while mast cells were concentrated in fraction 6 (density:

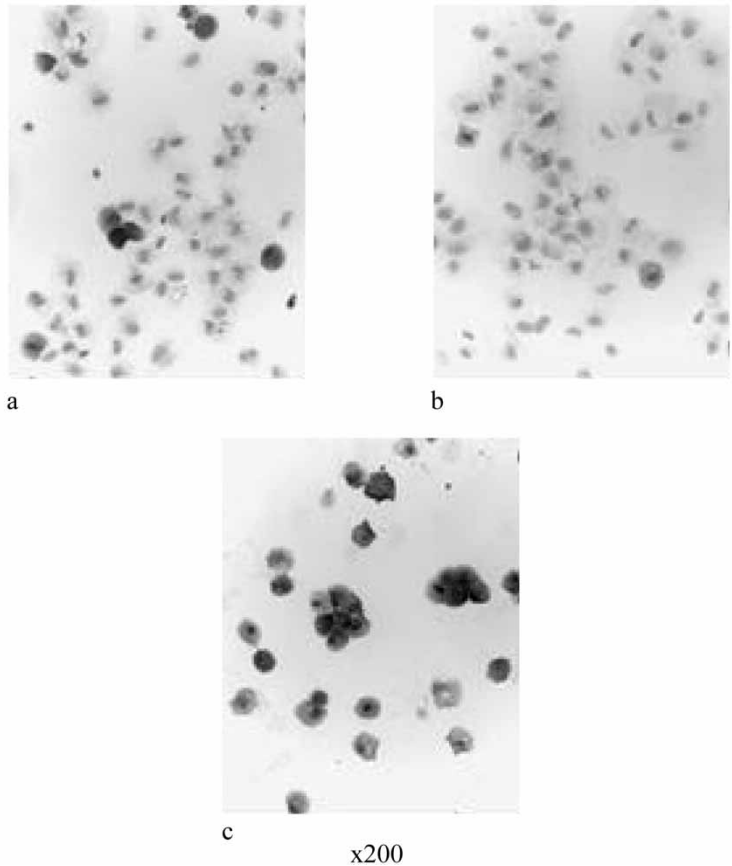

Figure 5. Separation of cultured human mast cells. (a, d), Co-cultured cell suspension (mast cells: 10.9%). (b, e), Fraction 2 (density: 1.065, mast cells: 3.5%). (c, f), Fraction 6 (density: 1.085, mast cells: 90.4%). Mast cells contain metachromatic granules.

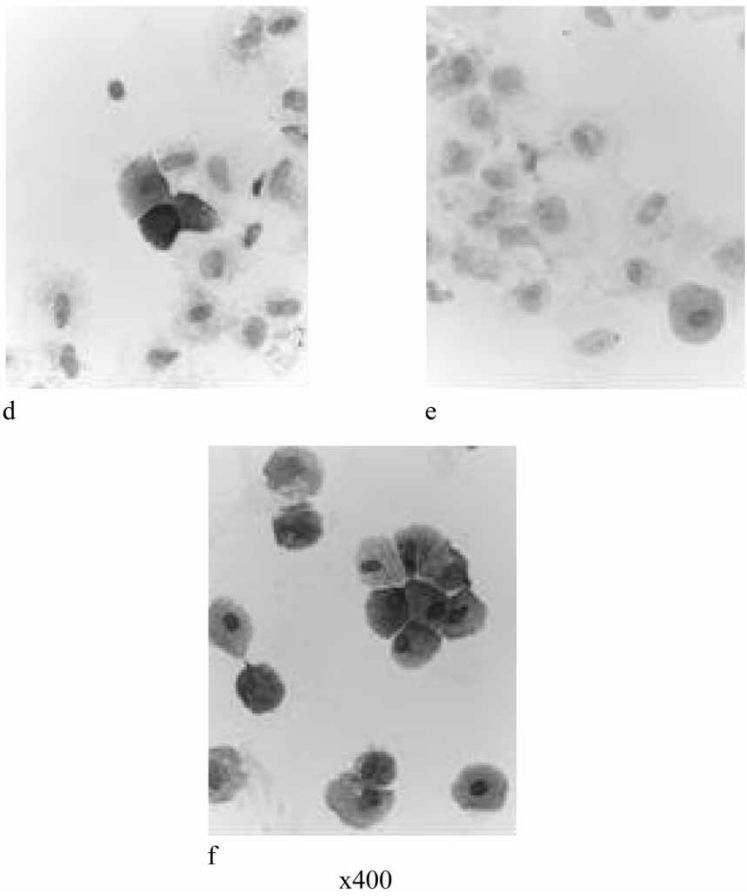

Figure 5. Continued.

1.085). It appears that heavily granulated mast cells in fraction 6 are more mature than those present in fraction 2.

Mast cells specifically produce and store tryptase; hence, tryptase detection is used for the identification of human mast cells. As shown in Fig. 6, it appears that mast cells were separated and concentrated in fraction 6 without loss of tryptase. The average cell recovery from these two co-cultured cell separations was 76.6%. The cell viability in each fraction was over 95%.

CONCLUSIONS

The surface marker antigen, CD34, is believed to be present on all hematopoietic stem cells. However, the existence of CD34-negative hematopoietic

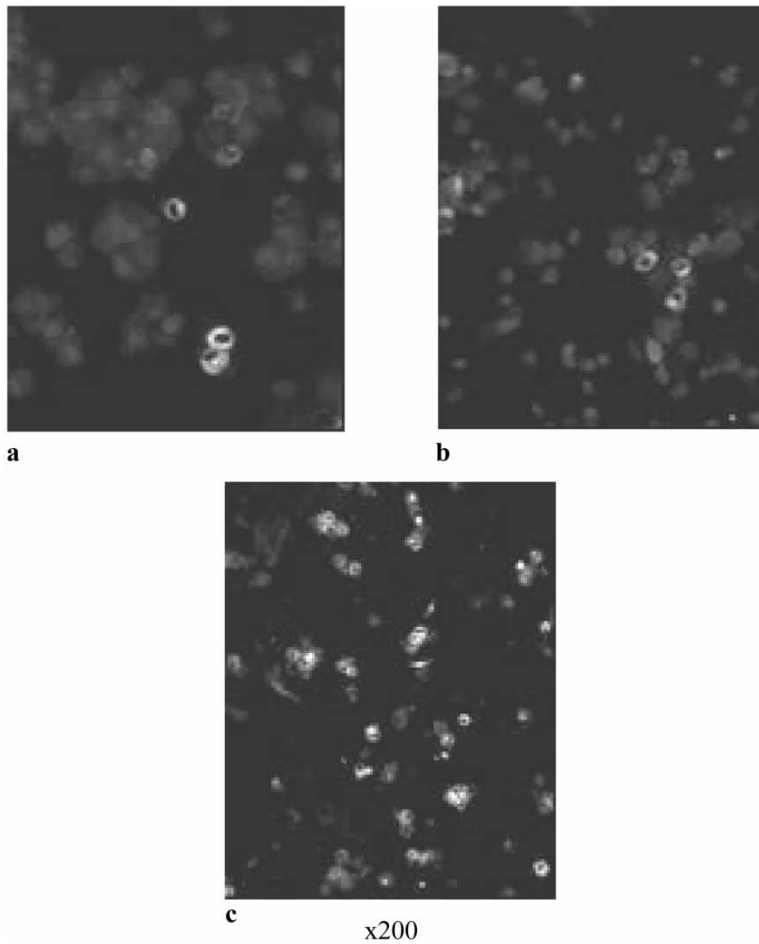

Figure 6. Separation of cultured human mast cells detected by immunochemical staining. a: Co-cultured cell suspension (mast cells: 9.3%,). b: Fraction 2 (density: 1.065, mast cells: 3.5%,). c: Fraction 6 (density: 1.085, mast cells: 92.0%,).

stem cells has been reported.[4] More recently, it has been shown that the CD34-positive cells may represent activated hematopoietic stem cells.[5] Currently, stem cells are harvested from the donor using apheresis after mobilizing the stem cells from the bone marrow by growth factors. The harvested cells in this manner consist of predominantly mature granulocyte, lymphocytes and monocytes: immature hematopoietic progenitor cells including stem cells usually comprise $<1\%$ of the total number of cells.[6] In the present method, nucleated cells in the sample are further separated into 5 fractions free of erythrocytes where CD34-positive stem cells are concentrated in one fraction with density of 1.060. From three flow cytometry analyses, the

number of CD34-positive stem cells in fraction 2 was 15.5%, 1.3%, and 2.4% among the total cell population manually determined with a hemocytometer. In order to harvest stem cells free of platelets without losing CD34-negative stem cells, the separation may be improved by focusing the media densities close to those of stem cells. For example, one may use a set of media with densities of 1.056, 1.058, 1.060, 1.062 and 1.064. In this way, the present method may be efficiently applied for enrichment of hematopoietic stem cells from human umbilical cord blood which contains a much greater number of stem cells than the peripheral blood.

Nucleated cells of human umbilical cord blood differentiate into mast cells under a special condition when co-cultured with fibroblasts.[7] In our method, matured mast cells with a large number of dense granules or expressing a large amount of tryptase antigens are concentrated in fraction 6 (density: 1.085), while fibroblasts are eluted in fraction 2 (density: 1.065). The method may be used for separation of various other types of cultured cells.

We propose that our new method may also be used to detect subtle changes in cell density under various physiological and pathological conditions.

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